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Prenatal programming of adult hypertension in the rat

V. Matti Vehaskari, Diego H. Aviles, and Jennifer Manning

Department of Pediatrics, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA

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Background. Epidemiological studies have suggested that low birthweight is a risk factor for the development of essential hypertension in adulthood, but the mechanism is unknown.

Methods. A rat model of intrauterine growth retardation was employed. Pregnant Sprague-Dawley rats were kept on 6% protein or on control isocaloric 20% protein diet from gestational day 12 until term. Systolic blood pressures of the offspring were monitored by the tail cuff method. Apoptosis was determined by the TUNEL method, cell proliferation by anti-Ki67 antibody, and the total number of glomeruli by the maceration method. Results are mean ± SD.

Results. The kidney and body sizes of the offspring from the low-protein pregnancies (LP) were proportionately decreased at birth. Full catch-up growth occurred during the first two weeks of life. The kidneys were normal by standard histology but exhibited increased apoptosis without increased cell proliferation at eight weeks of age. The total number of glomeruli per kidney was decreased by 28% in males ($P < 0.001$) and by 29% in females ($P < 0.01$). By eight weeks of age, both male and female LP had systolic blood pressures that were 20 to 25 mm Hg higher than those of control animals ($P < 0.001$), and their 18-month survival was significantly decreased (44 vs. 93%, $P < 0.01$). During the prehypertensive stage, at four weeks of age, PRA in LP was low (1.7 ± 1.4 vs. 19.7 ± 5.5 ng/mL/hour in males, $P < 0.0001$; 4.9 ± 2.2 vs. 14.9 ± 7.2 ng/mL/hour in females, $P < 0.0005$), and aldosterone was high (93 ± 15 vs. 54 ± 27 pg/mL in males, $P < 0.005$; 93 ± 20 vs. 48 ± 20 pg/mL in females, $P < 0.0001$). Smaller but significant differences persisted at eight weeks of age.

Conclusions. Adult blood pressure profile is susceptible to prenatal programming by maternal low-protein diet in the rat. The mechanism may involve an altered renin-aldosterone axis and a deficit in total nephron number.

The etiology of human essential hypertension is believed to be multifactorial. Because heredity can explain no more than 20 to 40% of essential hypertension [1], environmental factors have been the object of intense study for decades. Diet, smoking, physical activity, and obesity, among others, probably play a role in the development of essential hypertension. More recently, it has been suggested that prenatal nongenetic influences may predispose an individual to the development of hypertension in later life [2–4]. The evidence comes from epidemiological studies that have examined the association between birthweight and hypertension in adulthood. Most such studies have found that small birthweight is a risk factor for the development of hypertension [5–9]. The risk of hypertension does not appear to be related to prematurity but rather to low birthweight relative to gestational age [9]. The mechanisms by which small birthweight and essential hypertension may be linked are unknown; however, interestingly, the correlation between birthweight and blood pressure seems to strengthen as a person ages [7].

Animal models have been extensively used for the study of hypertension. Most are either genetic models involving selective breeding or models created by surgical or pharmacological manipulations; therefore, they are not suited for the study of the prenatal mechanisms that may predispose one to the development of essential hypertension. The goal of the current study was to examine the characteristics and mechanism of hypertension induced by fetal growth retardation in a genetically normotensive rat strain. The maneuver of prenatal maternal protein deprivation in the Sprague-Dawley rat was chosen because it has been shown to result in growth-retarded but otherwise healthy-appearing offspring [10]. The results indicate that maternal protein deprivation results in transient impairment of growth, reduced number of nephrons, alterations in renin-angiotensin-aldosterone axis, hypertension, accelerated nephrosclerosis, and shortened life span in the offspring.

METHODS

Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) were used for all experiments. Female timed-pregnant animals arrived on gestational day 10 from the vendor and were housed individually in a 12-hour light/dark cycle with free access to standard rat chow and tap water. After acclimatization, they were placed on either low-

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protein or control diet on gestational day 12 for the remainder of the pregnancy. Their food intake was not measured. The dams delivered spontaneously at term and were then immediately switched back to standard rat chow. The offspring were nursed by their mothers until weaned at four weeks of age to standard chow.

Synthetic low-protein and control rat diets were used (Purina Test Diets). The two diets were isocaloric. The low-protein diet derived 5.89% of total calories from protein, 19.79% from fat, and 74.32% from carbohydrate; the corresponding figures for the control diet were 18.87, 21.98, and 59.15%. Both diets contained equal amounts of standard vitamins and minerals.

Kidneys for standard histology as well as for the detection of apoptosis and cell proliferation were fixed in formaldehyde and embedded in paraffin; most kidneys were perfused retrograde via the abdominal aorta with 0.9% NaCl, followed by 3% glutaraldehyde before harvesting. Coronal sections of whole kidneys were processed and stained with hematoxylin and eosin. Apoptosis was determined using a colorimetric detection kit (DeadEnd; Promega, Madison, WI, USA) based on the TUNEL method. The vendor's instructions for paraffin-embedded tissue were followed. Biotinylated dUTP is incorporated to DNA at the 3′-OH ends using the enzyme terminal deoxynucleotidyl transferase (TdT), and horseradish peroxidase-labeled streptavidin is then bound to the biotinylated nucleotides, which are detected using the peroxidase substrate, hydroxen peroxide, and the stable chromogen diaminobenzidine. Apoptotic nuclei are stained dark brown. Positive controls, consisting of specimens treated with DNase to produce DNA fragments, yielded consistently strong staining of all cells. In negative controls, the TdT enzyme was omitted to prevent the end labeling of DNA fragments; the results were consistently negative. For the detection of proliferating cells, immunohistochemistry with a monoclonal antibody against nuclear antigen(s) associated with cell proliferation (anti-Ki67, clone MM1; Ventana Medical Systems, Inc., Tucson, AR, USA) was used. Antigen retrieval by microwave was achieved by 16 minutes of high power and 10 minutes of 30% power, followed by cooling for 20 minutes before incubation with the primary antibody for 32 minutes.

The total number of glomeruli per kidney was determined at the age of eight weeks by the maceration method [11, 12]. The left kidney was flushed free of blood with 0.9% NaCl, followed by perfusion with 5% Alcian blue to stain the glomeruli. The kidney was weighed, cut into small pieces in 1% NH_4Cl, and then gently agitated in 50% HCl for 90 minutes at 37°C. After slow-speed centrifugation, the supernatant was discarded, and the loose pellet containing the glomeruli was resuspended in 50 mL of water. The surpernatant was frequently examined microscopically and contained no glomeruli. The resuspended glomeruli were thoroughly mixed; twenty 20 μL aliquots were pipetted onto concave slides, and all glomeruli were counted under a microscope. The Alcian blue-stained glomeruli were easily recognized. All determinations were done by the same observer with intraindividual variation of less than 5%.

Table 1. Kidney and body weights

	Low protein	Control	*P*
Newborn	$N = 24$	$N = 22$	
Kidney weight *mg*	43 ± 4	53 ± 15	< 0.005
Body weight *g*	5.5 ± 0.5	6.5 ± 0.8	< 0.0001
Kidney weight/body weight	7.7 ± 0.7	8.0 ± 1.3	NS
14-day-old	$N = 24$	$N = 23$	
Kidney weight *mg*	295 ± 39	274 ± 46	NS
Body weight *g*	29 ± 3	29 ± 5	NS
Kidney weight/body weight	10 ± 0.4	9 ± 1.8	0.05

Systolic blood pressures were measured by the tail cuff method using a pletysmograph, varying size cuffs, and thermostatically warmed restrainers designed for rodents (Kent Scientific Corporation, Litchfield, CT, USA). The mean of four to six recordings was obtained on each occasion.

Trunk blood for chemistries, plasma renin activity (PRA), and plasma aldosterone level was obtained from the abdominal aorta of each animal under pentobarbital anesthesia before sacrifice. Blood for PRA was collected in ethylenediaminetetraacetic acid (EDTA) tube, and the PRA was measured by radioimmunoassay using a commercial kit (GammaCoat; Incstar Corporation, Stillwater, MN, USA). Aldosterone was measured in heparinized plasma by a radioimmunoassay kit (Coat-A-Coat; Diagnostic Products Corporation, Los Angeles, CA, USA).

All results are expressed as mean ± SD. Comparisons between the experimental and control groups were made using the *t* test with Welch correction, which does not require equal standard deviations.

RESULTS

The litter sizes varied from 3 to 17 pups with a tendency of the low-protein litters to have fewer pups. The offspring of the low-protein diet pregnancies (LP) appeared healthy. Their body and kidney weights were, however, 15 to 20% lower at birth than those of the control group, resulting in similar kidney weight to body weight ratios in the two groups (Table 1). By 14 days of age, the differences in both kidney weights and body weights had disappeared, and there were no differences between males and females. By six to eight weeks of age, the males weighed significantly more than the females, but no consistent differences in kidney or body weights between LP and control animals were observed after 14 days of age. At eight months of age, the LP females weighed 395 ± 21 g ($N = 16$), control females

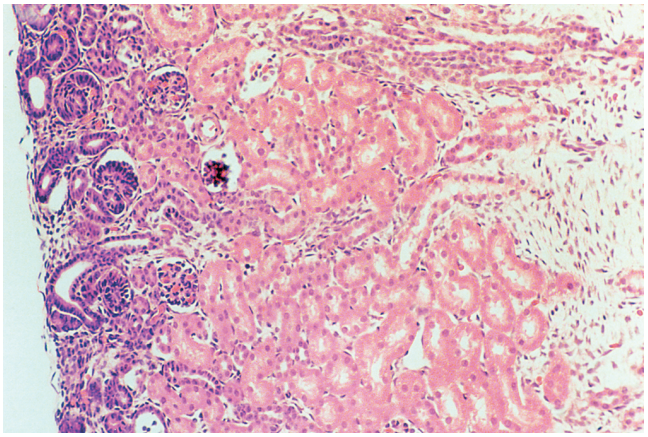

Fig. 1. Photomicrograph of a kidney from a one-day-old offspring from the low protein pregnancy (LP) group. A normal developmental pattern is present, with the outermost layer consisting of immature nephrogenic zone and primitive tubules. Small immature glomeruli are seen below the nephrogenic zone. Hematoxylin and eosin stain (magnification ×100).

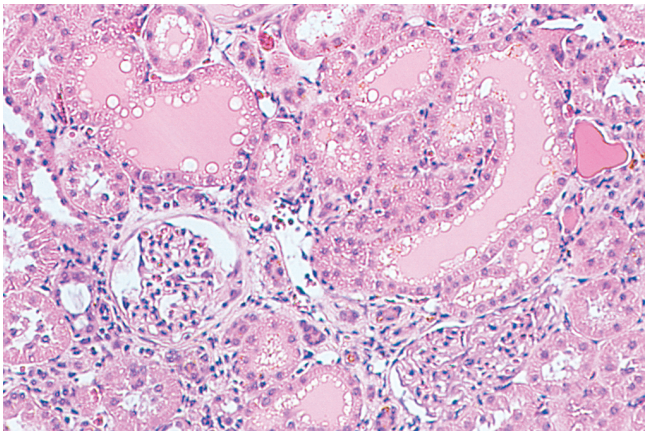

Fig. 7. Photomicrograph of kidney from an 18-month-old offspring from a low-protein pregnancy. Extensive tubular atrophy, interstitial fibrosis, and glomerular sclerosis are present (magnification ×400).

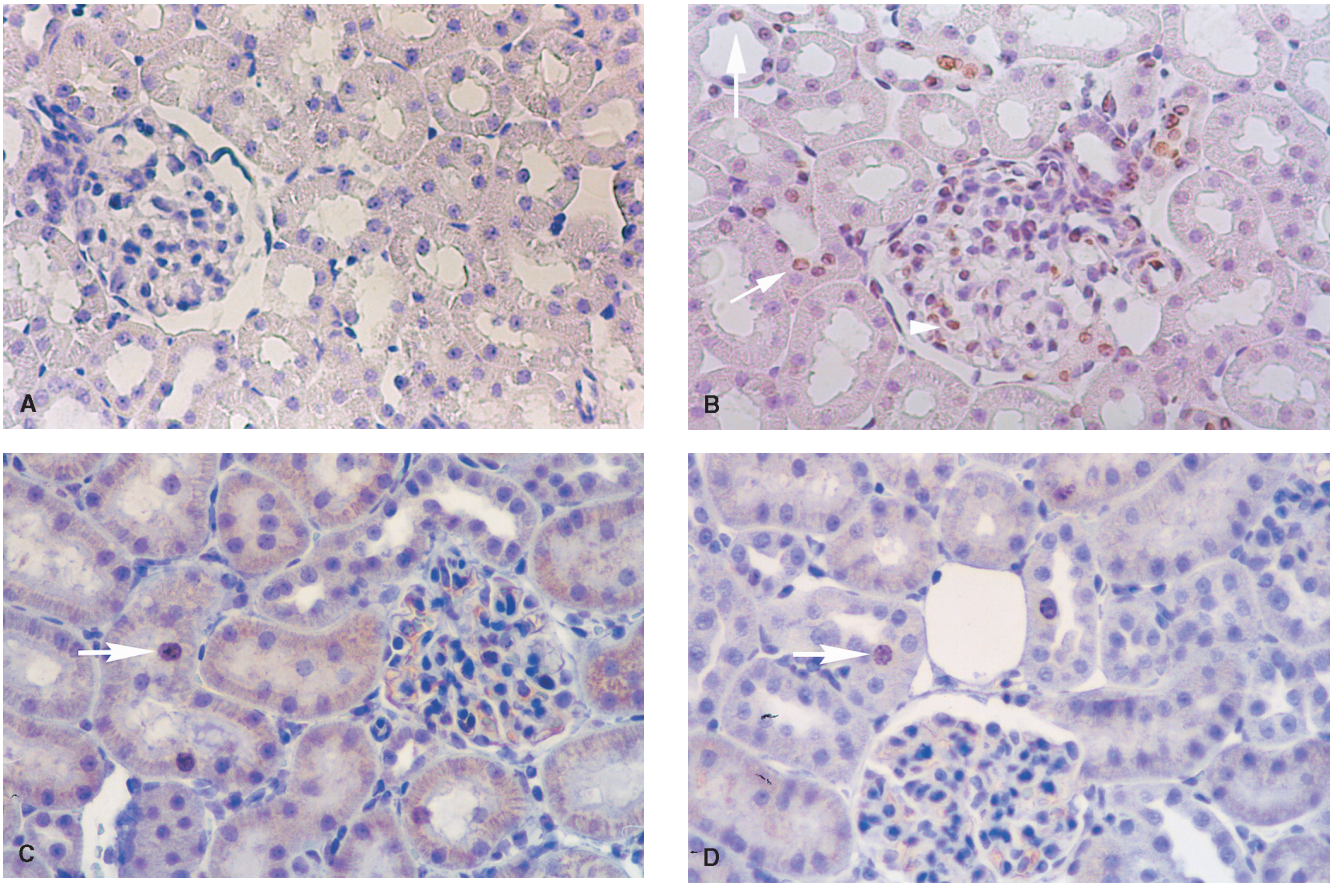

Fig. 2. Apoptosis and cell proliferation in kidneys from eight-week-old animals. No apoptosis is present in the control kidney (*A*), while the kidney from a low protein (LP) animal shows abundant apoptosis (brown stain) in glomerular (arrowhead), proximal tubule (small arrow), and distal tubule (large arrow) cells (*B*). Occasional proliferating cells (arrows) are seen in kidneys from both control (*C*) and LP (*D*) animals. (Magnification ×800).

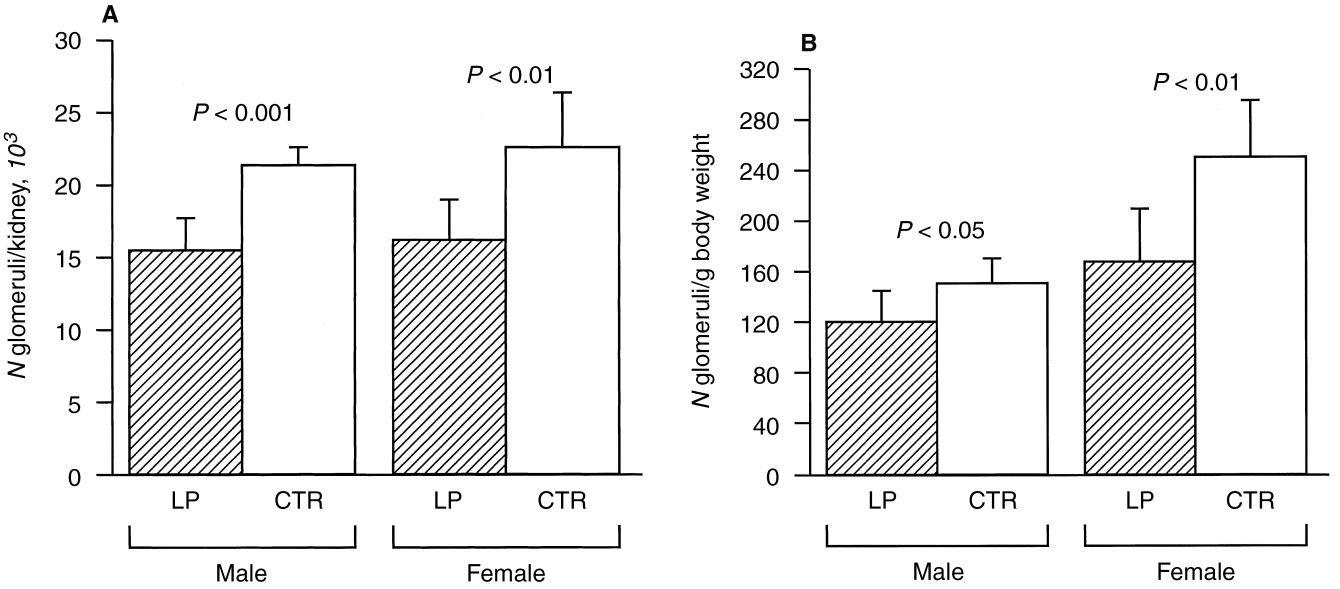

Fig. 3. Number of glomeruli in male ($N = 7$) and female ($N = 6$) offspring from low protein (LP) pregnancies and in male ($N = 7$) and female ($N = 7$) control rats (CTR) at eight weeks of age. Total number of glomeruli per kidney (*A*) and per gram of body weight (*B*) was decreased in the LP rats of each sex. Data are mean ± SD.

449 ± 19 g ($N = 20$), LP males 490 ± 15 g ($N = 17$), and control males 498 ± 12 g ($N = 13$).

The kidneys from the LP animals appeared normal by standard histology at birth (Fig. 1), at 14 days, and at 8 weeks of age. Apoptosis and cell proliferation were examined in kidneys from eight-week-old animals. An apoptotic cell was rarely seen in control kidneys (Fig. 2A), while the LP kidneys exhibited frequent apoptosis (Fig. 2B). Apoptosis was present in cortex and medulla and was not confined to any one cell type of the kidney, but was found in glomeruli, in both proximal and distal nephron cells and in interstitial cells. Occasional proliferating cells were seen in many sections of both control (Fig. 2C) and LP kidneys (Fig. 2D) without any clear differences between the two.

The number of glomeruli in the left kidney was determined at eight weeks of age, approximately six weeks after nephrogenesis was completed. At that age, small immature glomeruli were no longer present, making identification easy. The number of glomeruli per kidney was decreased in the LP groups by 28% in males and by 29% in females compared with controls (Fig. 3A). The total number of glomeruli was also related to the body size at eight weeks. Factored by body weight, the glomerular number was the highest in the female control group and the lowest in the male LP group; however, the ratio was similar in the male control group and the female LP group (Fig. 3B).

Systolic tail blood pressures were monitored beginning at age four weeks in conscious animals (Fig. 4). There were no differences between the LP and control groups of either sex at four weeks. By six and eight weeks of age, the males and the females, respectively, in the LP group had developed significantly higher pressures than the respective control animals. At two months, the mean systolic pressures in LP and control males were 130 ± 25 mm Hg ($N = 25$) and 109 ± 10 mm Hg ($N = 21$), respectively ($P < 0.001$). The corresponding pressures for females were 132 ± 26 mm Hg ($N = 24$) and 107 ± 9 mm Hg ($N = 28$, $P < 0.001$). The hypertension worsened with age in the LP groups and reached a level of 165 ± 14 mm Hg in males and 169 ± 19 mm Hg in females by age 10 months, while there was only a slight age-dependent increase in blood pressure in the control groups (Fig. 4). The blood pressure profiles were virtually identical in LP males and LP females.

At eight weeks of age, plasma creatinine concentrations were similar in all groups averaging 0.3 to 0.4 mg/dL. Plasma renin activity and plasma aldosterone levels were measured at four and eight weeks of age (Fig. 5). Before the onset of hypertension, at four weeks, PRA was strikingly low in the LP animals of both sexes, with only minimal overlap when compared with the control groups. In contrast, aldosterone levels were significantly higher in the LP groups. Statistically significant differences in both PRA and plasma aldosterone persisted after the hypertension was established, at eight weeks, but the magnitude of the difference was smaller with significant overlap between the LP and control values. In all groups, aldosterone levels decreased with age, consistent with the reported high mineralocorticoid activity during early life [13].

A subgroup of 18 LP and 14 control animals was followed for 18 months to compare the mortalities between

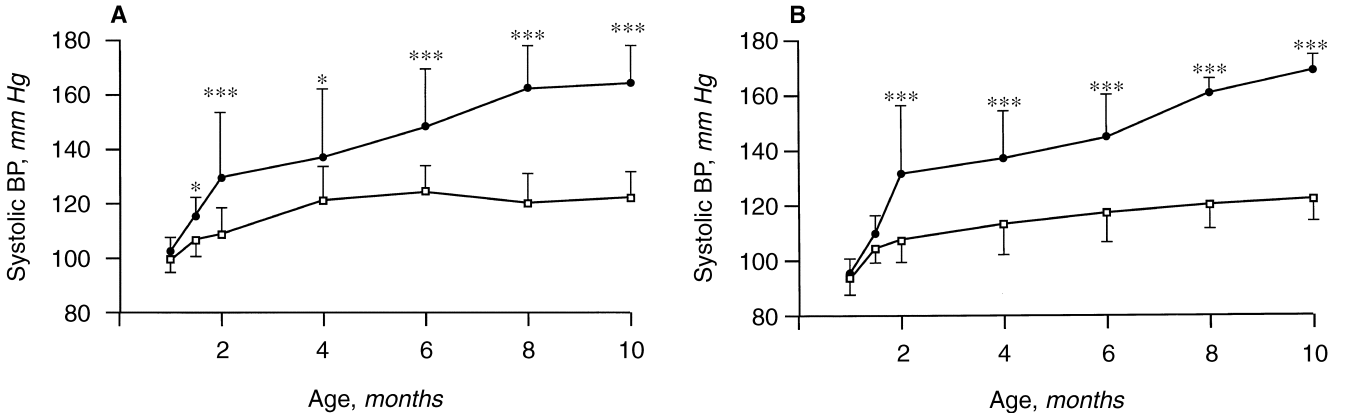

Fig. 4. Systolic blood pressures in offspring from low protein (●) and control (□) pregnancies. Both males (*A*) and females (*B*) in the low protein groups had progressively worsening hypertension beginning at six to eight weeks of age. Data are mean ± SD; $*P < 0.05$; $***P < 0.001$.

the two groups. The 18-month survival of the LP group was 44%, and that of the control group was 93% (Fig. 6). There was no significant difference in mortality between the males and females. The remaining animals were killed at 18 months to obtain renal histology. Kidneys from both groups of animals exhibited tubular atrophy and dilation, interstitial fibrosis, and glomerular sclerosis. Although no quantitative morphometric assessment was done, these changes appeared qualitatively more pronounced in the LP group (Fig. 7).

DISCUSSION

Our results show that maternal 6% protein diet during the second half of pregnancy in Sprague-Dawley rats results in offspring that develop hypertension by two months of age and have a moderately shortened life span. Both the severity of hypertension and the mortality were similar in males and females. The hypertension was associated with a modestly reduced number of nephrons, increased renal cell apoptosis, and alterations in systemic renin-angiotensin-aldosterone status.

The model of hypertension in the current study is unique because the only manipulation required for the development of hypertension is prenatal LP diet. The results convincingly demonstrate that adult blood pressure profile is, at least in the rat, susceptible to permanent programming by prenatal nongenetic factors. This is in agreement with the results of Langley-Evans et al, who used a similar dietary manipulation [14–16]. In their studies, the severity of hypertension decreased with time [14]. The current study shows a progressively worsening hypertension as the rats age, leading to premature death. The reason for the difference is not apparent, although a role for a difference in the rat strains (Sprague-Dawley vs. Wistar) cannot be excluded. In our experience, with a total of approximately 200 offspring of low-protein pregnancies, the blood pressure profiles have been remarkably reproducible in both sexes.

The mechanism by which prenatal events program future hypertension is not clear. Our results suggest that the nephron number and/or the renin-angiotensin-aldosterone axis may play a role. Previously, the most extensively used models for essential hypertension have been selectively bred rat strains with either spontaneous or salt-induced hypertension [17–19]. Several lines of evidence implicate the kidney in the pathogenesis of hypertension in these models, including cross-transplantation experiments in which a kidney from a nonhypertensive strain abolishes the hypertension in some genetically hypertensive strains [20, 21], and reports of multiple intrarenal abnormalities, such as alterations in renal hemodynamics [22, 23], in tubuloglomerular feedback [24–26], in intrarenal renin-angiotensin system [27], and in expression or activity of renal Na transporters [28–32].

If primary renal dysfunction is responsible for the development of hypertension, a likely mechanism is inappropriate renal Na retention leading to expanded extracellular volume. In the genetic rat models, the findings of increased intravascular volume during the generation of hypertension [33] and abnormal pressure-natriuresis relationship [22, 34, 35] support this concept. Experience with both experimental and human genetic abnormalities illustrates that primary abnormalities in tubular Na transport are capable of causing hypertension. Transgenic animals overexpressing the proximal tubule Na/H exchanger are prone to hypertension [36]. Mutations in the epithelial Na channel (ENaC) in Liddle's syndrome [37] and in the 11-β-hydroxysteroid dehydrogenase type 2 enzyme in the apparent mineralocorticoid excess (AME) syndrome [38] are examples of up-regulated Na transport as a cause of hypertension in humans. Whether prenatal reprogramming of renal Na reabsorption could be responsible for the low birthweight-associated hypertension requires further study.

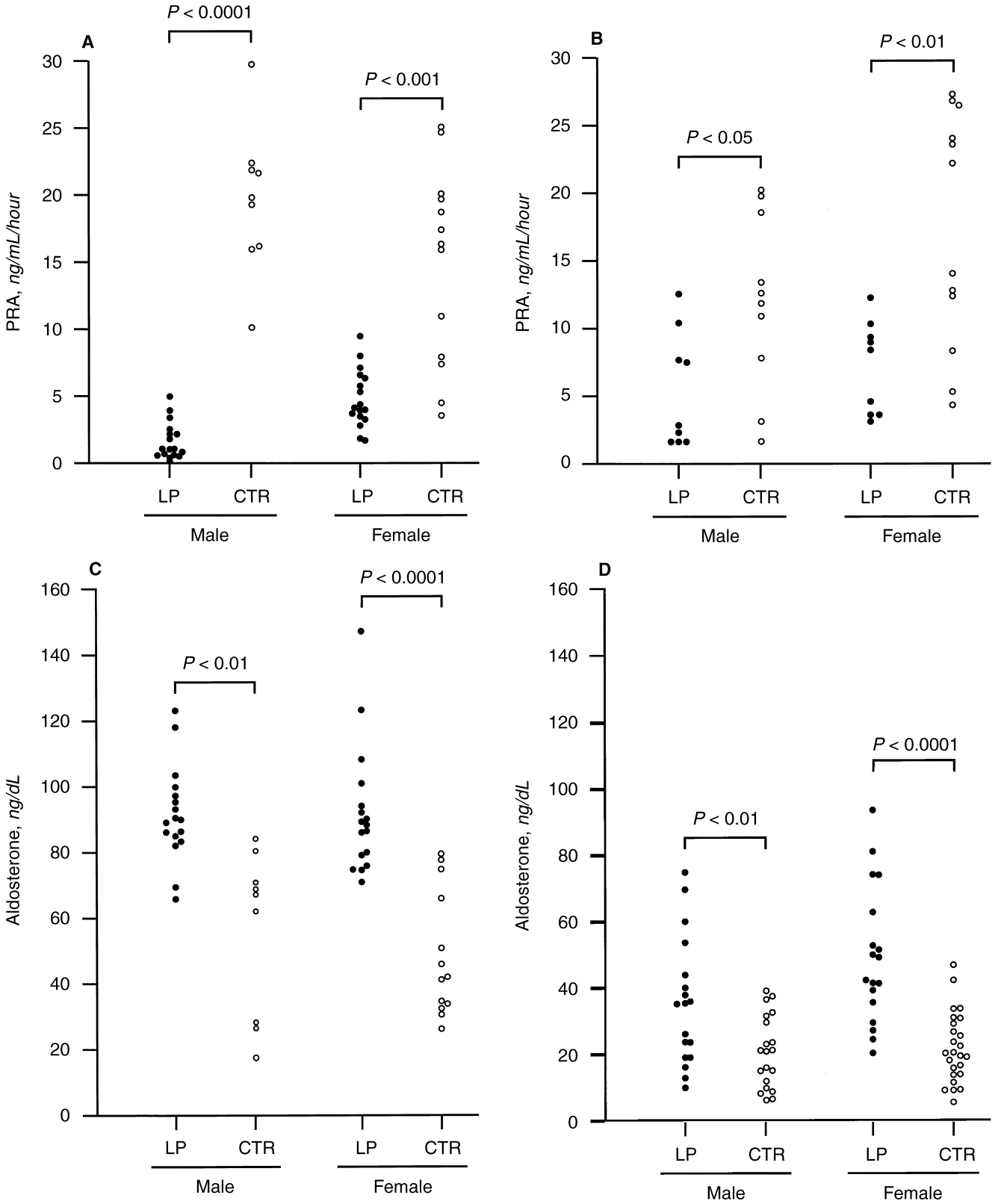

Fig. 5. Plasma renin activity (PRA) and plasma aldosterone at four and eight weeks of age. Compared with control animals (CTR), the PRA was very low in both male and female LP animals at four weeks (*A*); a smaller difference persisted at eight weeks (*B*). In contrast, plasma aldosterone was higher in the LP rats at four (*C*) and eight weeks (*D*).

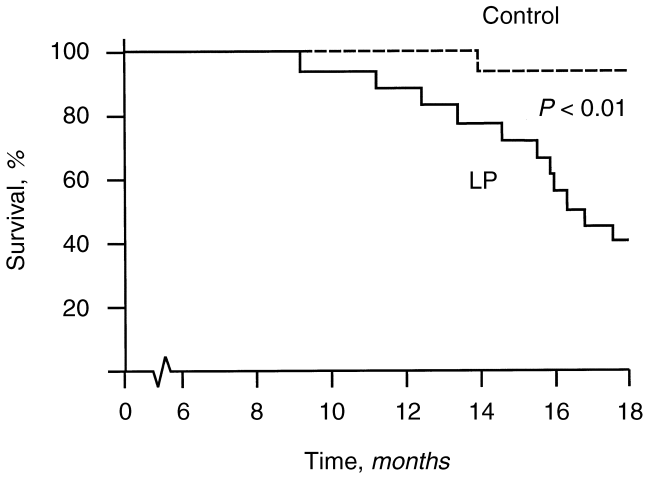

Fig. 6. Survival of the offspring from control (dashed line) and low protein (LP; solid line) pregnancies. The LP rats had a shortened life span; there was no difference between males and females.

The decreased PRA and increased plasma aldosterone concentration even before the onset of hypertension suggest a role for the renin-aldosterone axis in our model. The PRA became less discriminating after the hypertension was established, at eight weeks, in our studies (Fig. 5), and others have reported the PRA to be normal at 13 weeks of age [39]. Thus, the renin-angiotensin-aldosterone system may be important during the generation of hypertension until a new steady state is reached. Either a primary renal Na and water retention or a primary mineralocorticoid excess could be postulated; both mechanisms would lead to extracellular fluid expansion and explain the suppressed PRA. The increased plasma aldosterone concentration is consistent with a primary adrenal pathophysiology, but could also be the result non–volume-related stimuli such as the potassium balance. Determining the role of the adrenal gland in the pathogenesis will require detailed studies on adrenal function and morphology during the development of the hypertension.

A decrease in the total number of nephrons has been reported in some of the genetically hypertensive rat strains [12, 40], giving rise to the theory that low total nephron count leads to decreased filtration of Na, to increased extracellular volume, and, consequently, to development of hypertension [2–4]. Others, however, have been unable to confirm the relationship between the total nephron number and blood pressure [41, 42]. The theory has been further refined by relating the total number of glomeruli to body weight, suggesting that in hypertensive rat strains, the total filtering area relative to body size is disproportionately low [4, 12]. Our results confirm the presence of fewer nephrons compared with controls in the current model. However, we did not find a consistent relationship between hypertension and relative number of nephrons. Although the number of glomeruli per gram of body weight in hypertensive animals was lower than in control animals of the same sex, the nephron number-to-body weight ratio in hypertensive females was no lower than in nonhypertensive males at the age when hypertension first manifested (Table 1). Moreover, because new nephrons are not formed after this age, the ratio becomes more "disadvantageous" in males who gain more weight as they age, yet the control males had only a marginal increase in blood pressure with age. Thus, although reduced nephron number may play a role, it seems unlikely that reduced filtration surface area relative to body size is the sole pathophysiological factor in the development of hypertension. It is possible, however, that early reduction in filtration initiates a cascade of events, for instance, inappropriate tubuloglomerular balance and Na reabsorption, that ultimately is responsible for the generation of hypertension. Whether the observed increase in apoptosis is part of this putative sequence is unclear.

In summary, our results confirm in an experimental model that hypertension can be programmed prenatally on a nonhypertensive genetic background. They are in agreement with the epidemiological studies documenting an inverse relationship between birth weight and development of hypertension in later life [5–9]. The epidemiological correlation is relatively weak and not found in all studies [43, 44]; however, the well-known multiple other human risk factors may have masked the effect of birthweight in some of the retrospective surveys. Beyond the period of infancy, the relationship between weight and blood pressure becomes a direct one rather than inverse [9], reflecting the well-recognized risk of hypertension in obese individuals. The offspring from the protein-restricted pregnancies in our study were mildly growth retarded at birth but appeared healthy otherwise and soon caught up with their control counterparts in weight, mimicking the catch-up growth occurring in human prenatally growth-retarded infants. We did not observe any association between low birth weight and later excessive weight gain, suggesting that intrauterine growth retardation is not a risk factor for later obesity.

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Reprint requests to V. Matti Vehaskari, M.D., Department of Pediatrics, LSU Health Sciences Center, Box T8-1, 1542 Tulane Avenue, New Orleans, Louisiana 70112, USA.
E-mail: vvehas@lsuhsc.edu

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