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Programming of adult blood pressure by maternal protein restriction: Role of nephrogenesis

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Programming of adult blood pressure by maternal protein restriction: Role of nephrogenesis.

Background. Modest maternal protein restriction leads to hypertension and a reduced number of glomeruli in adult male but not female offspring. This study determined whether a more severe protein restriction has equivalent effects on male and female rat offspring, and examined the role of nephrogenesis in this programming.

Methods. Sprague-Dawley rats were fed a protein-restricted (5% protein) diet throughout (LLP), or during the first (LLP/NP) or second (NP/LLP) half of pregnancy. Controls ate a normal diet (NP, 19% protein). Adult offspring were chronically instrumented at 22 weeks; glomerular number and volume were estimated using stereologic techniques.

Results. Mean arterial pressures in male offspring were significantly higher in LLP (136 ± 2 mm Hg) or NP/LLP (137 ± 2 mm Hg) than in LLP/NP (125 ± 1 mm Hg) or NP (125 ± 2 mm Hg). Moreover, the hypertension was salt-sensitive (increase of 16 ± 4 mm Hg in LLP on a high Na^+ diet compared to 2 ± 2 mm Hg in NP). Glomerular number (per kidney) was reduced (15,400 ± 2,411 in LLP vs. 27,208 ± 1,534 in NP) but average individual glomerular volume was not different ($1.98 \pm 0.18\ 10^6\ \mu^3$ in LLP vs. $2.01 \pm 0.14\ 10^6\ \mu^3$ in NP). Female offspring showed qualitatively similar results.

Conclusion. Severe maternal dietary protein restriction reduces glomerular number and programs for salt-sensitive adult hypertension in both female and male offspring. The window of sensitivity of adult blood pressure to prenatal protein restriction falls within the period of nephrogenesis in the rat. These data are consistent with the hypothesis that maternal protein restriction causes adult hypertension in the offspring through impairment of renal development.

Epidemiologic evidence indicates that babies that are born smaller or grow more slowly during the first year of life have an increased risk for adult diseases, including heart disease and hypertension, than do larger babies [1–8]. This indicates that factors in the prenatal and early postnatal environment, that influence growth, can cause permanent changes in the morphology and physiology of specific organ systems, thus "programming" the individual for increased risk of disease later in life. One such factor that is known to cause this programming is maternal undernutrition. In the rat, maternal dietary protein restriction during pregnancy leads to hypertension in adult offspring [9–11], but the precise physiologic mechanisms by which this occurs remain controversial. We have previously hypothesized that maternal protein restriction causes suppression of the fetal/newborn intrarenal renin-angiotensin system (RAS), and thus impaired renal development, leading to permanent alterations in kidney structure and function, including a reduced number of nephrons, resulting in hypertension. In support of this postulate, we found that renal renin gene expression, renin protein, and angiotensin II levels are suppressed in newborn male offspring of modestly protein-restricted mothers [11], and that total glomerular number is reduced in adult male offspring [11]. We have not found adult female offspring of modestly protein-restricted mothers to have either hypertension or a reduced number of nephrons [12], suggesting that females are either insensitive or less sensitive to the renal and hypertensive effects of perinatal protein restriction than males.

If maternal protein restriction indeed causes adult hypertension through impairment of nephrogenesis, one would expect that the future blood pressure set point of an animal would be sensitive to maternal protein intake only during the specific window of development in which nephrogenesis occurs. In the rat, this period spans approximately the last half of gestation and the first half of lactation [13]. The initial purpose of this study was to determine whether more severe maternal dietary protein restriction during pregnancy causes hypertension in adult female offspring similarly to its effects in males. We then sought to determine whether the hypertension in this model is salt-sensitive and/or age-dependent. Finally, we tested the hypothesis that the effects of maternal protein

Key words: fetal origins of adult disease, glomerular filtration rate, renal plasma flow, nephron number, gender.

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deprivation to increase offspring blood pressure may be related to its effects on nephrogenesis.

METHODS

Female Sprague-Dawley rats (Simonsen, Gilroy, CA, USA) weighing ~250 to 300 g were bred at Oregon Health & Science University (OHSU) and maintained on either a normal protein (NP, 19% protein) (Purina basal diet 5755), or a low protein (LLP, 5% protein) (Purina 5767, modified from 5755) (Purina, Richmond, IN, USA) diet ad libitum during specific time windows in pregnancy and lactation. The two diets were isocaloric and had the same Na^+ content (0.20%). Dams were kept on the NP diet before breeding. The day sperm were seen in a vaginal smear was designated as day 1 of pregnancy. One group of dams was maintained on NP throughout pregnancy and lactation (NP, control group). A second group of dams was placed on LLP on day 1 of pregnancy, and switched to NP on day 11 of pregnancy (LLP/NP). A third group of dams was maintained on NP until day 11 of pregnancy, when they were placed on LLP (NP/LLP). They were then returned to the NP diet the day of delivery. A fourth group of dams was maintained on LLP throughout pregnancy and switched to NP the day of delivery. Food intake was not significantly different among the groups, either during the first or last half of pregnancy, or throughout pregnancy. No dams were maintained on LLP after delivery because of concerns that pup survival would be jeopardized. Thus, the third and fourth groups were exposed to maternal protein restriction during the prenatal portion of the window of nephrogenesis, whereas the first and second groups were not. All pups were weaned to the normal diet at 22 days of age and maintained on that diet until adulthood. The animals were housed in a room with a controlled temperature and a 12:12 hour light:dark cycle. Some newborn animals were used for histologic analysis of renal tissue; littermates were allowed to grow until adulthood for physiologic measurements. Some LLP and NP pregnant animals were euthanized at 23 days' gestation for collection of fetal kidneys.

Collection of fetal and newborn tissues

Fetal and newborn kidneys were collected at 23 days' gestation and 1 to 15 days of postnatal age for examination of renal histology. They were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and 5μm sections were stained with hematoxylin and eosin.

Surgical preparation of adult animals

At approximately 21 weeks of age, adult male and female animals were chronically instrumented for measurements of arterial pressure and renal function as described previously [11]. Briefly, they were anesthetized with a mixture of 55% ketamine (100 mg/mL), 28% xylazine (20 mg/mL), 11% acepromazine (10 mg/mL), and 6% sterile water, administered at 1.0 mL/kg intraperitoneally. A stainless steel Silastic-covered catheter was implanted in the bladder, flushed with chloramphenicol sodium succinate (30 mg/mL), and plugged. Sterile catheters made of Tygon microbore tubing were implanted into the left femoral artery and vein and tunneled under the skin to exit on top of the head. The catheters were filled with heparin (500 U/mL) following surgery, and plugged with stainless steel wire pins. For the first 24 hours after surgery, a mixture of rat chow and 5% dextrose was provided in a bowl to encourage eating. Animals were allowed to recover in individual cages for at least 6 days before any experiments were conducted and were maintained on the normal protein, normal sodium diet. Vascular catheters were flushed every 2 or 3 days to maintain patency. During the recovery period, the animals were placed in a wire restrainer in the study room for at least 2 hours on at least three occasions to allow them to become acclimatized to the study conditions. A few additional animals were allowed to grow to ~34 weeks of age before instrumentation.

Experimental protocol

At the time of study, the animals were either 22 or 35 weeks of age. For measurement of physiologic variables, the rat was placed in a wire restrainer in the study room. Urine was allowed to drain continuously from the bladder catheter into a tube throughout the experiment. Mean arterial pressure was measured through the arterial catheter using a pressure transducer (Statham, Oxnard CA, USA) connected to a polygraph (Grass Instruments, Quincy, MA, USA), and a reading was taken after at least 30 minutes, once the pressure had stabilized. Arterial pressures were always measured between 6:00 and 9:00 a.m. Following the pressure measurement, a small blood sample was taken from the arterial catheter for measurement of microhematocrit and plasma protein. Inulin (Sigma, St. Louis, MO, USA) and paraaminohippuric acid (PAH) (Sigma) in 5% dextrose were given intravenously as a bolus (0.45 mL containing 56 mg inulin and 5.6 mg PAH) followed by a continuous infusion (0.024 mL/min of 74 mg/mL inulin and 7.4 mg/mL PAH) throughout the rest of the experiment. At least 60 minutes after beginning the inulin/PAH infusion, urine was collected in a series of three or four successive 20-minute clearance periods, with a blood sample taken at the midpoint of each. Blood was collected in sterile heparinized syringes. Urine volume was determined gravimetrically. After centrifuging the blood and removing the plasma, the red cells were resuspended in an equivalent volume of saline and returned to the animal. The plasma was frozen at −20°C for later analysis.

Some animals from the NP and LLP groups were placed on a high (3.15%) Na^+ diet (Purina diet 5883, modified from diet 5755), and the arterial pressure measurements were repeated at 7 to 14 days after the diet was changed. Body weights plateaued by 7 to 10 days, and equilibration on the diet was verified by constancy of arterial pressures between 10 and 14 days.

When all experiments were completed or when the instrumentation was no longer functional, the rats were killed with a commercial euthanasia solution. The left kidney was fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 mol/L phosphate, and embedded in Technovit. These kidneys were cut in 2 mm thick horizontal slices, and glomerular number was determined using the disector method as described previously [11, 14, 15]. The average volume of all glomeruli (V_G) was estimated by the following equation

$$V_G = V_V(g/k) \times K_V/n$$

where V_V (g/k) is the volume density of glomeruli in the kidney, K_V is the kidney weight, and n is the number of glomeruli in one kidney.

The right kidney was sectioned coronally, fixed in 10% phosphate-buffered formalin and totally embedded in a paraffin block. Approximately three sections were cut from each block and stained with hematoxylin and eosin. Sections were evaluated for renal pathology using the following scoring system: 0, normal kidney; 1+, minimal focal tubulointerstitial injury (including tubular atrophy, dilation and fibrosis); 2+, moderate tubulointerstitial injury, involving multiple microscopic fields at 100×; and 3+, extensive tubulointerstitial injury, involving more than half of microscopic fields at 100×.

The observer was blinded to the codes indicating treatment. A total of 33 LLP, 22 NP/LLP, 17 LLP/NP, and 20 NP kidneys were examined.

Analytical measurements

Inulin in plasma and urine was assayed by a modification of the method of Waugh [16] after deproteinization with zinc sulfate. PAH was assayed on the same samples using the method of Brun [17]. Glomerular filtration rate (GFR) was calculated as the renal clearance of inulin

$$GFR = (U_{in}/P_{in}) \times V$$

where U_{in} and P_{in} are the urine and arterial plasma inulin concentrations, respectively, and V is the urine flow rate. Effective renal plasma flow (ERPF) was calculated as the renal clearance of PAH. The values obtained for the three or four clearance periods were averaged to give a single value for each animal. Plasma protein was measured by refractometry (National Instrument, Baltimore, MD, USA).

Statistical analysis

The data are expressed as means ± SE. Data for the groups were compared using two-way analysis of variance (ANOVA), followed by a post hoc test (Bonferroni). Histopathology scores were analyzed using a Kruskal-Wallis nonparametric ANOVA. Responses to a high-salt diet were compared using a paired *t* test. Statistical significance was assumed with a value of $P < 0.05$ or better.

RESULTS

Effects of maternal protein restriction on growth in the offspring

Perinatal growth data are shown in Table 1.The average weight gain during pregnancy was significantly reduced in mothers fed LLP either during the last half of (NP/LLP) or throughout (LLP) gestation, but not in those fed LLP only during the first half of gestation (LLP/NP). Birth weights were also significantly lower in the groups that received LLP during late gestation, although the number of pups per litter was not different. Figure 1 shows early postnatal growth in LLP and NP offspring. LLP animals were ~30% smaller at birth, and their kidney-to-body weight ratio was also reduced. Kidney-to-body weight caught up to control by 5 days of age, whereas body weight itself did not. The kidney-to-body weight ratio at 1 day of age was not reduced in offspring of mothers fed LLP during only the first or last half of pregnancy (Table 1). Body weights were still reduced in the LLP group at weaning and throughout life, although this reached statistical significance only in males. NP/LLP offspring also tended to be smaller than controls, although this difference did not always reach statistical significance. Thus, this degree of maternal dietary protein restriction, when it occurs during the last half of gestation, has lifelong consequences for growth in both male and female offspring. Kidney weights were significantly reduced in adult LLP males, but the kidney-to-body weight ratios were generally not different among groups. Heart weights and heart-to-body weight ratios were not significantly different among groups. Males always had significantly higher body and organ weights than their female littermates, regardless of maternal diet treatment.

Effects of maternal protein restriction on physiologic variables in the offspring

Arterial pressures and renal hemodynamics in adult offspring of rats fed normal or protein-restricted diets during all or part of pregnancy are shown in Figure 2. Overall, as well as within each maternal diet group, males had significantly higher blood pressures, GFRs, and ERPFs than their female littermates. Mean arterial pressure was significantly increased in both male and female offspring of mothers that were protein-restricted either

Table 1. Growth in pregnant rats exposed to different protein intakes and their offspring

	NP	LLP/NP	NP/LLP	LLP
Pregnancy weight gain *g*	149 ± 8 ($N = 9$)	144 ± 8 ($N = 7$)	50 ± 8 ($N = 9$)[a,b]	41 ± 6 ($N = 6$)[a,b]
Number of pups per litter	12 ± 1 ($N = 9$)	10 ± 1 ($N = 7$)	9 ± 1 ($N = 9$)	10 ± 2 ($N = 6$)
Birth weight *g*	6.62 ± 0.08 ($N = 9$)	6.27 ± 0.11 ($N = 7$)	4.93 ± 0.20 ($N = 9$)[a,b]	4.47 ± 0.29 ($N = 6$)[a,b]
Gestation length *days*	23.7 ± 0.3 ($N = 9$)	23.9 ± 0.1 ($N = 7$)	23.9 ± 0.2 ($N = 9$)	23.5 ± 0.3 ($N = 6$)
Kidney weight/body weight at 1 day %	1.008 ± 0.023 ($N = 20$)	1.058 ± 0.031 ($N = 8$)	1.013 ± 0.012 ($N = 4$)	0.787 ± 0.027 ($N = 4$)[a]
Weaning weight *g*				
Males	71 ± 1 ($N = 14$)	69 ± 1 ($N = 6$)	66 ± 1 ($N = 10$)	56 ± 3 ($N = 17$)[a,b,c]
Females	64 ± 2 ($N = 15$)[d]	68 ± 2 ($N = 10$)	61 ± 3 ($N = 9$)	53 ± 2 ($N = 16$)[a,b,c]
Weight at study *g*				
Males (22 weeks)	461 ± 11 ($N = 8$)	458 ± 8 ($N = 6$)	431 ± 8 ($N = 10$)[a]	415 ± 10 ($N = 12$)[a,b]
Males (35 weeks)	511 ± 12 ($N = 6$)			449 ± 7 ($N = 5$)[a,e]
Females (22 weeks)	271 ± 5 ($N = 11$)[d]	275 ± 5 ($N = 10$)[d]	259 ± 5 ($N = 9$)[d]	247 ± 4 ($N = 9$)[d]
Females (35 weeks)	306 ± 13 ($N = 4$)[d,e]			280 ± 9 ($N = 7$)[d,e]
Kidney weight *g*				
Males (22 weeks)	3.025 ± 0.102 ($N = 8$)	3.333 ± 0.120 ($N = 6$)	3.033 ± 0.122 ($N = 10$)	2.631 ± 0.082 ($N = 12$)[a,b,c]
Males (35 weeks)	2.894 ± 0.135 ($N = 6$)			2.531 ± 0.059 ($N = 5$)[a]
Females (22 weeks)	1.981 ± 0.081 ($N = 11$)[d]	2.023 ± 0.114 ($N = 10$)[d]	2.122 ± 0.085 ($N = 7$)[d]	1.704 ± 0.028 ($N = 9$)[c,d]
Females (35 weeks)	1.805 ± 0.085 ($N = 4$)[d]			1.694 ± 0.038 ($N = 7$)[d]
Kidney/body weight %				
Males (22 weeks)	0.680 ± 0.012 ($N = 8$)	0.732 ± 0.020 ($N = 6$)	0.745 ± 0.038 ($N = 10$)	0.663 ± 0.017 ($N = 12$)
Males (35 weeks)	0.578 ± 0.026 ($N = 6$)[e]			0.576 ± 0.017 ($N = 5$)[e]
Females (22 weeks)	0.737 ± 0.028 ($N = 11$)	0.730 ± 0.038 ($N = 10$)	0.828 ± 0.042 ($N = 7$)	0.687 ± 0.013 ($N = 9$)[c]
Females (35 weeks)	0.622 ± 0.014 ($N = 4$)[e]			0.611 ± 0.016 ($N = 7$)[e]
Heart weight *g*				
Males (22 weeks)	1.495 ± 0.056 ($N = 6$)	1.608 ± 0.058 ($N = 6$)	1.469 ± 0.041 ($N = 10$)	1.434 ± 0.057 ($N = 7$)
Females (22 weeks)	1.035 ± 0.029 ($N = 8$)[d]	1.046 ± 0.018 ($N = 10$)[d]	1.071 ± 0.062 ($N = 6$)[d]	0.942 ± 0.032 ($N = 7$)[d]
Heart/body weight %				
Males (22 weeks)	0.325 ± 0.008 ($N = 6$)	0.353 ± 0.009 ($N = 6$)	0.361 ± 0.015 ($N = 10$)	0.360 ± 0.018 ($N = 7$)
Females (22 weeks)	0.373 ± 0.005 ($N = 8$)[d]	0.378 ± 0.006 ($N = 10$)	0.414 ± 0.023 ($N = 6$)[d]	0.380 ± 0.013 ($N = 7$)

Abbreviations are: NP, normal protein; LLP/NP, low protein switched to normal protein on day 11 of pregnancy; NP/LLP, normal protein switched to low protein on day 11 of pregnancy; LLP, low protein. Mean ± SE.

[a]Significantly different than NP; [b]Significantly different than LLP/NP; [c]Significantly different than NP/LLP; [d]Significantly different than males of the same diet type and age; [e]Significantly different than value in 22-week-old animals of the same gender.

during the last half of (NP/LLP) or throughout (LLP) pregnancy, however blood pressures in offspring of mothers that were protein-restricted only during the first half of pregnancy (LLP/NP) were not different from controls. Likewise, GFR and ERPF were reduced by 15% to 25% in offspring of mothers maintained on a low protein diet either during the last half of or throughout pregnancy; this reached significance only in males. The GFR normalized to body weight also tended to be reduced in these groups (Table 2). The mean filtration fraction was not affected. Hematocrits and plasma protein levels were not different among diet groups, although both were lower in females than males (Table 2).

Figure 3 shows the renal function curves (changes in mean arterial pressure on a high salt diet) for male and female adult offspring of mothers maintained on normal or protein-restricted diets during pregnancy. In normal male rats, mean arterial pressure was not significantly increased after ~2 weeks on a high Na^+ diet (change of 2 ± 2 mm Hg, $P = 0.34$). In contrast, in male offspring of protein-restricted mothers, arterial pressure increased by 16 ± 4 mm Hg on the high Na^+ diet ($P = 0.006$), a response that was significantly greater ($P = 0.025$) than in control animals. Female animals showed a qualitatively similar result. On the high Na^+ diet arterial pressure was increased by 10 ± 3 mm Hg in female offspring of protein-restricted mothers ($P = 0.01$), which was significantly greater ($P = 0.03$) than the small increase of 3 ± 1 mm Hg in normal animals ($P = 0.024$). Thus, in both male and female offspring, perinatal protein restriction caused a salt-sensitive hypertension. The salt sensitivity (net increase in arterial pressure on a high Na^+ diet) tended to be greater in male than female offspring, although this was not statistically significant ($P = 0.32$).

Effects of maternal protein restriction on glomerular number and volume in offspring

The total number of glomeruli and glomerular volume are shown in Figure 4. Glomerular number was not significantly different in male compared to female offspring, either overall or within maternal diet groups. However, the individual glomerular volumes were generally higher in males. Total glomerular number was significantly reduced by ~30% to 45% in adult offspring of mothers protein-restricted throughout gestation, and in male but not female offspring exposed to LLP only during the last half of pregnancy. The average individual glomerular volume

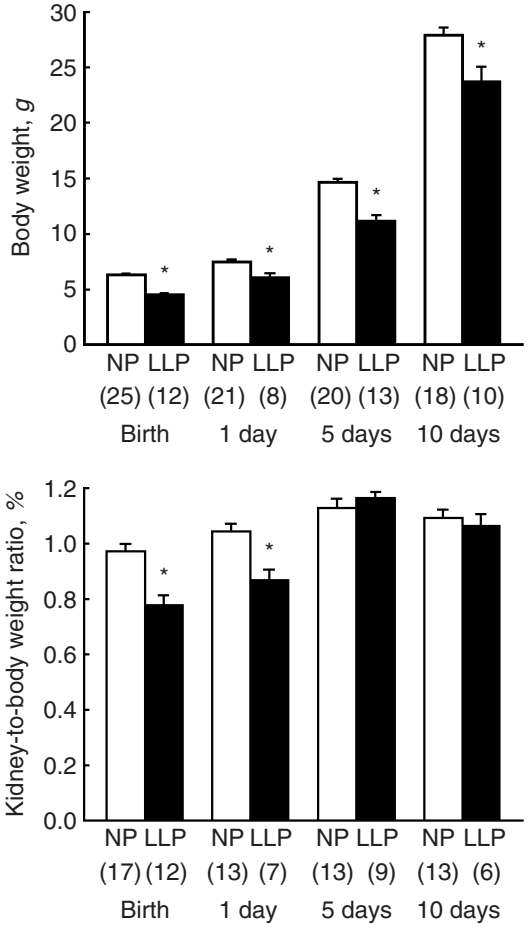

Fig. 1. Postnatal growth in offspring (males and females combined) of mothers maintained on a normal (NP) or low protein diet (LLP) throughout pregnancy. Mean ± SE. Number for each group in parentheses. *$P < 0.05$ compared to NP.

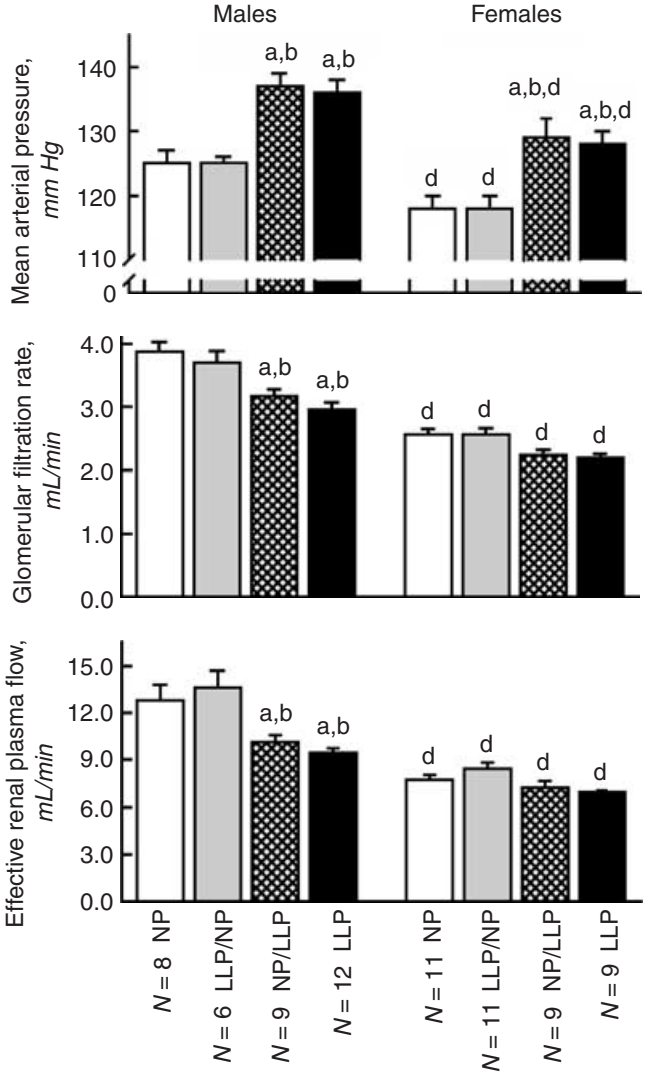

Fig. 2. Arterial pressure and renal hemodynamics in adult (22 weeks) offspring of mothers maintained on a normal protein diet (NP) throughout pregnancy, or on a low protein diet during either the first half (LLP/NP) or the last half of pregnancy (NP/LLP), or throughout pregnancy (LLP). Mean ± SE. a, Significantly different than NP; b, significantly different than LLP/NP; c, significantly different than NP/LLP; d, significantly different than males of the same diet type and age.

was not significantly different among groups, although in females it tended to be larger in NP/LLP and LLP animals. Thus, in females the total volume of all glomeruli was not significantly different among groups, whereas in males it was significantly reduced in NP/LLP and LLP animals. Out of all the variables in the study, the only one that showed a significant interaction between diet and gender was total glomerular volume ($P = 0.002$).

Effect of age on arterial pressure and renal structure in offspring of protein-restricted mothers

We allowed some animals from the NP and LLP litters to grow to ~35 weeks of age before study. These results are shown in Tables 1 and 2. Arterial pressure did not change significantly with age in offspring of mothers on the normal protein diet. However, in both male and female offspring of protein-restricted mothers, arterial pressure increased significantly between 22 and 35 weeks of age. The kidney-to-body weight ratio was also reduced in older (35 weeks) animals compared to younger animals of the same group, due primarily to increases in body weight.

Histopathologic findings

Photomicrographs of representative sections of kidneys of 1-day-old offspring of NP and LLP litters are shown in Figure 5 (a total of 25 NP and 11 LLP fetuses and newborns were studied). The cortex appeared thinner in the LLP vs. the NP animals at all ages examined. The nephrogenic zone is the compact outer layer of immature tubules and glomeruli. The proportion of cortex that was in the nephrogenic zone was significantly higher ($P <$ 0.001) in LLP (41% ± 3% in LLP fetuses vs. 32% ± 1%

Table 2. Cardiovascular and renal parameters in adult rats exposed prenatally to different protein intakes

	NP	LLP/NP	NP/LLP	LLP
Mean arterial pressure *mm Hg*				
Males (22 weeks)	125 ± 2 ($N = 8$)	125 ± 1 ($N = 6$)	137 ± 2 ($N = 9$)[a,b]	136 ± 2 ($N = 12$)[a,b]
Males (35 weeks)	129 ± 2 ($N = 6$)			144 ± 3 ($N = 5$)[a,e]
Females (22 weeks)	118 ± 2 ($N = 11$)[d]	118 ± 2 ($N = 11$)[d]	129 ± 3 ($N = 9$)[a,b,d]	128 ± 2 ($N = 9$)[a,b,d]
Females (35 weeks)	118 ± 2 ($N = 4$)[d]			137 ± 3 ($N = 7$)[a,d,e]
GFR/kidney weight *mL/min/g*				
Males (22 weeks)	1.290 ± 0.056 ($N = 8$)	1.093 ± 0.063 ($N = 5$)	1.062 ± 0.063 ($N = 10$)[a]	1.128 ± 0.051 ($N = 12$)
Females (22 weeks)	1.304 ± 0.048 ($N = 11$)	1.208 ± 0.053 ($N = 8$)	1.039 ± 0.052 ($N = 6$)[a]	1.282 ± 0.045 ($N = 7$)
ERPF/kidney weight *mL/min/g*				
Males (22 weeks)	4.278 ± 0.384 ($N = 8$)	4.043 ± 0.405 ($N = 5$)	3.414 ± 0.262 ($N = 10$)[a]	3.631 ± 0.128 ($N = 8$)
Females (22 weeks)	3.911 ± 0.121 ($N = 11$)	3.970 ± 0.145 ($N = 8$)	3.432 ± 0.248 ($N = 6$)	4.053 ± 0.071 ($N = 7$)
GFR/body weight *mL/min/100 g*				
Males (22 weeks)	0.842 ± 0.026 ($N = 8$)	0.799 ± 0.030 ($N = 5$)	0.736 ± 0.025 ($N = 10$)	0.710 ± 0.026 ($N = 12$)[a]
Females (22 weeks)	0.942 ± 0.024 ($N = 11$)[d]	0.933 ± 0.039 ($N = 8$)[d]	0.859 ± 0.026 ($N = 7$)[d]	0.874 ± 0.031 ($N = 7$)[d]
ERPF/body weight *mL/min/100 g*				
Males (22 weeks)	2.78 ± 0.21 ($N = 8$)	2.94 ± 0.20 ($N = 5$)	2.36 ± 0.10 ($N = 10$)[b]	2.41 ± 0.04 ($N = 8$)
Females (22 weeks)	2.84 ± 0.09 ($N = 11$)	3.07 ± 0.14 ($N = 8$)	2.77 ± 0.14 ($N = 7$)[d]	2.76 ± 0.06 ($N = 7$)
Filtration fraction				
Males (22 weeks)	0.314 ± 0.025 ($N = 8$)	0.275 ± 0.013 ($N = 5$)	0.314 ± 0.007 ($N = 10$)	0.301 ± 0.010 ($N = 8$)
Females (22 weeks)	0.334 ± 0.011 ($N = 11$)	0.304 ± 0.006 ($N = 8$)	0.313 ± 0.014 ($N = 7$)	0.316 ± 0.010 ($N = 7$)
Hematocrit %				
Males (22 weeks)	43 ± 2 ($N = 8$)	45 ± 1 ($N = 6$)	43 ± 2 ($N = 9$)	42 ± 2 ($N = 12$)
Females (22 weeks)	35 ± 2 ($N = 11$)[d]	39 ± 1 ($N = 11$)[d]	37 ± 1 ($N = 8$)[d]	35 ± 1 ($N = 9$)[d]
Plasma protein g/dL				
Males (22 weeks)	6.6 ± 0.1 ($N = 8$)	6.4 ± 0.1 ($N = 6$)	6.5 ± 0.1 ($N = 9$)	6.4 ± 0.1 ($N = 12$)
Females (22 weeks)	6.2 ± 0.1 ($N = 11$)[d]	6.1 ± 0.2 ($N = 11$)	6.1 ± 0.1 ($N = 8$)[d]	6.2 ± 0.1 ($N = 9$)
Histopathology score (0–3+)				
Males (22 weeks)	1.2 ± 0.3 ($N = 10$)	1.2 ± 0.2 ($N = 6$)	2.2 ± 0.1 ($N = 13$)[a,b]	2.1 ± 0.2 ($N = 14$)[a,b]
Females (22 weeks)	0.3 ± 0.2 ($N = 10$)[d]	0.8 ± 0.2 ($N = 11$)	1.4 ± 0.3 ($N = 9$)[a,d]	1.4 ± 0.2 ($N = 19$)[a,d]

Abbreviations are: NP, normal protein; LLP/NP, low protein switched to normal protein on day 11 of pregnancy; NP/LLP, normal protein switched to low protein on day 11 of pregnancy; LLP, low protein.; GFR, glomerular filtration rate; ERPF, effective renal plasma flow. Mean ± SE.

[a]Significantly different than NP; [b]Significantly different than LLP/NP; [c]Significantly different than NP/LLP; [d]Significantly different than males of the same diet type and age; [e]Significantly different than value in 22-week-old animals of the same gender.

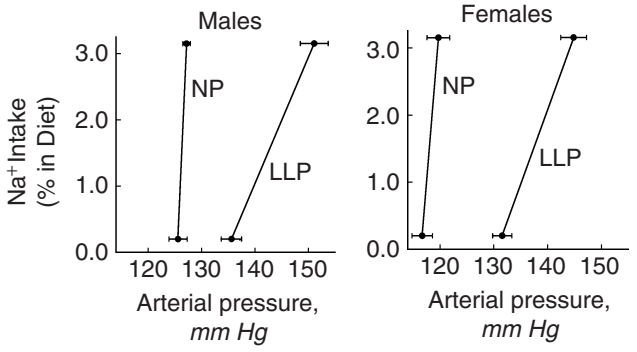

Fig. 3. Renal function curves showing salt sensitivity of mean arterial blood pressure in male and female offspring of mothers maintained on normal (NP) or low protein (LLP) diets throughout pregnancy. The lower points represent arterial pressure on the normal sodium diet, and the upper points represent arterial pressure in the same animals after approximately 2 weeks on the high sodium diet. The increase in blood pressure on a high salt diet was significantly greater in LLP animals than in NP animals ($P = 0.03$). Mean ± SE ($N = 5$ NP males, 9 LLP males, 7 NP females, and 7 LLP females).

in NP fetuses) and fell significantly with age ($P < 0.001$) in both groups (to 30% ± 3% in LLP and 20% ± 2% in NP at 5 days of age). Thus, not only is the cortex thinner in very young LLP animals compared to controls, but the proportion of cortex that is in the nephrogenic zone is increased, indicating that the kidneys of young LLP offspring are less mature than those of young NP offspring.

Histopathologic analysis of adult kidneys (Table 2) showed significantly more tubular dilatation, tubular atrophy, interstitial fibrosis, and scarring in the NP/LLP and LLP animals than in the NP and LLP/NP groups. In general, this injury was more prominent in males. Also, in LLP males the older animals appeared to be more affected, with no apparent age difference in other groups (data not shown because the numbers of older animals were small).

DISCUSSION

The most important findings of the present study are that (*1*) although female rats, unlike males, have a normal number of glomeruli and normal blood pressure when exposed perinatally to a modestly protein-restricted (8.5% protein) diet throughout gestation, a more severe maternal protein restriction causes a reduced number of glomeruli and hypertension in both males and females; (*2*) this hypertension is salt-sensitive and worsens with age, but is approximately equivalent in males and females; and (*3*) the window of development during which

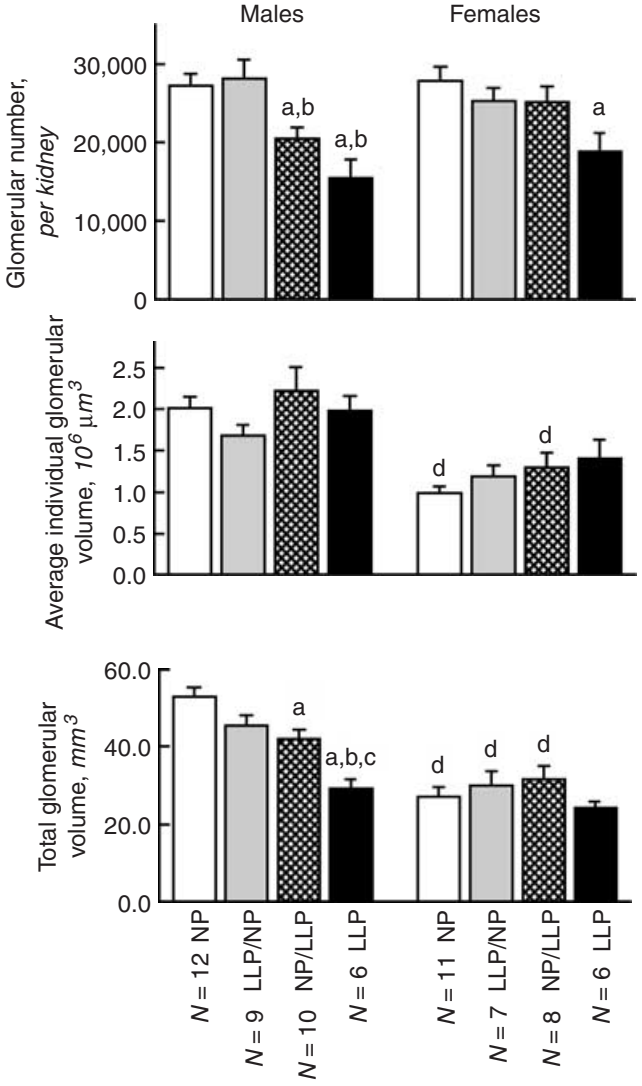

Fig. 4. Glomerular number and volume in adult (22 weeks) offspring of mothers maintained on a normal protein diet (NP) throughout pregnancy, or on a low protein diet during either the first half (LLP/NP) or the last half of pregnancy (NP/LLP), or throughout pregnancy (LLP). Mean ± SE. a, Significantly different than NP; b, significantly different than LLP/NP; c, significantly different than NP/LLP; d, significantly different than males of the same diet type and age.

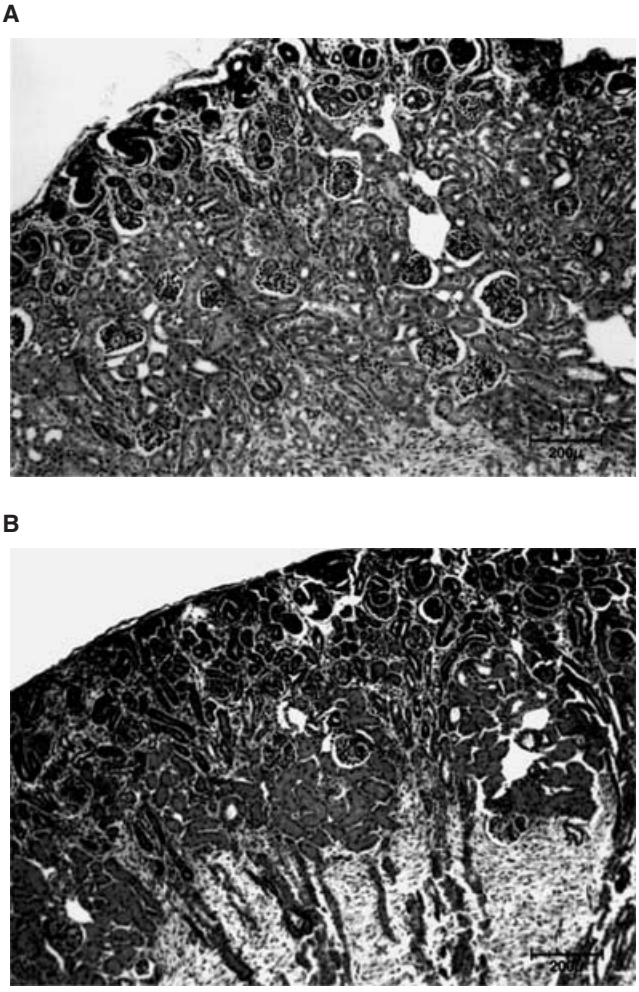

Fig. 5. Representative photomicraphs of kidney. (*A*) Kidney from 1-day-old normal protein (NP) offspring. The immature nephrogenic zone (top) comprises 20% of cortical thickness (hematoxylin and eosin, ×500 final magnification). (*B*) Kidney from 1-day-old low protein (LLP) offspring taken at same magnification as above. The nephrogenic zone (top) comprises 35% of a thinner cortex (hematoxylin and eosin, ×500 final magnification).

the future blood pressure set point is sensitive to maternal protein restriction falls within the period of nephrogenesis. Together with our previous work, these data provide strong evidence that maternal dietary protein restriction causes an increased arterial pressure in adult offspring through its effects on nephrogenesis.

We have previously postulated an important role for the fetal/newborn intrarenal RAS and nephrogenesis in programming for adult hypertension [11]. In support of this hypothesis, we found that newborn male offspring of rats maintained on an 8.5% protein diet during pregnancy have a suppressed intrarenal RAS, and have a reduced number of nephrons and hypertension when they reach adulthood [11]. This suggested the possibility that suppression of the RAS in the developing animal and consequent impaired nephrogenesis may be important in "programming" for an increased future blood pressure set point. Unlike males, female offspring of modestly protein-restricted mothers have a normal intrarenal RAS in the neonatal period, and a normal number of nephrons and normal blood pressure in adulthood, suggesting that these variables change in parallel [12]. Studies from our laboratory have also shown that pharmacologic suppression of the RAS during the period of nephrogenesis results in a reduced nephron number and adult hypertension, thus supporting a cause-and-effect relationship among the findings in protein-restricted offspring [18]. Finally, we have shown that a surgical reduction in the number of nephrons, when done during development,

also results in adult hypertension [19, 20], supporting a direct link between a reduced nephron endowment and adult hypertension. Results from the present study provide several new pieces of evidence supporting the hypothesis that perinatal protein restriction programs for adult hypertension through its effects on the developing RAS and nephrogenesis.

First, both male and female offspring exposed to LLP throughout gestation showed a markedly reduced number of nephrons (glomeruli) in adulthood, and both were hypertensive. These findings of reduced nephron number using unbiased stereologic techniques (the gold standard) are qualitatively in agreement with a previous report in which the maceration method was used to estimate glomerular number, although the magnitude of the reduction in the present study is somewhat larger despite a similar degree of protein restriction [21] (the importance of using unbiased techniques has recently come to the forefront [22]). Importantly, the degree of reduction of nephron number in the present study as well as the degree of hypertension were similar to that caused by perinatal uninephrectomy [19, 20]. Thus, when the degree of protein restriction was severe enough to reduce glomerular number in female offspring, which did not happen with the 8.5% protein diet in our earlier study [12], these animals also showed adult hypertension. However, although female offspring of protein-restricted mothers showed a similar degree of hypertension as males, the total number of nephrons was more severely reduced in males, and the salt sensitivity of blood pressure appeared to be greater in males. Thus, female gender still appears to afford some protection against the long-term detrimental effects of perinatal protein restriction. Although the present study does not address possible mechanisms for this protective effect, it is well known in humans and animals that males are more prone to develop cardiovascular and renal disease than are females. The fact that this is also true in the present model, in which the stimulus for hypertension occurs during development, suggests that whatever the mechanism for the protective effect, it is already present in early life. These results are also consistent with our findings in several other models of perinatally programmed hypertension, in which female offspring appear to require a longer or stronger stimulus than their male littermates to be programmed for renal and blood pressure abnormalities later in life (unpublished results).

The second new piece of information from the present study is that fetal exposure to the maternal dietary insult during the last half of gestation was required for programming of hypertension, and that the severity of the hypertension was independent of whether or not exposure had also occurred during early gestation. Thus, the window of sensitivity of future blood pressure to maternal protein restriction is within the period of nephrogenesis, and it is apparently not events occurring earlier in development that program for adult hypertension. This finding is in contrast to a report by Langley-Evans et al [23] suggesting that male offspring of protein-restricted mothers had increased systolic blood pressures whether exposure was during only the first, second, or third week of gestation. Furthermore, this group found that the blood pressure effects of maternal protein restriction were greater if exposure encompassed all of pregnancy than if it occurred in any single week. Finally, Langley-Evans et al reported gender differences in the response to maternal protein restriction (females insensitive to exposure during early gestation whereas males became hypertensive) which we did not see in the present study. The reasons for these discrepancies are not clear, although there were some technical differences between the two studies. Langley-Evans et al used the tail-cuff method and measured only systolic pressure, within a few minutes after handling the animals, whereas we measured mean arterial pressure directly in trained, conscious animals for at least 30 minutes after handling. Thus, the degree of stress to which the animals had been subjected may have differed. The study of Langley-Evans et al used a 9% casein diet, which is less restrictive than the 5% protein one in the present study. However, in our hands, a 9% casein diet did not cause hypertension in female offspring even when given throughout gestation [12]. It might be expected that a more restrictive diet such as that used in the present study would if anything cause greater effects than the diet used by Langley-Evans et al, rather than fewer or no effects. Perhaps the most important difference between the two studies is the age of the animals; our animals were 22 weeks old, whereas Langley-Evans et al studied animals that were 4 weeks of age. Thus, the differences they found may have been transient, whereas our data represent more long-term, steady-state responses to maternal protein deprivation. In any case, we show clearly in this study that exposure to maternal protein restriction has to occur within the last half of pregnancy (during nephrogenesis) to program the offspring, both male and female, for adult hypertension.

The third important new finding of the present study is that the hypertension in offspring exposed to LLP during nephrogenesis was salt-sensitive, consistent with a reduced renal mass type of hypertension [24]. Indeed, in normal rat offspring in which the number of nephrons was surgically reduced by 50% on the first day of postnatal life, we showed that the subsequent hypertension is also salt-sensitive [19, 20]. In contrast to our present findings, Langley-Evans and Jackson [25] reported that hypertensive adult offspring of protein-restricted mothers did not increase blood pressure further when sodium intake was increased. However, the degree of protein restriction in that study was different from that in our present work, and other components of the diets also differed. Furthermore, those investigators also report that blood

pressure of their control animals was salt-sensitive, which is not what one would expect in animals with normal kidneys [24]. Taken together with our previous work indicating a cause-and-effect relationship among newborn RAS suppression, reduced nephron number, and adult hypertension, the present studies strongly support the hypothesis that maternal protein restriction programs offspring for hypertension through its effects on the fetal/newborn RAS and renal development.

We also found in the present study that the hypertension in both male and female offspring of protein-restricted mothers worsened with age, whereas blood pressure in normal animals did not increase between the two ages studied. One other study has reported an increase in systolic blood pressure with age in offspring of rats fed a severely protein-restricted diet for only the last half of pregnancy [10]. This worsening of hypertension in offspring of protein-restricted mothers is also consistent with our previous findings in animals in which the nephron number was surgically reduced at birth [20]. These findings suggest that a perinatal insult resulting in a reduced endowment of nephrons sets into motion a vicious cycle of progressive renal injury and increasing arterial pressure. The fact that GFR was only reduced by 24% (males) and 15% (females) in LLP animals compared to controls, whereas glomerular number was reduced by 43% (males) and 32% (females), indicates that the GFR per nephron was increased in LLP animals. This hyperfiltration in the remaining nephrons is a well-known response to nephron loss, and is thought to contribute to the progressive nature of renal disease [26].

We also found that arterial pressure, body weight, GFR, ERPF, kidney weight, and heart weight were higher in males than in females, overall as well as within each individual maternal diet group. This is consistent with many previous studies [11, 12, 19, 20]. Although glomerular number was not different between males and females, males had larger glomeruli, which is consistent with their larger kidney size.

Epidemiologic data emerging over the last ten years have provided increasingly compelling evidence of an inverse relationship between an individual's size at birth and cardiovascular risk in adulthood. Even across the spectrum of normal birth weights, smaller babies have an increased risk for hypertension, type II diabetes, and death from cardiovascular disease when they reach adulthood [1–8]. These findings suggest that factors in the perinatal environment that affect fetal growth, and are probably related at least in part to maternal nutrition, cause permanent changes in the structure and function of the body, thus "programming" the individual for increased risk later in life. Although the precise factors in the maternal diet that may provide a key to this programming are not known, and the mechanisms by which they act are not well understood, the present and previous studies suggest that one important factor may be dietary protein. Furthermore, taken together with our previous work, these studies suggest that maternal dietary protein restriction may program the offspring for increased arterial pressure later in life by suppressing the RAS during development, resulting in impaired nephrogenesis, which leads in turn to a reduced number of nephrons at birth, resulting in hypertension in adulthood. Brenner et al [27–29] have hypothesized a cause-and-effect link between nephron endowment at birth and essential hypertension in humans, and individuals with hypertension have recently been shown to have fewer than normal nephrons [30]. The results of the present studies support such a link and provide insight into possible mechanisms by which it might occur.

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