The effect of a low protein diet in pregnancy on offspring renal calcium handling

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Running title: Fetal programming of calcium excretion

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Abstract

Low birth weight humans and rats exposed to a low protein diet in utero have reduced bone mineral content. Renal calcium loss during the period of rapid skeletal growth is associated with bone loss. As young low protein rats display altered renal function, we tested the hypothesis that renal calcium excretion is perturbed in this model. Pregnant Wistar rats were fed isocalorific diets containing either 18% (Control) or 9% (low) protein throughout gestation. Using standard renal clearance techniques, Western blotting for renal calcium transport proteins and assays for Na⁺:K⁺ATPase activity and serum calcitropic hormones, we characterised calcium handling in 4 week old male offspring. Histomorphometric analysis of femurs revealed a reduction in trabecular bone mass in low protein rats. Renal calcium (Control 10.4±2.1 vs low protein 27.6±4.5 nmol min⁻¹ 100g bwt⁻¹ \textit{P}<0.01) and sodium excretion were increased, but glomerular filtration rate was reduced in low protein animals. Total plasma calcium was reduced in low protein rats (\textit{P}<0.01), but ionised calcium, serum calcitropic hormone concentrations and total body calcium did not differ. There was no significant change in plasma membrane Ca²⁺-ATPase pump, epithelial calcium channel or calbindin-D₂₈K expression in low protein rat kidneys. However, Na⁺:K⁺ATPase activity was 36% lower (\textit{P}<0.05) in low protein rats. These data suggest that the hypercalciuria of low protein rats arises through a reduction in passive calcium reabsorption in the proximal tubule rather than active distal tubule uptake. This may contribute to the reduction in bone mass observed in this model.

\textbf{Keywords} Calcium, bone, kidney, programming
Introduction

The concept that an adverse intrauterine environment may have a long term effect on adult health and disease arose through observations made by Barker and colleagues that low birth weight infants were more likely to develop cardiovascular disease as adults (3, 4). There is now a large body of evidence which supports the view that poor maternal nutrition during pregnancy programmes the fetus to develop a range of chronic diseases in adulthood. Both epidemiological studies and animal models have shown that offspring exposed to a poor maternal diet in utero are at increased risk of developing cardiovascular disease, non-insulin dependent diabetes and metabolic syndrome (22). It has also become apparent that development of the skeleton and the risk of osteoporosis in later life are influenced by the maternal environment (13).

A number of epidemiological studies have reported associations between poor fetal growth and decreased bone mass in adulthood. Bone mineral content (BMC) at several sites, including the lumbar spine, femoral neck, forearm and hip, has been shown to be positively correlated with birth weight in young women in their early 20s (11) and several cohorts of older men and women aged 60-75 yr (12, 20, 45). These observations have led to the suggestion that an adverse intrauterine environment programmes bone growth and hence represents a risk factor for osteoporosis (13).

Animal studies support this hypothesis. Prenatal dexamethasone exposure, which is known to induce hypertension in rat offspring (5), has been reported to have a gender-specific effect on skeletal growth (41). In male offspring, dexamethasone induced a transient increase in tibia and femur length, whereas in females there was a reduction in cortical bone thickness in
the femur and an increase in periosteal and endosteal thickness. Intrauterine growth retardation, induced by uterine vessel ligation, has also been shown to influence skeletal development. Axial skeletal development was affected most, with a permanent reduction in long bone width (35). Exposure to a low protein diet during pregnancy, a manoeuvre most commonly associated with the development of hypertension in the offspring (27), has been reported to reduce bone area and BMC in late adulthood (34).

Bone mass in later life is dependent upon the peak bone mass achieved during skeletal growth and the subsequent rate of bone loss (23). Several longitudinal studies have shown that bone mass tracks throughout life: individuals in the upper quartile of bone mass in childhood and adolescence are likely to remain in the upper quartile in old age (16, 32). Hence changes in bone growth resulting in a reduction in peak bone mass increase the subsequent likelihood of developing osteoporosis. Peak bone mass is influenced both by genetic and environmental factors. During fetal development, calcium accretion and the proliferation and differentiation of chondrocytes are regulated by a number of factors, including parathyroid hormone related peptide (PTHrP) (25) and 1,25 (OH)2 vitamin D3 (42).

Postnatally, bone mineral accrual is enhanced by dietary calcium supplementation (29). Conversely, increased renal calcium loss can lead to a reduction in bone mineral density. Hypercalciuria is a common feature in postmenopausal bone loss (21) and is also associated with a reduction in bone mineral density in children (17). We have shown that, in the rat, exposure to a low protein diet in utero alters renal function in the offspring (37, 38). However, the effect of maternal protein restriction on renal calcium excretion has not been reported. As bone area and BMC have been shown to be reduced in old low protein (LP) rats, (34), we hypothesised that calcium homeostasis may be perturbed in this model.
Renal calcium reabsorption occurs in both the proximal tubule, via a passive paracellular pathway, and in the distal nephron, via a hormone-regulated transcellular route (18). The majority of calcium is reabsorbed in the proximal tubule and the thick ascending limb (TAL), with smaller quantities being reabsorbed in the distal tubule. Passive reabsorption is linked to sodium and water transport. Active calcium reabsorption is stimulated by parathyroid hormone (PTH) and vitamin D (18). The principal transport proteins in the distal nephron are the apical epithelial calcium channel (ECaC1, TRPV5) (24), the intracellular binding protein calbindin-D$_{28K}$ (8) and the basolateral plasma membrane Ca$^{2+}$-ATPase pump (PMCA) (28).

In this study we measured renal calcium excretion in young LP rats during the period of rapid growth. We used Western blots for semi-quantitative assessment of calcium transport proteins involved in active calcium reabsorption, and measured the activity of the Na$^{+}$:K$^{+}$ATPase pump which provides the driving force for passive calcium reabsorption. In order to determine whether hormonal control of renal calcium excretion is affected by a maternal low protein diet, we quantified serum calcitropic hormone concentrations in the offspring. Finally, we sought to confirm that exposure to a low protein diet in utero affects bone architecture in young animals by performing histomorphometric analysis of the femur.
Methods

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, UK) 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, UK) to one which contained either 9% protein (low protein, LP) or 18% protein (control). The semi-synthetic diets were formulated as described previously (38). On the day of birth, the dam’s diet reverted to standard chow; pups were weaned onto this standard diet. Male animals were studied at 4 weeks of age.

Bone morphology

The right femur was removed from thirty 4 week old male animals (control n = 15 from n = 7 litters and LP n = 15 from n = 7 litters). The femora were fixed immediately in 4% neutral buffered formalin for 24 hours. The bones were then decalcified in formic acid under radiographic control. Once decalcification was complete the bones were carefully bisected in the coronal plane. The halves were dehydrated, embedded in paraffin wax and sectioned at 4µm. Two ‘step serial sections’ (tissue sections taken at intervals through the tissue) were taken from the surface of each of the two bone halves and at 10µm below the surface.
Analysis further from the coronal plane is not possible because the curvature of the bone, which grossly distorts relative bone volumes, cannot be reproducibly eliminated. The 4 sections from each animal were stained with H&E and then examined using an Olympus BX60 microscope attached to a Leica QWin image analysis system. All tissue processing techniques lead to changes in the overall size of the bone but, with careful sectioning and mounting of the tissue sections, not in the relative proportions of the soft and hard tissues in sample cohorts. Thus the following parameters (expressed as ratios of total tissue volume) were measured: cortical bone volume, trabecular bone volume and area of primary spongiosa (spongiosa). Cortical and trabecular bone were measured in the distal half of the shaft and excluded the spongiosa. The spongiosa was defined as the actively remodelling bone extending proximally from the cartilaginous growth plate.

**Renal clearance**

Anaesthetised (Intraval, 100 mg/kg body weight, thiopentone sodium BP, Link Pharmaceuticals Ltd, Horsham, West Sussex, UK) control \( (n = 6 \text{ from } n = 5 \text{ litters}) \) and LP \( (n = 7 \text{ from } n = 5 \text{ litters}) \) rats were prepared for euvoalaemic fluid replacement of spontaneous urine output using a servo-controlled fluid replacement system, as described previously (1). Following surgery, a bolus dose of \( ^3 \text{H} \) inulin (1 \( \mu \text{Ci} \)) was injected via a jugular vein cannula and servo-infusion replacement of 2.5% dextrose was initiated at a rate matching spontaneous urine output. \( ^3 \text{H} \) inulin (0.3 \( \mu \text{Ci} / \text{h} \)) in 2.5% dextrose was infused continuously via a second, slow infusion pump (1ml/h). 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 saline and clearance markers was replaced via the jugular catheter. Arterial blood pressure was measured throughout the experiment via a carotid artery catheter (PowerLab
800/s; ADInstruments, Hastings, East Sussex, UK). Urine and plasma sodium and potassium concentrations were measured by flame photometry (model 480, Ciba Corning Diagnostics Ltd, Essex, UK), total calcium by atomic absorption spectrophotometry (model 3100, Perkin Elmer, Beaconsfield, Bucks, UK) and ionised calcium by autoanalyser (Rapidlab 865 blood gas analyser, Bayer, Newbury, Berkshire, UK). \(^3\)H inulin was determined using a 1900CA Tri-Carb Liquid Scintillation Analyser (Canberra Industries, Meriden, CT) \(\beta\)-counter. Plasma protein concentration was determined using the Bradford method (Bio-Rad Assay Reagent, Bio-Rad Laboratories, Hercules, CA, USA).

**Total body calcium**

Total body calcium was measured in a separate group of rats killed at 4 weeks of age (control \(n = 6\) from \(n = 6\) litters; LP \(n = 6\) from \(n = 6\) litters). Animals were first dried to a constant body weight at 70°C and were then ashed in porcelain crucibles at 700°C for 24 hours (Carbolite Furnaces, Sheffield, South Yorkshire, UK). The ash was dissolved in nitric acid and calcium content measured by atomic absorption spectrophotometry (model 3100, Perkin Elmer, Beaconsfield, Bucks, UK).

**Western analysis of Ca\(^{2+}\) transporters and NHE-3**

Kidneys were harvested from a separate group of control (\(n = 5\) from \(n = 5\) litters) and LP rats (\(n = 5\) from \(n = 5\) litters). Tissue was homogenised and renal Ca\(^{2+}\) transporter expression was determined according to the method of Bond *et al.* (7). The antisera used were mouse monoclonal anti-calbindin-D\(_{28K}\) (1:2500 dilution; Sigma, UK), mouse monoclonal anti-PMCA 5F10 (1:1500 dilution; Cambridge Bioscience, Cambridge, UK), affinity purified
rabbit polyclonal anti-ECaC1 (1:1000 dilution; Alpha Diagnostic International, San Antonio, USA) and polyclonal rabbit anti-NHE-3 (Na\textsuperscript{+}:H\textsuperscript{+} exchanger-3, 1:300 dilution, Alpha Diagnostic International). Negative controls were performed by omitting primary antibody. Immunoreactive species were detected with sheep anti-mouse antibody (1:1000, Amersham) or goat anti-rabbit antibody (1:2000, Dako, UK) using enhanced chemiluminescence detection (ECL, Amersham). A pooled adult kidney protein sample was run on each blot to allow normalisation of density data between different blots.

**Renal Na\textsuperscript{+}:K\textsuperscript{+}ATPase activity**

Tissue Na\textsuperscript{+}:K\textsuperscript{+}ATPase enzyme activity was measured in freshly harvested kidneys as described previously (2). Briefly, rats (control $n = 6$ from $n = 6$ litters; LP $n = 5$ from $n = 5$ litters) were anaesthetised with isoflurane and killed by decapitation. The kidneys were rapidly excised, placed into ice-cold homogenisation buffer, sliced and homogenised mechanically. Protein concentration was determined using the Bradford method (Bio-Rad Assay Reagent, Bio-Rad Laboratories, Hercules, CA, USA).

The homogenate was mixed 1:1 with SDS (0.75 mg/ml) and then mixed with incubation solution (150 mM histidine, 640 mM NaCl, 40 mM MgCl\textsubscript{2}, 200 mM KCl). Non-specific and Na\textsuperscript{+}:K\textsuperscript{+}ATPase-specific phosphate production were determined in the presence or absence of 10 mM ouabain, respectively. Following 10 minutes pre-incubation at 37 °C, 30 mM ATP was added; 5 minutes later the reaction was quenched with perchloric acid. Samples were centrifuged at 1200 g for 15 minutes at 2 °C, after which equal volumes of supernatant and distilled water were mixed with colour reagent (1 g (NH\textsubscript{4})\textsubscript{6}Mo\textsubscript{7}O\textsubscript{24}.4 H\textsubscript{2}O, 94.7 mL H\textsubscript{2}O, 3.3 mL concentrated sulphuric acid and 4 g FeSO\textsubscript{4}.7H\textsubscript{2}O). 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 ouabain.

Serum calcitropic hormone assays

Serum was collected from a separate group of control ($n = 7$ from $n = 5$ litters) and LP ($n = 7$ from $n = 5$ litters) rats. Serum parathyroid hormone (PTH) concentration was determined by ELISA using a commercial assay kit (Rat intact PTH ELISA kit, Immunodiagnostics Systems Ltd, Boldon, Tyne & Wear, UK). The intra-assay co-efficient of variation was 8.0%. Serum 25-hydroxyvitamin D₃ (25(OH) D₃) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂ D₃) were measured by in-house assays. Serum samples were extracted with acetonitrile and separated using C₁₈ Sep-Pak cartridges followed by straight phase HPLC (Waters Associates, Milford, MA). (33) 25(OH) D₃ was measured separately by application to a second straight phase HPLC column measuring absorbance at 265 nm; the results were corrected for recovery (33). 1,25(OH)₂ D₃ was measured by in-house radioimmunoassay (6). The intra- and inter-assay coefficients of variation for the 25(OH) D₃ assay were 3.0% and 4.2% respectively; those for the 1,25(OH)₂ D₃ assay were 7.8% and 10.5% respectively.

Statistical Analysis

All data are presented as the mean ± SEM. Statistical analysis was by two-tailed independent samples t-test; analysis of the urinary calcium:sodium excretion rate ratios, calcium clearance rates and fractional excretion of calcium were performed following log₁₀ transformation as
these data were not normally distributed. A correlation correction was combined with a Sidak-Holm stepdown procedure to control for the family-wise error rate (39). A two-tailed partial correlation coefficient was calculated for urinary calcium and sodium excretion, controlling for mean arterial blood pressure. Significance was assumed at $P \leq 0.05$ (SPSS for Windows, version 11.5.0, SPSS UK Ltd, Surrey, UK).

Results

**Bone morphology**

Figure 1a shows a typical coronal section through the lower femur of a control and an LP rat. Total bone volume was measured in 3 bone compartments (Shaft cortex [cortex], Shaft trabecular bone [trabecular bone], primary spongiosa [spongiosa]) and comparisons made between the 2 groups of animals. No significant difference was found in cortical bone volume between the groups. The volume of primary spongiosa was greater in LP than control rats ($P < 0.05$, Fig. 1b). By comparison trabecular bone mass in the lower femur was reduced in the LP rats ($P < 0.05$, Fig. 1c).

**Renal clearance**

LP rats were smaller ($P < 0.01$) than age matched control rats and had higher mean arterial blood pressure ($P < 0.01$, Table 1), consistent with earlier reports (27, 38). The plasma concentration of total calcium ($P < 0.01$) was significantly lower in LP rats, but plasma ionised calcium, sodium, potassium and protein did not differ from that of control rats. Total body calcium did not differ between control and LP rats (Table 1).
Glomerular filtration rate was significantly lower ($P < 0.01$) in LP rats, but urine flow rate was comparable with that of control rats (Table 2). In contrast, urinary calcium and sodium excretion rates were increased significantly in LP rats ($U_{Ca}V P < 0.01$, $U_{Na}V P < 0.05$, Table 2). The urinary calcium: sodium excretion rate ratio tended to be greater in LP rats, but this did not reach statistical significance ($C 0.016 \pm 0.003$ vs. LP $0.053 \pm 0.018$, $P > 0.05$). Partial correlation analysis, controlling for mean arterial blood pressure, revealed significant positive correlations between calcium and sodium excretion in both control ($r = 0.808$, $P < 0.001$) and LP rats ($r = 0.5$, $P < 0.05$). The clearance rate and fractional excretion of calcium were significantly higher in LP rats (Table 3, $C_{Ca} P < 0.01$, $F_{ECa} P < 0.001$). Similarly for sodium, clearance and fractional excretion rates were higher in LP rats (Table 3, $C_{Na} P < 0.05$, $F_{ENa} P < 0.01$). In contrast, the urinary excretion rate of potassium was significantly lower in LP rats (control, $n = 6$, $1.4 \pm 0.2$ vs LP, $n = 7$, $0.8 \pm 0.1 \mu$mol min$^{-1}$ 100g body weight$^{-1}$, $P < 0.01$), as was the potassium clearance rate (Table 3, $P < 0.01$). The fractional excretion of potassium did not differ between the two groups (Table 3).

**Renal $Ca^{2+}$ transporter expression**

The polyclonal anti-ECaC1 antibody detected a single protein band at 84 kDa in kidney membrane fractions. There was no difference in the relative density of this band between control and LP rats ($P > 0.05$, Fig. 2a). The monoclonal anti-calbindin-D$_{28K}$ antibody detected a single protein band at 28 kDa in the renal post-nuclear supernatant. There was no difference in the relative density of this band between control and LP rats ($P > 0.05$, Fig. 2b). The monoclonal anti-PMCA antibody detected a broad protein band around 140 kDa (~133 to 147 kDa) as well as a smaller band at 92 kDa, consistent with previous observations (7):
these bands were absent from negative control blots (data not shown). The relative density of the major immunoreactive species at ~140 kDa, representing PMCA (7), tended to be greater in LP compared with control kidney membrane fractions, but this did not reach statistical significance (Fig. 2c).

Renal NHE-3 expression and Na⁺:K⁺ATPase activity

The polyclonal anti-NHE-3 antibody detected a single band at 83 kDa. There was no difference in the relative expression of NHE-3 between control and LP rats (relative density, control \( n = 5 \) from \( n = 5 \) litters 100 ± 8 vs LP \( n = 5 \) from \( n = 5 \) litters 105 ± 16%, \( P > 0.05 \)). Renal Na⁺:K⁺ATPase enzyme activity was significantly lower in LP rats compared with controls (control \( n = 6 \) from \( n = 6 \) litters 8.0 ± 0.9 vs LP \( n = 5 \) from \( n = 5 \) litters 5.1 ± 0.7 nM PO₄.µg protein⁻¹.h⁻¹, \( P < 0.05 \)).

Serum calcitropic hormone concentrations

There were no significant differences between control and LP rats in the serum concentrations of PTH, 25(OH) D₃ and 1,25(OH)₂ D₃ (Table 4).

Discussion

This study has confirmed that calcium homeostasis is perturbed in young rats following exposure to a maternal low protein diet. Although total body calcium content did not differ between control and LP rats, there is evidence that long bone structure was altered in the LP group. This was associated with a marked increase in renal calcium loss at a stage of
development critical to the achievement of peak bone mass. The mechanisms underlying the observed changes in bone structure are not clear. However, it is likely that the high blood pressure of the LP animals contributed to the observed hypercalciuria and natriuresis through the pressure natriuresis process.

Urinary calcium excretion is closely linked to sodium excretion (18) so pressure-related increases in urinary sodium excretion are associated with hypercalciuria (10). In the proximal tubule, calcium is reabsorbed via a paracellular route, as a passive process coupled to sodium and water transport. Hence sodium and calcium are reabsorbed in parallel in this part of the nephron. Calcium is also reabsorbed by a passive process in the thick ascending limb (TAL), however, the driving forces differ from those of the proximal tubule. In the TAL, sodium uptake via the Na⁺:K⁺:2Cl⁻ co-transporter (NKCC2) creates an electropositive lumen, which drives paracellular calcium transport. Active, transcellular calcium transport is seen in the distal portion of the nephron. Here, calcium crosses the apical membrane via the epithelial calcium channel (ECaC). It binds to the vitamin D-dependent intracellular binding protein calbindin-D_{28K} (8) and is extruded by a combination of the plasma membrane Ca²⁺ATPase (PMCA) and a Na⁺:Ca²⁺ exchanger, the former being the dominant transport system (15). PTH stimulates this process by hyperpolarising the membrane voltage, activating ECaC and enhancing Na⁺:Ca²⁺ exchanger activity. In contrast to the proximal tubule, calcium and sodium reabsorption are inversely related in the distal nephron (18).

It is unlikely that the hypercalciuria observed in the LP rats in the current study was caused by a reduction in distal tubular calcium uptake. While acknowledging that immunoblots are semiquantitative at best, it appears that expression of the distal tubular Ca²⁺ transport proteins ECaC1, calbindin-D_{28K} and PMCA did not differ between control and LP rats. Furthermore,
calcium and sodium reabsorption are inversely related in the distal nephron (18), yet LP rats exhibited both hypercalciuria and a natriuresis by comparison with control animals, suggesting that the reduction in calcium reabsorption is linked to sodium excretion. Passive calcium reabsorption in the TAL parallels that of sodium (19), so the TAL could be the potential site of altered tubular function. This seems unlikely, though, as maternal protein restriction has been reported to increase NKCC2 expression (31), which would favour increased sodium reabsorption. Therefore the most likely site is the proximal tubule and the most likely cause of hypercalciuria in the LP rat is the 36% reduction in Na⁺:K⁺ATPase activity.

Active sodium transport in the proximal tubule is driven by the basolateral Na⁺:K⁺ATPase pump which creates an electrochemical gradient favouring sodium entry across the apical membrane via the Na⁺:H⁺ exchanger, NHE-3. As water follows the sodium, the luminal calcium concentration rises, providing the driving force for paracellular calcium transport (18). Hence a reduction in sodium transport will result in a parallel reduction in calcium transport. We observed no difference in NHE-3 expression in LP rats but we did see a significant reduction in Na⁺:K⁺ATPase activity, which leads us to conclude that the hypercalciuria of LP rats arises through a reduction in passive calcium reabsorption. We cannot be certain that this is entirely due to changes in proximal tubule function, as the Na⁺:K⁺ATPase pump is expressed ubiquitously by the nephron and we measured pump activity in whole kidney homogenates, but it seems to be the most likely explanation based on the available data.

A reduction in Na⁺:K⁺ATPase pump activity in not restricted to the maternal low protein model of fetal programming. Similar reductions have been reported in the kidneys of rats
exposed to a high fat diet in utero (2) and in the erythrocytes of young men with low birth weight (43). The mechanisms underlying the reduction in Na\textsuperscript{+}:K\textsuperscript{+}ATPase activity in the LP rat kidney are unknown, but may be related to their elevated blood pressure. Acute hypertension results in a reduction in proximal tubule Na\textsuperscript{+}:K\textsuperscript{+}ATPase activity (47), leading to a pressure-related natriuresis and diuresis. Furthermore, chronic exposure to hypertension has also been shown to reduce Na\textsuperscript{+}:K\textsuperscript{+}ATPase activity in spontaneously hypertensive rats (SHR) (30). Interestingly, these authors also reported that brush border NHE-3 activity was reduced by internalisation, without a change in protein expression, further reducing sodium reabsorption by the proximal tubule, (30). Our observation that NHE-3 expression in whole kidney homogenates did not differ between control and LP rats is consistent with this concept of transporter redistribution, however further studies are required to quantify NHE-3 activity in the LP rat kidney.

Redistribution of NHE-3 and reduction in Na\textsuperscript{+}:K\textsuperscript{+}ATPase activity in the adult SHR are thought to contribute to a resetting of the pressure natriuresis set point (30). Little is known about the pressure natriuresis relationship in LP rats. However, it is clear that the hypercalciuria and natriuresis observed in the current study could not be sustained unless intake of electrolytes matched the urinary losses. We did not measure electrolyte balance (dietary intake minus urinary output) in the current study, but it is interesting to note in this context that 4 week old LP rats eat more food than controls (S.H. Al-Wasil and N. Ashton, unpublished observations).

The hypercalciuria of LP rats was associated with a reduction in plasma total calcium, although the ionised calcium concentration did not differ from that of control animals. The majority of calcium is transported in plasma bound to protein, so a reduction in binding
capacity could account for the reduction in total plasma calcium of the LP rat. However, plasma protein concentrations did not differ between control and LP rats, suggesting that this was not the case. The endocrine profile of the LP group does not offer an explanation for the observed differences either, as the serum concentrations of PTH, 25(OH) D₃ and 1,25(OH)₂ D₃ in LP rats were no different from those of the control group. Hypocalcaemia coupled with hypercalciuria is a feature of a number of genetic models of hypertension but this is usually associated with an increase in serum PTH concentration (40). It has been suggested that, in the rat, PTH secretion by the parathyroid glands is mediated by 1,25(OH)₂ D₃ acting on the calcium-sensing receptor (CaR) rather than the plasma calcium concentration (9). However, serum 1,25(OH)₂ D₃ did not differ between LP and control rats either. Further investigation of the expression and activity of the CaR in LP rats is required before these anomalies can be resolved.

Clearly, calcium homeostasis is perturbed in the LP rat. Despite the maintenance of total body calcium, bone formation was adversely affected in the young LP rat. The structure and volume of bone being formed at the growth plate was altered, leading to a marked reduction in trabecular bone, one of the key structural elements of bone. The effects of maternal protein restriction on bone structure appear to persist throughout the offspring’s life, as bone mineral content has been reported to be lower in aged LP rats compared with controls (34). The consequences for the risk of developing osteoporosis or other diseases of bone associated with aging require further investigation.

It is important to note that many of the variables described in this study were corrected for bodyweight, as the LP animals were 20% lighter than the age-matched control group. Such an adjustment assumes that the animals differ only in size and that body composition does not
differ, which may not be the case. It is difficult to take any potential difference into account without performing an exhaustive analysis of body composition, which is beyond the scope of the current investigation. Hence some caution must be applied when interpreting the ‘corrected’ variables.

In conclusion, this study has shown that renal calcium excretion is perturbed in rats exposed to protein restriction in utero. LP rats also had elevated blood pressure which, given the known effects of raised arterial pressure on renal sodium excretion, is likely to have had a major influence on the combined hypercalciuria and natriuresis seen in these animals. Assessment of transporter expression and activity linked to calcium reabsorption in the proximal and distal tubules suggests that a reduction in Na\(^+\):K\(^+\)ATPase activity underlies the loss of calcium. This, in turn, may be a consequence of the high blood pressure exhibited by the LP rat, as hypertension is known to inhibit proximal tubule Na\(^+\):K\(^+\)ATPase activity (30). However, this defect in renal transport may not be the only mechanism contributing to altered calcium homeostasis, as there appears to be a mismatch between plasma total calcium concentration and serum calcitropic hormones. Bone structure, particularly at the growth plate, is also altered in young LP rats lending further support to the suggestion that skeletal development is programmed in utero.
References


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Figure Legends

Figure 1 (A) Typical coronal section of the lower femur of a 4 week old male rat exposed to either a normal (control) or low (LP) maternal protein diet during pregnancy. (B) Area of spongiosa, as a proportion of total tissue volume, and (C) trabecular bone volume, as a proportion of the total tissue in the medulla, in control (n = 15 from n = 7 litters, open bars) and LP rats (n = 15 from n = 7 litters, solid bars). Data are shown as the mean ± SEM. Statistical comparisons were by independent samples t-test, adjusted by correlation correction and Sidak-Holm stepdown procedure. * P < 0.05 control vs LP.

Figure 2 (A) Renal epithelial calcium channel (ECaC1), (B) calbindin-D_{28K} and (C) plasma membrane Ca^{2+}ATPase (PMCA) expression in 4 week old male rats exposed to either a normal (control, n = 5 from n = 5 litters, open bars) or low (LP, n = 5 from n = 5 litters, solid bars) maternal protein diet during pregnancy. Data were normalised to a positive control (PC) and are shown as the mean ± SEM. Statistical comparisons were by independent samples t-test, adjusted by correlation correction and Sidak-Holm stepdown procedure. * P < 0.05 control vs LP.
Table 1. Body weight, mean arterial blood pressure, plasma electrolyte concentrations and total body calcium in anaesthetised, 4 week old male rats exposed to either a normal (control) or low (LP) maternal protein diet during pregnancy.

<table>
<thead>
<tr>
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<th>control ($n = 6$)</th>
<th>LP ($n = 7$)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>114.5 ± 3.6</td>
<td>90.7 ± 5.2**</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>82 ± 6</td>
<td>111 ± 5**</td>
</tr>
<tr>
<td>Plasma total Ca$_{2+}$ (mmol/L)</td>
<td>2.87 ± 0.21</td>
<td>2.09 ± 0.10**</td>
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<tr>
<td>Plasma ionised Ca$_{2+}$ (mmol/L)</td>
<td>1.19 ± 0.03</td>
<td>1.23 ± 0.03</td>
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<tr>
<td>Plasma Na$^+$ (mmol/L)</td>
<td>142.3 ± 1.1</td>
<td>138.0 ± 1.4</td>
</tr>
<tr>
<td>Plasma K$^+$ (mmol/L)</td>
<td>4.16 ± 0.20</td>
<td>4.54 ± 0.40</td>
</tr>
<tr>
<td>Plasma protein (mg/mL)</td>
<td>5.6 ± 0.4</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>Total body calcium (mmol/g ash)</td>
<td>14.1 ± 1.2</td>
<td>10.7 ± 1.4</td>
</tr>
<tr>
<td>Total body calcium (mmol/g wet weight)</td>
<td>0.36 ± 0.03</td>
<td>0.30 ± 0.05</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM. Statistical comparisons were by independent samples $t$-test, adjusted by correlation correction and Sidak-Holm stepdown procedure. ** $P < 0.01$ control vs LP.
Table 2 Glomerular filtration rate (GFR), urine flow rate (V), urinary calcium excretion (UCaV) and urinary sodium excretion (UNaV) in anaesthetised, 4 week old male rats exposed to either a normal (control) or low (LP) maternal protein diet during pregnancy.

<table>
<thead>
<tr>
<th></th>
<th>control (n = 6)</th>
<th>LP (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR (µl min⁻¹ 100g bwt⁻¹)</td>
<td>1.3 ± 0.9</td>
<td>0.9 ± 0.1**</td>
</tr>
<tr>
<td>V (µl min⁻¹ 100g bwt⁻¹)</td>
<td>38.1 ± 6.5</td>
<td>53.8 ± 6.0</td>
</tr>
<tr>
<td>UCaV (nmol min⁻¹ 100g bwt⁻¹)</td>
<td>10.4 ± 2.1</td>
<td>27.6 ± 4.5**</td>
</tr>
<tr>
<td>UNaV (µmol min⁻¹ 100g bwt⁻¹)</td>
<td>0.76 ± 0.15</td>
<td>1.55 ± 0.28*</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM over 1 hour of 2.5% dextrose infusion at a rate matching spontaneous urine output. Statistical comparisons were by independent samples t-test, adjusted by correlation correction and Sidak-Holm stepdown procedure. * P < 0.05, ** P < 0.01 control vs LP.
Table 3 Renal clearance and fractional excretion of calcium, sodium and potassium in anaesthetised, 4 week old male rats exposed to either a normal (control) or low (LP) maternal protein diet during pregnancy.

<table>
<thead>
<tr>
<th></th>
<th>control (n = 6)</th>
<th>LP (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(_{\text{Ca}}) (µl min(^{-1}) 100g bwt(^{-1}))</td>
<td>7.7 ± 1.5</td>
<td>20.0 ± 3.0**</td>
</tr>
<tr>
<td>FE(_{\text{Ca}}) (%)</td>
<td>0.6 ± 0.1</td>
<td>2.1 ± 0.4***</td>
</tr>
<tr>
<td>C(_{\text{Na}}) (µl min(^{-1}) 100g bwt(^{-1}))</td>
<td>5.3 ± 1.0</td>
<td>11.1 ± 2.0*</td>
</tr>
<tr>
<td>FE(_{\text{Na}}) (%)</td>
<td>0.41 ± 0.07</td>
<td>1.32 ± 0.23**</td>
</tr>
<tr>
<td>C(_{\text{K}}) (µl min(^{-1}) 100g bwt(^{-1}))</td>
<td>384.6 ± 45.7</td>
<td>237.2 ± 23.9**</td>
</tr>
<tr>
<td>FE(_{\text{K}}) (%)</td>
<td>30.1 ± 3.0</td>
<td>26.7 ± 2.9</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM over 1 hour of 2.5% dextrose infusion at a rate matching spontaneous urine output. Statistical comparisons were by independent samples \(t\)-test, adjusted by correlation correction and Sidak-Holm stepdown procedure. * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\) control vs LP.
Table 4  Serum parathyroid hormone (PTH), 25-hydroxyvitamin D$_3$ (25(OH) D$_3$) and 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$ D$_3$) concentrations in 4 week old male rats exposed to either a normal (control) or low (LP) maternal protein diet during pregnancy.

<table>
<thead>
<tr>
<th></th>
<th>control ($n = 7$)</th>
<th>LP ($n = 7$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH (pg/ml)</td>
<td>125.8 ± 23.8</td>
<td>121.9 ± 24.8</td>
</tr>
<tr>
<td>25(OH) D$_3$ (ng/ml)</td>
<td>10.8 ± 0.8</td>
<td>9.7 ± 0.7</td>
</tr>
<tr>
<td>1,25(OH)$_2$ D$_3$ (pg/ml)</td>
<td>107.4 ± 10.5</td>
<td>98.6 ± 5.6</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM. Statistical comparisons were by independent samples $t$-test, adjusted by correlation correction and Sidak-Holm stepdown procedure.
Cortex
Trabecular bone
Actively remodelling new bone
Growth plate

Control
Low protein

(B) Area of spongiosa (%)

(C) Trabecular bone volume (%)

(A)
(A) ECaC1

(B) Calbindin-D$_{28K}$

(C) PMCA