Modest Maternal Protein Restriction Fails to Program Adult Hypertension in Female Rats

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ABSTRACT

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 males following maternal protein restriction. Pregnant rats were fed a normal (19%, NP) or low (8.5%, LP) protein diet throughout gestation. Renal renin protein and ANGII levels were reduced by 50-65% in male LP compared to NP pups, but were not suppressed in female LP compared to female NP. Mean arterial pressure in conscious, chronically instrumented adult female offspring (22 wks) was not different in LP (120±3 mmHg, LP vs. 121±2 mmHg, NP), 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 (26,050±2,071, LP vs. 26,248±1,292, NP), and individual glomerular volume was also not different (0.92±0.11 10^6 µ^3, LP vs. 1.07±0.11 10^6 µ^3, NP); 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 RAS during development may play a key role in this protective effect of female gender.

Keywords: perinatal programming, nephron number, gene expression, gender, renin-angiotensin system
INTRODUCTION

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 physiologic and morphologic 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 in the developing animal, and consequent impairment of nephrogenesis (39). Adult male offspring of mothers maintained on a low (8.5%) protein diet throughout pregnancy have about 25% fewer nephrons than those of mothers maintained on a normal protein (19%) diet (39), and mean arterial pressure in conscious, chronically instrumented animals averages approximately 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 are men. This has been attributed at least in part to 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, and 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 if female rats are resistant to developing the adult hypertension seen in males following 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 renin-angiotensin system in this phenomenon.

METHODS

Animals. Female Sprague-Dawley rats (Simonsen) were bred at OHSU 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 LP diet, additional sucrose was substituted for the missing casein. Maternal food intake was not different between groups (421±9 g, NP and 419±15 g, LP), but maternal gestational weight gain was
significantly reduced in LP animals (152±6 g, NP vs 97±5 g, LP). At delivery, all dams were placed on the normal diet, and pups were weaned to the normal diet at 22 d of age and maintained on that diet thereafter. The animals were housed in a room with a controlled temperature and a 12:12 hr light:dark cycle. Some newborn animals were used for measurement of tissue renin protein and ANGII 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-hr 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 renin-angiotensin system activity.** Renal tissue ANGII 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 Na₄EDTA (Sigma), 1 mmol/l enalaprilat (Merck), 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 and 90% methanol, 10 mmol/l sodium acetate, 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, 0.1% TFA and lyophilized. Recovery of ANGII standard (Sigma) over a range of 10-200 fmol/ml by this procedure was 95%. An ANGII radioimmunoassay was then performed using a commercial rabbit anti-ANGII 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 4mM), sodium tetrathionate (5mM), phenylmethylsulfonyl fluoride (0.1mM), and Triton X-100 were added. The tissue homogenate was incubated for 1 hour 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 angiotensin I generated was measured by RIA (17). Data were normalized by protein content (BioRad, Burlingame, California, 4).

**Surgical preparation of adult animals.** At approximately 20 wks 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 intraperitoneally. 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 hrs 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 two or three 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 hrs on at least 3 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 wks of age.

**Physiological studies.** At the time of study, the younger adult female animals were 21.5±0.2 wks 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. Mean arterial pressure 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 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) 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, 2.5% glutaraldehyde in 0.1 M phosphate. Glomerular number and volume were determined using stereologic 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). GFR was calculated as the renal clearance of inulin \[\text{GFR} = (U_{in}/P_{in}) \times V\], 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 3 or 4 clearance periods were averaged to give a single value for each animal. Plasma protein was measured by refractometry (National Instrument, Baltimore MD). Urine protein was determined on 24-hr 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. Renin-angiotensin system 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 (5.70±0.17 g, LP vs 6.41±0.09 g, NP, p<0.001, n=13 LP litters and 16 NP litters), but the number of pups per litter was not different (12±1, LP vs. 11±1, NP). 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 to their NP counterparts (6.35±0.22 g, LP vs 7.25±0.17 g, NP in males, and 6.16±0.22 g, LP vs 6.76±0.18 g, NP 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 (59±2 g, LP vs. 66±3 g, NP). Adult body weights at 22 wks of age were also not different (262±8 g, LP vs. 273±9 g, NP). In adult animals, total kidney weight (1.94±0.10 g, LP vs. 1.88±0.11 g, NP) and the kidney-to-body weight ratio (0.746±0.035, LP vs. 0.736±0.034%, NP) were not significantly different.

*Maternal protein restriction and the newborn intrarenal RAS in the offspring.*

Intrarenal renin protein and ANGII levels in newborn offspring are shown in Figure 1. Both variables were significantly suppressed in LP compared to NP male pups, but there was no difference between LP female and NP male or female pups.

*Maternal protein restriction and physiologic variables in the younger adult offspring.* Hematocrits (41±1%, LP vs. 38±2%, NP) and plasma protein levels (6.5±0.1 g/dl, LP vs. 6.4±0.1 g/dl, NP) were not different in 22-wk-old LP and NP female offspring. Urine protein excretions were also not different (3±1 mg/d, LP vs 4±1 mg/d, NP). Arterial pressures and renal hemodynamics in adult female offspring of rats fed normal or protein-restricted diets during pregnancy are shown in Figure 2. Mean arterial pressure was not significantly different in female offspring of protein-restricted mothers.
compared to controls (120±3 mmHg, LP vs 121±2 mmHg, NP); 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 wks of age (278±10 g, LP vs 344±13 g, NP), as were kidney weights (1.46±0.03 g, LP vs 1.79±0.06 g, NP). Thus, the kidney-to-body weight ratios were not different between these groups (0.527±0.014%, LP vs 0.524±0.023%, NP). Mean arterial pressure was also not different between the two groups (123±2 mmHg, LP vs 123±3 mmHg, NP), nor were these values different than 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 Figure 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.

**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 renin-angiotensin system 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 renin-angiotensin system during development and consequent endowment with a normal nephron number.

Langley and Jackson 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 (18). 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 renin-angiotensin system during development, leading to impaired nephrogenesis and a reduced number of nephrons at birth which persists into adulthood (39). In support of this hypothesis, we showed that renal renin mRNA, renin, and angiotensin 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 about 25% (39). Additional studies from our laboratory have shown that pharmacologic suppression of the renin-angiotensin system 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 renin-angiotensin system, 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 following 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 ANGII 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 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 unpublished observations). Although the degree of protein restriction was similar in the study of Langley et al and our present work, the other components of the diets used were not identical. Additionally, Langley et al measured only systolic blood pressure, using the indirect tail cuff method, whereas we measured mean arterial pressure directly in trained, chronically instrumented animals. Thus, a number of technical differences could contribute to the differences between our findings and those of Langley 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 have also recently reported a reduction in nephron number in 4-wk-old male and female offspring in their model (19). 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 have reported approximately a 30% reduction in the number of glomeruli in both male and female offspring (35). 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.

In order 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 mean arterial pressures than male littermates of control females (136±2 mmHg, LP vs. 125±2 mmHg, NP, 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 suggests that both male and female offspring of 
severely protein-restricted mothers have reduced numbers of glomeruli, with the males 
being more markedly affected (11). Vehaskari et al 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 weeks 
of age (35). 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 renin-angiotensin system (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 one year of age. It appears that this difference is due in large part to differences in the intrarenal renin-angiotensin system 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 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 (5). In support of this idea, Keller et al 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 (16). 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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FIGURE LEGENDS

Figure 1. Renal renin concentration and ANGII concentration in newborn rat offspring exposed prenatally to normal (19%) or low (8.5%) protein maternal diets. Mean ± SE, number of animals in parentheses. * p< 0.05 compared to NP of the same gender.

Figure 2. Arterial pressure and renal hemodynamics in adult female rats exposed prenatally to normal (19%) or low (8.5%) protein maternal diets. Mean ± SE, n=7 NP, n=8 LP. There were no significant differences between NP and LP animals.

Figure 3. Glomerular number and volume in adult female rats exposed prenatally to normal (19%) or low (8.5%) protein maternal diets. Mean ± SE, n=10 NP, n=10 LP. There were no significant differences between NP and LP animals.
Figure 1
Figure 3