Nutrient Restriction Impairs Nephrogenesis in a Gender-Specific Manner in the Ovine Fetus

JEFFREY S. GILBERT, STEPHEN P. FORD, A. LEE LANG, LINDSAY R. PAHL, MARK C. DRUMHILLER, SARA A. BABCOCK, PETER W. NATHANIELSZ, AND MARK J. NIJLAND

Center for the Study of Fetal Programming [J.S.G., S.P.F., A.L.L., L.R.P., M.C.D., P.W.N., M.J.N.], Departments of Zoology and Physiology [J.S.G., A.L.L., L.R.P.] Animal Science [S.P.F., M.C.D.], University of Wyoming, Laramie, Wyoming 82071; and Department of Obstetrics and Gynecology [J.S.G., S.A.B., P.W.N., M.J.N.] Center for Pregnancy and Newborn Research, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229

ABSTRACT: Inadequate nutrition compromises fetal development and poses long-term health risks for the offspring, even without decreased birth weight. The present study sought to 1) establish the ontogeny of fetal renal glomerulus number (GN) in sheep and 2) evaluate the effects of 50% global nutrient restriction (NR) during early to midgestation on GN and the renin-angiotensin system in the fetal kidney. GN increased from 78 dG (68,560 ± 3802) to 135 dG (586,118 ± 25,792). NR increased combined kidney weight (29 ± 0.6 g *versus* 23 ± 1.1 g), whereas decreased GN relative to right kidney weight approached significance in males (26,000 ± 5300 *versus* 39,000 ± 2800 GN/g) compared with control (C) males and females. NR decreased immunoreactive angiotensin II (Ang II) type 1 receptor (AT1) in the NR kidneys at 78 dG and increased renin at 135 dG. Immunoreactive renin decreased from 78 to 135 dG. Female fetuses had more immunoreactive Ang II type 2 receptor (AT2) than male fetuses at 78 dG and males had more AT1 at 135 dG. The present study demonstrates gender-specific differences in fetal growth and development and in fetal kidney development in pregnancies affected by NR. (*Pediatr Res* **61:** 42–47, 2007)

The American Kidney Foundation estimates the rates of chronic renal diseases are increasing rapidly, such that 20 million Americans (one in nine adults) have some form of chronic renal disease. Similarly, epidemiologic and animal studies showing a developmental origin for renal dysfunction are also increasing (1–3). This growing body of evidence clearly shows that the kidney is sensitive to perturbations of the fetal environment. Viewed in concert with factors in modern society that may collude to impair fetal nutrition, such as poverty and drug abuse, and clinical conditions, such as hyperemesis gravidarum, anorexia nervosa, and bulimia, it is clear that decreased nutrient delivery to the fetus will affect a large number of pregnancies across socioeconomic strata (4–6).

The acquisition of renal GN is a morphogenic process that reflects sensitivity to a suboptimal intrauterine environment (7,8). Reduced GN has been shown when fetal growth restric-

tion begins early in gestation but not when the insult is restricted to late gestation (9). Interestingly, a reduction in GN following uninephrectomy during fetal life is strongly associated with increased postnatal blood pressure (10,11), whereas adult uninephrectomy is not (12). Factors that affect nephrogenesis during fetal life therefore appear to have a greater impact on blood pressure than the hypertrophic responses induced in the adult to facilitate the maintenance of filtration surface area. In accordance with this perspective, we have recently demonstrated decreased GN associated with hypertension in the male offspring of sheep NR from early to midgestation (7). From these and other studies, it is clear that although the period in which kidney development may be affected is broad, late gestation is not a part of the critical nephrogenic window in large animals such as sheep (7,9,13,14).

Previous studies have shown the importance of the renin-angiotensin system (RAS) in fetal renal development (15,16). Several studies have shown that the intrarenal RAS of the rat neonate is perturbed as a result of maternal low-protein diets (8,17). Little work in this area has been done in long-gestation species, such as the sheep, that complete nephrogenesis *in utero*. Further, there is a paucity of data describing the ontogeny of absolute GN and protein levels of the RAS in sheep. Thus, there remains a need to characterize the ontogeny not only of GN but also of the biochemical changes that occur during renal organogenesis to gain insights into the etiology of renal dysfunction in both the fetus and the adult.

Previous work from our group has shown 50% global maternal NR between 0.19 and 0.50 gestation alters fetal ovarian, skeletal muscle, and cardiac development in sheep (7,18–20). The intent of this study was to first document the ontogeny of renal GN and the expression of key intrarenal RAS proteins in the fetal sheep from mid- to late gestation and then to place these observations in the context of our model of maternal NR.

Abbreviations: ACE, angiotensin-converting enzyme; **Ang II,** angiotensin II; **AT1,** angiotensin II type 1 receptor; **AT2,** angiotensin II type 2 receptor; **C,** control fed 28–78 dG; **CC,** control fed 28–135 dG; **dG,** days of gestational age; **GN,** glomerulus number; **NR,** nutrient restriction/restricted; **RAS,** renin-angiotensin system; **R,** NR fed 28–78 dG; **RC,** NR 28–78 dG, control fed 79–135 dG

METHODS

Animals. All experimental procedures in this study were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Wyoming Institutional Animal Care and Use Committee. Multiparous Western white-faced ewes (Rambouillet × Columbia breeding) bred naturally to the same Suffolk ram were used in this study.

Animal groupings for two different experiments are described. 1) Thirty-two pregnant ewes were selected for use in the NR study and divided into two cohorts with 16 ewes each. Age- and weight-matched ewes were placed into two study cohorts destined for study at either midgestation [78 d of gestation (dG)] or late gestation (135 dG). In the 78-dG cohort, there were two experimental groups: animals fed from 28 to 78 dG (C) and animals fed 50% of the C diet from 28 to 78 dG (R). Two experimental groups were also defined in the 135-dG cohort: animals fed from 28 to 135 dG (CC) and animals fed 50% of the C diet from 28 to 78 dG followed by re-alimentation to C from 79 to 135 dG (RC). Diets were calculated based on C = 100% of National Research Council daily rations for a pregnant ewe while assuming 150% lambing rate. The NR protocol has been described previously in detail (7,18–20). The four groups (C, R, CC, and RC) each contained ewes carrying four singleton and four twin pregnancies. One fetus from each twin pregnancy was randomly selected for study. The gender distribution of the fetuses in the C, CC, R, and RC groups at mid- and late gestation is shown in Table 1.

[2] In addition to the control animals (dG 78 and 135) from the NR study (described above) fetal kidneys were obtained from other ongoing studies at dG 90 ($n = 4$), 100 ($n = 6$), and 120 ($n = 15$) for assessment of the ontogeny of glomerulus number. On day of necropsy, pregnant ewes were euthanized as described previously (7, 18–20). Kidneys were cleansed of extraneous connective tissues and snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Acid maceration and glomerulus counting. The method of acid maceration used in the present study is an adaptation of a method published previously (7). Right kidneys were decapsulated and placed in 50% HCl at room temperature for 1–6 h (depending on kidney weight). HCl was decanted, the kidneys were rinsed with three volumes of tap water, and stored overnight at 4°C. The kidney was dispersed and brought to a final volume of 750–2000 mL of tap water (depending on kidney weight). Kidneys were coded to blind the investigator to the experimental groups to which each sample belonged. The kidney solution was thoroughly mixed, and four 250-μL aliquots were each counted in duplicate as described previously (7). Several kidneys from each age were counted in triplicate to establish an 8% coefficient of variation for the procedure.

Western blot analysis. Total soluble protein was obtained and quantified as described previously (7,21). Fifty micrograms of total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane as described previously (7,21). Membranes were blocked for 1 h to reduce nonspecific binding using 5% nonfat milk in Tris-buffered saline with 0.01% Tween (TBS-T). Immunodetection of the following proteins was accomplished as described previously (7,21), using commercially available polyclonal antibodies raised in rabbit against human peptide sequences for AT1 (306), AT2 (H-143), renin (H-105) (Santa Cruz Biotechnology; Santa Cruz, CA), or monoclonal antibody raised in mice against full length human ACE (3500; Chemicon International, Temecula, CA) and a peptide segment of human β-actin (Ab8227; Abcam, Cambridge, MA). In brief, membranes were incubated 1 h in primary antibody solutions (renin 1:400, AT1 and AT2, 1:500; ACE: 1:1000) and then washed 3 × 5 min each. Membranes were then incubated in secondary horseradish peroxidase–conjugated antibodies [AT1, AT2, and renin 1:1000 goat anti-rabbit (Santa Cruz); ACE, 1:5000 goat anti-mouse (Chemicon)] for 1 h and washed 3 × 5 min. Proteins were revealed using West Pico Supersignal (Pierce) chemiluminescent substrate and exposed to film 10–120 s as needed (Kodak Biomax).

Table 1. *Fetal morphometry at mid- and late gestation*

	C			NR		
Mid-gestation (78 dG)	M + F (8)	M (5)	F (3)	M + F (7)	M (3)	F (4)
Fetal Weight (g)	280 ± 7.8	290 ± 3.3§	250 ± 7.0	280 ± 11	300 ± 1.3§	260 ± 15
Combined Kidney Weight (KW, g)	2.9 ± 0.13	3.0 ± 0.16§	2.7 ± 0.19	2.7 ± 0.22	3.3 ± 0.19§	2.3 ± 0.15
Glomerulus Number (GN) × 10³	62.0 ± 3.8	59.0 ± 4.5	68.0 ± 6.4	58.0 ± 5.5	63.0 ± 4.4	55.0 ± 8.6
GN/Right KW × 10³	43.0 ± 2.2	39.0 ± 2.3	48.0 ± 1.3	43.0 ± 2.6	43.0 ± 4.5	44.0 ± 3.6
	CC			NRR		
Late-gestation (135 dG)	M + F (8)	M (4)	F (4)	M + F (8)	M (4)	F (4)
Fetal Weight (kg)	5.2 ± 0.2	5.3 ± 0.3	5.0 ± 0.3	5.2 ± 0.2	5.6 ± 0.2	4.8 ± 0.1
Combined Kidney Weight (KW, g)	24 ± 1.1	23 ± 1.1	24 ± 2.1	26 ± 1.1	29 ± 0.6*	23 ± 0.9
Glomerulus Number (GN) × 10³	490 ± 49	540 ± 10	450 ± 49	420 ± 60	370 ± 84	460 ± 92
GN/Right KW × 10³	40 ± 2.8	39 ± 2.8	41 ± 6.1	33 ± 5.4	26 ± 5.3**	41 ± 8.3

Fetal weight, total kidney weights, and GN during midgestation in C and 50% NR sheep fetuses at mid- (78 dG) and late (135 dG) gestation; § Different from females of same dietary group ($p < 0.05$); * Different from C group of the same sex ($p < 0.05$); ** Different from all groups at mid- and late gestation ($p < 0.05$).

β-Actin was used as a loading control, and measurement was performed after each membrane was tested for the primary protein of interest (AT1, AT2, ACE, renin). Membranes were stripped of previously applied antibodies using a commercially available buffer (Restore; Pierce). Membranes were washed 3 x 5 min and blocked for 1 h before developing for β-actin as described above.

Images were optically scanned (Hewlett-Packard 5200C with HP PrecisionScan software v2.02) and digitized, and antigen signal was quantified by pixel density (Un Scan It gel v5.1; Silk Scientific, Orem, UT). Quantification was performed only after linearity was established between the amount of protein and film exposure time.

Statistical analysis and calculations. All data are presented as mean ± SEM except where indicated. Western blot data are presented as the ratio of each protein of interest to β-actin. Kidney weight (KW) is presented as the sum of both kidneys. GN/KW was calculated per gram of right KW. Statistical comparisons were made using a *t* test, analysis of variance (ANOVA) or two-way ANOVA where appropriate with significance accepted when $p < 0.05$ (GraphPad Prism v4.00 for Windows; GraphPad Software, San Diego, CA).

RESULTS

Kidney and Fetus Morphometry

Midgestation. Across dietary groups, male fetuses were 16% heavier than female fetuses in both the C and R groups at midgestation. In accordance with increased body weight, the male fetuses also had larger kidneys than the female fetuses ($p < 0.05$). NR did not reduce either fetal weight or KW at midgestation in the present study (Table 1).

Late gestation. RC male fetuses had heavier kidneys than male C fetuses ($p < 0.05$; Table 1). Fetal weights were not different between CC and RC animals at 135 dG (Table 1).

Glomerulus Number

Ontogeny. Figure 1 illustrates the change in GN across the last half of gestation in the fetal sheep: 78 dG (0.53 of gestation), 90 dG (0.60), 100 dG (0.68), 120 dG (0.82), and 135 (0.92). No differences were observed in GN between male and female fetuses at any gestational age. Therefore, data were pooled across sex.
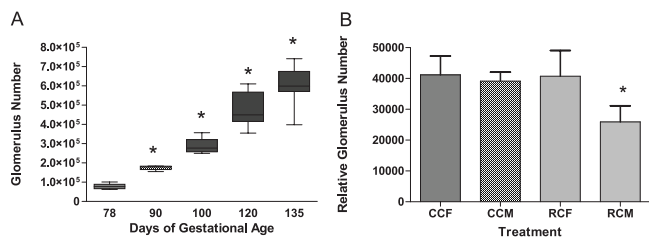

Figure 2. Ontogeny of AT1 (*A*) and AT2 (*B*) expression in male and female fetuses at mid- and late gestation. Immunoreactive AT1 (*A*) and AT2 (*B*) at 78 and 135 dG. AT1 was greater (*$p < 0.05$) in the control male 135 dG (CM135, $n = 4$) fetal kidneys than in the CM78 ($n = 5$), control female (CF78, $n = 3$) or CF 135 ($n = 4$) fetal kidneys. AT2 was greater (*$p < 0.05$) in the CF78 ($n = 3$) than the CM78 fetal kidneys. Figure depicts both observed AT2 bands (78 and 44 kD), and data represent the sum of both bands.

Effects of NR. Absolute GN and GN relative to right KW were not different between C and R groups at 78 dG, nor was any difference apparent between the sexes (Table 1). The number of glomeruli expressed relative to right KW was constant between 78 and 135 dG in all groups except the RC male, in which it was decreased ($p < 0.05$). Absolute GN was decreased 18% in the male RC kidneys compared with the male CC kidney, although this was not significant (Table 1).

Protein Expression

Ontogeny. Immunoreactive AT1 was observed as a 67-kD band and increased from 78 to 135 dG in the male fetuses but not in the female fetuses (Fig. 2*A*). Total immunoreactive AT2, observed as two bands at 44 and 78 kD, was higher in the female 78-dG fetuses than in the 78-dG male fetuses and remained unchanged at 135 dG in both male and female fetuses (Fig. 2*B*). Immunoreactive ACE was observed as a single 170-kD band and remained unchanged from 78 to 135 dG in the kidneys of both the male (0.80 ± 0.19 *versus* 0.69 ± 0.10) and female (0.93 ± 0.12 *versus* 0.53 ± 0.15) fetuses. Immunoreactive renin was observed as two bands, prorenin at 52 kD and active renin at 37 kD, and decreased from 78 to 135 dG (Fig. 3*A*).

Effects of NR. At midgestation, immunoreactive AT1 decreased in the R fetal kidney compared with C (0.17 ± 0.03 *versus* 0.31 ± 0.05; $p < 0.05$), although no difference was found when fetal gender was considered. AT2 (2.95 ± 0.46 *versus* 2.56 ± 0.26), ACE (0.85 ± 0.12 *versus* 0.69 ± 0.09),

Figure 1. Glomerulus number. Ontogeny (*A*) and effect of NR on relative GN (*B*). (*A*) GN increased between each gestational age evaluated. Data are shown as a box and whisker plot indicating the median and the first and third quartiles. (*Different from previous gestational age; $p < 0.05$, ANOVA). (*B*) 50% NR during 28–78 dG resulted in fewer glomeruli per gram of KW in the male but not female kidneys at 135 dG (*$p < 0.05$, ANOVA).
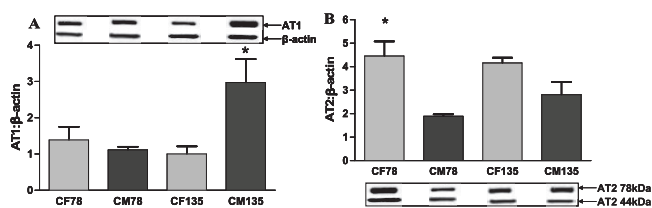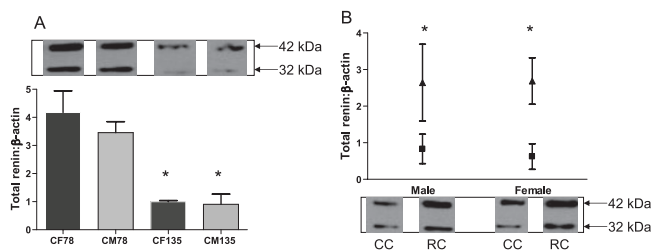

Figure 3. Renal renin immunoreactivity. Ontogeny (*A*) and effect of NR (*B*). Immunoreactive renin–to–β-actin ratio decreased from 78 to 135 dG. (*B*) Immunoreactive renin was increased in the male and female RC (▲) compared with the CC (■) fetuses at 135 dG (*$p < 0.05$). Figure depicts both observed prorenin and renin (52 and 37 kD), and data represent the sum of both bands.

and renin immunoreactivity was not different between C and R fetuses, and no gender effects was observed.

Immunoreactive renin was increased in the RC male and female fetuses at dG 135 (Fig. 3*B*). No differences were noted in immunoreactive AT1, AT2 (3.85 ± 0.65 *versus* 3.02 ± 0.33), and ACE (0.58 ± 0.09 *versus* 0.59 ± 0.09) between the CC and RC groups at 135 dG.

DISCUSSION

The present study was designed to evaluate the ontogeny of GN and the intrarenal RAS in the ovine fetal kidney and the effects of 50% global NR during early to midgestation on GN and the intrarenal RAS. We report several important findings in this study: 1) there are gender differences in fetal weight and in the ontogeny of protein expression for AT1 and AT2, a phenomenon only recently described for intrarenal gene expression in a study using murine gene arrays (22); 2) 50% global NR of the pregnant ewe from 28 to 78 dG elicited decreased AT1 at 78 dG and increased renin at 135 dG that is independent of a reduction in body weight; and 3) following re-alimentation, 135-dG male fetuses exhibit a reduction in GN (whereas female fetuses do not).

The present data indicate that GN in the sheep rapidly increases between midgestation and achieves its culmination at 0.9 of gestation. No significant increase in GN was observed between 135 dG and our previously reported measurement in wethers at 9 mo of age (7), indicating that the generation of new glomeruli has largely been completed by late gestation. These findings are in agreement with those of previous studies in the sheep (23) and the baboon (24), both species in which glomerulogenesis terminates before birth.

In the present study, we evaluated steady-state protein expression as an assessment of the molecular phenotype of the fetal kidney. Consequently, it is beyond the scope of the present study to ascribe possible transcriptional, posttranscriptional, or translational regulatory mechanisms for the gene products studied. The RAS is known to be important in renal development (25,26). Recent work has demonstrated innate gender differences in gene transcription of osmoregulatory and drug-metabolizing proteins in the kidney (22), and a previous study reported a trend toward higher AT2 and lower AT1 in fetal kidneys from female microswine (27). Moreover, investigators have held for some time that the RAS contributes significantly to the observed differences between the sexes in the progression of renal disease (28).

Presently, AT1 immunoreactivity was increased from 78 to 135 dG in male but not female fetuses. Previous studies have reported increased AT1 in late gestation, but no discrimination has been made between male and female fetuses (15,29). We show that immunoreactive AT2 decreased in the female but not male fetuses from mid- to late gestation, a finding in females that is in agreement with previous studies that have reported decreased AT2 mRNA from mid- to late gestation (15). Although immunoreactive ACE did not change from mid- to late gestation, renin decreased from 78 to 135 dG, but no gender effect was observed. A previous study reported no change in renin mRNA from 90 to 134 dG but did

show a significant decrease between late gestation and post partum (30).

Previous studies have not evaluated the effects of fetal gender on gene expression or reported numbers of male and female fetuses in the study cohorts, making retrospective comparison impossible. The mechanisms underlying the observed gender dichotomy in AT1 and AT2 remain unclear. Previous authors have recognized a gender dichotomy in the progression of renal disease and have suggested a central role for the RAS (28). The present data support this hypothesis, although further studies are needed to elucidate the sex differences in the regulation of AT1 expression.

No effect of NR was seen at midgestation on fetal or kidney weight or on GN when evaluated for gender differences or when gender data were combined. This is not unreasonable because previous studies have been equivocal on fetal weight after NR (7,18–20,31) and it appears that only 10% of the final complement of glomeruli has been attained at this point. It is recognized that a possible limitation of the methodology used in this study is a loss of immature glomeruli during sample preparation. Nevertheless, this is unlikely to have changed our findings because only a small portion of glomerulogenesis has occurred by midgestation.

The present data show that 135-dG male fetuses that were NR from early to midgestation and then re-alimented have fewer glomeruli per gram of KW than C males and females from both dietary treatment groups. Absolute GN in these animals decreased 18%. Although this reduction was not statistically significant, it is similar to previous studies with greater sample sizes reporting reduced GN from maternal NR from d 12 to term in rats (8). In contrast, a previous study that used a low-protein diet during pregnancy demonstrated similar decreases in nephron number in both male and female offspring (32). The reasons underlying the gender differences observed in fetal nephron endowment following maternal NR reported in the present study remain unclear. It is possible that there are innate differences between male and female kidneys, as recently reported by Rinn *et al.* (22), and therefore it is conceivable that male and female fetuses respond to NR differently. Further, Tamimi *et al.* (33) reported mothers of male babies eat more than mothers of females. Viewed together with the present study, it appears that the male fetuses require a higher plane of nutrition than female fetuses and may therefore be more susceptible to NR. It also appears that, in contrast to maternal low-protein diets in rats (32), the NR stress in the present study may not have been severe enough to elicit effects in both fetuses.

Several changes were observed with respect to protein expression as a consequence of NR. Immunoreactive AT1 was decreased at 78 dG, whereas renin was increased in the RC kidneys at dG 135 but not at 78 dG. Previous authors have suggested AT1 as an important mediator of branching morphogenesis in the fetal kidney (34). Others have demonstrated dysregulation of the intrarenal RAS in rat models of programmed hypertension (8,17,35,36). The present finding of reduced immunoreactive AT1 in the NR kidney is consistent with the hypothesis that NR mediates impaired nephron development *via* AT1 and Ang II signaling (37). Similarly,

disruption of Ang II signaling by ACE inhibition or AT1 antagonism both result in altered renal development (25). This finding is in agreement with a recent report by Vehaskari *et al.* (17) demonstrating decreased AT1 protein levels in 1-d-old rat pups of protein-restricted dams in an established model of *in utero* programmed hypertension. The renin increase in the late gestation kidney of the RC group is difficult to interpret at present, although it may presage the onset of hypertension that we have previous reported in the male offspring (7). Previous work has shown that renin mRNA levels are sensitive to alterations in the intrarenal kinin-kallikrein system (KKS) (38); thus, the potential for crosstalk between the KKS and the RAS cannot be ruled out in our model of NR.

A driving force for the study of the ontogeny of nephrogenesis in the fetal kidney is to gain better understanding of the critical time points in development at which perturbation of the *in utero* environment can alter the trajectory of renal development. This has implications not only for fetal NR and the teratogenic effects of substances/activities encountered by the mother during pregnancy, but also for renal development in premature infants. Preterm delivery has increased substantially over the past 10 y, increasing to as high as 10% of births in the Western world (39). Because recent advances in neonatal care have increased the chances of survival to 80% (40), understanding the effects of prematurity on fetal renal development is important and clinically relevant. The premature infant is a fetus that will finalize renal maturation *ex utero*. Although this is the norm for kidney development in species such as the rat and the pig, long-gestation species such as the sheep and the human normally complete renal development *in utero*. Clinical data indicate that neonatal mortality of neonates with acute renal failure remains quite high (30%–60%), suggesting that renal immaturity has severe physiologic consequences and that much remains unknown about the transition from intrauterine to extrauterine renal function (40).

Summary. The data reported in the present study support the hypothesis that maternal NR during early to midgestation alters the trajectory of renal organogenesis and renal maturation in the ovine fetus. These effects were observed at both the molecular and organ levels demonstrating a systemwide disruption of renal organogenesis. Decreased GN has been related to increased blood pressure in the sheep (7) and other animal models (32,37). These results show definitively that the decreased nephron number is generated *in utero* and is not a postnatal effect of elevated blood pressure (7). We also report NR during early to midgestation increased renin in the late-gestation fetal kidney, an event that may presage later hypertension in these animals. The present study demonstrates altered renal development *in utero* and in doing so provides a substrate for the future development of hypertension. These findings may also provide insight for further studies examining gender differences in the progression of renal disease.

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