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Prenatal Programming of Renal Sodium Handling in the Rat

Saleh H. Alwasel¹ and Nick Ashton

Faculty of Life Sciences, University of Manchester, Manchester, M13 9PT, United Kingdom

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Author for correspondence:

Dr Nick Ashton Faculty of Life Sciences University of Manchester 1.124 Stopford Building Oxford Road Manchester M13 9PT United Kingdom

Phone: +44 (0)161 275 5454 Fax: +44 (0)161 275 3938 Email: nick.ashton@manchester.ac.uk

¹ current address:

Department of Zoology College of Science King Saud University PO Box 2455 Riyadh 11451 Saudi Arabia

Abstract

Prenatally programmed hypertension induced by maternal protein restriction is associated with increased expression of the renal tubular $Na^+:K^+:2Cl^-$ co-transporter (NKCC2) and the Na⁺:Cl⁻ co-transporter (NCC). This has led to the suggestion that renal sodium retention contributes to the development of hypertension in the low protein (LP) rat. However, this hypothesis has not been tested *in vivo*. Renal clearance measurements in hypertensive 4 week old male and female LP rats showed that while glomerular filtration rate remained unaltered, urine flow (P < 0.01) and urinary sodium excretion (control male 1.6 ± 0.3 vs LP male $3.0\pm0.4 \,\mu\text{mol.min}^{-1}.100 \,\text{bwt}^{-1}$, P<0.001) rates were increased. Sodium excretion was positively correlated with mean arterial pressure in both males (P < 0.01) and females (P<0.05) but neither the slope nor intercept differed between control and LP rats. Fractional excretion of sodium was increased in male (control 1.5±0.2 vs LP 3.0±0.5%, P<0.001) and female LP rats, implying reduced tubular reabsorption of sodium. Western blot and quantitative PCR showed that NKCC2 expression was increased; NCC mRNA was not upregulated. Na⁺:K⁺ATPase α_1 subunit expression did not differ from controls, however, there was a significant reduction in whole kidney pump activity (control male 23.4±1.8 vs LP male 17.7±1.2 nM PO₄.µg protein⁻¹.h⁻¹, P<0.001); immunohistochemistry showed that the α_1 subunit was virtually absent from the inner medulla. The greater sodium excretion of LP rats can be explained, in part, by a pressure natriuresis mechanism. However, loss of the Na⁺:K⁺ATPase α_1 subunit from the inner medulla and upregulation of NKCC2 suggests that altered renal sodium handling is also programmed prenatally.

Introduction

An adverse environment during key stages of fetal and neonatal development can increase the risk of developing cardiovascular disease, non-insulin dependent diabetes and metabolic syndrome [1]. The mechanisms underlying what has become known as developmental programming are, as yet, unknown. The physiology of a number of organs appears to be affected by the maternal environment, however, studies employing maternal protein restriction in the rat have shown that the kidney is a central target of developmental programming [2]. Initial studies have linked the development of hypertension in rats exposed to prenatal protein restriction to an increase in renal sodium retention [3, 4]. A chronic increase in sodium retention would lead to an expansion of extracellular fluid volume which ultimately increases blood pressure [5]. One potential cause of increased renal sodium retention is a reduction in glomerular filtration rate (GFR), as a result of a reduction in nephron number [6]. Fewer nephrons may result in a reduction in the filtration surface area, which may impair sodium excretion and thus lead to an expansion of extracellular fluid volume [6]. Previous studies have shown that rat offspring exposed to a maternal low protein (LP) diet in utero develop fewer nephrons [7, 8]. However, it has been argued that the reduction in nephron number observed in LP rats does not affect total GFR, because of an increase in individual glomerular volume [9] or hyperfiltration [10].

The other major determinant of urinary sodium excretion is renal tubular sodium reabsorption. A recent report showed that the expression of the apical bumetanide-sensitive Na⁺:K⁺:2Cl⁻ co-transporter (NKCC2 or BSC1) and the thiazide-sensitive Na⁺:Cl⁻ co-transporter (NCC or TSC) is increased in LP rats [4]. Interestingly, neither the sodium: hydrogen exchanger type-3 (NHE3) nor the amiloride-sensitive epithelial sodium channel (ENaC) were altered in LP rat offspring [4]. Increased NKCC2 and NCC expression have also been reported in rats exposed to dexamethasone *in utero* [11]. In this model NHE-3 expression and activity were also increased, resulting in greater tubular fluid absorption in isolated proximal tubules *in vitro* [12]. Taken together with the report that exposure to a low protein diet increased renal Na⁺:K⁺A TPase α_1 and β_1 subunit mRNA expression by 2- to 3-fold [3], these observations have led to the suggestion that renal sodium retention contributes to the higher blood pressure of the LP rat [13].

However, this hypothesis has not been tested *in vivo*. Accordingly, the aim of the current study was to quantify renal sodium handling by the LP rat. We studied both male and female rats, as some aspects of developmental programming are gender-specific [14], at 4 weeks of age in order to minimise the potentially confounding impact of chronic exposure to high blood pressure on renal function. In order to confirm that exposure to a low protein diet induced increases in NKCC2 and NCC expression in our hands, we used real-time quantitative PCR and Western blotting to quantify expression of the principle sodium transporters in the LP rat kidney. We also quantified expression of the Na⁺:K⁺ATPase α_1 subunit, the principle renal α subunit isoform and catalytic unit of the pump [15], localised its renal distribution by immunohistochemistry and measured activity of the Na⁺:K⁺ATPase pump as the driving force for renal sodium reabsorption. Finally, we measured the plasma aldosterone concentration to determine whether endocrine control of sodium reabsorption was altered in LP rats.

Methods

Ethical approval

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and received local ethical approval.

Animals

Wistar rats (Harlan UK Ltd, Belton) were paired in individual breeding cages and held in a room at 22-24°C with a 12 hour light : 12 dark cycle. As soon as mating was confirmed by the presence of a sperm plug, the female's diet was switched from standard chow (Rat & Mouse Standard Diet, Bantin & Kingman Ltd, Hull, North Humberside) to one which contained either 9% protein (low protein, LP) or 18% protein (control). The semi-synthetic diets were formulated as described previously [7]. On the day of birth, the dam's diet reverted to standard chow and the litter size was reduced to 8 animals (4 male and 4 female). The offspring were studied at 4 weeks of age. As the experimental manipulation was of the dams rather than the offspring *per se*, no more than 2 rats of either sex were studied per litter. Separate litters were bred for each of the methodologies described below.

Renal clearance measurements

Inactin (thiobutabarbital sodium, 100 mg/kg i.p.) anaesthetised control male (CM, n = 15), control female (CF, n = 13), low protein male (LPM, n = 15) and low protein female rats (LPF, n = 12) were prepared for euvolaemic fluid replacement of spontaneous urine output using a servo-controlled fluid replacement system, as described previously [16]. Following surgery, a bolus of ³H inulin (4 μ Ci) was injected via the jugular vein cannula and servocontrolled replacement of 0.154 M NaCl was initiated. Infusion of ³H inulin (0.3 µCi/h) continued via a second, slow (1 ml/h) infusion pump. After a 2h equilibration period, four 15 min urine collections were made. 0.3 ml of blood was withdrawn via a carotid artery catheter at 30 min; a similar volume of 0.154 M NaCl and ³H inulin was replaced via the jugular catheter. Blood pressure was recorded throughout the experiment via a catheter implanted in the left carotid artery (PowerLab 800/s, ADInstruments, Hastings, East Sussex). Animals were killed humanely at the end of the experiment. Urine and plasma sodium and potassium concentrations were measured by atomic absorption spectrophotometry (Solaar S series, Thermo Elemental, Cambridge). ³H inulin was determined using a 1900CA Tri-Carb Liquid Scintillation Analyser (Canberra Industries, Meriden, CT, USA) β-counter. Plasma aldosterone concentration was determined using a commercial radioimmunoassay kit (Coata-Count, Diagnostic Products Corporation, Caernarfon, Gwynedd). The intra-assay coefficient of variation was 3% (n = 10).

Expression of renal sodium transporters

Real-time quantitative PCR

Total RNA was extracted from kidneys harvested from a separate group of offspring (n = 8 per group) using TRIzol reagent and reverse-transcribed using SuperScript II RT (200 U, Invitrogen Ltd., Paisley). All primers and TaqMan probes (Table 1) were designed using Primer Express (ABI Instruments, Foster City, CA, USA); β -actin was used as an internal control. Reverse transcriptase-polymerase chain reaction was performed using the ABI Prism

7000 system (ABI Prism, Foster City, CA, USA). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [17] and normalised to a control adult kidney.

Western blot analysis

Western blot analysis of renal sodium transporters was also performed using the following antisera: polyclonal rabbit anti-rat NHE-3 (1:300 dilution, Alpha Diagnostic International, San Antonio, TX, USA), polyclonal rabbit anti-rat NKCC2 (1:150 dilution, Alpha Diagnostic International), polyclonal rabbit anti-rat ENaC (1:5000 dilution, Sigma-Aldrich) and monoclonal mouse anti-rat Na⁺:K⁺ATPase (α_1 subunit, 1:5000 dilution, ab2867, Abcam, Cambridge). Commercially available NCC antibodies proved unsatisfactory for Western blotting, hence the NCC transporter was quantified by qPCR alone. Labelling was identified by application of horseradish peroxidase-conjugated goat anti-rabbit or swine anti-mouse immunoglobulin (1:5000 or 1:20,000, DakoCytomation Ltd, Cambridge) and enhanced chemiluminescence detection (ECL-plus, Amersham Pharmacia Biotech Ltd, Little Chalfont, Buckinghamshire). Whole kidney homogenates (n = 16 per group) were normalised against a reference adult kidney sample [18]. Equality of protein loading was confirmed by Ponceau staining; antibody controls were performed by pre-adsorption of the primary antibody with antigen or omission of primary antibody (data not shown).

Immunohistochemistry

Kidneys were perfused with paraformaldehyde *in situ*, removed and embedded in paraffin wax (n = 8 per group). Sections (5 µm) were incubated overnight at 4°C with primary antibodies (polyclonal rabbit anti-rat NCC (TSC11-a, Alpha Diagnostic International); all others as above for Western blotting) diluted 1:100 in 0.1% BSA with 0.3% Triton X-100. Labelling was identified by application of a goat anti-rabbit or swine anti-mouse HRP-conjugated secondary antibody (1:100, DakoCytomation Ltd., Cambridgeshire) before incubation with diaminobenzidine (Sigma Fast DAB (3, 3'-Diaminobenzidine) to detect peroxidise activity.

Na⁺:*K*⁺*ATPase activity*

Renal Na⁺:K⁺ATPase activity was determined in whole kidney homogenates of freshly collected tissue (n = 12 per group). The homogenate was mixed 1:1 with SDS (0.75 mg/ml) and an incubation solution (150 mM histidine, 640 mM NaCl, 40 mM MgCl₂, 200 mM KCl) containing 30 mM ATP at 37 °C. Non-specific and Na⁺:K⁺ATPase-specific phosphate production were determined in the presence or absence of 10 mM ouabain. Samples were quenched with perchloric acid, centrifuged at 1200 g (15 min, 2 °C) after which equal volumes of supernatant and distilled water were mixed with colour reagent (1 g (NH₄)₆MO₇O₂₄.4H₂O, 94.7 mL H₂O, 3.3 mL concentrated sulphuric acid and 4 g FeSO₄.7H₂O). Absorbance of the final solution was read at 690 nm on a microplate reader (Titertek Multiskan MC, Flow Laboratories Australasia, North Ryde, Australia) with a phosphate standard curve (10-250 nM KH₂PO₄). Total Na⁺:K⁺ATPase activity (nM PO₄.µg protein⁻¹ h⁻¹) was calculated as the difference between inorganic phosphate liberated in the presence and absence of 10 mM ouabain [19].

Statistical analysis

Data were analysed by 2-way ANOVA (SPSS 13.0 for Windows, SPSS UK Ltd, Surrey) and are presented as the mean \pm SEM. The correlation between urinary sodium excretion and mean arterial pressure was assessed by linear regression analysis. The effect of maternal diet on the slopes and intercepts of the calculated regression lines were compared by analysis of covariance (ANCOVA) using Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Significance was assumed when P < 0.05.

Results

Renal clearance measurements

Gender had no effect on any of the measured variables, with the exception of body weight which was significantly greater in males compared with females (P < 0.001). Mean arterial blood pressure (MAP) was significantly higher (P < 0.001) in rats exposed to a low protein (LP) diet *in utero* (Table 2). MAP remained stable in all groups throughout the experiment. Plasma sodium concentration was significantly greater in LP rats (P = 0.04), but plasma potassium, osmolality and haematocrit did not differ between dietary groups (Table 2). Despite the increase in plasma sodium concentration, plasma aldosterone did not differ between control and LP rats (Table 2).

Glomerular filtration rate (GFR, Figure 1A) did not differ between control and LP rats, however, urine flow rate was significantly greater in the LP animals (Figure 1B). This was associated with significant increases in urinary sodium (P < 0.001, Figure 1C) and chloride (P < 0.001, Table 3) excretion rates; in contrast, potassium excretion did not differ between groups (Table 3). Sodium clearance was also increased significantly in both male (control 11.5 ± 1.9 vs LP $22.3 \pm 3.1 \ \mu l \cdot \min^{-1} \cdot 100 \text{g bwt}^{-1} P < 0.001$) and female (control 11.5 ± 2.1 vs LP $21.0 \pm 4.5 \ \mu l \cdot \min^{-1} \cdot 100 \text{g bwt}^{-1} P < 0.001$) LP rats. The basis for the natriuresis and chloruresis was tubular, as the fractional excretion rates of both ions were significantly greater in LP rats (FE_{Na} P < 0.001, Figure 1D; FE_{Cl} P < 0.001, Table 3). Consistent with the increase in sodium excretion, os molar excretion was also greater (P < 0.001) in the LP group (Table 3). Free water clearance (C_{H2O}) was negative for both groups (Table 3), indicating that the excreted urine was hyperosmotic to plasma; C_{H2O} was significantly lower (i.e. more negative) in LP rats compared with controls (P < 0.001, Table 3).

There were significant correlations between MAP and urinary sodium excretion for control $(r^2 = 0.46, P = 0.006, Figure 2A)$ and LP males $(r^2 = 0.45, P = 0.006)$. A similar relationship was also evident in control $(r^2 = 0.44, P = 0.014, Figure 2B)$ and female LP rats $(r^2 = 0.56, P = 0.005)$. Surprisingly, there were no significant differences between control and LP rats for either the slope (ANCOVA: males $F_{1,26} = 1.81, P = 0.19$; females $F_{1,21} = 1.59, P = 0.22$) or intercept (males $F_{1,27} = 0.48, P = 0.49$; females $F_{1,22} = 0.30, P = 0.59$) of the regression line for either gender. This implies that the pressure natriuresis relationship was not altered in young LP rats, despite their higher systemic blood pressure.

Renal sodium transporters

Gender had no effect on sodium transporter expression or activity. Western blot (Figure 3) and qPCR (Table 4) analysis were in agreement, indicating that renal NHE-3, ENaC and Na⁺:K⁺ATPase expression did not differ between LP and control animals. NCC mRNA

expression did not differ between control and LP rats either (Table 4). However, NKCC2 mRNA and protein levels were significantly greater (P < 0.001) in LP rats compared with controls. Immuno-localisation did not reveal any notable differences between the groups in the distribution of NHE-3, NKCC2, NCC or ENaC (data not shown). However, there was a striking difference in the distribution of Na⁺:K⁺ATPase in the kidneys of LP rats. While present in the cortex and outer medulla, the Na⁺:K⁺ATPase α_1 subunit was virtually absent from the inner medulla of LP rats (Figure 4). At high power, faint staining was observed in inner medullary collecting ducts (compare Figure 4 E and F), indicating very low levels of Na⁺:K⁺ATPase α_1 subunit expression, but this was considerably lower than that seen in tissue from control animals. This striking reduction in Na⁺:K⁺ATPase expression was observed in both male and female LP rats. Furthermore, there was a significant reduction in Na⁺:K⁺ATPase activity, measured in whole kidney homogenates, of both male (CM, n = 11, 23.4 ± 1.8 vs LPM, n = 10, 17.7 ± 1.2 nM PO₄.µg protein⁻¹.h⁻¹, P < 0.001) and female (CF, n = 16, 21.9 ± 1.3 vs LPF, n = 14, 17.5 ± 1.1 nM PO₄.µg protein⁻¹.h⁻¹, P < 0.001) LP rats.

Discussion

On the basis of previous molecular studies, which showed up-regulation of NKCC2, NCC [4] and Na⁺:K⁺ATPase [3] in LP rats, we hypothesised that urinary excretion of sodium would be reduced in the LP model of hypertension. However, this was not the case: urinary sodium excretion was increased in both male and female LP rats. In view of this apparent inconsistency, and because the low protein diet employed by Manning et al. [4] differs from that used by ourselves (6% protein vs 9% protein used herein), we sought to confirm the outcome of their earlier study of renal sodium transporter expression. Our results are in broad agreement, however, there are some important differences. Manning et al. [4] reported marked increases in both NKCC2 and NCC mRNA and protein expression at 4 weeks of age; by 8 weeks of age, NCC mRNA expression had returned to control levels, however NKCC2 mRNA remained elevated. We observed a similar increase in NKCC2 expression at both the protein and mRNA level in 4 week old rats, however, we did not see any difference in NCC expression. Furthermore, our rats were hypertensive at 4 weeks of age, in common with other workers using the same 9% protein diet formulation as that used in the current study [3, 20, 21], whereas Manning's animals were normotensive at 4 weeks and did not show elevated blood pressure until 8 weeks of age [4]. Hence the pattern of sodium transporter expression and blood pressure in our 4 week old animals more closely resembles Manning's rats at 8 weeks old. The reason for the difference between the two models is not clear, but could reflect different degrees of protein restriction (6% vs 9%), differing periods of protein restriction (from day 12 of pregnancy vs the whole of gestation), litter size, and thus milk availability (litter size not controlled vs litters standardised to 8 pups) and the strain of rats used (Sprague-Dawley vs Wistar).

The basis for the natriuresis displayed by LP rats appears to be tubular in origin rather than being due to increased filtration of sodium at the glomerulus. GFR did not differ between control and LP rats and although the plasma sodium concentration was somewhat higher in LP animals, the filtered load of sodium was not significantly greater in the LP group. In contrast, the fractional excretion of sodium was markedly elevated in LP rats (Figure 1 D), which implies that tubular reabsorption was diminished. A reduction in tubular sodium reabsorption is consistent with the observed increases in osmolar excretion and both total and fractional excretion of chloride. The loss of NaCl does not appear to be due to a wash-out effect of the accompanying diuresis. Rather the opposite seems to be the case, with increased sodium loss driving the diuresis. Free water clearance was more negative in the LP animals. Free water clearance is a measure of the concentration of osmolytes in excreted water compared with plasma: a negative value indicates that the water: osmolyte ratio is lower in the urine than plasma whereas a positive value indicates that the ratio is higher in urine. As the LP rats had more negative free water clearances compared with controls, this implies that they were excreting proportionately less water than osmolytes. A further indication that the natriuresis was due to specific alterations in tubular sodium transport comes from the potassium handling data. Neither total nor fractional excretion of potassium differed between control and LP rats, suggesting that the diuresis was not washing out electrolytes in a non-specific manner.

The natriuresis does not appear to be due to a reduction in aldosterone activity either, as plasma aldosterone concentrations in LP rats of both sexes were comparable with control animals, consistent with our previous report [18]. This is somewhat surprising in view of the hypernatraemia exhibited by the LP animals; however, there is evidence from other studies that the relationship between plasma sodium and aldosterone is perturbed in the LP rat. Plasma aldosterone was elevated at both 4 and 8 weeks in Manning's model [22], but had returned to control levels by 16 weeks [23]. Interestingly, LP pups fed a high salt diet had elevated plasma aldosterone concentrations in the latter study, suggesting that aldosterone may be regulated inappropriately in LP rats. Of the three main regulators of aldosterone synthesis, plasma potassium, angiotensin II (Ang II) and adrenocorticotrophin (ACTH), the former does not appear to be altered in LP rats. However, expression of the adrenal AT_{1b} receptor, which mediates Ang II-induced aldosterone secretion in the rat, is increased in the LP rat [24]. Diurnal changes in ACTH secretion have been reported to be blunted in LP animals; however, plasma corticosterone still followed a diurnal pattern, leading to the suggestion that the adrenal gland is hyper-responsive to ACTH [25]. Increases in either of these regulators could account for the apparent mis-match between plasma sodium and aldosterone concentrations in LP rats. The effects of maternal protein restriction of offspring aldosterone synthase activity, which catalyses the 11-hydroxylation and 18-oxidation of 11deoxycorticosterone to aldosterone, are unknown.

What, then, is responsible for the reduction in tubular sodium reabsorption by LP rats? One possibility is that hyperfiltration leads to increased tubular fluid flow rates and a reduction in sodium reabsorption. We [7] and others [8] have shown that LP rats have fewer nephrons than control animals. Since total GFR is unaltered in LP rats, single nephron GFR (SNGFR) should be increased, although this has not been measured directly. High tubular fluid flow rates impair sodium reabsorption, particularly when linked with elevated systemic blood pressure which stimulates sub-cellular redistribution of NHE-3 in the proximal tubule leading to a reduction in sodium transport [26]. Tubuloglomerular feedback (TGF) might be expected to correct SNGFR to reduce fluid delivery to the distal nephron. However, evidence suggests that TGF may be reset to accommodate long term changes in SNGFR and distal tubular flow, thereby maintaining both hyperfiltration and high tubular fluid flow rates [27].

Another explanation, which may also be linked to hyperfiltration, is the 20-24% reduction in renal Na⁺:K⁺ATPase activity, which would reduce the driving force for sodium reabsorption as well as that for other solutes. A similar reduction in renal Na⁺:K⁺ATPase activity has been reported in rats exposed to a fat-rich diet *in utero* [19] and in the erythrocytes of low birthweight men [28]. Acute increases in blood pressure have also been reported to result in a 50% reduction in basolateral Na⁺:K⁺ATPase activity and internalisation of α_1 and β_1 subunits [29]; a similar decrease in cortical Na⁺:K⁺ATPase activity was observed in

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hypertension [26]. However, these data are at odds with Bertram *et al.*'s [3] observations in LP rats exposed to the same synthetic diet as that used in the current study. Bertram reported increased renal mRNA expression of both the α_1 and β_1 subunits of Na⁺:K⁺ATPase in 12 week old LP rats, whereas we saw no difference in α_1 subunit mRNA between 4 week old LP and control animals. Age differences between the studies may account, at least in part, for this discrepancy: De Assis *et al.* [30] reported that Na⁺:K⁺ATPase activity was diminished in the outer medulla of weanling rats exposed to a high cholesterol diet *in utero* whereas pump activity in adult rats did not differ from that in controls.

Immunohistochemistry showed that the Na⁺:K⁺ATPase α_1 subunit was virtually absent from the inner medullary collecting ducts (IMCD) of both male and female LP rats in the present study. Loss of Na⁺:K⁺ATPase activity from the IMCD is probably not enough on its own to account for the 20-24% reduction in pump activity measured in whole kidney homogenates. However, as the IMCD are responsible for the final 2% of sodium reabsorption, loss of Na⁺:K⁺ATPase pump activity in this segment could account for the 1.5-2.4% increase in fractional excretion of sodium seen in LP rats. The mechanism responsible for the loss of the α_1 subunit is not clear. Cell type-specific transcription factors have been described which allow differential expression of the α_1 subunit gene, Atp1a1, in various tissues [31] which may explain how α_1 subunit expression was down-regulated in inner but not outer medullary collecting ducts. Several physiological stimuli have been described which result in upregulation of renal α_1 subunit mRNA, including increases in mineralocorticoid [32], glucocorticoid [33] and thyroid (T_3) [34] hormone concentrations. Therefore a reduction in one or more of these hormones might lead to a reduction in α_1 subunit expression. However, maternal protein restriction had no effect on plasma aldosterone concentration in the current study, renal glucocorticoid receptor expression is increased while 11B-hydroxysteroid dehydrogenase type 2 activity is diminished in LP rats [3] and serum T₃ concentrations are elevated in rats reared by a protein-restricted dam [35].

The other notable difference in sodium transporter expression between control and LP rats is an increase in NKCC2 expression seen both here and in Manning et al.'s [4] study. This increase in NKCC2 expression contrasts with reports of a nitric oxide (NO)-mediated decrease in NKCC2 activity [36] in other models of hypertension in which activation of endothelial nitric oxide synthase (eNOS) [37] has been suggested to result in a reduction in membrane expression of the transporter [38]. This is consistent with the report that chronic L-NAME treatment increased both NKCC2 protein expression and activity [39]. Little is known about renal NOS activity in the LP rat: urinary excretion of nitric oxide metabolites was increased in Manning's LP rat model at 4 weeks of age (prehypertensive) but was unchanged at 8 weeks (hypertensive) [40]. Endothelial NOS mRNA expression has been reported to be reduced in the thoracic aorta of LP rats exposed to the same synthetic diet that we employed in the current study [41]. In the same study, the impaired endotheliumdependent vasodilatation of LP rat mesenteric arteries was shown to be resistant to eNOS and cyclooxygenase blockade, leading the authors to conclude that the NO pathway is down regulated in LP rats. If such a reduction in NO occurs in the kidney, this could account for the observed increase in NKCC2 expression. Sodium reabsorption by the thick ascending limb (TAL) is also regulated by prostaglandin E_2 (PGE₂) which has been reported to inhibit NKCC2 activity in cultured mouse TAL cells [42]. Urinary PGE₂ excretion is increased in 4 week old LP rats [43], which raises the possibility that net NKCC2 transport is inhibited, despite increased transporter expression.

Despite the changes in Na⁺:K⁺ATPase activity and NKCC2 expression, the pressure natriuresis relationship does not appear to be altered in the LP rat. Plotting sodium excretion against mean arterial pressure (Figure 2) shows that the LP rat kidney appears to be able to excrete sodium in a manner very similar to that seen in control animals. This is somewhat surprising, as in the majority of models of hypertension either the slope of the regression and / or the intercept are shifted to the right. As a result, elevated systemic pressure is required to achieve renal sodium loss at a rate matching dietary intake. However in LP rats of both sexes, the relationship between mean arterial pressure and sodium excretion was identical to that seen in control animals. This lack of difference in the pressure natriures is curve implies that the natriuresis and the elevated blood pressure of the LP rat are driven by increased sodium intake rather than an impaired ability to excrete sodium, despite LP rats having fewer nephrons than control animals [7, 8]. Certainly, continued loss of sodium could only be sustained in the long term if it is matched by a similar increase in sodium intake. Preliminary experiments show this to be the case, with LP rats eating (CM 14.6 ± 0.2 vs LPM 16.0 ± 0.3 g food $\cdot 24h^{-1} \cdot 100g$ bwt⁻¹, P < 0.01) and drinking (CM 22.2 ± 0.4 vs LPM 24.2 \pm 0.8 ml water \cdot 24h⁻¹ \cdot 100g bwt⁻¹, P < 0.01) more than control animals when provided with food and water ad libitum (S.H. Alwasel & N. Ashton, unpublished observations).

Taken together, the data presented in this study suggest that renal sodium handling is perturbed in the LP rat model of hypertension. In young animals with elevated blood pressure sodium excretion is increased, despite the presence of fewer nephrons in the kidney [7, 8] and greater expression of NKCC2 which might be expected to favour sodium retention. These observations have parallels with a study in young low birth weight (LBW) Caucasian men in which hypertension, natriuresis and reduced Na⁺:K⁺ATPase activity were recorded [28]. The underlying cause of the natriuresis in both the LP rat and LBW men appears to be due to a pressure natriuresis response. However, this appears to be ineffective in lowering blood pressure. There is also evidence of a programming effect on renal sodium transport in the LP rat: expression of the Na⁺:K⁺ATPase α_1 subunit in the inner medulla, which is thought to play only a minor role in pressure natriuresis [44], is markedly reduced in the hypertensive LP rat. In prehypertensive LP rats NKCC2 and NCC expression are increased [4]: whether this results in enhanced sodium reabsorption is not known, but it raises the possibility that sodium retention could contribute to the onset of hypertension in the LP rat.

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References

- 1 Gluckman, P.D. and Hanson, M.A. (2004) The developmental origins of the metabolic syndrome. Trends. Endocrinol. Metab. **15**, 183-187
- 2 Marchand, M.C. and Langley-Evans, S.C. (2001) Intrauterine programming of nephron number: the fetal flaw revisited. J. Nephrol. **14**, 327-331
- 3 Bertram, C., Trowern, A.R., Copin, N., Jackson, A.A. and Whorwood, C.B. (2001) The maternal diet during pregnancy programs altered expression of the glucocorticoid receptor and type 2 11β-hydroxysteroid dehydrogenase: potential molecular mechanisms underlying the programming of hypertension *in utero*. Endocrinol. **142**, 2841-2853

4 Manning, J., Beutler, K., Knepper, M.A. and Vehaskari, V.M. (2002) Upregulation of renal BSC1 and TSC in prenatally programmed hypertension. Am. J. Physiol. **283**, F202-206

ical

- 5 Guyton, A.C. (1992) Kidneys and fluids in pressure regulation. Small volume but large pressure changes. Hypertension **19**, I2-8
- 6 Brenner, B.M., Garcia, D.L. and Anderson, S. (1988) Glomeruli and blood pressure. Less of one, more the other? Am. J. Hypertens. **1**, 335-347
- 7 Sahajpal, V. and Ashton, N. (2003) Renal function and angiotensin AT₁ receptor expression in young rats following intrauterine exposure to a maternal low-protein diet. Clin. Sci. (Lond). **104**, 607-614
- 8 Langley-Evans, S.C., Welham, S.J. and Jackson, A.A. (1999) Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. Life Sci. **64**, 965-974
- 9 Woods, L.L., Ingelfinger, J.R., Nyengaard, J.R. and Rasch, R. (2001) Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. Pediatr. Res. **49**, 460-467
- 10 Manning, J. and Vehaskari, V.M. (2001) Low birth weight-associated adult hypertension in the rat. Pediatr. Nephrol. **16**, 417-422
- 11 Dagan, A., Kwon, H.M., Dwarakanath, V. and Baum, M. (2008) Effect of renal denervation on prenatal programming of hypertension and renal tubular transporter abundance. Am. J. Physiol. Renal Physiol. **295**, F29-34
- 12 Dagan, A., Gattineni, J., Cook, V. and Baum, M. (2007) Prenatal programming of rat proximal tubule Na⁺/H⁺ exchanger by dexamethasone. Am. J. Physiol. Regul. Integr. Comp. Physiol. **292**, R1230-1235
- 13 Vehaskari, V.M. (2007) Developmental origins of adult hypertension: new insights into the role of the kidney. Pediatr. Nephrol. 22, 490-495
- 14 Woods, L.L., Ingelfinger, J.R. and Rasch, R. (2005) Modest maternal protein restriction fails to program adult hypertension in female rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289, R1131-1136
- 15 Lingrel, J.B. (1992) Na,K-ATPase: isoform structure, function, and expression. J Bioenerg. Biomembr. 24, 263-270
- 16 Ahmed, M.H., Ashton, N. and Balment, R.J. (2003) The effect of chloroquine on renal function and vasopressin secretion: a nitric oxide-dependent effect. J. Pharmacol. Exp. Ther. 304, 156-161
- 17 Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25**, 402-408
- 18 Sahajpal, V. and Ashton, N. (2005) Increased glomerular angiotensin II binding in rats exposed to a maternal low protein diet *in utero*. J. Physiol. **563**, 193-201
- 19 Armitage, J.A., Lakasing, L., Taylor, P.D., Balachandran, A.A., Jensen, R.I., Dekou, V., Ashton, N., Nyengaard, J.R. and Poston, L. (2005) Developmental programming of aortic and renal structure in offspring of rats fed fat-rich diets in pregnancy. J. Physiol. 565, 171-184
- 20 Sherman, R.C. and Langley-Evans, S.C. (2000) Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure in the rat. Clin. Sci. 98, 269-275
- 21 Nwagwu, M.O., Cook, A. and Langley-Evans, S.C. (2000) Evidence of progressive deterioration of renal function in rats exposed to a maternal low-protein diet *in utero*. Br. J. Nutr. 83, 79-85
- 22 Vehaskari, V.M., Aviles, D.H. and Manning, J. (2001) Prenatal programming of adult hypertension in the rat. Kidney Int. **59**, 238-245



- 23 Manning, J. and Vehaskari, V.M. (2005) Postnatal modulation of prenatally programmed hypertension by dietary Na and ACE inhibition. Am. J. Physiol. Regul. Integr. Comp. Physiol. **288**, R80-84
- 24 Bogdarina, I., Welham, S., King, P.J., Burns, S.P. and Clark, A.J. (2007) Epigenetic modification of the renin-angiotensin system in the fetal programming of hypertension. Circ. Res. **100**, 520-526
- 25 Langley-Evans, S.C., Gardner, D.S. and Jackson, A.A. (1996) Maternal protein restriction influences the programming of the rat hypothalamic-pituitary-adrenal axis. J. Nutr. 126, 1578-1585.
- 26 Magyar, C.E., Zhang, Y., Holstein-Rathlou, N.H. and McDonough, A.A. (2000) Proximal tubule Na transporter responses are the same during acute and chronic hypertension. Am. J. Physiol. Renal Physiol. 279, F358-369
- 27 Thomson, S.C., Bachmann, S., Bostanjoglo, M., Ecelbarger, C.A., Peterson, O.W., Schwartz, D., Bao, D. and Blantz, R.C. (1999) Temporal adjustment of the juxtaglomerular apparatus during sustained inhibition of proximal reabsorption. J. Clin. Invest. **104**, 1149-1158
- 28 Vásárhelyi, B., Dobos, M., Reusz, G.S., Szabó, A. and Tulassay, T. (2000) Normal kidney function and elevated natriuresis in young men born with low birth weight. Pediatr. Nephrol. **15**, 96-100
- 29 Zhang, Y., Mircheff, A.K., Hensley, C.B., Magyar, C.E., Warnock, D.G., Chambrey, R., Yip, K.P., Marsh, D.J., Holstein-Rathlou, N.H. and McDonough, A.A. (1996) Rapid redistribution and inhibition of renal sodium transporters during acute pressure natriuresis. Am. J. Physiol. **270**, F1004-1014
- 30 De Assis, S.M., Seguro, A.C. and Helou, C.M. (2003) Effects of maternal hypercholesterolemia on pregnancy and development of offspring. Pediatr. Nephrol. 18, 328-334
- 31 Suzuki-Yagawa, Y., Kawakami, K. and Nagano, K. (1992) Housekeeping Na,K-ATPase α_1 subunit gene promoter is composed of multiple *cis* elements to which common and cell type-specific factors bind. Mol. Cell Biol. **12**, 4046-4055
- 32 Tsuchiya, K., Giebisch, G. and Welling, P.A. (1996) Aldosterone-dependent regulation of Na-K-ATPase subunit mRNA in the rat CCD: competitive PCR analysis. Am. J. Physiol. 271, F7-15
- 33 Celsi, G., Nishi, A., Akusjarvi, G. and Aperia, A. (1991) Abundance of Na⁺-K⁺-ATPase mRNA is regulated by glucocorticoid hormones in infant rat kidneys. Am. J. Physiol. 260, F192-197
- 34 McDonough, A.A., Brown, T.A., Horowitz, B., Chiu, R., Schlotterbeck, J., Bowen, J. and Schmitt, C.A. (1988) Thyroid hormone coordinately regulates Na^+-K^+ -ATPase α and β -subunit mRNA levels in kidney. Am. J. Physiol. **254**, C323-329
- 35 Passos, M.C., da Fonte Ramos, C., Dutra, S.C., Mouco, T. and de Moura, E.G. (2002) Long-term effects of malnutrition during lactation on the thyroid function of offspring. Horm. Metab. Res. **34**, 40-43
- 36 Ortiz, P.A., Hong, N.J. and Garvin, J.L. (2001) NO decreases thick ascending limb chloride absorption by reducing Na⁺-K⁺-2Cl⁻ cotransporter activity. Am. J. Physiol. Renal Physiol. **281**, F819-825
- 37 Ortiz, P.A., Hong, N.J. and Garvin, J.L. (2004) Luminal flow induces eNOS activation and translocation in the rat thick ascending limb. Am. J. Physiol. Renal Physiol. 287, F274-280
- 38 Herrera, M., Ortiz, P.A. and Garvin, J.L. (2006) Regulation of thick ascending limb transport: role of nitric oxide. Am. J. Physiol. Renal Physiol. **290**, F1279-1284



- 39 Wangensteen, R., Rodriguez-Gomez, I., Moreno, J.M., Vargas, F. and Alvarez-Guerra, M. (2006) Chronic nitric oxide blockade modulates renal Na-K-2Cl cotransporters. J. Hypertens. 24, 2451-2458
- 40 Stewart, T., Jung, F.F., Manning, J. and Vehaskari, V.M. (2005) Kidney immune cell infiltration and oxidative stress contribute to prenatally programmed hypertension. Kidney Int. **68**, 2180-2188
- 41 Torrens, C., Brawley, L., Anthony, F.W., Dance, C.S., Dunn, R., Jackson, A.A., Poston, L. and Hanson, M.A. (2006) Folate supplementation during pregnancy improves offspring cardiovascular dysfunction induced by protein restriction. Hypertension 47, 982-987
- 42 Kaji, D.M., Chase, H.S., Jr., Eng, J.P. and Diaz, J. (1996) Prostaglandin E₂ inhibits Na-K-2Cl cotransport in medullary thick ascending limb cells. Am. J. Physiol. **271**, C354-361
- 43 Sherman, R.C., Jackson, A.A. and Langley-Evans, S.C. (1999) Long-term modification of the excretion of prostaglandin E₂ by fetal exposure to a maternal low protein diet in the rat. Ann. Nutr. Metab. **43**, 98-106
- 44 Sonnenberg, H., Honrath, U. and Wilson, D.R. (1990) Effects of increased perfusion pressure on medullary collecting duct function. Can. J. Physiol. Pharmacol. **68**, 402-407

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Table 1 Taqman probe and primer sequences (5' to 3') used for qPCR.

Gene name	TaqMan Probe	Forward 0	Reverse
β-actin	CACCATGAAGATCAAGATCATTGCTCCTCCT	GCAAGGATCCAGAAGGAGATTAC	GAGCCACCAATCCACACAGA
NHE-3	CCACTGACAATGCCAGGGTCAGTAATTTCAC	CATTTGTCCCTTTCCGAATTG	CCAAATGGCAGCTCCAAATAG
NKCC2	TCCTGGACATAACCCATGCCTTTACGAAGA	ACAGGAGGACCCATGACAAGA	GCAGCAGATACAGAGGCCACTA
NCC	TGTGATCCTCTATCTGCGGCTGCCC	TGATCCGATGCATGCTCAA	CGCCTGCGCCGTAATC
ENaC	TCTGCCGGCTACTCACGGTGGC	GCCTTGTAGTGTGATCAACTACA	CCAATCCTGGGACTTCACAGAT
(a subunit)		NON	
Na ⁺ :K ⁺ ATPase	ATCATCGTAGCCAACGTGCCGGA	GGCTGTCATCTTCCTCATTGG	CGGTGGCCAGCAAACC
$(\alpha_1 \text{ subunit})$			

NHE-3, Na⁺/H⁺ antiport; NKCC2, Na⁺:K⁺:2Cl⁻ cotransporter; NCC, Na⁺:Cl⁻ cotransporter; ENaC, epithelgel Na⁺ channel

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	Male		Female		qoi	P value	
	СМ	LPM	CF	LPF	Diet	Gender	Interaction
	(<i>n</i> = 15)	(<i>n</i> = 15)	(<i>n</i> = 13)	(<i>n</i> = 12)	ORD		
Body weight (g)	99.4 ± 1.6	103.5 ± 1.6	90.8 ± 1.8	93.3 ± 18	NS	<i>P</i> < 0.001	NS
MAP (mmHg)	88 ± 3	109 ± 3	85 ± 3	108 ± 4	P <mark>u</mark> < 0.001	NS	NS
Plasma Na ⁺ (mmol/l)	133.5 ± 2.3	139.8 ± 1.9	133.1 ± 3.9	140.5 ± 3.6	$\underline{B} = 0.04$	NS	NS
Plasma K ⁺ (mmol/l)	3.5 ± 0.2	3.4 ± 0.2	3.3 ± 0.2	3.4 ± 0.4	NS NS	NS	NS
Plasma Cl ⁻ (mmol/l)	108.2 ± 0.8	111.3 ± 0.8	107.4 ± 1.0	110.2 ± 1.9	> ₩NS	NS	NS
Plasma osmolality (mOsm/kg)	295.9 ± 4.3	292.6 ± 2.7	292.4 ± 3.0	292.1 ± 1.8		NS	NS
Haematocrit	0.38 ± 0.01	0.39 ± 0.01	0.38 ± 0.01	0.39 ± 0.01	ອັ _{NS}	NS	NS
Plasma aldosterone (nmol/l)	1.12 ± 0.12	1.09 ± 0.11	1.16 ± 0.15	1.23 ± 0.12	SINS	NS	NS

Table 2. Body weight, mean arterial pressure (MAP) and plasma composition of 4 week old rats undergoing renal clearance measurements

Data are shown as the mean \pm SEM; statistical analysis was by 2-way ANOVA

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NCCex



Table 3. Urinary excretion of potassium, chloride and total osmolytes.

	Male		Fen	Female		P value	
	СМ	LPM	CF	LPF	ⁱⁱⁱ oDiet	Gender	Interaction
	(<i>n</i> = 15)	(<i>n</i> = 15)	(<i>n</i> = 13)	(<i>n</i> = 12)	- Set		
$U_{\rm K} V \ (\mu { m mol} \cdot { m min}^{-1} \cdot 100 { m g \ bwt}^{-1})$	0.50 ± 0.06	0.51 ± 0.05	0.43 ± 0.06	0.45 ± 0.03	NS	NS	NS
FE _K (%)	19.7 ± 2.2	17.8 ± 1.3	17.7 ± 1.8	16.8 ± 1.8	Щ NS	NS	NS
$U_{Cl}V \ (\mu mol \cdot min^{-1} \cdot 100g \ bwt^{-1})$	2.5 ± 0.3	4.2 ± 0.5	2.6 ± 0.3	5.5 ± 0.8	Pu 0.001	NS	NS
FE _{C1} (%)	3.3 ± 0.3	4.1 ± 0.4	3.1 ± 0.4	5.8 ± 0.6	<i>P</i> <u></u>	NS	NS
$U_{Osm}V (mOsm \cdot min^{-1} \cdot 100g bwt^{-1})$	9.0 ± 1.0	12.1 ± 1.1	8.5 ± 1.0	13.9 ± 1.6	$P_{\underline{u}}^{o} = 0.001$	NS	NS
$C_{H2O} \left(\mu l \cdot min^{-1} \cdot 100g \text{ bwt}^{-1}\right)$	-11.1 ± 1.0	-15.2 ± 1.0	-10.6 ± 1.3	-18.3 ± 1.7	> ₽ <u>u</u> < 0.001	NS	NS

U_XV urinary excretion rate of X; FE_x fractional excretion of X; C_{H20} free water clearance. Data are show by as the mean ± SEM; statistical analysis was by 2-way ANOVA



Table 4. Expression of renal sodium transporter mRNA in 4-week-old rats.

	Male		Female		10.	P value		
	СМ	LPM	CF	LPF	Diet	Gender	Interaction	
	(<i>n</i> = 16)	- Set						
NHE-3	1.01 ± 0.06	0.94 ± 0.06	1.09 ± 0.10	1.13 ± 0.13	R NS	NS	NS	
NKCC2	1.08 ± 0.16	7.48 ± 0.87	1.04 ± 0.11	6.93 ± 1.18	о щ0.001	NS	NS	
NCC	1.05 ± 0.11	1.26 ± 0.23	1.09 ± 0.12	1.50 ± 0.30	Ц NS	NS	NS	
ENaC (α subunit)	1.04 ± 0.12	0.90 ± 0.10	0.94 ± 0.15	0.96 ± 0.07	Z NS	NS	NS	
$Na^+:K^+ATPase (\alpha_1 subunit)$	1.02 ± 0.07	1.01 ± 0.06	0.95 ± 0.08	1.11 ± 0.10	NS (ERS	NS	NS	
NHE-3, Na ⁺ /H ⁺ antiport; NKCC2, Na ⁺ :K ⁺ :2Cl ⁻ cotransporter; NCC, Na ⁺ :Cl ⁻ cotransporter; ENaC, epitheled Na ⁺ channel. Data are shown as the mean ± SEM; statistical analysis was by 2-way ANOVA								

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Figure Legends

Figure 1. (A) Glomerular filtration rate (GFR), (B) urine flow rate (UV), (C) urinary sodium excretion ($U_{Na}V$) and (D) fractional excretion of sodium (FE_{Na}) in 4 week old rats exposed to either a control (CM, n = 15; CF, n = 13, open bars) or low (LPM, n = 15; LPF n = 12 solid bars) maternal protein diet during pregnancy. Data are shown as the mean \pm SEM. Two-way ANOVA showed that gender has no effect, but maternal diet had a significant influence on UV, $U_{Na}V$ and FE_{Na} . ** P < 0.01, *** P < 0.001.

Figure 2. Correlation between mean arterial pressure and urinary sodium excretion $(U_{Na}V)$ in (A) male and (B) female control and LP rats.

Figure 3. Western blot analysis of (A) Renal Na⁺/H⁺ antiport (NHE-3), (B) Na⁺:K⁺:2Cl⁻ cotransporter (NKCC2), (C) epithelial Na⁺ channel (ENaC) and (D) Na⁺:K⁺ATPase expression in 4 week old rats exposed to either a control (CM, n = 16; CF, n = 16) or low (LPM, n = 16; LPF n = 16) maternal protein diet during pregnancy. Representative blots are shown in the left panel; data normalised to adult rat kidney (Adult) are shown as scatter plots in the right panel; the horizontal bar represents the mean (control male \Box , LP male \blacksquare , control female \circ , LP female \bullet). Two-way ANOVA showed that gender had no effect, but maternal diet had a significant influence on NKCC2 expression. *** P < 0.001

Figure 4. Na⁺:K⁺ATPase (α_1 subunit) immuno-staining in kidneys from 4 week old male rats exposed to either a control (CM, n = 8) or low (LPM, n = 8) maternal protein diet during pregnancy. Low power images show that Na⁺:K⁺ATPase was present in all regions of the CM kidney (A), but was virtually absent from the inner medulla of the LPM kidney (B). Higher magnification reveals Na⁺:K⁺ATPase-immunoreactivity on the inner medullary collecting ducts of CM rats (C, E), but little or no staining on LPM tubules (D, F). Positive staining is indicated by arrows in E and F. C – cortex, OM – outer medulla, IM – inner medulla. Scale bars A-B = 600µm; C-D = 300 µm; E-F = 100µm.



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Figure 3



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Figure 4



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