Altered dietary iron intake is a strong modulator of renal DMT1 expression

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Divalent metal transporter1 (DMT1; also known as DCT1 or NRAMP2) is an important component of the cellular machinery responsible for dietary iron absorption in the duodenum. DMT1 is also highly expressed in the kidney where it has been suggested to play a role in urinary iron handling. In this study, we determined the effect on renal DMT1 expression of feeding an iron-restricted diet (50 mg/kg) or an iron-enriched diet (5 g/kg) for 4 wk and measured urinary and fecal iron excretion rates. Feeding the low-iron diet caused a reduction in serum iron concentration and fecal iron output rate with an increase in renal DMT1 expression. Feeding an iron-enriched diet had the converse effect. Therefore, DMT1 expression in the kidney is sensitive to dietary iron intake, and the level of expression is inversely related to the dietary iron content. Changes in DMT1 expression occurred intracellularly in the proximal tubule and in the apical membrane and subapical region of the distal convoluted tubule. Increased DMT1 expression was accompanied by a decrease in urinary iron excretion rate and vice versa when DMT1 expression was reduced. Together, these findings suggest that modulation of renal DMT1 expression may influence renal iron excretion rate.

iron is an essential metal for life because it is a key constituent of a family of fundamental proteins, which includes hemoglobin, cytochromes, and NADH-enzyme Q reductase. Maintaining the correct balance of iron is paramount to health because iron deficiency or excess results in morbidity and mortality. The molecular characterization of membrane-bound iron transporter proteins, in particular divalent metal transporter 1 (DMT1; 9), also known as DCT1 (14) or NRAMP2 (13), has shed new light on some of the mechanisms of body iron homeostasis. DMT1 is the product of the NRAMP2 gene (13) and it is a broad-spectrum DMT that has the capacity to transport Fe2+, Mn2+, Cu2+, Cd2+, Ni2+, and Co2+, but not Ca2+ or Mg2+ (14, 25). It has been shown to be of fundamental importance in iron metabolism because Belgarde (b) rats (7, 9, 11, 22) and mk mice (5, 8) suffer from microcytic hypochromic anemia caused by mutations in the DMT1 gene. This mutation results in a functional deficit of DMT1 due to a G185R point mutation in the DMT1 protein (23).

DMT1 is expressed in the duodenum where it transports divalent metals across the apical membrane of enterocytes (18, 26). It is also expressed in late endosomes (7) and lysosomes (24) in some cell types and functions as a vesicular iron transporter in the transferrin cycle (12, 20).

To date, four splice variants derived from the DMT1 gene have been identified (7, 14–16, 21). These differ with respect to whether they encompass either exon 1A or exon 1B and also whether they include an iron-responsive element (IRE) in the 3′-UTR. The net result is four possible protein products, two being responsive to regulation by iron via the IRE and two so-called non-IRE forms.

DMT1 has been extensively studied in the duodenum, where modulation of dietary iron absorption is the major means of regulating body iron balance. When iron intake is restricted, DMT1 expression is increased (29); the converse is true when iron-enriched diets are fed in that DMT1 expression is reduced. A similar relationship has been reported in the placenta to deliver the large amounts of iron required by the developing fetus (10).

In the liver, where iron is stored bound to ferritin, the situation is reversed. Reducing dietary iron causes a reduction in DMT1 expression, which in turn causes a reduction in liver iron accumulation (17). Feeding an iron-enriched diet brings about an increase in DMT1 expression (26), which in turn promotes iron acquisition. These responses serve to modulate serum iron levels and allow the liver to act as a reservoir for iron. It also means that in condi-
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ctions of iron overload, the liver can be damaged, as occurs in haemochromatosis.

The kidney is generally not thought to be involved in modulating serum iron concentration. However, it is clear from previous findings that DMT1 is highly expressed in the kidney (1, 6, 14). In rats, DMT1 is located in the cytoplasm of proximal tubule cells and at the apical membranes of late thick ascending limbs, early distal tubules, and in intercalating cells of cortical collecting ducts (6). These findings suggest that DMT1 may play a role in renal iron handling, possibly reabsorption of filtered iron.

In the current study, we investigated the renal response to dietary iron. We altered dietary consumption of iron to determine the effect on urinary iron excretion rates and the level and pattern of renal DMT1 expression. We found that increasing dietary iron decreased DMT1 expression and increased urinary iron excretion rate. Decreasing dietary iron caused an increase in DMT1 expression and a decrease in urinary iron excretion.

MATERIALS AND METHODS

Metabolic cage studies. All experiments were performed within guidelines set down in the UK Animals (Scientific Procedures) Act 1986. Three groups of male Wistar rats, obtained from Harlan UK (Bicester, UK), were maintained for 4 wk either on a iron-restricted diet (restricted diet (RD) 50 mg iron/kg dry weight), or a control diet (CD; 185 mg iron/kg dry weight), or an iron-enriched diet (enriched diet (ED) 5,000 mg iron/kg dry weight) and were given deionized water ad libitum. Diets were manufactured by Special Diet Services (Whitman, Essex, UK). After this period, animals were individually housed in metabolism cages for 8 days while maintained on the same diet. For the first 5 days of the experiment (an acclimatization period), food and water were changed daily, but no data collection was made. The experimental period was started on day 6 and continued through days 7 and 8 of the experiment. Body weight, food, and water intake were measured. Twenty-four-hour collections of feces and urine were measured. Twenty-four-hour collections of feces were averaged to days 6–8.

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Histology and immunohistochemistry. To study intrarenal distribution of DMT1 in Wistar rats fed either the RD, CD, or ED, animals were anesthetized with Inactin (Sigma RBI, Poole, UK) at a dose of 100–110 mg/kg ip. Perfusions were carried out as described elsewhere (6) and 4-μm-thick cryosections were cut using a Leica CM3050 cryostat (Leica Instrumente GmbH, Nussloch, Germany), thaw-mounted onto Superfrost Plus slides (BDH, Poole, UK), and kept at −20°C until used. For histological examination, slides were thawed, dipped into hematoxylin-eosin (BDH), dehydrated through ethanol series, and mounted using Eukitt (Kindler, Freiburg, Germany). DMT1 immunodetection was performed as previously described using affinity-purified anti-DMT1 polyclonal antiserum (6). Secondary antibody was goat anti-rabbit Alexa 594 (Molecular Probes) diluted into phosphate-buffered saline containing 5% normal goat serum and 1% bovine serum albumin. Intrarenal distribution of DMT1 was assessed on cryosections from animals perfusion-fixed as described above. After being rinsed with PBS, the sections were mounted using DAKO-Glycergel (Dokapatts) with 2.5% 1,4-diazabicyclo-[2.2.2]octane added (Sigma, St. Louis, MO). Negative controls were carried out by preincubating the DMT1 antiserum with an excess of antigenic peptide and by omitting the primary antibodies. Slides were viewed using a Zeiss Axioplan 2 microscope with ×2.5–63 objectives. Images were acquired using a Hamamatsu digital camera and processed using the software package KS300 version 3.0 (Carl Zeiss, Welwyn Garden City, UK). To determine if iron deposits were present, rat kidney sections were stained using the Accustain Iron Stain Kit (SIGMA Diagnostics, St. Louis, MO) following the manufacturer’s instructions.

Statistical analysis. For serum, urine, and fecal iron values, statistical significance was assessed using single-factor ANOVA. Bonferroni’s multiple comparison post hoc test was used to indicate significant differences between groups. Data are expressed as means ± SE throughout the text, where n = number of animals unless otherwise stated. Significance was accepted at P ≤ 0.05.
RESULTS

Metabolic cage studies. To measure the daily rates of dietary intake and excretion, we performed metabolic cage studies on groups of rats fed three diets that were identical except for the iron content. The mean weight of the rats in the three groups (n = 4 rats per group) at the end of the experiment was as follows: CD 291.8 ± 2.4 g, RD 289.5 ± 5.0 g, and ED 260.8 ± 1.4 g. Measurements of uptake and excretion represented the mean daily rates taken on 3 consecutive days at the end of the 8 days in metabolic cages. Over this period, the mean daily food intake was the same for each group (Fig. 1A), showing that altering the iron content of the diet had no effect on feeding. The amounts of iron consumed are shown in Fig. 1B. Animals on the RD diet ingested nine times less iron per day than rats fed the CD (10.8 ± 0.2 μmol·day⁻¹·100 g body wt⁻¹ compared with 95.7 ± 0.9 μmol·day⁻¹·100 g body wt⁻¹), whereas animals on the ED ingested 15 times more iron per day than rats fed the CD (1,430.8 ± 34.4 μmol·day⁻¹·100 g body wt⁻¹). These different dietary loads resulted in a significantly different amount of iron in the feces that paralleled the amount of iron consumed. The rate of fecal iron output from animals fed the RD was 6.4 ± 0.4 μmol·day⁻¹·100 g body wt⁻¹ compared with 50.0 ± 1.8 μmol·day⁻¹·100 g body wt⁻¹ for CD animals and 781.4 ± 11.0 μmol·day⁻¹·100 g body wt⁻¹ for rats fed the ED (P < 0.05). Expressing the rates of fecal output as percentage of the rate of iron ingested gave values of 59% for the RD group, 52% for the CD group, and 54% for the ED group. Thus irrespective of the iron intake between 50 and 60% of the ingested iron was voided in the feces. Subtracting daily fecal output from the rate of iron intake gave an indication of iron retained. For rats fed the RD, this was 4.4 μmol·day⁻¹·100 g body wt⁻¹ compared with 45.7 μmol·day⁻¹·100 g body wt⁻¹ for CD-fed rats and 649.4 μmol·day⁻¹·100 g body wt⁻¹ for ED-fed rats. The fact that the three groups of animals experienced different iron loads was reflected in significant differences in serum iron (P < 0.05; Fig. 1D). Rats fed the RD had a mean serum iron concentration of 12.2 ± 0.8 μmol compared with 37.2 ± 2.1 μmol for the CD group and 60.8 ± 6.6 μmol for the ED group.

Urinary volume and urinary osmolar output were measured to ensure that feeding the different diets did not have an adverse effect on renal function. There were no significant differences in the volume of urine excreted between experimental groups (Fig. 1E). ED-fed animals did, however, excrete less solute (4.2 ± 0.3 mosM·day⁻¹·100 g body wt⁻¹) compared with ED rats (5.7 ± 0.3 mosM·day⁻¹·100 g body wt⁻¹), although this was deemed to be within the normal range.

Measurement of urinary iron revealed that feeding the different diets had a significant effect on urinary iron excretion. ED rats excreted six times more iron per day (0.76 ± 0.14 μmol·day⁻¹·100 g body wt⁻¹) than CD rats (0.12 ± 0.03 μmol·day⁻¹·100 g body wt⁻¹), P <

![Fig. 1. Physiological variables measured over 3 days in metabolic cages. All values are normalized per 100 g body wt. A: food intake in g/day. B: dietary iron intake. Amount of iron is in μmol/day. C: rate of fecal iron output in μmol/day. D: serum iron concentration in μmol. E: rate of urinary output (ml/day) and osmolar output (mosM/day). F: rate of urinary iron output in μmol/day. *Significantly different from restricted diet (RD)-fed animals (ANOVA, Bonferroni’s multiple comparison post hoc test P ≤ 0.05, n = 4).](Image)
0.05). In contrast, RD-fed rats excreted three times less iron per day than CD rats (0.04 ± 0.02 \( \mu \text{mol} / \text{day}^{-1} \cdot 100 \text{ g body wt}^{-1} \), \( P < 0.001 \)). These data strongly suggest that feeding different amounts of iron caused changes in urinary iron excretion.

**Western analysis.** Western analysis of protein from total kidney microsomal membranes showed that feeding the RD caused a significant increase in DMT1 expression compared with kidneys from animals fed a CD (\( P < 0.0001 \); Fig. 2). Conversely, DMT1 levels were decreased in animals fed the ED compared with controls. These data strongly suggest that the renal expression of DMT1 is sensitive to dietary iron intake.

**Histology and iron deposits staining.** Low-magnification micrographs of hematoxylin-eosin staining of paraformaldehyde-fixed cryosections showed marked structural changes in the kidneys of animals fed the ED (Fig. 3, A and C) compared with those from animals fed a RD (Fig. 3, B and D). ED kidneys had an ill-defined corticomedullary junction, with projections of the medullary collecting epithelium into the cortex of high-iron rats (Fig. 3A). Higher magnification revealed evidence of tubular hypertrophy and enhanced granularity of the tubular epithelium in the kidney cortex. In addition, it was evident that glomeruli of ED rats had increased cellularity, although without fibrosis or sclerosis (Fig. 3C) or irregularity in the capillary loops. These changes were not observed in kidneys of rats kept on a RD (Fig. 3D) or CD (data not shown). Iron deposits were not detected in any of the experimental groups when kidneys were stained using the Accustain Iron Stain Kit (data not shown).

**Immunostaining.** The overall pattern of immunoreactive DMT1 expression in kidneys from animals fed the CD was the same as we observed in two previous studies (6, 9a). Low-magnification images show that the strongest staining was observed in tubular structures in the cortex (Fig. 4A). As previously observed, this intense staining extended to the outer medulla/inner medulla junction. In the inner medulla, staining was considerably less intense.

Low-magnification images showed that there were no overall differences in the pattern of staining among the experimental groups. In all experimental groups, the staining was predominantly in the cortex and outer medulla. Importantly, there were distinct differences in the intensity of staining between the experimental groups (Fig. 4). Kidneys from rats fed the RD showed strong DMT1 immunoreactivity in cortical tubules (Fig. 4B). In comparison, control animals (Fig. 4A) showed less intense staining of tubules and staining was greatly reduced in animals fed the ED (Fig. 4C).

Analysis of images captured at higher magnification revealed punctate intracellular staining of proximal tubules in control animals (Fig. 5A). This cellular pattern of DMT1 expression was identical to that observed in our previous study (6). DMT1 immunoreactivity was greatly increased in the low-iron group, indicating that expression of DMT1 had increased in response to dietary iron restriction (Fig. 5B). As observed in the control group, staining was cytoplasmic, and apical or basolateral plasma proximal tubule membranes were not labeled.

In contrast to CD- and RD-fed animals, the immunostaining of proximal tubules was much weaker in the ED group (Fig. 5C). Thus feeding a diet enriched in iron resulted in a decrease in expression of DMT1 in proximal tubule cells. Overall, the differences in im-

![Fig. 2. Western blot analysis of kidney microsomal membrane fraction protein from rats fed iron-restricted diet (RD), control iron diet (CD), and iron-enriched diet (ED). A: Western blot showing marked increase in DMT1 protein in RD-fed rats compared with CD-fed rats. Compared with CD animals, ED-fed rats showed a significant decrease in DMT1 protein. B: densitometric analysis of Western blots. *Significantly different from RD-fed animals (ANOVA \( P \leq 0.0001, n = 3 \)).](http://ajprenal.physiology.org/)

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munoreactivity in response to dietary intake agree with the changes quantified by Western analysis.

The immunostaining observed in the midcortical region of rats fed the CD was as follows (Fig. 6A): 1) the cytoplasm of proximal tubules showed a punctate DMT1 signal, which was not associated with either plasma membrane. 2) The apical and subapical regions of distal convoluted tubules (DCTs) were stained. 3) Collecting ducts showed a heterogeneous pattern of DMT1 labeling in that in some cells staining was apical, in others it was cytoplasmic and in another subset it was basolateral. These patterns of staining were consistent with those we previously reported (6).

As in the superficial region, proximal tubule labeling was increased in response to the RD (Fig. 6B) and reduced in response to the ED (Fig. 6C). The RD also increased apical staining of DCTs, whereas the ED had the converse effect. The density and intensity of immunostaining of cortical collecting ducts were not different in low and control animals but were reduced in the ED. This suggests that the RD did not affect overall expression of DMT1 in cortical collecting ducts, but feeding the ED reduced DMT1 expression in this segment.

As we previously reported, there was much less DMT1 immunoreactivity in the outer medulla or inner medulla than in the cortical regions. The iron content of the diet had no discernible effect on DMT1 immunoreactivity in these medullary regions (data not shown).

**DISCUSSION**

In a previous study (6), we determined the renal distribution of DMT1 using an affinity-purified antiserum targeted to the NH2 terminal 21 amino acids of rat DMT1. This antiserum recognizes the iron-respon-
sive and noniron-responsive DMT1 isoforms as well as the recently discovered exon 1A and exon 1B splice variants (15). With the use of this antiserum, we observed punctate intracellular expression of DMT1 in proximal tubule cells. In DCTs, and in principal and A-intercalated collecting duct cells, DMT1 was expressed in the apical plasma membranes. From these findings, we concluded that DMT1 was unlikely to be responsible for transport of iron across the apical proximal tubule apical membrane, although in more distal nephron segments DMT1 may fulfill this role.

In the present study, we set out to determine the effect of dietary iron intake on the renal expression of DMT1. We found that feeding a diet high in iron caused changes in the morphology of the kidney compared with the other diets we administered. In particular, we observed changes in the cortex and at the corticomedullary junction in the high-iron-fed animals. Although we do not know the underlying mechanism of these changes, it is worthy of note for future studies employing enriched iron diets.

Specific to the aims of the project, we found that altering the iron content of the diet had a profound effect on DMT1 protein expression. DMT1 immunoreactivity increased in response to feeding an iron-restricted diet and decreased in response to feeding an iron-enriched diet. Therefore, there is an inverse relationship between dietary iron content and the level of renal DMT1 expression. This corroborates data in mice fed iron-restricted or iron-enriched diets (3, 15). In the iron-restricted mice, an increase in the amount of the two IRE-containing DMT1 transcripts (15) and DMT1 protein (3) was observed, and in mice fed the iron-enriched diet decreases in DMT1 mRNA and protein were reported. We suggest that of the four DMT1 isoforms expressed in the kidney, the changes we observed reflect changes in the proteins derived from the transcripts containing IREs. Others showed that DMT1 expression in the duodenum, placenta, and liver is also responsive to dietary iron load (10, 29). In duodenum and placenta, as in the kidney, low-iron diets induce an increase in DMT1 expression, whereas the inverse is true for iron-enriched diets. In the liver, low-iron diets cause a reduction in the amount of DMT1, and iron-enriched diets cause an increase in DMT1 expression (26). Therefore, expression of DMT1 in the kidney responds in the same way as the duodenum and placenta, but the opposite to the liver, to altered dietary iron intake.

In the duodenum, DMT1 is directly responsible for translocation of iron across the apical mucosal mem-

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**Fig. 4.** Indirect immunofluorescence micrographs of divalent metal transporter1 (DMT1) distribution in 4-μm cryosections from PFA-fixed rat kidney superficial cortex (magnification ×85). A: cortex from CD-fed rat showing typical DMT1-specific fluorescence in proximal tubule within cortical labyrinth and extending along collecting ducts. B: cortex from RD-fed rats showing much increased DMT1-specific fluorescence throughout cortical labyrinth. C: cortex from ED-fed rats showing much decreased DMT1-specific fluorescence throughout cortical labyrinth. G, glomerulus.

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brane (26). Transport of iron out of the gastrointestinal lumen via DMT1 is driven by a proton gradient directed inward to the cell interior (25). The increase in DMT1 expression in response to low-iron diet is suggested to increase the amount of iron absorbed from the diet to maintain iron balance (29). The changes in DMT1 expression we observed in the kidney support a role for DMT1 in renal handling of iron, perhaps in preserving body iron levels by increasing reabsorption of filtered iron. If this is true, then changes in DMT1 expression would be expected to affect the rate of renal iron excretion.

In this study, renal iron excretion rate decreased in iron-restricted animals, as would be expected if iron reabsorption had increased, due to the increased expression of DMT1. In contrast, the inverse was observed for animals fed an iron-enriched diet, because DMT1 expression was reduced in this group. The reduction in DMT1 expression may have caused a reduction in iron reabsorption, hence the observed increase in urinary iron excretion. On the other hand, the different diets caused changes in the serum concentration of iron that could result in different rates of iron being filtered by the glomerulus. By normalizing the rate of urinary excretion to the plasma iron concentration (UV/P), it is possible to gain a valuable insight into the contribution of glomerular and tubular effect. Performing these calculations gave values of 0.0033 l/day for the RD group and 0.0126 l/day for the ED group. Because the value for the ED group is 3.8 times greater than that of the RD group, this signifies that, although plasma iron was increased in the ED group, significantly more iron was excreted than can be accounted for purely due to glomerular events. This strongly suggests a causal link between the changes in DMT1 expression we observed and altered urinary iron excretion.

It is acknowledged that the majority of iron is bound to transferrin and serum iron concentration is not the filtered iron concentration. However, we would argue that the rate of iron filtration is related to the serum (transferrin bound) iron and therefore it is valid to normalize urinary excretion to serum iron concentration. It is also worthy of note that even if the assumption was made that the amount of iron filtered did not differ between the groups, then we would have severely underestimated UV/P, especially in the ED group and consequently the differences between the groups would be much greater.

The changes in expression we observed were most marked in the proximal tubule. Of note is the finding that despite significant changes in the level of DMT1 expression, there was no evidence of DMT1 expression in apical or basolateral proximal tubule membranes.

Fig. 5. High-magnification (×340) images of DMT1 immunostaining in 4-μm cryosections from PFA-fixed rat kidney superficial cortex. A: proximal tubules (P) from CD-fed rat showing typical intracellular DMT1-specific fluorescence without staining on brush-border (arrowheads) or basolateral (small arrows) membranes. B: proximal tubules from RD-fed rats showing much increased, compared with CD rats, punctate intracellular DMT1 staining. No staining of brush-border (arrowheads) or basolateral (small arrows) membranes was evident. Inset: staining was not observed in the absence of DMT1 antisera, when only the secondary antiserum was included. C: proximal tubules from ED-fed rats showing much decreased intracellular DMT1 staining. Brush-border (arrowheads) or basolateral (small arrows) membranes showed no staining.
under any of the experimental regimens we employed. Thus DMT1 is unlikely to play a role in transport of iron across the plasma membranes of these cells. Also, these data oppose the notion that DMT1 expressed in the intracellular compartment of proximal tubule cells represents transporters that under certain conditions traffic to the plasma membrane to increase iron transport into or out of the cell. In contrast, Canonne-Hergaux and Gros (3) reported DMT1 to be expressed on brush-border membranes of proximal tubule cells in mice. Therefore, our data further highlight possible differences between the mouse and rat in terms of renal DMT1 distribution.

If DMT1 is not responsible for moving iron across the apical or basolateral membranes of the proximal tubule, what role does it play in these cells? In Hep-2...
cells, DMT1 localized to late endosomes and lysosomes and functions to transfer endosomal free iron into the cytoplasm (24). In a previous study, we showed that a high proportion of the DMT1 expressed in the proximal tubule colocalized with the lysosomal protein LAMP1 (6). DMT1 may therefore serve a similar intracellular function in the proximal tubule, possibly mediating exit of sequestered iron from vesicles into the cytosol. Given the very high metabolic activity of proximal tubule cells, inferring a considerable demand for iron-containing enzymes, upregulation of DMT1 perhaps represents a means of maintaining a supply of iron to the cells when serum iron is low. When serum iron levels are high, downregulation would also make sense because cells would be iron replete and downregulation of DMT1 may function as a protective measure against iron-induced damage. The question as to the origin of the iron handled by DMT1 expressed in the proximal tubule, is it from the basolateral membrane or the apical membrane remains unclear. Although we would suggest that this iron originates from the lumen of the proximal convoluted tubule and is the result of reabsorption of filtered iron, it is also possible that iron is taken up across the basolateral membrane. Clearly, further investigation is necessary to resolve this question.

Is DMT1 responsible for iron reabsorption in distal nephron segments? The mean pH in the DCT is 6.7 (4) and the optimal pH for human DMT1 has been reported to be pH 6.7 (28), although at lower pH DMT1 is also active. Therefore, a proton gradient orientated into the cell is present in the DCT and because DMT1 uses a proton gradient to translocate iron, this would favor transport of iron across the apical membrane and into the cell by DMT1. Alfrey and Hammond (2) suggested that iron is filtered by the glomerulus complexed to other molecules, and it may dissociate as it passes down the nephron into the acidic environment of the DCT. Our finding that DMT1 is expressed in the apical region of the DCT and iron is reabsorbed in distal nephron segments (27) suggests that DMT1 may mediate iron movement across apical DCT membranes. Furthermore, we observed enhanced DMT1 expression in DCT in animals fed the RD and decreased DMT1 expression in DCT and cortical collecting ducts of ED-fed animals. These changes are congruent with increased iron reabsorption when dietary iron is restricted and the converse when iron is plentiful.

In conclusion, renal DMT1 expression is strongly modulated in response to altered dietary iron intake and these responses are associated with changes in urinary iron excretion rate. Together, these findings suggest that changes in DMT1 expression influence urinary iron excretion and potentially provide the body with one means of conserving or excreting iron.

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DISCLOSURES
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