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Modest maternal protein restriction fails to program adult hypertension in female rats

Lori L. Woods,[1] Julie R. Ingelfinger,[2] and Ruth Rasch[3]

[1]*Division of Nephrology and Hypertension, Oregon Health & Science University, Portland, Oregon;*
[2]*Pediatric Nephrology Unit, Massachusetts General Hospital, Boston, Massachusetts; and*
[3]*Department of Cell Biology, Institute of Anatomy, University of Aarhus, Aarhus, Denmark*

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Woods, Lori L., Julie R. Ingelfinger, and Ruth Rasch. Modest maternal protein restriction fails to program adult hypertension in female rats. *Am J Physiol Regul Integr Comp Physiol* 289: R1131–R1136, 2005. First published June 16, 2005; doi:10.1152/ajpregu.00037.2003.—Modest maternal dietary protein restriction in the rat leads to hypertension in adult male offspring. The purpose of this study was to determine whether female rats are resistant to developing the increased blood pressure seen in male rats after maternal protein restriction. Pregnant rats were fed a normal protein (19%, NP) or low-protein (8.5%, LP) diet throughout gestation. Renal renin protein and ANG II levels were reduced by 50–65% in male LP compared with NP pups, but were not suppressed in female LP compared with female NP. Mean arterial pressure in conscious, chronically instrumented adult female offspring (22 wk) was not different in LP (LP: 120 ± 3 mmHg vs. NP: 121 ± 2 mmHg), and glomerular filtration rate was also not different in LP vs. NP. The number of glomeruli per kidney was similar in adult LP and NP female offspring (LP: 26,050 ± 2,071 vs. NP: 26,248 ± 1,292, NP), and individual glomerular volume was also not different (LP: 0.92 ± 0.11 10^6 μm^3, LP vs. NP: 1.07 ± 0.11 10^6 μm^3); the total volume of all glomeruli per kidney was also not significantly different. Thus female rats are relatively resistant to the programming for adult hypertension by perinatal protein restriction that we have described in males. This resistance may be due to the fact that modest maternal protein restriction does not reduce the number of glomeruli with which females are endowed as it does in males. The intrarenal renin-angiotensin system during development may play a key role in this protective effect of female gender.

perinatal programming; nephron number; gene expression; gender; renin-angiotensin system

A GROWING BODY OF EVIDENCE from various populations around the world indicates an inverse relationship between birth weight and cardiovascular risk in adulthood (1, 2, 3, 32). These findings have led to the postulate that environmental factors in the perinatal period can cause permanent changes in the physiology and structure of the body, thus "programming" the individual for increased cardiovascular risk in adulthood. However, the particular aspects of the perinatal environment that contribute to this programming and the precise physiological and morphological mechanisms by which they operate are not well understood.

One factor known to play a role in perinatal programming is maternal diet, and, in particular, the protein content of the diet. We and others have reported that male offspring of mothers that were modestly protein-restricted during pregnancy are hypertensive in adulthood (18, 39). Recently, we provided evidence that this hypertension is programmed during development through suppression of the intrarenal renin-angiotensin system (RAS) in the developing animal and consequent impairment of nephrogenesis (39). Adult male offspring of mothers maintained on a low-protein (8.5%) diet throughout pregnancy have about 25% fewer nephrons than those of mothers maintained on a normal protein (19%) diet (39), and mean arterial pressure (MAP) in conscious, chronically instrumented animals averages ~10 mmHg above normal (39). However, the effect of perinatal protein or calorie restriction on adult blood pressure in females is controversial, and the relationship between nephron number and hypertension in female offspring of modestly protein-restricted mothers has not been defined.

Before menopause, women are less likely to develop cardiovascular disease than men. This has been attributed, at least in part, to the protective effects of estrogen, as a woman's cardiovascular risk increases after menopause, and estrogen replacement therapy may reduce that risk (15, 31). Indeed, both systolic and diastolic blood pressures are lower in women than men before middle age, but they become higher after menopause (34, 37). Hypertension is a major risk factor for coronary heart disease, and estrogen replacement in postmenopausal women reduces blood pressure (21). However, the mechanisms underlying the protective effect of female gender on cardiovascular risk remain poorly understood. As in utero events are now known to play a role in programming an individual for cardiovascular risk, it seems likely that female gender may impart a protective effect at this level as well.

The purpose of this study was to determine whether female rats are resistant to developing the adult hypertension seen in males after perinatal dietary protein restriction, and if so, to ascertain whether this resistance could be due to a gender-related difference in nephron number. We also examined a possible role for the intrarenal RAS in this phenomenon.

METHODS

Animals. All procedures were approved by the Institutional Animal Care and Use Committee. Female Sprague-Dawley rats (Simonsen) were bred at Oregon Health & Science University and maintained on either a normal protein (19% protein = 21% casein, NP), normal sodium (0.20%), diet (Purina basal diet 5755) or a modestly protein-restricted (8.5%, LP), normal sodium diet (Purina diet 5769, modified from 5755) ad libitum throughout pregnancy. The diets were isocaloric, and in the low-protein (LP) diet, additional sucrose was substituted for the missing casein. Maternal food intake was not different

Address for reprint requests and other correspondence: L. L. Woods, Div. of Nephrology and Hypertension, L463, Oregon Health & Science Univ., 3181 S.W. Sam Jackson Park Rd., Portland, OR 97239–3098 (e-mail: woodsl@ohsu.edu).

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between groups (NP: 421 ± 9 g and LP: 419 ± 15 g), but maternal gestational weight gain was significantly reduced in LP animals (NP: 152 ± 6 g vs. LP: 97 ± 5 g). At delivery, all dams were placed on the normal diet, and pups were weaned to the normal diet at 22 days of age and maintained on that diet thereafter. The animals were housed in a room with a controlled temperature and a 12:12-h light-dark cycle. Some newborn animals were used for measurement of tissue renin protein and ANG II levels; littermates were allowed to grow until adulthood for physiological measurements. Some adult animals were housed overnight in metabolic cages just before surgery for 24-h urine collections.

Collection of newborn tissues. Newborn male and female pups were euthanized with commercial euthanasia solution given intraperitoneally. Kidneys were rapidly harvested, rinsed in saline, blotted, and snap-frozen in liquid nitrogen. They were kept frozen at −70°C and shipped on dry ice to the site where measurements were done.

Measurement of intrarenal RAS activity. Renal tissue ANG II levels were measured in kidneys from 1-day-old newborn rat pups as follows. Kidneys were rapidly harvested as described above, rinsed in ice-cold inhibitor solution [125 mmol/l Na_4 EDTA (Sigma, St. Louis, MO), 1 mmol/l enalaprilat (Merck, Whitehouse Station, NJ), 25 mmol/l phenanthroline (Sigma), and 1 mmol/l pepstatin A (Sigma) in 2% ethanol], and snap-frozen in liquid nitrogen. The tissue was homogenized in cold 8 mmol/l urea, 0.1% Triton X-100, 90% methanol, 10 mmol/l sodium acetate, and 0.1% trifluoroacetic acid (TFA) in a Dounce homogenizer. Samples were centrifuged at 30,000 rpm (913,000 *g*) for 10 min at 4°C, and the supernatant was filtered through a Sep-Pak column (Waters, Millis, MA). Then peptides were eluted in 80% methanol and 0.1% TFA and then lyophilized. Recovery of ANG II standard (Sigma) over a range of 10–200 fmol/ml by this procedure was 95%. An ANG II radioimmunoassay was then performed using a commercial rabbit anti-ANG II antibody (Arnel, New York, NY) and a donkey anti-rabbit second antibody (Amersham, Arlington Heights, IL) for magnetic separation of bound and unbound tracer (33).

Renal tissue renin activity (12) was measured in kidneys from 1-day-old newborn rat pups, as follows. Kidneys were rapidly harvested and snap-frozen, as described above. They were homogenized in 0.1 M Tris, pH 7.4, to which EDTA (final concentration 4 mM), sodium tetrathionate (5 mM), phenylmethylsulfonyl fluoride (0.1 mM), and Triton X-100 were added. The tissue homogenate was incubated for 1 h at 37°C, following the addition of exogenous excess substrate (anephric plasma from rats), pH 7.4 with additional protease inhibitors (3.4 mM 8-hydroxyquinolone sulfate and 1.6 mM dimercaprol). The ANG I generated was measured by RIA (17). Data were normalized by protein content (Bio-Rad, Burlingame, CA) (4).

Surgical preparation of adult animals. At ~20 wk of age, 8 LP and 7 NP adult female offspring were chronically instrumented for measurements of arterial pressure and renal function, as previously described (38–40). 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 ip. A midline abdominal incision was made, and a stainless steel Silastic-covered catheter was inserted through a puncture hole at the apex of the bladder and secured by a purse string suture. The muscle was sutured closed around the catheter, which was allowed to exit through the skin on the ventral surface of the abdomen. The bladder was flushed with chloramphenicol sodium succinate (30 mg/ml), and the catheter was plugged with a stainless steel pin covered with Silastic tubing. Sterile Tygon catheters were placed into the left femoral artery and vein and tunneled under the skin to exit on top of the head, where they were secured, filled with heparin (500 U/ml), and plugged. A mixture of rat chow and 5% dextrose was provided in a bowl for the first 24 h after surgery to encourage eating. Animals were maintained on the normal protein, normal sodium diet and allowed to recover for at least 7 days before experiments. Vascular catheters were flushed every 2 or 3 days to maintain patency. Animals were acclimatized to the study conditions by placing them in a wire restrainer in the study room for at least 2 hr on at least three occasions during the recovery period. Two additional groups of female animals ($n = 6$ LP, $n = 6$ NP) were chronically instrumented for measurement of arterial pressure at ~49 wk of age.

Physiological studies. At the time of study, the younger adult female animals were 21.5 ± 0.2 wk of age. To make physiological measurements, the rat was placed in a wire restrainer, and urine was allowed to drain continuously from the bladder catheter into a tube. MAP was measured through the arterial catheter using a pressure transducer (Statham, Oxnard, CA) connected to a polygraph (Grass Instruments, Quincy, MA). A reading was taken after at least 30 min, once the pressure had stabilized. Arterial pressures were always measured between 6:00 AM and 9:00 AM. After the pressure measurement, a small blood sample was taken from the arterial catheter for measurement of microhematocrit and plasma protein. Inulin (Sigma) and 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 min after beginning the inulin/PAH infusion, a series of three or four successive 20-min urine collection periods was begun, with a blood sample taken at the midpoint of each. Blood was collected in sterile heparinized syringes, and 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.

Stereology. 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 and 2.5% glutaraldehyde in 0.1 M phosphate. Glomerular number and volume were determined using stereological methods, as previously described (39).

Analytical measurements. Inulin in plasma and urine was measured by a modification of the method of Waugh (36) after deproteinization with zinc sulfate, and PAH was measured on the same samples using the method of Brun (6). 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 Instruments, Baltimore MD). Urine protein was determined on 24-h samples by precipitation with sulfosalicylic acid using albumin standards.

Statistical analysis. The data are expressed as means ± SE. Values from LP and NP offspring were compared using an unpaired *t*-test. RAS components were analyzed using a two-way ANOVA, followed by a post hoc test (Bonferroni). Statistical significance was assumed with a value of $P < 0.05$.

RESULTS

Effects of maternal protein restriction on growth in the offspring. Birth weights were significantly lower in offspring of protein-restricted mothers (LP: 5.70 ± 0.17 g vs. NP: 6.41 ± 0.09 g, $P < 0.001$, $n = 13$ LP litters and 16 NP litters), but the number of pups per litter was not different (LP: 12 ± 1 vs. NP: 11 ± 1). In the 1-day-old pups used for tissue harvest, body weights, corrected for the weight of stomach contents, were also reduced in both male and female LP animals compared with their NP counterparts (LP: 6.35 ± 0.22 g vs. NP: 7.25 ± 0.17 g in males, and LP: 6.16 ± 0.22 g vs. NP: 6.76 ± 0.18 g in females). This suggests that both male and female LP pups were likely growth retarded at birth. Body

weights of females at weaning were not significantly different in the two groups (LP: 59 ± 2 g vs. NP: 66 ± 3 g). Adult body weights at 22 wk of age were also not different (LP: 262 ± 8 g vs. NP: 273 ± 9 g). In adult animals, total kidney weight (LP: 1.94 ± 0.10 g vs. NP: 1.88 ± 0.11 g) and the kidney-to-body weight ratio (LP: 0.746 ± 0.035 vs. NP: 0.736 ± 0.034%) were not significantly different.

Maternal protein restriction and the newborn intrarenal RAS in the offspring. Intrarenal renin protein and ANG II levels in newborn offspring are shown in Fig. 1. Both variables were significantly suppressed in LP compared with NP male pups, but there was no difference between LP female and NP male or female pups.

Maternal protein restriction and physiological variables in the younger adult offspring. Hematocrits (LP: 41 ± 1% vs. NP: 38 ± 2%) and plasma protein levels (LP: 6.5 ± 0.1 g/dl vs. NP: 6.4 ± 0.1 g/dl) were not different in 22-wk-old LP and NP female offspring. Urine protein excretions were also not different (LP: 3 ± 1 mg/day vs. NP: 4 ± 1 mg/day). Arterial pressures and renal hemodynamics in adult female offspring of rats fed normal or protein-restricted diets during pregnancy are shown in Fig. 2. MAP was not significantly different in female offspring of protein-restricted mothers compared with controls (LP: 120 ± 3 mmHg vs. NP: 121 ± 2 mmHg); GFR, ERPF, and filtration fraction were also not significantly different.

Weights and blood pressure in 50-wk-old offspring. Body weights were significantly lower in LP than in NP female offspring at 50 wk of age (LP: 278 ± 10 g vs. NP: 344 ± 13 g), as were kidney weights (LP: 1.46 ± 0.03 g vs. NP: 1.79 ± 0.06 g). Thus the kidney-to-body weight ratios were not different between these groups (LP: 0.527 ± 0.014% vs. NP: 0.524 ± 0.023%). MAP was also not different between the two groups (LP: 123 ± 2 mmHg vs. NP: 123 ± 3 mmHg), nor were these values different from those in the younger adult females.

Maternal protein restriction and renal structure in offspring. The total number of glomeruli and glomerular volume are shown in Fig. 3. Female offspring of protein-restricted mothers had a similar number of glomeruli per kidney and similar individual and total glomerular volumes as normal animals.

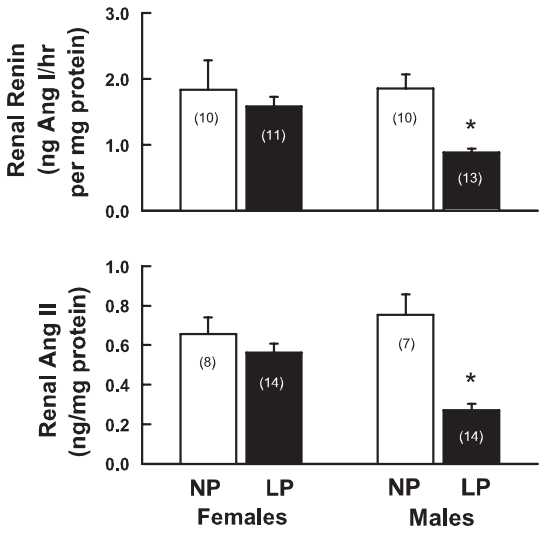

Fig. 1. Renal renin concentration and ANG II concentration in newborn rat offspring exposed prenatally to normal protein (NP; 19%) or low-protein (LP; 8.5%) maternal diets. Values are presented as means ± SE; number of animals are given in parentheses. *$P < 0.05$ compared with NP group of the same gender.

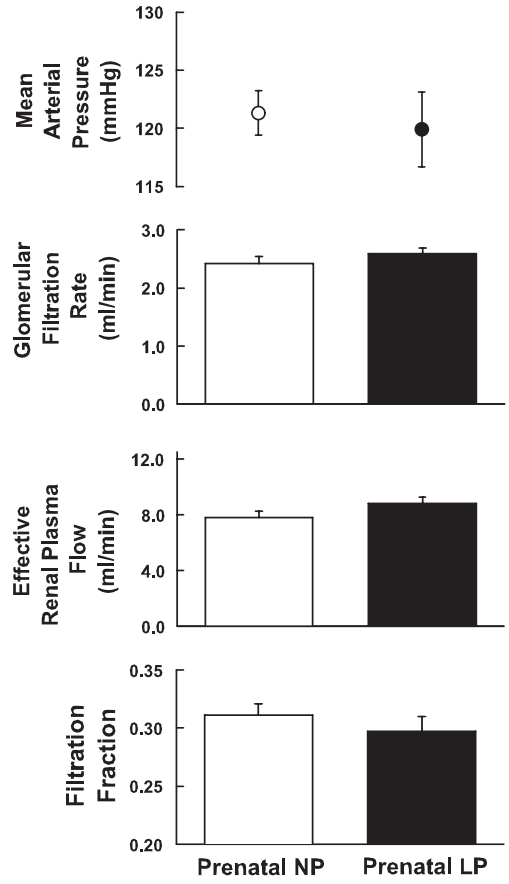

Fig. 2. Mean arterial pressure and renal hemodynamics in adult female rats exposed prenatally to NP (19%) or LP (8.5%) maternal diets. Values are presented as means ± SE; NP: $n = 7$, LP: $n = 8$. There were no significant differences between NP and LP animals.

DISCUSSION

The most important findings of the present study in the rat are that, unlike male offspring, female offspring of mothers subjected to modest dietary protein restriction are normotensive as adults and have a normal number of glomeruli. The intrarenal RAS is also normal in newborn female LP offspring, whereas it is suppressed in male LP newborns. Thus female gender is protective against the development of hypertension in this model, and this may at least in part be programmed in utero through maintenance of a normal RAS during development and consequent endowment with a normal nephron number.

Langley and Jackson (18) first reported several years ago that female rats that were mildly to severely protein-restricted in pregnancy produced offspring that had increased systolic blood pressures in adulthood. We recently confirmed that modest maternal protein restriction (8.5% protein) during pregnancy leads to hypertension (directly measured in conscious, chronically instrumented animals) in adult male offspring (39). We proposed the hypothesis that maternal protein restriction causes offspring hypertension by suppressing the intrarenal RAS during development, leading to impaired nephrogenesis and a reduced number of nephrons at birth, which persists into

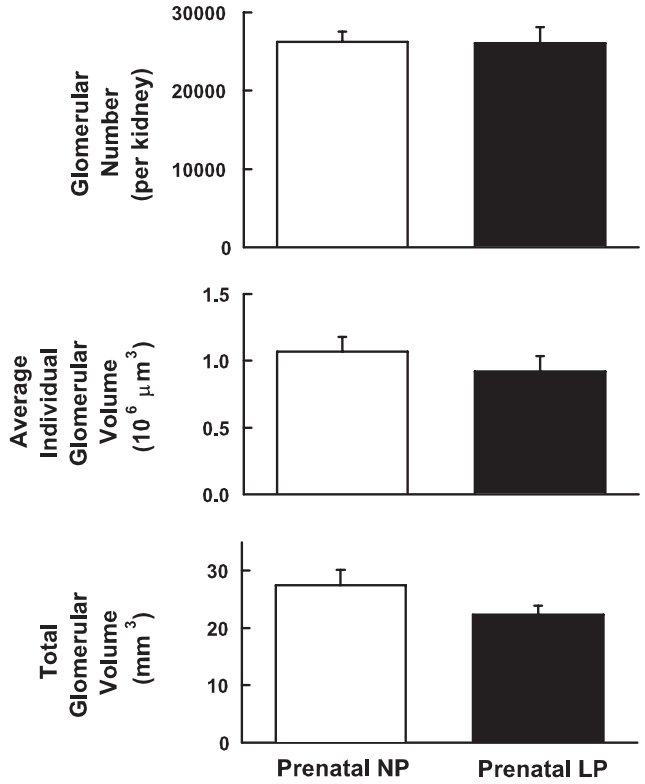

Fig. 3. Glomerular number and volume in adult female rats exposed prenatally to NP (19%) or LP (8.5%) maternal diets. Values are presented as means ± SE; NP: $n = 10$, LP: $n = 10$. There were no significant differences between NP and LP animals.

adulthood (39). In support of this hypothesis, we showed that renal renin mRNA, renin, and ANG II levels are reduced in newborn male offspring of modestly protein-restricted mothers and that the number of glomeruli per kidney in males is reduced by ~25% (39). Additional studies from our laboratory have shown that pharmacologic suppression of the RAS during development leads to a reduced number of glomeruli and hypertension in adulthood in both genders (40). Finally, we showed that surgical reduction in the number of glomeruli at birth also results in adult hypertension in both males and females (38, 41). These results strongly support a cause-and-effect relationship among the alterations in the intrarenal RAS, glomerular number, and blood pressure in the physiological model of maternal protein restriction.

An important consideration in our own previous work and that of others is the gender of the animals studied. The offspring in which we reported hypertension and a reduced nephron number after modest maternal protein restriction were males (39). In the present study, we found that modest maternal protein restriction during pregnancy fails to cause hypertension in adult female offspring. Thus female gender appears to afford at least some protective effect against the development of this type of hypertension. Our finding that these female animals also have a normal number of nephrons, taken together with our previous work, strongly suggests that the females are protected from developing hypertension, at least in part, by their normal endowment of nephrons. Furthermore, our present findings that renal renin protein and ANG II levels are normal in LP female pups, whereas they are suppressed in LP males, and our previous findings (40), support an important role for the fetal/newborn RAS in this protective effect of female gender.

In contrast to our findings in the present study, Langley-Evans and colleagues reported that both male and female offspring of modestly protein-restricted mothers developed hypertension (18, 20). The reason for this discrepancy is not clear. They used the Wistar strain, whereas we used Sprague-Dawley rats, although it seems unlikely that strain differences account for the presence or absence of gender differences (23, and our unpublished observations). Although the degree of protein restriction was similar in the studies of Langley-Evans et al. and our present work, the other components of the diets used were not identical. Additionally, Langley-Evans et al. measured only systolic blood pressure, using the indirect tail-cuff method, whereas we measured MAP directly in trained, chronically instrumented animals. Thus a number of technical differences could contribute to the differences between our findings and those of Langley-Evans et al. However, consistent with our present findings, other investigators have reported that restriction of total food intake by 30% during rat pregnancy does not lead to hypertension in adult female offspring, although vascular function appears to be altered (14, 26). Langley-Evans and colleagues (19) have also recently reported a reduction in nephron number in 4-wk-old male and female offspring in their model. An issue of concern is that the technique used to estimate nephron number in that study is subject to considerable bias. [The importance of using unbiased techniques in renal research has recently come to the forefront (22).] Indeed, absolute numbers of nephrons per kidney reported by those investigators are only ~60% of the numbers other investigators have reported for the Wistar strain (9). Furthermore, data for males and females were combined in that study, so the reader is unable to assess possible gender differences. In a model of more severe maternal protein restriction in the last half of pregnancy, Vehaskari et al. (35) have reported an ~30% reduction in the number of glomeruli in both male and female offspring. At any rate, we show clearly in the present work that, in contrast to males, adult female offspring of modestly protein-restricted mothers are normotensive and have a normal number of nephrons.

To verify that our findings in female animals could not be due to an error in diet composition, we compared our present findings in females to those in their male littermates studied in parallel using identical techniques, some of which were included in our previous study (39). Male littermates of these perinatally protein-restricted females had significantly higher MAPs than male littermates of control females (LP: 136 ± 2 mmHg vs. NP: 125 ± 2 mmHg, $P = 0.004$). Thus, in offspring of the same LP pregnancies, males were clearly hypertensive in adulthood, whereas females were not.

The precise connection between birth weight and adult hypertension remains unclear. In the present study, although the birth weights of LP females were likely reduced, the animals were not programmed for adult hypertension. In contrast, Langley-Evans and colleagues have shown a hypertensive programming effect of a similar level of dietary protein without a consistent reduction in birth weight (25). Thus, although the findings of an association between birth weight and hypertension in humans are what drew attention to this phenomenon of programming, there are clearly cases in which

dissociation between these two parameters occurs. It is likely that birth weight in humans is not itself the critical factor, but rather that it is a surrogate for other, more subtle, aspects of fetal growth.

The level of protein in the protein-restricted diet in our present and previous studies was intentionally chosen to reflect a modest restriction that would consistently yield a reduction of about 10% in birth weight. It is possible that a more severe or a more extended maternal dietary restriction (i.e., into the lactation period, during which nephrogenesis continues) would also lead to hypertension in female offspring. Indeed, in one study in women, the inverse association between birth weight and adult hypertension or cardiovascular disease was marked only in the lowest birth weight category, which represented birth weights more than 25% below average (8, 30). A preliminary report by Gurnani et al. (11) suggested that both male and female offspring of severely protein-restricted mothers have reduced numbers of glomeruli, with the males being more markedly affected. Vehaskari et al. (35) reported that both male and female rats exposed to a severe maternal protein intake (6% protein) during the last half of gestation are born 15% smaller and have elevated systolic blood pressures by 8 wk of age. We have also recently reported hypertension and reduced nephron number in female offspring of more severely protein-restricted dams (42). Thus it appears that the resistance of females to the hypertensive effects of perinatal insults breaks down when the insults are more severe.

The existence of gender differences in blood pressure has been widely recognized, and both androgens and estrogens have been postulated to play a role. In humans, as well as normotensive and hypertensive rat models, males generally have higher blood pressures than do females (7, 10, 13, 37). Various studies in rats suggest that it is the presence of androgens rather than the absence of estrogens that contributes to the normally higher blood pressure in males (10, 24, 28, 29). Androgens may, in turn, act through changes in the RAS (27). In the present study, we found that not only is the blood pressure in female offspring of modestly protein-restricted mothers lower than that of their male littermates, but it is also not different from that in female offspring of normal mothers. Thus, unlike other animal models, in this model of maternal protein restriction, the differences between males and females appear to be not only quantitative, but qualitative. Males are hypertensive, whereas females are not, even up to nearly 1 year of age. It appears that this difference is due, in large part, to differences in the intrarenal RAS during a critical period in development, resulting in impaired nephrogenesis in males. The possible roles of sex hormones in programming for adult hypertension during development or in maintaining the hypertension later in life in this model are not known and will require further study.

Perspectives

The causes of essential hypertension in humans are not well understood. Brenner and colleagues (5) have postulated that the number of nephrons with which an individual is endowed at birth is an important factor in determining the level of blood pressure in adulthood. In support of this idea, Keller et al. (16) recently reported that there were indeed fewer glomeruli on autopsy in persons who had been diagnosed as hypertensive than in persons who had normal blood pressures. Our present data also support this concept, as males, with a 25% reduction in nephron number, became hypertensive, whereas females, with a normal number of nephrons, did not. It is well recognized that premenopausal women have a lower incidence of coronary heart disease and hypertension than men and that estrogen replacement therapy in postmenopausal women may lower their risk of these conditions (15, 31). Thus estrogen has been thought to play a major role in the gender-related differences in cardiovascular risk. The present study suggests that, in addition to the protective effect in adulthood, female gender also provides some protective effect against the long-term hypertensive effects of in utero insults. Importantly, this protective effect occurs during development, and is thus programmed into the female from before birth, presumably through endowment with a normal number of nephrons.

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