

Postnatal early overnutrition causes long-term renal decline in aging male rats

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BACKGROUND: We evaluated the influence of postnatal early overnutrition on renal pathophysiological changes in aging rats.

METHODS: Three or 10 male pups per mother were assigned to either the small litter (SL) or normal litter (control) groups, respectively, during the first 21 d of life. The effects of early postnatal overnutrition were determined at 12 mo.

RESULTS: SL rats weighed more than controls between 4 d and 6 mo of age ($P < 0.05$). However, between 6 and 12 mo, body weights in both groups were not different. In the SL group, at 12 mo, systolic blood pressure was higher and creatinine clearance was lower than the same in controls ($P < 0.05$). Numbers of CD68 (ED1)-positive macrophages and apoptotic cells in renal cortex were higher in SL rats ($P < 0.05$). Furthermore, index scores for glomerulosclerosis and tubulointerstitial fibrosis were higher in the SL group ($P < 0.05$). Significantly less glomeruli per section area were found in aging SL rats ($P < 0.05$). Immunoblotting and immunohistochemistry showed decreased intrarenal renin expression in SL rats ($P < 0.05$).

CONCLUSION: Early postnatal overnutrition can potentiate structural and functional abnormalities in the aging kidney and can lead to systolic hypertension with reduced intrarenal renin activity.

The neonatal era is a critical period because the environmental conditions at this stage can permanently influence the structures and functions of various organs, including the kidney (1). The role of rapid postnatal growth on long-term consequences has recently emerged, and deleterious long-term renal effects of postnatal overnutrition and/or rapid postnatal growth have been reported (2,3).

The perinatal renin–angiotensin system (RAS) plays a key role in long-term renal physiological regulation (4). With total nephron endowment, the RAS has been shown to be involved in “fetally programmed” cardiovascular and renal diseases (5). Perinatal protein restriction in rats has been shown to (i) suppress the newborn intrarenal RAS, (ii) reduce the numbers of glomeruli, and (iii) induce glomerular enlargement and hypertension (HTN) in the adult (4). Adult offspring of dams fed a lard-rich diet during pregnancy and suckling also develop HTN,

increased aortic stiffness, blunted endothelium-dependent vasodilatation, and reduced renal renin activity (6). Notably, the RAS has been implicated in the natural aging process of the kidney (7), and inappropriate stimulation of the RAS is viewed as a potential mechanism of renal injury in obese individuals (8).

The kidneys often represent a state of chronic inflammation during aging and during many forms of chronic kidney disease, including obesity-related renal pathology (9,10). Infiltrating macrophages play critical roles during the initiation and progression of kidney aging and renal scarring (10,11). Irrespective of the initial trigger, progressive tubulointerstitial fibrosis represents a common pathway for the development of injury and progression to end-stage renal disease. Obesity also has effects consistent with accelerated, “premature” aging (12), and aging causes progressive structural and functional changes in the kidney, that is, progressive nephron loss, which eventually leads to age-related nephrosclerosis (13).

We have demonstrated that early postnatal overfeeding during the suckling period in rats results in (i) the development of hyperleptinemia and overweight, in addition to the acquired reset of key intrarenal hormone systems at 28 d of age; (ii) the upregulation of renin and angiotensin II type 2 receptor (AT2); and (iii) the downregulation of plasminogen activator inhibitor-1 and matrix metalloproteinase-9 (14). Increased cellular turnover and interventricular septal hypertrophy were also seen with increased activities of renin and AT2 in the hearts of small litter (SL) rats (15). In addition, we have recently reported that postnatal overfeeding results in persistent overweight, increased renal expression of macrophages and inflammatory cytokines, augmented cortical apoptosis and glomerulosclerosis, renal dysfunction, and systemic HTN in adult male rats (16). The goals of this study were to test the hypothesis that early postnatal overfeeding and/or accelerated neonatal growth induces long-standing overweight, HTN, and chronic renal dysfunction, in addition to identifying intrarenal molecular changes associated with aging.

RESULTS

On day 1 of life, body weights were similar in the SL and normal litter (NL) groups (NL 7.56 ± 0.05 vs. SL 7.56 ± 0.09 g). However, between 4 d and 6 mo after birth, SL rats were significantly

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heavier than NL rats ($P < 0.05$), and at 1 mo after birth, SL rats were 37.3% heavier than NL rats (NL 77.3 ± 1.7 vs. SL 123 ± 2.3 g; **Figure 1a**); at 6 mo after birth, mean body weights in the SL and NL groups were 653 ± 14 and 600 ± 13 g, respectively ($P < 0.05$). However, at more than 6 up to 12 mo, body weights were similar in the two groups. At 12 mo, mean body weights in the SL and NL groups were 838 ± 24 and 842 ± 31 g, respectively (**Figure 1b**). Furthermore, mean kidney weight/body weight ratios were also similar at 12 mo (**Table 1**). No specific events were detected during the observation period except the death of two NL rats at 7 mo because of pneumonia.

Functional Findings

At 12 mo after birth, systolic blood pressure (BP) levels were 15.1% higher in SL rats ($P < 0.05$). Serum creatinine was higher and creatinine clearance was significantly lower in the SL group than in the NL group ($P < 0.05$). The mean urinary albumin to creatinine ratio was increased in SL rats compared

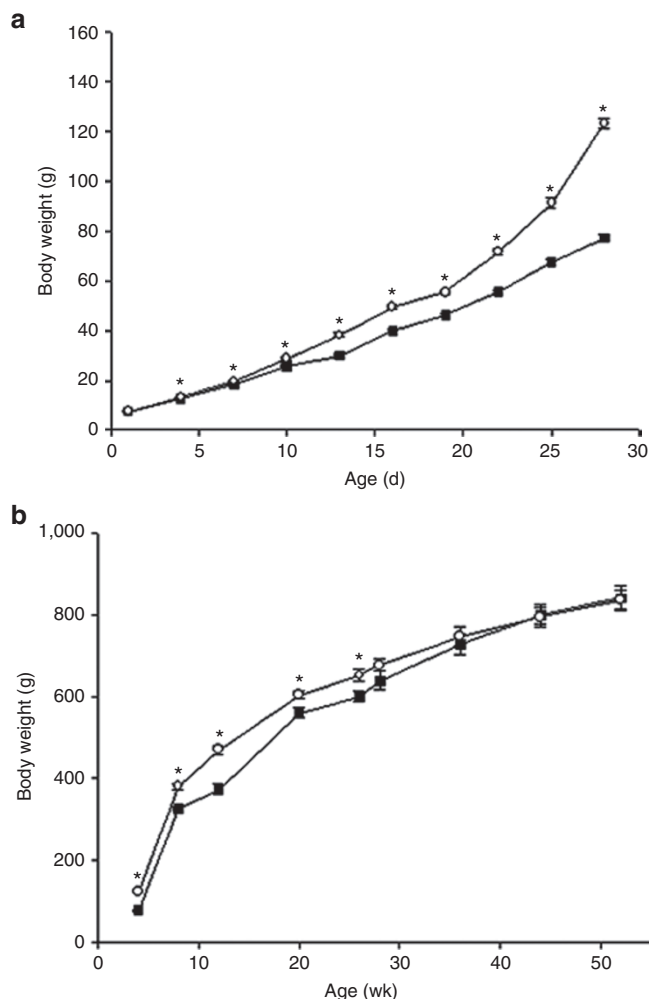


Figure 1. Body weight from (a) day 1 to day 28 of life and (b) day 28 to day 365 of life in normal litter (NL) and small litter (SL) rats. Significant overweight was observed between 4 d and 6 mo after birth in postnatally overfed rats. However, between 6 and 12 mo, body weights of both groups were not different (black squares: NL rats; white circles: SL rats; * $P < 0.05$ vs. normal litter rats).

Table 1. Body weight, kidney weight, and functional parameters

	NL	SL
Body weight (g)		
1 d	7.56 ± 0.05	7.56 ± 0.09
1 mo	77.3 ± 1.7	123 ± 2.3*
6 mo	600 ± 13	653 ± 14*
12 mo	842 ± 31	838 ± 24
Kidney weight/body weight ratio	0.004 ± 0.0002	0.003 ± 0.0002
Blood pressure (mmHg)		
Systolic	125 ± 7.4	147 ± 5.3*
Diastolic	92 ± 4.4	106 ± 4.8
Mean	102 ± 5.3	119 ± 5.0
Urine albumin/creatinine ratio (µg/mg)	380 ± 101	646 ± 72
Serum creatinine (mg/dl)	1.22 ± 0.12	2.17 ± 0.39*
Creatinine clearance (ml/min)	0.69 ± 0.08	0.3 ± 0.09*
Blood glucose (mg/dl)	121 ± 4.8	112 ± 2.1
Plasma leptin (ng/ml)	7.28 ± 0.68	7.35 ± 0.73

NL, normal litter; SL, small litter.

Data are shown as mean ± SEM. * $P < 0.05$ vs. NL rats.

with the same in NL rats, although it was not statistically significant. Blood glucose and plasma leptin levels were not different between the two groups (**Table 1**).

Cell Apoptosis, Macrophage Infiltration, Glomerulosclerosis, Tubulointerstitial Fibrosis, and Glomerular Number

Numbers of both CD68 (ED1)-positive macrophages in renal cortexes (**Figure 2a–c**) and terminal deoxynucleotidyl transferase nick-end labeling (TUNEL)-positive apoptotic cortical cells were significantly higher in SL rats at 12 mo ($P < 0.05$; **Figure 2d–f**), and SL rats had a higher average glomerulosclerosis score ($P < 0.05$; **Figure 3a**). NL rat kidneys at 12 mo showed mild segmental sclerosis in the glomeruli (**Figure 3b**), whereas the glomeruli of SL rats demonstrated diffuse global sclerosis (**Figure 3c**). Distinct tubular dilatation and some tubular atrophy were observed in the renal cortexes of SL rats (**Figure 3c**). In addition, the mean tubulointerstitial fibrosis index of the kidneys of SL rats was significantly higher at 12 mo than that of NL rats ($P < 0.05$; **Figure 3d**). Interstitial fibrosis and some periglomerular sclerosis were observed in the renal cortexes of NL rats (**Figure 3e**), but these fibrotic changes were severer; moderate tubular atrophy was also detected in the kidneys of SL rats (**Figure 3f**). The number of glomeruli per microscopic field was significantly reduced by 27.5% in the kidneys of SL rats compared with that in the kidneys of NL rats ($P < 0.05$; **Figure 4a–c**).

Cytokines

Immunoblotting showed that intrarenal renin/ β -actin protein expression was lower in SL rats than in NL rats at 12 mo after birth ($P < 0.05$; **Figure 5a**). Immunohistochemistry (IHC) showed moderate renin expression in juxtaglomerular cells and tubular epithelial cells in the kidneys of NL rats

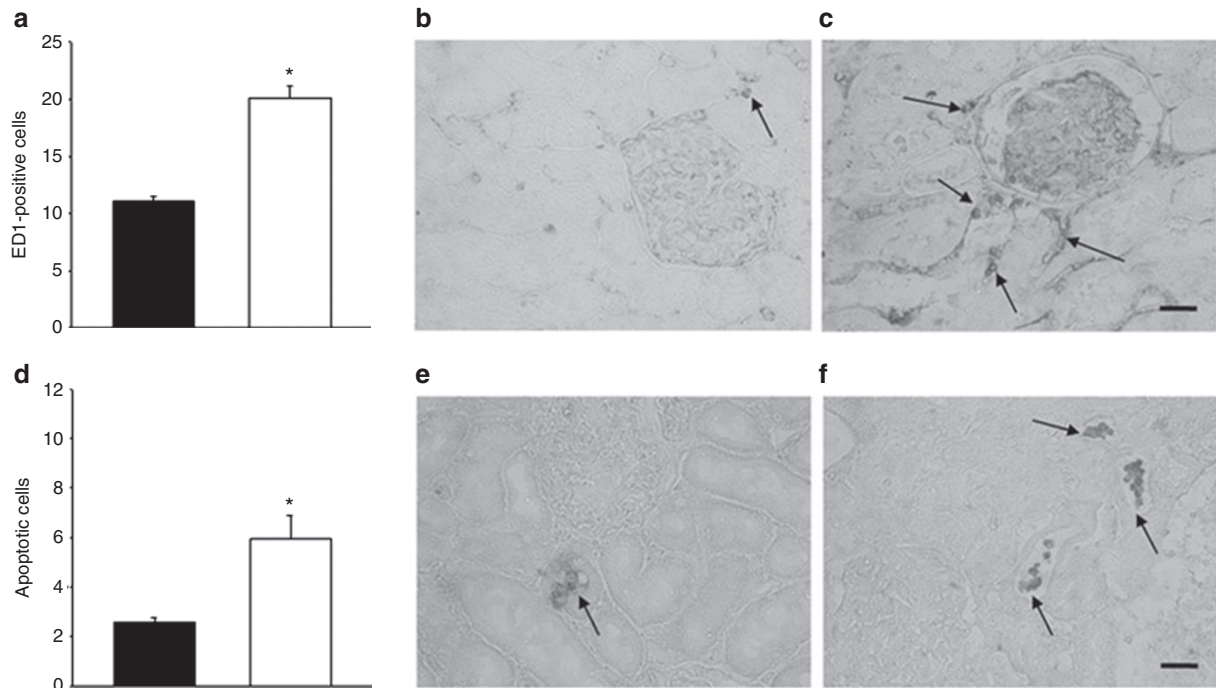


Figure 2. Intrarenal macrophage infiltration and cell apoptosis in normal litter (NL) and small litter (SL) rats at 12 months. (a–c) ED1-positive macrophages and (d–f) terminal deoxynucleotidyl transferase nick-end labeling (TUNEL)-positive apoptosis in renal cortex. Macrophage infiltration and apoptotic cell numbers were increased in SL rats at 12 mo (arrows; $n = 5$ for each group; (b,e): NL rats; (c,f): SL rats; black bars: NL rats; white bars: SL rats; $*P < 0.05$; scale bar = 100 μm , magnification = $\times 400$).

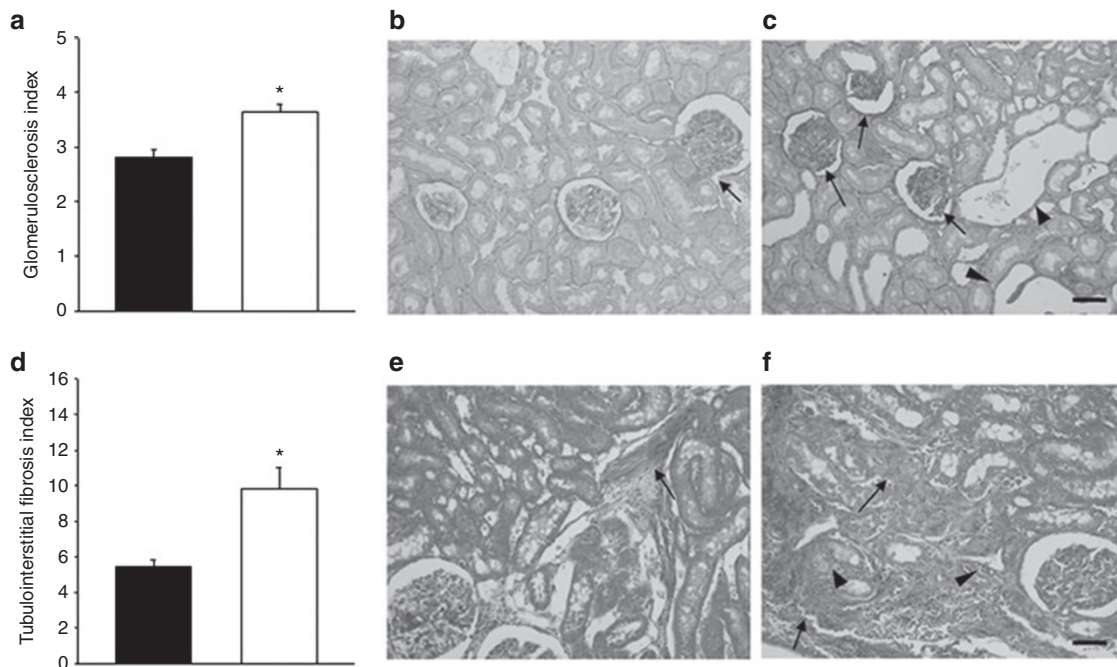


Figure 3. Glomerulosclerosis and tubulointerstitial fibrosis in normal litter (NL) and small litter (SL) rats at 12 mo. (a) The index for glomerulosclerosis and (d) the fibrotic index for tubulointerstitium were increased in SL rats compared with the same in NL rats. (b) The aging kidneys of the NL offspring showed mild focal sclerosis in the glomeruli (arrow). (c) However, the glomeruli of postnatally overfed aging rats demonstrated diffuse global sclerosis (arrows). Apparent tubular dilatation was also identified in the renal cortex of SL rats (arrowheads). (e) Normal aging kidneys showed some interstitial fibrosis and mild periglomerular sclerosis (arrow), whereas (f) the aging kidneys of neonatally overfed SL rats demonstrated more evident interstitial fibrosis (arrows) and some tubular atrophy (arrowheads; $n = 5$ per group; black bar: NL rats; white bar: SL rats; $*P < 0.05$; (b, c, e, f) scale bar = 50 μm ; magnification: $\times 200$).

(Figure 5b), whereas in SL rats, it was rarely detected in tubular cells (Figure 5c). On the other hand, immunoblots of angiotensin II type 1 receptor, AT2, matrix metalloproteinase-9,

plasminogen activator inhibitor-1, osteopontin, and tumor necrosis factor- α revealed no intergroup difference (Figure 6a–f). IHC was not performed for these cytokines.

DISCUSSION

The major findings of this study are that early postnatal overnutrition in male rats does not induce continued obesity but that it does result in systolic HTN, renal dysfunction, and increased macrophage infiltration, apoptosis, glomerulosclerosis, and tubulointerstitial fibrosis in the renal cortex during late adulthood. At 12 mo, postnatally overfed rats also had significantly less glomeruli than controls. A decrease in intrarenal renin expression was observed in neonatally overfed aging rats. This study confirms and strengthens a series of previous data (14–16) and provides novel findings concerning the harmful long-term renal effects of early postnatal overfeeding given a normal birth weight.

SL rats promptly become overweight because milk is readily available. In this study, overweight in SL rats persisted from the early postnatal period through the juvenile stage into adulthood; previous studies have reported continued overweight for periods ranging from 2 to 8 mo (2,17,18). Moreover, at 1 mo, SL rats were 37.3% heavier than NL rats and remained heavier until 6 mo after birth. Body weights became similar in the two groups over the next 6 mo. Nevertheless, systolic HTN and impaired renal function in SL rats persisted at 12 mo, following that at 6 mo of age (16).

Chronic inflammation has recently emerged to be involved in the development and progression of physiological renal

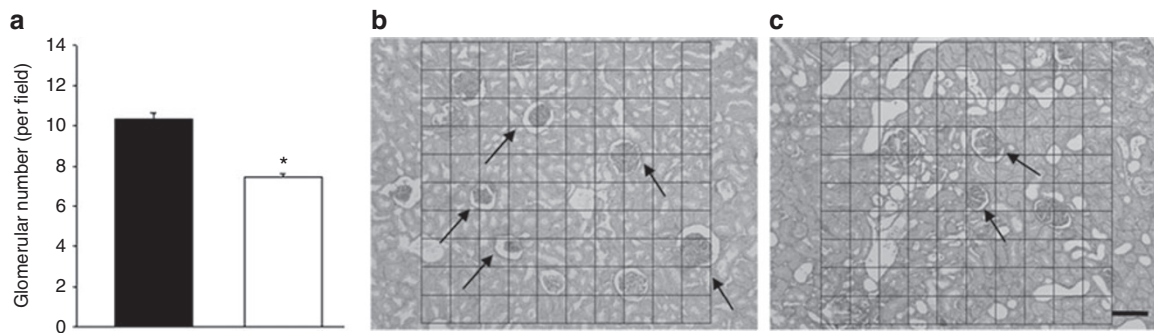


Figure 4. Glomerular number in normal litter (NL) and small litter (SL) rats at 12 months. (a) Glomerular number and photomicrographs showing a visual field from a sampled kidney section of (b) NL rats and (c) SL rats at 12 mo. Glomeruli were counted if they were contained completely or partly in the frame of 25 × 25 μm² of cortex. The aging kidneys of the SL offspring showed a significantly lower number of the glomeruli per section area (n = 5 kidneys × 5 serial sections × 20 fields for each group; black bar: NL rats; white bar: SL rats; *P < 0.05; scale bar = 25 μm; magnification: ×100).

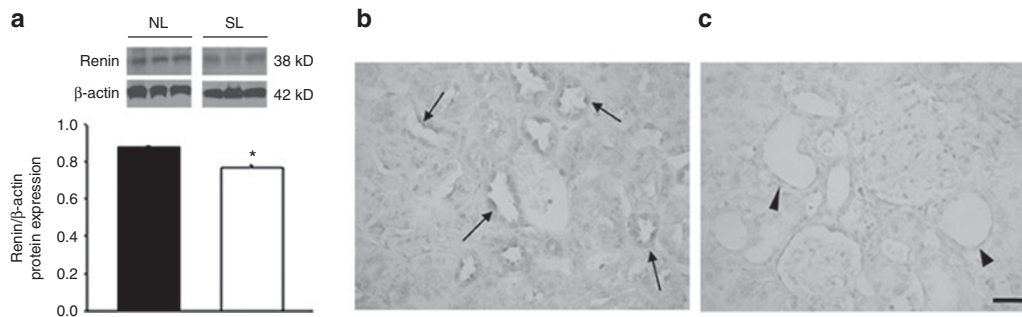


Figure 5. Intrarenal renin expression in 12-mo-old rats. (a) Immunoblots, (b) cortical expression in the normal litter (NL) group (arrows), and (c) cortical expression in the small litter (SL) group. Renin expression was significantly decreased in the SL rat kidneys compared with the same in control rats. Some tubular dilatation was also observed in the SL rat kidneys (arrowheads; n = 5 for each group; black bar: NL rats; white bar: SL rats; *P < 0.05; scale bar = 100 μm; magnification: ×400).

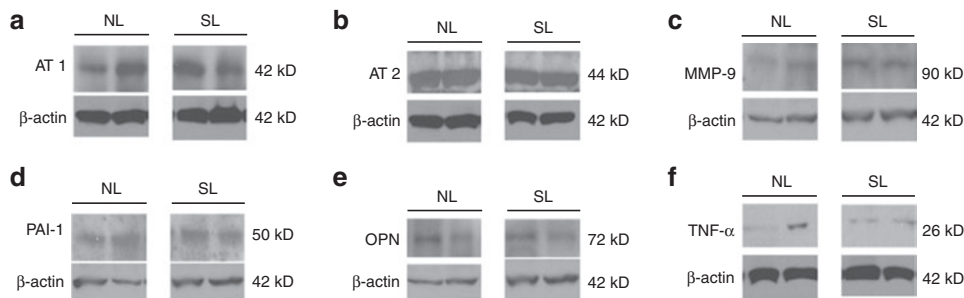


Figure 6. Intrarenal cytokine expression in 12-mo-old rats. (a) Angiotensin II type 1 receptor (AT1), (b) angiotensin II type 2 receptor (AT2), (c) matrix metalloproteinase-9 (MMP-9), (d) plasminogen activator inhibitor-1 (PAI-1), (e) osteopontin (OPN), and (f) tumor necrosis factor-α (TNF-α). There were no differences between the normal litter (NL) and small litter (SL) groups (n = 5 for each group).

aging and obesity-related renal pathology diseases (10,19). Although the cellular basis of chronic inflammation during aging has not been determined, age-related abnormalities in T cells and macrophages may contribute to the increased levels of serum inflammatory markers during aging (20). In our experimental model, SL rats showed a steady increase in macrophage infiltration into the renal cortex at 3, 6, and 12 mo after birth, and apoptotic cell numbers were also significantly higher at 6 and 12 mo, which suggests that early postnatal overfeeding enhances the progression of kidney aging. Previously, we also found the upregulation of intrarenal inflammatory cytokines in neonatally overfed 3- and 6-mo-old rats (16).

Glomerulosclerosis, tubular atrophy, interstitial fibrosis, and arterial intimal fibrosis are well-known histological findings of renal aging (21). Early postnatal overnutrition enhanced and accelerated these structural changes in aging kidneys of our experimental model. Glomerulosclerosis in neonatally overfed SL rats persisted from 3 mo to 1 y after birth (16). In this study, interstitial fibrosis, tubular dilatation, and atrophy were also greater in aging kidneys of SL rats. Possibly, increased renal workload due to postnatal overnutrition induces single-nephron glomerular hyperfiltration in the same way as a reduced nephron count does in the intrauterine growth restriction setting, and this can lead to glomerulosclerosis, progressive renal dysfunction, and HTN (22). Impairment of the glomerular capillary bed, as occurs in glomerulosclerosis, reduces peritubular perfusion and tubular oxygen supply and eventually contributes to tubulointerstitial damage (23). Regardless of the nature of any initial insult that causes renal dysfunction, tubulointerstitial fibrosis is one of the important characteristics of disease progression (24). A lower glomerular number in neonatally overfed aging rats in this study supports this theory even further.

Traditionally, low nephron number has been proposed to play a vital role in the pathogenesis of HTN in the setting of intrauterine growth restriction (25). Low birth weight with a reduced nephron number has been reported to predict later cardiovascular and renal disease, acting as a “first hit” (5). However, recent studies have shown that final nephron endowment in the rat can be profoundly affected by adequate nutrition during the later stages of nephrogenesis in the early postnatal period (2,26,27). Postnatal food restriction obtained by enlarged litter size during the suckling period (20 vs. 10 pups) was associated with glomerular number reduction in 75-d-old rats (26), whereas improved postnatal nutrition achieved by 70% reduced litter size (3 vs. 10 pups) enhanced glomerular number in 22-mo-old aging offspring (2). A 50% reduction in litter size at birth (5 vs. 10 pups) induced a nephron deficit in 22-week-old-adult male offspring (27). In our study, a 70% decrease of litter size showed a 27.5% decline in glomerular number at 12 mo of age. Regardless of dissimilar glomerular numbers, sustained HTN, glomerulosclerosis, and chronic renal dysfunction have been commonly observed in all these studies (2,27). These findings suggest that the nephron number alone can be insufficient to mediate “perinatally programmed” cardiovascular or renal disease; rather, altered nephrogenesis certainly plays a vital role in the early origins of cardiovascular and renal diseases in adulthood.

Therefore, altered early postnatal nutrition in rats can act as an important “second hit,” leading to later chronic renal dysfunction and HTN. A limitation in these studies might be that glomeruli were counted in different-aged offspring, in that nephron number may be influenced by the aging process. Glomerular counting just after the weaning period can be more valuable to confirm the effects of postnatal nutrition on the later stages of nephrogenesis in the early postnatal period.

Although early postnatal overfeeding elicits a long-term renal decline, molecular alterations induced by early overnutrition became fainter with time (14,16). In this study, we found that intrarenal renin levels were depressed in SL rats but that angiotensin II type 1 receptor, AT₂, matrix metalloproteinase-9, plasminogen activator inhibitor-1, tumor necrosis factor- α , and osteopontin expressions were unaffected. Low renin status is frequently associated with essential HTN (28), and sustained HTN in SL rats may contribute to downregulation of renin. Comparably, renal renin and Na⁺, K⁺-ATPase (adenosine triphosphatase) activity were significantly reduced in adult offspring of dams that were fed a lard-rich diet in pregnancy and suckling and these also developed HTN, increased aortic stiffness, and reduced endothelium-dependent relaxation (6). Decreased intrarenal renin activity in this study could also just be a consequence of fewer renin-secreting cells secondary to renal fibrosis.

Several studies have addressed the roles of perinatal RAS in the programming of long-term renal physiological regulation and cardiovascular risk during later life. Woods *et al.* (4) demonstrated that maternal dietary protein restriction during pregnancy leads to reduced birth weight, downregulation of the newborn intrarenal RAS, a reduction in glomeruli numbers and renal function, and an elevated BP in adulthood. Woods and Rasch (29) showed that inhibition of the RAS during the developmental period has long-standing effects on the physiological regulation of BP and renal function. We found that overnutrition during the early postnatal period results in RAS dysregulation, as indicated by elevated intrarenal and intracardiac renin and AT₂ levels during the juvenile period (14,15) and by the depressed intrarenal renin levels during late adulthood. Inappropriate RAS stimulation at 28 d of life was accompanied by altered activities of intrarenal extracellular matrix-related proteins and increased intracardiac cellular turnover along with interventricular septal hypertrophy (14,15), suggesting disturbance in renal and cardiac maturation during juvenile period. Furthermore, HTN and renal impairment progressed insidiously, which suggests that altered RAS control from the neonatal stage can malprogram long-standing renal physiological regulation and BP levels. Persistent HTN and glomerulosclerosis may aggravate tubulointerstitial damage, which can result in the acceleration of renal aging and the downregulation of intrarenal renin in aging rats.

Then, what are the implications of this study for humans? A series of our observations underline the effects of nutritional programming along the life. Early postnatal gains in weight can affect persistent overweight through the juvenile stage until adulthood, and the disease outcome, such as chronic kidney disease, HTN, and other cardiovascular risks, may be delayed

by 30–60 y. In the rat, nephrogenesis is unfinished at birth and continues into the early postnatal period. By contrast, human nephrogenesis is complete by the 34th wk of gestation. The period of vulnerability to renal injury from overnutrition may therefore extend from the third trimester of pregnancy to the first several months of life. Our study suggests that perinatal exposure to hypernutrition and rapid postnatal weight gain can have an independent significant effect in terms of susceptibility to obesity, HTN, and chronic kidney disease throughout life. Careful prospective clinical trials will be needed to identify the “optimal nutrition” in early life regarding early prevention of obesity and related renal diseases.

Some authors may say that the reliance on tail-cuff pressures remains controversial. Telemetry measurement of BP may give greater power to this study. It allows for longer periods of the measurement of BP (30). However, it requires an invasive surgical procedure that could affect renal hemodynamics and cause inflammatory molecular changes, which are the key parameters of our study. Additionally, telemetered BP measurements in conscious rats have been reported to be highly variable in a strain-dependent manner (31). The CODA tail-cuff system that we used applies volume pressure recording to measure the BP by determining the tail blood volume. Volume pressure recording tail-cuff method can provide accurate BP measurements over the physiological range of BP in animals (32). The method of glomerular counting can also be a weakness of this study. Quantification of nephron number in the kidney can be performed using several techniques (33,34). Because glomerular counting, using unbiased stereological counting methods, was unavailable, it was quantified directly on serial sections from each group. The estimates obtained could be associated with some problems, such as dimensional changes or deformation artifacts, and could have rarely provided estimates of total number; however, even though the new stereological techniques are unbiased, most of the methods are based on approximations and assumptions that are unproven and therefore possibly biased (34).

Taken together with previous observations, RAS dysregulation and resulting renal impairment may explain in part the mechanism whereby early postnatal overfeeding deteriorates physiological kidney aging in later life. Most importantly, from a clinical point of view, these findings point at the possibility of primary prevention of lifelong obesity, HTN, and renal impairment by avoiding early postnatal overnutrition and/or rapid postnatal growth.

METHODS

Animals

All experimental procedures were carried out as described previously (14–16). Twenty virgin Sprague Dawley rats were time mated with normal males at 3 mo of age. Almost all pups were delivered after 21 d of pregnancy. On day 1 of life, male pups were randomly distributed among mothers to achieve cross-fostering, and litter sizes were adjusted to 10 male newborns (NL, $n = 60$) to induce normal feeding or to 3 male newborns (SL, $n = 30$) to induce overfeeding. Litters with extremely small or large body weights of >2 SEs were excluded. Early postnatal overfeeding was induced by the reduction of litter size down to three pups per mother during the lactation period. This model is

known to induce increased weight gain during the suckling period due to higher availability of milk to each pup (14,35). Rats were weaned at 21 d, and body weights were monitored every 3 d from day 1 to day 28; and then every 7 d until killing. After weaning, rats had free access to tap water and standard chow (Purina, Cargil Agri Purina, Jeolla-do, South Korea). Rats were killed at 12 mo (pentobarbital sodium: 50 mg/kg, intraperitoneally), and kidneys were rapidly harvested. Right kidneys were used for light microscopy and IHC, and left kidneys were used for western blot analysis. All experimental procedures were approved by the Animal Experimentation Ethics Committee of Korea University Guro Hospital and conformed with Korean national guidelines for the care and handling of animals and the guidelines published by the Korean National Institute of Health.

BP Measurements

At 12 mo, systolic, diastolic, and mean BP levels were measured in nonanesthetized prewarmed rats by volume pressure recording tail-cuff plethysmography (CODA, Kent Scientific, Torrington, CT). BP measurements were performed as previously described (14,16).

Biochemical Analysis

At 12 mo, rats were placed in metabolic cages for urine collection. Urinary albumin was measured with a commercially available ELISA (enzyme-linked immunosorbent assay) kit (Alpco, Salem, MA). Urine creatinine was also determined using an ELISA kit (Assay Designs, Ann Arbor, MI), and the urine albumin levels were adjusted for urine creatinine. Cardiac blood was obtained from deeply anesthetized rats (isoflurane inhalation) at 12 mo. Blood glucose was determined automatically using a photometric glucose oxidase-peroxidase method (Optium Xceed, Abbott, IL). Serum creatinine (Luminos, Ann Arbor, MI) and plasma leptin (Assay Designs) were determined using commercial ELISA kits. Endogenous creatinine clearance was determined in 12-mo-old animals.

Cell Apoptosis, Macrophage Infiltration, Glomerulosclerosis, and Tubulointerstitial Fibrosis

To detect cellular changes, TUNEL staining was carried out as described previously (36,37). IHC was performed against the rat monocyte-specific marker CD68 (ED1; 1:100; Abdsertec, Kidlington, UK) to analyze monocyte/macrophage infiltrations. To evaluate degree of glomerulosclerosis, sections were also stained with periodic acid-Schiff reagent and scored semiquantitatively, as described by Ma et al. (38). Numbers of TUNEL-positive apoptotic cells and CD68 (ED1)-positive cells and the average scores of glomerulosclerosis were calculated as described in previous studies (14,16). To assess degrees of tubulointerstitial fibrosis, sections were stained with Masson's trichrome and semiquantitative fibrotic indexes were determined. The areas stained blue (collagen) were identified by point detection, and the average collagen fiber index in at least 20 fields of cortex per kidney section from each of five rats was determined at a magnification of $\times 400$. All histological quantification of renal injury was conducted by two different investigators who were unaware of the specimen groups.

Glomerular Counting

At 12 mo of age, the glomerular count was quantified directly on 4- μ m-thick serial sections from five kidneys of each group. The numbers of glomeruli were determined by counting 20 nonoverlapping consecutive fields ($25 \times 25 \mu\text{m}^2$ of cortex) in each of the five serial sections stained with periodic acid-Schiff reagent at $\times 100$ magnification by two different investigators who were unaware of the specimen groups. Glomerular counting of each NL and SL group was performed in 5 kidneys \times 5 serial sections \times 20 fields, respectively.

Western Blotting

Protein extraction and immunoblotting were performed as previously described (37). Membranes were first blotted for renin (dilution 1:200), angiotensin II type 1 receptor (1:200), AT2 (1:400), plasminogen activator inhibitor-1 (1:300), matrix metalloproteinase-9 (1:250), tumor necrosis factor- α (1:300), and osteopontin (1:400); all primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To control for equal loading, β -actin (1:2000; Sigma Aldrich, St. Louis, MO) and anti-mouse IgG-conjugated horseradish peroxidase (1:1000 dilution; Millipore, Temecula, CA) were used

as primary and secondary antibodies using a method identical to that described above. Developed blots were scanned using an Epson GT-9500 (Seiko, Nagano, Japan) and results were quantified using a computerized densitometer (Image PC alpha 9; National Institute of Health, Bethesda, MD). Because immunoblotting has inherent variability, the samples were run three to six times, and the mean of three independent immunoblots was used for analysis.

IHC

Five kidneys were selected for representative IHC of renin in 12-mo-old rats, using an avidin–biotin immunoperoxidase method (Vectastain ABC kit, Burlingame, CA). IHC was performed for protein directed against positive finding in the immunoblots, using paraffin sections, as described previously (36,37). Primary antibody against renin (1:400; Santa Cruz Biotechnology) was used in the study.

Statistical Analysis

Data are presented as mean \pm SEM. The significance of intergroup differences was determined using the *t*-test. Sigmapstat version 2.03 for Windows (SPSS Science, Chicago, IL) was used throughout, and significance was accepted for *P* values <0.05.

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