

Belgrade Rats Display Liver Iron Loading¹

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Abstract

Patients with mutations in divalent metal transporter-1 (DMT1), an intestinal nonheme iron transporter, suffer from microcytic anemia and hepatic iron loading. DMT1 is also mutated in Belgrade rats, an animal model with a thalassemic-like disorder of microcytic anemia with hyperferrinemia. However, aspects of hepatic iron loading in this genetic model are not well characterized. To more fully define the Belgrade rat's iron status, we compared the characteristics of homozygous (*b/b*) and heterozygous (*b/+*) rats fed an iron-supplemented diet for 3 wk postweaning. Dietary supplementation with ferrous iron improved the anemia of *b/b* rats insofar as hematocrits increased from 0.13 (21-d-old) to 0.31 (42-d-old). However, hematocrits remained significantly lower than those of age-matched *b/+* rats (0.36 and 0.41 in 21- and 42-d-old heterozygotes, respectively, $P < 0.05$). Wright's staining of *b/b* red cells confirmed the hypochromic microcytic nature of Belgrade rats' anemia. The liver iron concentration of 42-d-old *b/b* rats was greater than in age-matched *b/+* rats (5.97 vs. 2.24 $\mu\text{mol/g}$, $P < 0.05$). Whereas Perls' Prussian blue iron staining was evident in both periportal and centrilobular regions in 42-d-old *b/b* liver sections, no staining was observed in age-matched *b/+* tissue sections. Quantitative real-time PCR analysis showed that expression of liver hepcidin mRNA in 42-d-old *b/b* rats was 3-fold greater than age-matched *b/+* rats. These results indicate that, similar to human patients with DMT1 mutations, Belgrade rats also display hepatic iron loading. Our data suggest this condition arises from ineffective erythropoiesis. J. Nutr. 136: 3010–3014, 2006.

Introduction

The role of divalent metal transporter-1 (DMT1)⁴ (SCL11A2) in iron transport was established by positional cloning of the defective gene in the microcytic anemia (*mk*) mouse (1) as well as functional expression cloning (2). Mice with selective inactivation of this transporter in the intestine (*Slc11a2^{int/int}*) survive, but only with parental iron administration (3), indicating that DMT1 activity is essential for adequate dietary nonheme iron absorption. Recent studies report that human patients with DMT1 mutations suffer from microcytic anemia, but also show hepatic iron loading (4–6). In light of these data, Mims et al. (4) proposed that loss of intestinal DMT1 function can be compensated by upregulation of other pathways of iron absorption, possibly heme-iron uptake. The alternative possibility raised by Gunshin et al. (7) is that residual transporter activity, coupled with ineffective erythron iron utilization, results in increased liver iron in these patients.

DMT1 deficiency also exists in Belgrade (*b*) rats. The same glycine-to-arginine missense mutation found in the *mk* mouse

(G185R) cosegregates with the *b* phenotype (8). Belgrade rats were originally characterized as having a thalassemia-like disorder (9) and possessing some characteristics described in case reports for human patients with DMT1 mutations, including hypochromic microcytic anemia and hyperferrinemia. Subsequent investigations indicate severe defects in iron absorption by several different tissues and cell types from Belgrade rats, including transferrin-mediated iron uptake by reticulocytes (10–12). Administration of parental iron to *b/b* rats promotes tissue iron loading (9), but, to our knowledge, how diet affects the iron status of Belgrade rats has not been studied. This study demonstrates that dietary supplementation with ferrous iron increases the liver iron concentration in Belgrade rats. Thus, the characteristics of Belgrade rats resemble the liver iron-loading phenotypes associated with DMT1 mutations in humans.

Methods and Materials

Animals and diets. Animal protocols were approved by the Harvard Medical Area Animal Care and Use Committee. Belgrade rats were bred from animals obtained from Dr. Michael Garrick (State University of New York at Buffalo). They were maintained on a 12-h light/dark cycle and consumed food and water ad libitum. Mating pairs of female heterozygotes (*b/+*) and male Belgrade (*b/b*) rats were fed an iron-supplemented diet (TD02385, Harlan Teklad; Table 1). Female *b/+* rats were fed the iron-supplemented diet throughout pregnancy. At postnatal d 6, litters were cross-fostered to F344 Fischer dams (Harlan Sprague Dawley) fed a standard diet containing 3.76 mol/kg iron (Purina Lab Diet 5053, PharmaServ). Upon weaning, both *b/b* and *b/+* rats were fed the iron-supplemented diet. Pups were humanely killed by Halothane

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⁴ Abbreviations used: DAB, 3, 3'-diaminobenzidine tetrahydrochloride; DMT1, divalent metal transporter-1; Tf, transferrin; TIBC, total iron-binding capacity.

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TABLE 1 Composition of iron-supplemented diet

Diet components	g/kg
Casein, high protein	200.0
DL-Methionine	4.3
Sucrose	532.8
Corn starch	150.0
Corn oil	50.0
Mineral mix, Fe deficient, AIN-76 with added calcium phosphate, dibasic ¹	35.0
Calcium carbonate	6.9
Ferrous sulfate FeSO ₄ · 7H ₂ O	2.5
Magnesium oxide MgO	0.167
Cupric carbonate	0.004
Sodium selenite (0.0445% Na ₂ SeO ₃ in sucrose)	1.5
Vitamin mix, AIN-76A (40077) ¹	14.0
Phylloquinone	0.0005
Vitamin B-12 (0.1% in trituration)	0.025
Choline bitartrate	2.8
Ethoxyquin (antioxidant)	0.01

¹ Reeves et al. (30).

overdose at the time of weaning (21 d) and after being fed an iron-supplemented diet for 3 wk (42 d) for tissue collection. Belgrade (*b/b*) pups were identified at birth by their pale anemic features and their genotype was verified by PCR as described (8).

Iron analysis. Hematocrits and tissue nonheme iron concentrations were measured as previously described (13). Serum iron and total iron-binding capacity (TIBC) measurements were performed by Kansas State Veterinary Medical Center Comparative Hematology Department (Manhattan, KN). Iron and TIBC measurements of serum samples were quantified using the Ferene method (Raichem Iron/TIBC kit; Hemagen Diagnostics) and analyzed in a Cobas Mira Chemistry System (Roche Diagnostics). Blood cell morphology was analyzed by Wright's staining using the Diff-Quik Stain Kit (68100–408; VWR Scientific). Liver iron was stained using 3, 3'-diaminobenzidine tetrahydrochloride (DAB)-intensified Perls' Prussian blue. Briefly, paraffin-embedded liver tissue was sectioned (8 μm), and then sections were deparaffinized, rinsed in phosphate buffered saline, pH 7.4, and stained with a solution containing 2% HCl and 2% K₄Fe(CN)₆. After preincubation in a solution containing 0.125 g/L DAB and 0.06% NiCl₂, sections were then processed in the same DAB/NiCl₂ solution containing 0.3% H₂O₂.

RNA preparation. Total RNA from rat livers was prepared using RNA-Bee (IsoTex Diagnostics) following the manufacturer's instructions. RNA was treated with DNase (Promega) to remove any contaminating genomic DNA. Two μg DNase-treated RNA was used to synthesize cDNA with oligo-deoxythymidine (dT) primers (Promega) and M-MLV Reverse Transcriptase (Promega) for each sample.

Quantitative real-time RT-PCR analysis. Quantitative real-time RT-PCR analysis was carried out in triplicate for each sample on an iQ5 detection system using iQ SYBR Green (Bio-Rad). Analysis was performed in a 25 μL reaction volume according to Zhang et al. (14). Forty cycles of PCR amplification were carried out as follows: 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Primers for hepcidin were: 5'-TGACAGTGCCTGCTGATG (forward) and 5'-GGAATTCTTACAGCATTACAGCAGA (reverse). Primers for 36B4 were: 5'-AGATGCAGCAGATCCGCAT (forward) and 5'-GTTCTTGCCCATCAGCACC (reverse). The ΔC_T method was utilized for the data analysis where threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The ΔC_T (C_{T-target} - C_{T-reference}) is the difference in threshold cycles for target (hepcidin) and reference (36B4).

TABLE 2 Characteristics of weanling (21-d-old) homozygous (*b/b*) and heterozygous (*b/+*) Belgrade rats¹

	<i>b/b</i>		<i>b/+</i>	
	<i>n</i>		<i>n</i>	
Body weight, <i>g</i>	9	36.50 ± 3.88*	12	50.33 ± 3.42
Liver weight, <i>g</i>	9	3.09 ± 0.44	12	2.99 ± 0.30
Liver, % <i>body weight</i>	9	8.23 ± 0.95*	12	5.89 ± 0.50
Hematocrit, <i>volume fraction</i>	9	0.131 ± 0.006*	12	0.355 ± 0.010
Liver nonheme iron, μmol/g	6	0.670 ± 0.026	6	0.653 ± 0.048

¹ Values are means ± SEM. *Different from *b/+*, *P* < 0.05.

Statistical analysis. Values reported are means ± SEM (*n* = 5–12). Statistical significance was evaluated using a 2-tailed Student's *t* test (2-sample assuming unequal variances) and differences were considered significant at *P* < 0.05.

Results

Characteristics of weanling *b/b* and *b/+* rats. At the time of weaning (21 d of age), the body weight of homozygous Belgrade rats (*b/b*) was significantly lower than control heterozygous siblings (*b/+*) (Table 2). Although liver weights did not differ, the relative liver weight (% body weight) in *b/b* rats was significantly higher due to the difference in body weight between 21-d-old and *b/b* and *b/+* siblings. The liver nonheme iron concentration of weanling *b/b* rats did not differ from that of *b/+* siblings. However, the hematocrits of *b/b* weanlings were extremely low, consistent with past reports characterizing the hypochromic microcytic anemia of Belgrade rats (9–11,15).

Effects of dietary iron supplementation. After rats were fed an iron-supplemented diet containing 8.99 mol Fe/g for 3 wk, the total body weight of *b/b* rats remained significantly lower than control *b/+* siblings (Table 3; however, relative liver weights no longer differed). Hematocrits of iron-supplemented *b/b* rats were significantly reduced relative to the age-matched iron-supplemented *b/+* siblings, but they were markedly improved from those in *b/b* rats at the time of weaning. Wright's staining of blood smears from iron-supplemented rats showed reduced hemoglobin content in *b/b* red cells, with pronounced anisocytosis and poikilocytosis (Fig. 1).

Despite their anemic state, the liver nonheme iron concentration of *b/b* rats was more than double that of *b/+* rats fed the same iron-supplemented diet (Table 3). Serum iron and TIBC

TABLE 3 Characteristics of 42-d-old homozygous (*b/b*) and heterozygous (*b/+*) Belgrade rats fed an iron-supplemented diet for 3 wk¹

	<i>b/b</i>		<i>b/+</i>	
	<i>n</i>		<i>n</i>	
Body weight, <i>g</i>	12	124.21 ± 5.84*	12	156.38 ± 9.51
Liver weight, <i>g</i>	12	5.97 ± 0.18	12	6.93 ± 0.62
Liver, % <i>body weight</i>	12	4.79 ± 0.25	12	4.38 ± 0.24
Hematocrit, <i>volume fraction</i>	12	0.313 ± 0.001*	12	0.411 ± 0.005
Liver nonheme iron, μmol/g	7	5.97 ± 0.72*	7	2.24 ± 0.37
Serum iron, μmol/L	5	107.67 ± 4.02*	5	50.15 ± 6.99
Serum TIBC, μmol/L	5	114.52 ± 4.17*	5	85.97 ± 2.65
Serum Tf saturation, %	5	94.02 ± 0.33*	5	58.12 ± 7.62

¹ Values are means ± SEM, *Different from *b/+*, *P* < 0.05.

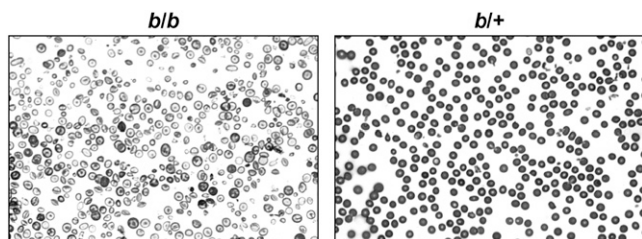


Figure 1 Wright's staining of blood indicates microcytic hypochromic anemia in *b/b* rats. Blood smears were stained with Wright's stain and viewed using an Ernst Leitz Wetzlar Laborlux D Microscope. Digital images were obtained with a Nikon D100 camera. Images were acquired by iPhoto software [version 5.0.4 (263), Apple software]. The original magnification was $\times 102$. Blood samples were taken from 42-d-old *b/b* rats fed an iron-supplemented diet for 3 wk postweaning.

were also significantly greater in *b/b* rats compared with *b/+* rats. Accordingly, transferrin (Tf) saturation was greater in *b/b* rats than in *b/+* controls. Perls' Prussian blue-stained liver sections showed iron deposition in *b/b* rat tissue (Fig. 2). No iron staining was observed for age-matched *b/+* rat tissue (data not shown). Iron staining in the *b/b* liver was evident in both periportal (zone 1) and centrilobular (zone 3) areas.

Hepcidin levels in iron-supplemented *b/b* and *b/+* rats. Liver iron-loading is associated with increased expression of the iron regulatory hormone hepcidin (16). Iron-supplemented *b/b* rats had hepcidin expression levels that were >3 -fold those of age-matched *b/+* controls (ΔC_T values of 6.160 ± 0.614 vs. -1.777 ± 0.454 , respectively ($n = 5/\text{group}$, $P = 0.000002$). These results are consistent with studies showing that hepcidin expression increases with liver iron loading despite severe anemia from experimentally induced inhibition of hematopoiesis (17).

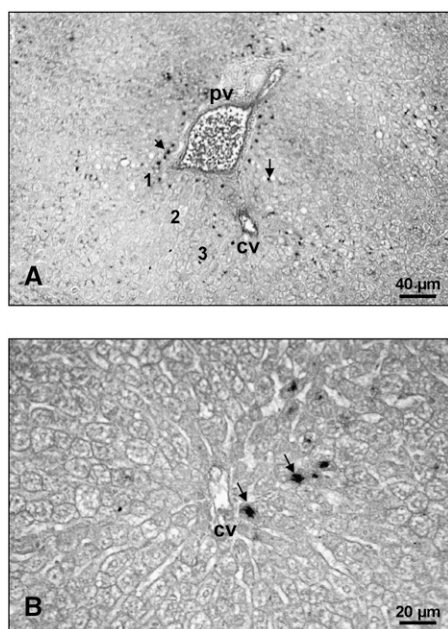


Figure 2 Perls' Prussian blue staining shows liver iron deposition in *b/b* rats. Liver tissue from *b/b* rats fed an iron-supplemented diet for 3 wk postweaning was processed, paraffin embedded, and sectioned ($8 \mu\text{m}$). An Ernst Leitz Wetzlar Laborlux D Microscope was used to view the sections. Digital images were obtained with a Nikon D100 camera. Images were acquired by iPhoto software [version 5.0.4 (263), Apple software]. pv = portal vein; cv = central vein. Hepatocyte iron staining is indicated by arrows.

Discussion

In an earlier study, Garrick et al. (15) showed that dietary ferrous iron increased liver iron content in *b/b* rats compared with *b/b* rats on a control diet, but unfortunately they did not report whether the values were compared with age- and/or diet-matched *b/+* sibling controls. The new information provided by our study provides a direct comparison and demonstrates that when fed a high iron diet, *b/b* rats develop hyperferrinemia compared with iron-supplemented age-matched heterozygote *b/+* sibling controls. Previous studies indicate that administration of parental iron to *b/b* rats promoted tissue iron loading (9); our study further confirms that iron-loading also occurs with dietary supplementation, indicating that Belgrade rat intestine can absorb iron despite defects in DMT1 function. Iron staining in the *b/b* liver was evident in both periportal (zone 1) and centrilobular (zone 3) areas. Weaker iron staining was present in zone 2, indicating that iron deposition in the region of the central vein results from hyperferrinemia (10). Based on the pattern of liver iron deposition, it appears that primary defects in erythron iron utilization caused by insufficient iron uptake by the hematopoietic system not only give rise to the Belgrade rat's microcytic anemia, but also promote liver iron loading.

DMT1 is expressed at the duodenal brush border, where it is responsible for the pH-dependent uptake of dietary iron from the lumen of the intestine. It also plays an important role in bringing iron into red blood cell precursors for hemoglobin biosynthesis. DMT1 colocalizes with Tf in the recycling endosomes of many cell types, including reticulocytes, where it transports iron from the acidified lumen of the endosomes into the cytoplasm. Recent studies of knockout mice have demonstrated that DMT1 is essential for intestinal nonheme iron absorption after birth and that it is required for hemoglobin synthesis during erythropoiesis (3). It has been shown that Belgrade rat reticulocytes have markedly reduced high affinity iron transport, consistent with impaired DMT1 function, whereas low affinity uptake of iron is not altered and probably contributes to the residual capacity to produce hemoglobin (18,19). The significantly lower uptake of iron by erythroid precursors accounts for the increased Tf saturation and serum iron levels in Belgrade rats. The liver appears to have an alternative way of acquiring iron independent of DMT1 and/or Tf so that iron accumulates in Belgrade rat liver when DMT1 activity is mutationally diminished. In fact, liver iron loading is observed in DMT1 knockout mice (3). How the liver takes up iron under these circumstances is not fully understood, but perfusion studies demonstrate that non-Tf bound iron is efficiently cleared (20). The zinc transporter Zip14 mediates the uptake of non-Tf bound iron into hepatocytes and is abundantly expressed in the liver (21), raising the possibility that this transporter may be responsible for iron uptake by the liver.

The fact that dietary iron supplementation is associated with liver iron loading in Belgrade rats further suggests that intestinal nonheme iron absorption persists despite the DMT1 mutation. Studies of mice with targeted disruption of the DMT1 gene show that the transporter is required for intestinal iron absorption after birth (3); however, immunohistochemical analyses indicate that minimal levels of DMT1 protein are present at postnatal d 0 and postnatal d 5 (22). By postnatal d 10 DMT1 becomes localized in the apical membrane of the maturing intestine and is predominantly expressed in its deglycosylated form until postnatal d 20 (22). In rats, expression of DMT1 still increases by postnatal d 40 (23). In our study, the *b/b* rats were severely anemic at the time of weaning, but after dietary iron

supplementation, hematocrits improved in a manner consistent with the pattern of developmental regulation of DMT1 expression in the duodenum (22,23). Thus, it is possible that residual activity of the DMT1 (G185R) mutant was responsible for dietary nonheme iron absorption. However, alternative DMT1-independent pathways for intestinal iron absorption have been reported (24). In particular, Zip14 is abundantly expressed in the duodenum and jejunum (21), suggesting this transporter may play a role in dietary iron absorption as well.

Our observations documenting liver iron loading in Belgrade rats help to address questions provoked by studies of patients with genetic defects in DMT1 (4–6). Clinical reports have made the assumption that the human phenotypes associated with DMT1 mutations (e.g., liver iron loading) are dissimilar to animal models of the DMT1 G185R mutation. Our study corrects this common misperception. The G185R mutation carried by Belgrade rats and *mk* mice impairs the subcellular localization and stability of DMT1, resulting in reduced transport function (25,26). Studies of the *mk* mutation on different strain backgrounds indicate that despite severe functional defects, the mutant G185R transporter displays residual activity that can be modified by genetic factors to improve survival (3). Our study confirms that when Belgrade rats are fed a high iron diet, they can incur liver iron loading, indicating that intestinal iron absorption must continue despite the defects in DMT1 activity. Although in vitro studies show that the human E399D mutant of DMT1 is stably expressed, fully processed, and retains transport activity (7,27,28), the G1285C substitution giving rise to this missense mutation causes exon skipping with a significant loss of function (7,27). Because the aberrantly spliced DMT1 isoform is predominantly expressed in the patient's duodenum, any residual function would also have to be provided by relatively low levels of DMT1 (E399D) or by other transport pathways as discussed above (21,24,29). The functional consequences of 2 other DMT1 mutations reported for a compound heterozygote patient have not yet been determined, but Western blot analysis showed significantly reduced transporter levels (5). Although low urinary hepcidin levels have been reported for 1 patient with the E399D mutation (4), our results show that hepcidin gene expression is appropriately regulated in Belgrade rats in response to iron loading. Based on our data, we conclude that loss of DMT1 function primarily disrupts iron delivery necessary for hemoglobin production during erythropoiesis and therefore contributes to liver iron loading observed in humans patients (4–6).

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ERRATUM

Thompson K., Molina R., Brain J., and Wessling-Resnick M. 2006 Belgrade rats display liver iron loading. *J. Nutr.* 136:3010–14.

The text gives incorrect amounts of iron of diets used in this study. The standard diet contained 3.76 $\mu\text{mol Fe/g}$ and the iron-supplemented diet contained 8.99 $\mu\text{mol Fe/g}$. The information in Table 1 is correct.