Impaired renal function and development in Belgrade rats

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1Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, Massachusetts; 2Department of Environmental Health, Harvard School of Public Health, Boston, Massachusetts; and 3Renal Division, Department of Medicine, Brigham and Women’s Hospital, Boston, Massachusetts

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Veuthey T, Hoffmann D, Vaidya VS, Wessling-Resnick M. Impaired renal function and development in Belgrade rats. Am J Physiol Renal Physiol 306: F333–F343, 2014. First published November 13, 2013; doi:10.1152/ajprenal.00285.2013.—Belgrade rats carry a disabling mutation in the iron transporter divalent metal transporter 1 (DMT1). Although DMT1 plays a major role in intestinal iron absorption, the transporter is also highly expressed in the kidney, where its function remains unknown. The goal of this study was to characterize renal physiology of Belgrade rats. Male Belgrade rats died prematurely with ~50% survival at 20 wk of age. Necropsy results indicated marked glomerular nephritis and chronic end-stage renal disease. By 15 wk of age, Belgrade rats displayed altered renal morphology associated with sclerosis and fibrosis. Creatinine clearance was significantly lower compared with heterozygote littermates. Urinary biomarkers of kidney injury, including albumin, fibrinogen, and kidney injury molecule-1, were significantly elevated. Pilot morphological studies suggest that nephrogenesis is delayed in Belgrade rat pups due to their low iron status and fetal growth restriction. Such defects in renal development most likely underlie the compromised renal metabolism observed in adult b/b rats. Belgrade rat kidney nonheme iron levels were not different from controls but urinary iron and transferrin levels were higher. These results further implicate an important role for the transporter in kidney function not only in iron reabsorption but also in glomerular filtration of the serum protein.

DMT1; kidney; iron deficiency anemia; renal development

IRON HOMEOSTASIS IS TIGHTLY regulated to limit both iron deficiency and overload (3, 20, 22, 73). A key player in regulation is divalent metal transporter 1 (DMT1), an iron transporter involved in intestinal absorption (19, 25). A glycine-to-arginine substitution (G185R) found in the fourth transmembrane region of DMT1 in Belgrade male rats and microcytic mk mice results in loss of DMT1 activity (18, 19). As a result, both animal models display anemia with less dietary iron absorption. Since the transporter also plays a major role in transferrin iron distribution to erythroid precursors, impaired erythropoiesis occurs leading to microcytic and hypochromic anemia (18, 57, 63).

Recently, we investigated metabolic complications displayed by the Belgrade rat (31, 32). In the course of these studies, we discovered that Belgrade rats display increased urinary glucose (31). Urinary iron excretion was also greater than control rats, and these effects were associated with glomerulosclerosis observed in Belgrade rat kidneys (31). Although DMT1 is highly expressed in the kidney (25), relatively little is known about its possible role in renal iron handling. It has been reported that a significant amount of iron is filtered by the glomerulus and that the majority of this iron is reabsorbed in renal tubules (72). Among the possible pathways involved in renal iron reabsorption, DMT1 has been proposed as one of the main transporters (16, 65). Because the serum iron-binding protein transferrin (Tf) is a ligand of cubulin, the potential reabsorption of the Fe3+-Tf complex via cubulin-mediated endocytosis has been postulated (1, 10). Neutrophil gelatinase-associated lipocalin or lipocalin 2 (LCN2) is also implicated in renal reabsorption of iron (6, 75). The aim of the present study was to more fully characterize renal metabolism and altered kidney morphology in Belgrade rats to better understand DMT1 function.

Our results demonstrate impaired renal metabolism in adult Belgrade rats in addition to renal injury. DMT1 is also expressed in placenta (21, 24), and our study shows that loss of DMT1 function in the Belgrade pups limits iron supply to the fetus in utero. During fetal growth, iron deficiency is known to affect the early stages of renal development (14). Our pilot study suggests that nephrogenesis is significantly impaired in Belgrade rat pups compared with heterozygous littermates. Defective renal development compromises renal metabolism in adult rats (14, 40, 76), thus our investigation underscores DMT1’s role in providing necessary iron for proper renal development of the kidney. Moreover, the lack of DMT1 function in older Belgrade rats is associated with increased Tf and iron in urine, implicating potential roles not only in iron reabsorption but also in glomerular filtration of the serum protein.

METHODS AND MATERIALS

Animals and diets. Animal protocols were approved by the Harvard Medical Area Animal Care and Use Committee. Belgrade rats were maintained on a 12:12-h light-dark cycle and consumed food and water ad libitum. To help relieve anemia and extend the life of Belgrade rats, mating pairs of female heterozygotes (+/−) and male Belgrade (b/b) rats were fed an iron-supplemented diet containing 500 mg ferrous sulfate iron/kg (TD 02385, Harlan Teklad) as previously described (32, 63, 64). At postnatal days 3–6, litters were cross-fostered to F344 Fischer dams (+/+; Charles River) fed standard diet containing 220 mg/kg iron (PicoLab 5053, PharmaServ). The b/b and +/− groups were verified by PCR genotyping (18) and both groups were maintained on the diet supplemented with iron (500 mg/kg). Life span of b/b male rats was recorded. Hematocrit, liver and kidney nonheme iron, and serum iron were measured as previously described (32). Serum creatinine was measured using a quantitative colorimetric assay (DICT-500, BioAssay Systems).

Urinary analysis. Rats were individually housed in metabolic cages for 24 h with free access to water and food. Urine samples were collected and the volume was measured. Urinary creatinine was assayed as described above for serum measurement. Creatinine clearance (CrCl) was calculated as Creat urine×Urine volume (ml)/Creat serum×time (min) and normalized to body weight (ml·min−1·kg−1 body wt). Albumin in urine was measured using a rat microalbumin ELISA (Kamiya Biomedical). Urinary fibrinogen (Fg) was measured...
Histology. Fresh kidneys were fixed in 10% neutrally buffered formalin (pH 7.2) and embedded in paraffin. Each 5-μm-thick kidney section was then stained with periodic acid Schiff (PAS) and Masson’s Trichrome. Briefly, so PAS staining could be performed, paraffin sections were deparaffinized, rinsed, and oxidized in 0.5% periodic acid solution. After incubation in Schiff reagent, sections were counterstained in hematoxylin. For Masson’s Trichrome, tissue sections were deparaffinized and refixed in Bouin’s solution for 1 h at 56–60°C. After being stained with Weigert’s hematoxylin and Bielich scarlet-acid fuschin, sections were treated in phosphomolybdic-phosphotungstic and finally stained with aniline blue. Images were acquired with a Zeiss Axioscope microscope equipped with Axxiovision software. A semiquantitative score for renal injury considering intensity of staining and proportion of positive staining was determined for each sample, using the multiplicative “quickscore” method described by Vaidya et al. (67). Intensity was categorized as negative = 0, weak = 1, moderate = 2, or intense = 3. This number was multiplied by the percent of tissue stained and categorized as 0 to 4%; 1; 2; 3; 4; 5; 6 to 19%; 20 to 39%; 40 to 59%; 60 to 79%; 80 to 100%; 6. Scoring was performed blinded to genotype.

Immunohistochemistry. Kidneys were fixed in 10% neutrally buffered formalin (pH 7.2) and embedded in paraffin. Sections of 5 μm were deparaffinized in xylene and rehydrated in a graded series of ethanol baths. Endogenous peroxidase activity was blocked with 3% H2O2. Incubation with goat anti-rat LCN2 antibody (AF3508 R&D System, 10 μg/ml) was carried out overnight at 4°C, followed by secondary antibody anti-goat conjugated to biotin (sc-2042 Santa Cruz Biotechnology, 1:100). Sections were incubated with streptavidin-coupled horseradish peroxidase (RPN1051 Amersham, 1:100) and then with 3,3′-diaminobenzidine tetrahydrochloride and finally counterstained with hematoxylin. Negative controls included incubation with phosphate-buffered saline without the primary antibody. The expression of LCN2 was evaluated by the same semiquantitative quickscore method described above, which considers intensity and the proportion of the area with positive immunostaining. Scoring was performed blinded to genotype.

RNA isolation and quantitative PCR analysis. Total RNA from kidneys was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. An additional step with phenol/chloroform/8-quinoilinol was used to further purify the RNA. Five micrograms of RNA were reverse transcribed using Superscript III-First Strand synthesis system (Invitrogen) and both random hexamers and oligo(dT)20 primers. Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7300 Real Time PCR System. The cDNA was diluted 1:40 and 6 μl were used as a template in 15 μl of reaction volume. The analysis was performed in triplicate for each sample. The cycles were as follows: 40 cycles 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s (36B4, IL-6); 34 cycles 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min (TNF-α); 50 cycles 95°C for 15 s, 58°C for 5 s, and 72°C for 20 s (TGF-β1). The following primers were used at a final concentration of 200 nM: IL-6 5′-AAGAGACCTCCAGCAGTTGCC-3′ (fwd), 5′-ACTGTCCTGTGGTGTTGATC-3′ (rev); TNF-α 5′-TTCCTACACACACATGGC-3′ (fwd), 5′-TGCCAGATTCAGCAATCTC-3′ (rev); 36B4 5′-AGATGACAGATCGCCAT-3′ (fwd), 5′-CTTGGCCAGCACTCCTGCA-3′ (rev). 36B4 was used as a reference gene. A dissociation curve was performed to detect nonspecific products. Calculations for relative quantification were done using the comparative Ct method (ΔΔCt).

Western blot analysis. Urinary protein content in +/b and b/b rats at 15 wk was determined by Bradford assay and 10 μg of proteins were electrophoresed on 8% gel and then transferred to nitrocellulose. The blot was incubated overnight with anti-Tf antibody (Rockland Immunocchemicals, 1:2,000). Frozen renal cortex from 15-wk-old +/b and b/b rats was homogenized in 20 mM HEPES (pH 7.4), 100 mM KC1, 85 mM sucrose, and 20 μM EGTA with protease inhibitors (Complete-mini, Roche) using a tissue tearor. The homogenate was centrifuged at 35,000 rpm for 10 min at 4°C and the supernatant was centrifuged at 95,000 rpm for 10 min at 4°C. The pellet was rinsed and resuspended with the same buffer and Triton X-100 was added to 1% final concentration. Samples were rotated for 1 h at 4°C and centrifuged at 95,000 rpm for 10 min at 4°C to obtain the soluble membrane protein preparation. Protein content was determined by Bradford assay and 80 μg of membrane proteins were electrophoresed on a 4–15% gradient gel and transferred to a nitrocellulose membrane overnight. The blot was incubated 1 h with anti-cubilin antibody (sc-20609, Santa Cruz Biotechnology, 1:200) or anti-Tf receptor-1 antibody (TIR1; 13– 6800, Invitrogen, 1:500). Protein signals were visualized with secondary IR Dye 800 donkey anti-goat and anti-mouse antibody (Li-Cor, Lincoln, NE) using a Li-Cor Odyssey infrared imaging system. The intensity of bands was quantified with Odyssey infrared imaging system.

Pilot study of nephrogenesis. At postnatal day 9 (PN9) total body iron content was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima DV 2000, PerkinElmer). Radial glomerular count (RGCs) was utilized to estimate the number of glomerular generations formed within the kidney. This method has been validated by Hinchliffe et al. (27) and utilized by several different groups (14, 52, 61, 62). Although this method has limitations, in this pilot study it provides an estimate of the number of glomerular generations that radiate from the center of the kidney with increasing maturation.

In the present study, the RGCs were obtained by counting the layers of glomeruli formed from the corticomedullary junction to the renal capsule on midlongitudinal tissue sections from X 100 photomicrograph fields. Generations were identified by drawing parallel lines perpendicular to a medullary ray starting at the first glomerulus seen at the corticomedullary junction, and ensuring every glomerulus traverses a line. In circumstances where clear medullary rays were not observed, a straight line was drawn from the corticomedullary junction to the capsule and glomeruli along the line were counted. To standardize the RGC, the counting was performed in both kidneys from three +/b rats and three b/b rats, analyzing two tissue sections from each right and left kidney, and counting eight different fields of each section to determine an average number of glomerular generations. In sum, 16 fields of view were analyzed from the left and right kidney of each animal. To obtain similar sections and analyze similar regions in both kidneys and in all the animals, a longitudinal cut was performed in each kidney in the middle of the organ. Thus, the sections sampled in all cases correspond to the central zone of the organ.

Statistical analysis. Reported values are expressed as means ± SE. Statistical significance was evaluated by two-tailed Student’s t-test. Differences between +/b and b/b rats were considered significant at P < 0.05.

RESULTS

Characteristics of b/b rats. Hematological and physiological parameters of Belgrade (b/b) rats were evaluated at 6, 10, and 15 wk of age (Table 1). Body weight and hematocrit were significantly lower in b/b rats compared with +/b rats at 6, 10, and 15 wk. Serum iron of b/b rats was higher compared with +/b at all ages studied but liver nonheme iron (NHI) was significantly higher in b/b only at 6 wk. These findings are similar to those previously reported (23, 57, 63). Notably, there were no differences detected in kidney NHI levels between b/b and +/b.
rats at all ages we studied. Garrick et al. (23) also observed no differences in kidney tissue iron.

**Belgrade rats have reduced life span.** Homozygous b/b rats displayed an abrupt decrease in survival between 15 and 25 wk of age, showing premature death with ~50% survival by 20 wk (Fig. 1A). Upon necropsy of 20-wk-old rats, it was recognized that the size of b/b kidneys was much greater than age-matched +/b rats (Fig. 1B). Belgrade rat kidneys were paler than control +/b rats, most likely due to the anemic state of the b/b group. In veterinarian-documented necropsies, marked glomerular nephritis and chronic end-stage renal disease were noted.

**Histological findings.** Kidney sections of 15-wk-old b/b rats stained with PAS exhibited disorganization in the renal cortex with an ill-defined corticomedullary junction, glomerulosclerosis, and interstitial sclerosis (Fig. 2, VI and IX). Previous studies by Ferguson et al. (17) also noted morphological differences between b/b and +/b rats. Our histological analysis also showed tubular dilation with flattened epithelium in some cortical tubules while others had occlusion of the luminal space (Fig. 2IX). In addition, Masson’s trichrome staining of 15-wk-old samples showed severe fibrosis in glomeruli of b/b rats accompanied by areas of tubulointerstitial fibrosis (Fig. 3, VI and IX). A semiquantitative numeric score confirmed significant renal fibrosis and sclerosis in homozygous Belgrade rats (Table 2). Similar histological findings were observed in b/b rats at 6 and 10 wk of age, although the fibrosis and sclerosis were less severe (Figs. 2 and 3IV–VIII). Moreover, kidney weight as a fraction of body weight was statistically higher in b/b rats than in control +/b rats above 10 wk of age (Table 2). Normal morphology and absence of fibrosis and sclerosis were noted in kidneys of +/b rats at all ages studied (Figs. 2 and 3I–III). Both groups of rats were fed the same iron-supplemented diet, indicating that defects observed were due to genotype.

**Renal function.** Renal function of Belgrade rats was evaluated for each age group. Serum creatinine and 24-h urine volumes were similar in b/b and +/b rats at 6, 10, and 15 wk (Figs. 4A and 5A). However, creatinine clearance was significantly reduced in b/b rats at all ages studied, suggesting a decrease in the glomerular filtration rate (GFR; Fig. 4B). The b/b rats also displayed increased albumin excretion that became more severe with age (Fig. 5B).

**Urinary biomarkers.** Urinary creatinine measurements showed significantly lower levels at 6 and 10 wk of age in b/b rats compared with age-matched controls (Fig. 6A). A significant increase in urinary Fg was observed in b/b rats compared with +/b rats at all ages studied (Fig. 6B). Moreover, Kim-1 levels were higher in Belgrade rats with significant differences observed at 6 and 15 wk of age (Fig. 6C). Kim-1 was measured using a previously developed microbead-based assay that has a dynamic range from 4 to 40,000 pg/ml. Other advantages of this assay include the ability to quantitate Kim-1 using only 30 μl of undiluted urine samples and while maintaining the intra- and interassay variability to <10% (58). For the experiment shown CV% = 5.22%, therefore variability most likely reflects phenotypic differences between individual rats.

**Renal LCN2.** Immunohistochemical detection of LCN2 in kidney at 15 wk showed cortical expression in control +/b rats, with intracellular localization in tubules with morphology of proximal and distal tubules (Fig. 7A, I and II). Kidneys from b/b rats also showed a similar staining pattern but in addition, strong LCN2 expression was observed in all abnormal cortical tubules, including those with flattened epithelium and those with decreased lumen (Fig. 7A, III and IV). Scoring the expression of LCN2 showed that both intensity and the proportion of the staining were stronger in b/b rats, with a slightly higher expression in the kidney of b/b rats compared with control +/b (Fig. 7B).

### Table 1. General characterization of +/b and b/b rats

<table>
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<tr>
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<th>6 wk</th>
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<th>10 wk</th>
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<th>15 wk</th>
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<tr>
<td></td>
<td>+/b</td>
<td></td>
<td>b/b</td>
<td></td>
<td>+/b</td>
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<tr>
<td>Body wt, g</td>
<td>140.2 ± 6.7</td>
<td>5</td>
<td>119.2 ± 4.9*</td>
<td>5</td>
<td>233.0 ± 19.0</td>
<td>5</td>
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<tr>
<td>Hematocrit, %</td>
<td>40.2 ± 0.5</td>
<td>5</td>
<td>29.2 ± 2.3†</td>
<td>5</td>
<td>42.5 ± 0.6</td>
<td>5</td>
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<tr>
<td>Serum iron, μg/ml</td>
<td>3.19 ± 0.37</td>
<td>5</td>
<td>4.92 ± 0.27†</td>
<td>5</td>
<td>2.47 ± 0.65</td>
<td>5</td>
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<tr>
<td>Liver NHL, μg/g</td>
<td>121.62 ± 26.29</td>
<td>5</td>
<td>210.68 ± 26.24*</td>
<td>5</td>
<td>76.22 ± 4.91</td>
<td>5</td>
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<tr>
<td>Kidney NHL, μg/g</td>
<td>17.19 ± 4.58</td>
<td>5</td>
<td>15.43 ± 3.55</td>
<td>5</td>
<td>52.48 ± 6.63</td>
<td>5</td>
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Data are means ± SE. Body weight and iron status were evaluated at 6, 10, and 15 wk in +/b and b/b rats. NHL, nonheme iron. *P < 0.05; †P < 0.01.
Renal tissue injury and inflammation biomarkers. Quantitative PCR was performed to identify changes in renal expression of genes known to be involved in renal injury. For this study, tissue from 15-wk-old rats was examined for expression levels of TGF-β1, TNF-α, and IL-6 mRNA. An increase was only observed for TGF-β1 in b/b rats compared with +/b rats (Fig. 8, A–C).

Urinary iron. Belgrade b/b rats displayed greater urinary iron excretion than +/b rats at 10 and 15 wk of age (Fig. 9A). Immunoblot experiments showed that urinary Tf was higher in 15-wk-old b/b rats compared with +/b rats (Fig. 9B). Total protein content in urine was also higher in b/b rats (data not shown), making it difficult to establish a proper loading control for immunoblotted samples. Therefore, we compared the fold increase of urinary Tf with the fold increase of urinary albumin measured by ELISA assay in 10 μg of total urinary protein from each group (Fig. 9C). Based on this analysis, a 48.5-fold increase was observed in urinary Tf while a 3.1-fold increase in urinary albumin was found in 15-wk-old b/b rats compared with +/b rats.

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The elevated urinary Tf levels led us to evaluate renal expression of cubilin, which is thought to be involved in tubular Fe\textsuperscript{3+}-Tf reabsorption. Immunoblot analysis indicated that cubilin protein levels in kidney membrane preparations from +/b and b/b rats at 15 wk were not significantly different (Fig. 9D). Moreover, immunoblot analysis of Tf receptor levels also did not indicate any difference between the two groups (data not shown). Since the Tf receptor is regulated by tissue iron levels (47), this observation is consistent with NHI measurements for +/b and b/b rat kidneys, which were similar (Table 1).

Pilot study of nephrogenesis. Body weight at birth, hematocrit, and total body iron in PND9 b/b pups were much lower than age-matched +/b littermates (Fig. 10A). Previous studies have suggested an association between human fetal growth restriction and decreased tissue iron content (9), and also between intrauterine growth restriction and abnormal renal development (42, 45). During kidney development, glomerular generations show radial distribution with the more immature nephrons located below the capsule and mature nephrons located close the renal medulla (14). In a pilot study, a RGC was performed in kidneys of heterozygous and homozygous PND9 pups (Fig. 10B). The determination of RGCs showed 10 glomerular generations in +/b rats while b/b rats displayed a 25% decrease in RGCs (Fig. 10C).

Table 2. Kidney weight and scoring of histological alterations in +/b and b/b rats

<table>
<thead>
<tr>
<th></th>
<th>6 wk +/b</th>
<th>6 wk b/b</th>
<th>10 wk +/b</th>
<th>10 wk b/b</th>
<th>15 wk +/b</th>
<th>15 wk b/b</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>Sclerosis</td>
<td>0.03 ± 0.02</td>
<td>0.25 ± 0.12</td>
<td>0.10 ± 0.02</td>
<td>0.20 ± 0.04</td>
<td>0.22 ± 0.09</td>
<td>4.28 ± 0.45*</td>
<td>3</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.20 ± 0.03</td>
<td>1.35 ± 0.16*</td>
<td>0.10 ± 0.01</td>
<td>0.88 ± 0.09*</td>
<td>0.15 ± 0.04</td>
<td>5.30 ± 0.65*</td>
<td>3</td>
</tr>
<tr>
<td>Kidney wt, % body wt</td>
<td>0.48 ± 0.28</td>
<td>0.47 ± 0.27</td>
<td>0.33 ± 0.01</td>
<td>0.43 ± 0.03*</td>
<td>0.32 ± 0.01</td>
<td>0.84 ± 0.16*</td>
<td>3</td>
</tr>
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Data are means ± SE. The degree of sclerosis and fibrosis was evaluated by a numeric score in +/b and b/b rats at 6, 10, and 15 wk. Kidney weight as a percentage of body weight was measured. *P < 0.05.
Compared with the strain background F344 Fischer rat, which has a mean lifetime of 92–108 wk (43, 59), male Belgrade rats have 50% survival at 20 wk. Veterinarian-documented necropsies of 22-wk-old rats indicated marked glomerular nephritis and chronic end-stage renal disease, with blood urea nitrogen as high as 80 mg/dl, much higher than values reported in normal Fischer, Wistar, and Sprague-Dawley rats (4, 33, 36). This genetic model has several unique pathophysiological features that could lead to premature death, including dyslipidemia (32), severe anemia (18, 58), and liver iron overload (63). The glomerulosclerosis and fibrosis observed in Belgrade rats suggest that renal defects further contribute to their shortened life span. The predominant peritubular distribution of the interstitial fibrosis found in b/b rats is characteristic of chronic kidney disease, suggesting that injured tubular cells may release fibrogenic signals to cortical fibroblasts (69). Increased levels of TGF-β1 mRNA found in b/b rat kidneys confirm ongoing fibrosis. Overall, the renal injury score observed in b/b rats from 6 to 15 wk indicates progressive decline in kidney function with age.

In the last few years, several reliable and sensitive early biomarkers of renal injury have been discovered (26, 50, 66). Fg levels are known to increase after renal damage (35), and our results suggest that tubular damage may be occurring as early as 6 wk of age. Kim-1 is a transmembrane protein highly expressed in dedifferentiated proximal tubule epithelial cells after injury, with the soluble form of cleaved Kim-1 present in urine after kidney damage (29, 66). The greater levels of urinary Kim-1 observed in b/b rats further support tubular renal injury. The predominant peritubular distribution of the interstitial fibrosis found in b/b rats is characteristic of chronic kidney disease, suggesting that injured tubular cells may release fibrogenic signals to cortical fibroblasts (69). Increased levels of TGF-β1 mRNA found in b/b rat kidneys confirm ongoing fibrosis. Overall, the renal injury score observed in b/b rats from 6 to 15 wk indicates progressive decline in kidney function with age.

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injury from 6 wk of age. LCN2, which is known for its interaction with iron (6), has also been reported as a biomarker of renal injury. The intracellular expression of LCN2 and its localization in proximal and distal tubules observed by immunohistochemistry in b/b rats are similar to that reported in nephrectomized mice and in rats with kidney injury (51, 56, 70). Semiquantitative scoring of the immunohistochemistry data suggests that chronic renal damage could induce LCN2 expression by injured renal cells (6).

Fig. 8. mRNA levels of transforming growth factor (TGF)-β1, TNF-α, and IL-6 in kidney of +/+ and b/b rats at 15 wk. TGF-β1 mRNA levels were measured in renal tissue of +/+ and b/b rats by quantitative PCR as described in METHODS AND MATERIALS (A). mRNA levels of TNF-α (B) and IL-6 (C) were also measured in kidney tissue. qPCR data are presented as relative to 36B4. Samples for this study were taken from the 15-wk-old cohort in Figs. 5 and 6. Data are means ± SE for control +/+ (open bars) and b/b rats (filled bars). *P < 0.05, n = 5.

Fig. 9. Urinary iron levels, urinary transferrin (Tf), and renal cubilin in +/+ and b/b rats. Urinary iron excretion (24 h) was measured in +/+ and b/b rats at 6, 10, and 15 wk; samples were taken from a subset of rats used in the longitudinal study in Figs. 5 and 6 (A). Urinary Tf was determined by Western blot analysis of samples from +/+ and b/b rats at 15 wk (B). Fold increase of Tf and albumin (Alb) was compared. Tf levels were determined in B and albumin levels were determined in equivalent 10-μl urine samples from b/b and +/+ using ELISA (C). Renal cubilin protein levels were determined by immunoblot analysis in cortical membrane preparations from 15-wk-old +/+ and b/b rats. D: data are means ± SE. *P < 0.05, n = 4.
Serum creatinine levels in b/b rats and /H11001 rats were similar, concordant with observations by Ferguson et al. (17). The fact that this parameter was unaffected despite the morphological changes is not surprising because it is known that serum creatinine only rises when a severe renal injury occurs and more than 50% of the nephrons are damaged (11, 49). Levey (38) discusses patient cases with similar serum creatinine values but markedly different levels of renal function. Serum creatinine fails to reflect kidney function at all stages of kidney diseases because the inverse relation between serum creatinine and GFR is nonlinear (39). When renal function starts to decline, relatively large changes in GFR (~30% decrease from the normal value) only produce small changes in serum creatinine. One way to estimate GFR is by CrCl (48, 53). The reliability of this parameter lies in the linear correlation between creatinine and inulin clearance (5, 13, 60). Significantly decreased CrCl in b/b rats at all ages compared with /H11001 rats suggests decreased GFR. It is important to note that the inverse correlation between serum creatinine and CrCl is also nonlinear, which explains the decline in CrCl seen in b/b rats even without changes in serum creatinine (13, 16).

Although urine output is usually low in end-stage renal disease, the absence of changes observed between b/b and /H11001 rats is comparable with other studies that have reported normal urine volume and normal urinalysis in nonterminal stages of renal failure (8, 41). Moreover, because urine volume does not only depend on GFR but also can be affected by tubular function, urine output has been described as a nonspecific parameter to evaluate kidney injury (37). We did find highly elevated levels of urinary albumin in b/b rats compared with control /H11001 rats at 6, 10, and 15 wk, suggesting damage in the glomerular membrane (54) and progressive interstitial fibrosis and tubular damage (15, 30, 71) leading to decreased albumin reabsorption, contributing to even greater urinary albumin (12, 74).

To better understand the origins of kidney defects in the Belgrade rat, we studied early development. Placental DMT1 expression has been reported (21, 24), and therefore we examined whether DMT1 deficiency limited iron supply in utero. We found that hematocrit values and total body iron were lower in b/b rat pups at PND9 compared with littermate /H11001 controls. Since rat renal development begins in the embryo and continues in the first 7–10 days postnatally (40, 44), we undertook a pilot study of nephrogenesis at PND9 using the RGC method to evaluate the potential effect of limited iron availability on Belgrade renal development. The decrease in glomerular generations observed in b/b rats compared with /H11001 rats supports the idea that due to limited iron supply during
early life, renal development is affected (14). It should be noted that the RGC method is limited because it does not provide an exact nephron count. Another limitation of this technique is the difficulty to analyze the entire organ, section by section (14). Future studies must verify our results across the full length of kidneys by stereology, for example.

Because the number of nephrons is set early in life and does not increase regardless of the demand on the number of functional units (2), the pilot study suggests that b/b pups have decreased nephron allotment. According to the hyperfiltration hypothesis described by Brenner et al. (7), these early defects could explain the compromised renal metabolism observed in adult b/b rats. Activity of remnant nephrons is an adaptive response to maintain GFR. Over time, the overloaded nephrons suffer an increase in the glomerular pressure that promotes fibrosis and sclerosis together with proteinuria, leading to glomerular injury. These alterations further induce nephron loss, thereby continuing a vicious cycle that finally decreases the glomerular filtration, ending in renal damage and poor kidney function. Compensatory hypertrophy of the Belgrade kidneys could reflect these conditions since they are increased in size compared with control +/+ rats. Others have reported electrolyte imbalance with low-serum K⁺ values for Belgrade rats (17), and therefore potassium depletion may further aggravate the situation.

Our results also agree with previous studies that suggest an association between intrauterine growth restriction and abnormal renal development outlined above (42, 45). Besides the iron deficiency we observe, Belgrade pups have significantly reduced body weight. A correlation between low birth weight, proteinuria, glomerulosclerosis, and impaired glomerular filtration has been reported, with increasing susceptibility to renal dysfunction with time (42, 55). Moreover, maternal iron restriction during pregnancy has been documented to induce similar renal morphology in adult offspring. Lisle et al. (40) reported altered renal morphology, reduced nephron number, and greater relative kidney weight as a fraction of body weight in offspring born to anemic dams. In our study, the pregnant +/+ dams are not anemic and are provided an iron-supplemented diet. Therefore, in the case of the developing b/b rat fetus, maternal iron is not restricting intrauterine development, but delivery to the b/b fetus is most likely restricted due to mutation in DMT1. The lack of maternal imprinting is confirmed by the normal development of +/+ littersmate. Thus, it is important for investigators studying DMT1 function to recognize these in utero effects contribute to pathophysiology in adult life. It is clear that a tissue-specific knockout of DMT1 function will be necessary to answer questions about the direct role of the transporter in iron handling by the kidney raised in our study.

How iron restriction influences kidney development in the fetus needs to be better understood. At least two mechanisms have been suggested. Under the low-iron conditions, poor oxygenation and/or fetal hypoxia could influence key transcription factors like hypoxia-inducible factor and vascular endothelial growth factor. Lisle et al. (40) point out that oxygenation influences the extent of nephrogenesis as one explanation for the effects of maternal iron restriction. A second possibility raised by their study is glucocorticoid levels, which increase in response to iron deficiency in the rat (40). Such changes could strongly influence growth factors known to be important for nephrogenesis (28).

Although renal iron handling is not fully understood, evidence points to the metal’s reabsorption by tubules (46, 72). The fact that more iron is excreted in b/b rat urine could result from the altered glomerular membrane function, but loss of tubular reabsorption is also possible. The idea that both pathways are affected is supported by histological observations discussed above. Urinary Tf was elevated in b/b at 15 wk, along with iron. Since total protein, in particular albumin, is also elevated, it is difficult to ascertain whether the levels of urinary Tf are specific for the chronic kidney condition of the Belgrade rat or reflect deficient DMT1 function. Nonetheless, the fold increase of Tf (48.5) is much greater than the fold increase of albumin (3.1) when b/b rat urinary values are compared with +/+ rat measurements. Tubular uptake of Fe³⁺ bound to Tf, which is filtered by the glomerulus, has been proposed (34). Tf has been described as a ligand for cubulin, a protein expressed in the renal proximal tubule that functions as an endocytic receptor (10, 34). Renal expression of cubulin was similar in +/+ and b/b rats at 15 wk, suggesting that increased urinary Tf seen in homozygous rats does not arise from changes in tubular reabsorption of Tf due to loss of cubulin. Levels of Tf receptor are also unchanged (data not shown). We speculate that the lack of DMT1 to export endosomal iron could disrupt reabsorption of iron from holo-Tf and/or enhance glomerular filtration of holo-Tf due to impaired recycling of apo-Tf, thereby leading to excess urinary Tf. Increased urinary iron excretion could account for the lack of iron loading by the kidney in the Belgrade rat. It is clear that further research is necessary to better understand iron handling in renal physiology.

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AUTHOR CONTRIBUTIONS

REFERENCES


