Iron absorption and hepatic iron uptake are increased in a transferrin receptor 2 (Y245X) mutant mouse model of hemochromatosis type 3

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1School of Medicine and Pharmacology and 2Western Australian Institute for Medical Research, Fremantle Hospital; 3School of Biomedical and Chemical Sciences; 4Laboratory for Cancer Medicine, Centre for Medical Research, Western Australian Institute for Medical Research and 5School of Medicine and Pharmacology, Royal Perth Hospital, The University of Western Australia, Perth, Western Australia, Australia; and Departments of 6Pediatrics and 7Internal Medicine, St. Louis University School of Medicine, St. Louis, Missouri

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Drape SF, Morgan EH, Herbison CE, Delima R, Graham RM, Chua AC, Leedman PJ, Fleming RE, Bacon BR, Olynyk JK, Trinder D. Iron absorption and hepatic iron uptake are increased in a transferrin receptor 2 (Y245X) mutant mouse model of hemochromatosis type 3. Am J Physiol Gastrointest Liver Physiol 292: G323–G328, 2007. First published August 24, 2006; doi:10.1152/ajpgi.00278.2006.—Hereditary hemochromatosis type 3 is an iron (Fe)-overload disorder caused by mutations in transferrin receptor 2 (TfR2). TfR2 is expressed highly in the liver and regulates Fe metabolism. The aim of this study was to investigate duodenal Fe absorption and hepatic Fe uptake in a TfR2 (Y245X) mutant mouse model of hereditary hemochromatosis type 3. Duodenal Fe absorption and hepatic Fe uptake were measured in vivo by 59Fe-labeled ascorbate in TfR2 mutant mice, wild-type mice, and Fe-loaded wild-type mice (2% dietary carbonyl Fe). Gene expression was measured by real-time RT-PCR. Liver nonheme Fe concentration increased progressively with age in TfR2 mutant mice compared with wild-type mice. Fe absorption (both duodenal Fe uptake and transfer) was increased in TfR2 mutant mice compared with wild-type mice. Likewise, expression of genes participating in duodenal Fe uptake (Dcytb, DMT1) and transfer (ferroportin) was increased in TfR2 mutant mice. Nearly all of the absorbed Fe was taken up rapidly by the liver. Despite hepatic Fe loading, hepcidin expression was decreased in TfR2 mutant mice compared with wild-type mice. Even when compared with Fe-loaded wild-type mice, TfR2 mutant mice had increased Fe absorption, increased duodenal Fe transport gene expression, increased liver Fe uptake, and decreased liver hepcidin expression. In conclusion, despite systemic Fe loading, Fe absorption and liver Fe uptake were increased in TfR2 mutant mice in association with decreased expression of hepcidin. These findings support a model in which TfR2 is a sensor of Fe status and regulates duodenal Fe absorption and liver Fe uptake.

Heredity hemochromatosis; iron metabolism; liver iron overload and transferrin receptor 2; hepcidin

Heredity Hemochromatosis (HH) type 1 is a genetic iron (Fe)-overload disorder that is usually caused by C282Y homozygosity in the HFE gene. Other types of HH have also been identified that have similar characteristics to HH type 1 and are caused by mutations in hemojuvelin (type 2a), hepcidin (type 2b), or transferrin receptor 2 (TfR2; type 3) (5, 33, 37). HH type 3 was initially identified in two Sicilian families and was found to be caused by a homozygous nonsense mutation (Y250X) in the TfR2 gene (5). Subsequently, a number of other mutations have been identified in TfR2 that cause Fe overload in both Caucasians and Japanese (3, 13, 16, 23, 25, 28, 34, 38). Phenotypic characteristics of this disorder include elevated serum transferrin saturation, serum ferritin levels, and liver Fe loading and clinical manifestations of HH such as liver cirrhosis, arthritis, and diabetes. The clinical phenotype of HH type 3 is usually more severe than classical HH type 1 but less severe than juvenile HH type 2 (6).

TfR2 is a type II transmembrane glycoprotein that is highly expressed by liver (22). It has a high degree of similarity to TfR1 but has a 30-fold lower affinity for diferric transferrin than TfR1 and unlike TfR1 does not bind to HFE in vitro (20, 40). Because of the similarity between TfR2 and TfR1, it is thought that TfR2 may have a role in the cellular uptake of Fe. Several lines of evidence suggest this is the case. When TfR2 was overexpressed in Chinese hamster ovary cells or K562 erythroleukemic cells, there was an increase in the cellular uptake of transferrin-bound Fe (22, 36). In addition, HuH7 human hepatoma cells express both TfR1 and TfR2 and take up transferrin-bound Fe by two saturable processes. During cellular proliferation, both TfR1 and TfR2 expression and transferrin-bound Fe uptake by these two processes were upregulated (26).

Unlike TfR1, TfR2 mRNA does not contain an Fe-responsive element and TfR2 mRNA expression is not regulated by intracellular Fe levels (20). However, recently, it has been shown that hepatic TfR2 protein is regulated posttranslationally by diferric transferrin, which increases stability and half-life of the receptor (17). In animal models of Fe overload, such as mice fed an Fe-supplemented diet or Hfe knockout mouse models of HH type 1, transferrin saturation, hepatic Fe levels, and hepatic TfR2 protein expression are all increased (35). However, in hypotransferrinemic mice, the hepatic Fe level is increased, whereas serum transferrin concentration and hepatic TfR2 levels are low (35). These findings suggest that the concentration of diferric transferrin and not hepatic Fe levels is important in the regulation of hepatic TfR2 protein.

In humans and animal models of HH type 3, mutations in TfR2 have been shown to cause liver Fe overload (5, 11). Therefore, in addition to mediating the uptake of Fe, it is likely that TfR2 is important in the regulation of Fe metabolism. The most common mutation found in human TfR2 is the Y250X

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mutation that introduces a stop codon into the mRNA, reducing TfR2 protein expression (11, 21). It is hypothesized that hepatic TfR2 senses body Fe status by reflecting transferrin saturation and that TfR2 controls the expression of the hepatic peptide hepcidin to regulate Fe absorption and hepatic Fe levels. Furthermore, in HH type 3, Fe sensing by TfR2 and signaling to hepcidin is impaired, disrupting Fe homeostasis.

In this study, we have investigated the mechanisms of Fe loading in HH type 3 by measuring Fe absorption and liver Fe uptake in vivo using a mouse model of HH type 3, which has a Y245X mutation in TfR2 (murine orthologue of human TfR2 Y250X). Our results show that the TfR2 Y245X mutation causes an upregulation in Fe absorption, with almost all absorbed Fe being incorporated into the liver. In contrast, Fe-loaded wild-type mice have reduced Fe absorption and liver Fe uptake. These results suggest that TfR2 acts as a sensor of Fe status that regulates Fe absorption and consequent liver Fe deposition and that this sensing process is impaired in the TfR2 mutant mouse model of HH type 3.

MATERIALS AND METHODS

Animals. TfR2 (Y245X) mutant mice were generated on a C57BL/6 × 129/SVJ hybrid strain background as described previously (11). The mice were backcrossed for five generations onto an AKR background, and homozygous mutant and wild-type mice were derived from TfR2 Y245X heterozygous mice. Female TfR2 mutant and wild-type mice were fed a control diet (containing 0.007% Fe) and studied between 4 and 21 wk of age. Another group of female wild-type mice were fed an Fe-supplemented diet containing 2% carboxyl Fe for 3 wk from 7 wk of age. This study was approved by The University of Western Australia Animal Ethics Committee.

Analytic measurements. Plasma nontransferrin-bound iron (NTBI) was determined with the use of Tris-carbonatocobaltate (III) trihydrate as described previously (27) and modified to measure mouse plasma NTBI (8). Plasma Fe concentration and transferrin saturation were measured by the method of Fielding (10). Liver nonheme Fe concentration was measured by the method of Kaldor (18).

Fe absorption. Mice were fasted overnight, and Fe absorption was measured in vivo by a method similar to that described for rats (32). The mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) for the duration of the experiment. A midline incision was made, and the stomach and duodenum were exteriorized. A ligature was loosely tied around the pylorus of the stomach, and a second ligature was tied securely around the small intestine 2–3 cm distal of the pyloric ligature. A small opening was made into the wall of the antrum of the stomach, above the pyloric ligature, and a cannula was then withdrawn, and the ligature was secured tightly. The abdominal contents were returned to the peritoneal cavity, and the wall of the abdomen was closed with sutures. Each mouse was placed in a whole body counter to measure gamma radiation to determine the dose of 59Fe injected. Thirty minutes after the injection, the abdominal cavity was opened and the duodenal sac was removed. The duodenal sac was washed with 1 ml of 5 mM EDTA-HEPES in 150 mM NaCl (pH 7.0). The duodenum and carcass were counted separately in the whole body gamma counter. The liver was then removed and counted for gamma radiation.

Total absorption of Fe was expressed as a percentage of the dose injected and calculated as follows: (cpm carcass/cpm injected) × 100. Percent Fe uptake by the duodenum was calculated by [(cpm duodenum + cpm carcass)/cpm injected] × 100. Percent Fe transfer from the duodenum to the body was calculated by [cpm carcass/(cpm duodenum + cpm carcass)] × 100. Liver Fe uptake was expressed either as percent dose calculated by (cpm liver/cpm injected) × 100 or as percent Fe absorbed calculated by (cpm liver/cpm carcass) × 100.

Gene expression. RNA was isolated from duodenum and liver using RNA-WIZ (Ambion, Austin, TX) according to the manufacturer’s instructions and then treated with DNase I (DNAfree; Ambion). Reverse transcription was performed with 1.5 μg of RNA, oligo(dT), and Superscript II reverse transcriptase (Invitrogen, Melbourne, Australia) as per manufacturer’s instructions with the addition of 15 U RNase inhibitor in each reaction (RNasin; Promega, Madison, Wisconsin, USA). mRNA transcripts were quantified by real-time PCR with the use of SYBR green (Molecular Probes, Sydney, Australia) on a Roche LightCycler instrument (Roche, Mannheim, Germany). Gene expression was standardized against 18S rRNA (GenBank accession number D03706) as an endogenous control.

RESULTS

Plasma Fe parameters. Plasma NTBI, Fe concentration, and transferrin saturation were elevated two- to threefold in TfR2 mutant mice compared with wild-type mice at 10 wk of age (Table 1). Plasma Fe parameters were also elevated in TfR2 mutant mice compared with wild-type mice at 6 and 15 wk of age (data not shown). In addition, wild-type mice fed an Fe-supplemented diet had similar plasma NTBI and Fe levels and transferrin saturation to TfR2 mutant mice at 10 wk of age (Table 1).

Fe absorption. To determine whether the elevated plasma Fe parameters in TfR2 mutant mice were due to changes in dietary Fe absorption, we measured duodenal Fe uptake and transfer in vivo in TfR2 mutant and wild-type mice and in wild-type mice fed an Fe-supplemented diet. At 10 wk of age, total duodenal Fe absorption (Fig. 1A) by TfR2 mutant mice was increased.
Iron Absorption by TfR2 Mutant Mice

Twofold compared with wild-type mice (TfR2 mutant, 63 ± 6%; wild-type, 30 ± 5%; P < 0.01). This was because of increased Fe uptake by the duodenum (TfR2 mutant, 100 ± 4%; wild-type, 87 ± 2%; P < 0.05; Fig. 1B) and Fe transfer from the duodenum to the plasma (TfR2 mutant, 63 ± 4%; wild-type, 37 ± 5%; P < 0.01; Fig. 1C). In contrast, there was a marked decrease in the total absorption of Fe by wild-type mice fed an Fe-supplemented diet (8 ± 4%; P < 0.01) compared with both TfR2 mutant and wild-type mice fed a control diet (Fig. 1A). This was due to a significant decrease in both Fe uptake (54 ± 7%; P < 0.01) and Fe transfer (14 ± 4%; P < 0.01) by the duodenum of the Fe-loaded wild-type mice compared with both TfR2 mutant and wild-type mice fed a control diet (Fig. 1, B and C). At 6 wk of age, compared with wild-type mice, total Fe absorption by TfR2 mutant mice was also significantly elevated to a similar degree, as seen at 10 wk of age (6 wk: TfR2 mutant, 62 ± 7%; wild-type, 24 ± 5%; P < 0.001).

Liver Fe uptake. $^{59}$Fe uptake by the liver was increased markedly in TfR2 mutant mice compared with wild-type mice when expressed as a percentage of the $^{59}$Fe dose given to the mice (TfR2 mutant, 55 ± 5%; wild-type, 5 ± 1%; P < 0.001; Fig. 2A). Also, liver Fe uptake expressed as a percentage of Fe absorbed from the diet by TfR2 mutant mice was about sevenfold greater than liver uptake by wild-type mice, and nearly all Fe absorbed by TfR2 mutant mice was taken up by the liver (TfR2 mutant, 87 ± 6%; wild-type, 13 ± 1%; P < 0.001; Fig. 2B). In contrast, hepatic Fe uptake by wild-type mice fed an Fe-supplemented diet (4 ± 1% dose; Fig. 2A) was greatly reduced compared with TfR2 mutant mice but was similar to Fe uptake by wild-type mice. However, when the differences in the amount of Fe absorbed by the mice was taken into account, and hepatic Fe uptake was expressed as a percentage of Fe absorbed, uptake by wild-type mice fed the Fe-supplemented diet (41 ± 13%; Fig. 2B) was greater than uptake by wild-type mice but less than TfR2 mutant mice (Fig. 2B). Hepatic Fe uptake in TfR2 mutant mice was also elevated at 6 wk of age compared with wild-type mice (TfR2 mutant, 57 ± 7%; wild-type, 2 ± 0.2% dose; P < 0.001) and was similar to that in TfR2 mutant and wild-type mice at 10 wk of age.

Liver Fe levels. Hepatic nonheme Fe concentration increased progressively in TfR2 mutant mice between 4 and 21 wk of age. Nonheme Fe levels increased two- to fivefold in TfR2 mutant mice compared with wild-type mice at 4 and 21 wk, respectively (Fig. 3A). At 10 wk of age, the liver nonheme Fe levels in wild-type mice fed an Fe-supplemented diet was similar to TfR2 mutant mice (TfR2 mutant, 23.8 ± 0.9; wild-type, 24.0 ± 1.2 μmol Fe/g wet wt liver). Also hepatic ferritin levels in TfR2 mutant mice and wild-type mice fed an Fe-supplemented diet were similar and increased by about fivefold compared with wild-type mice (data not shown).

Gene expression. Liver hepcidin mRNA expression was reduced significantly in TfR2 mutant mice compared with wild-type mice. However, when the differences in the amount of Fe absorbed by the mice was taken into account, and hepatic Fe uptake was expressed as a percentage of Fe absorbed, uptake by wild-type mice fed the Fe-supplemented diet (41 ± 13%; Fig. 2B) was greater than uptake by wild-type mice but less than TfR2 mutant mice (Fig. 2B). Hepatic Fe uptake in TfR2 mutant mice was also elevated at 6 wk of age compared with wild-type mice (TfR2 mutant, 57 ± 7%; wild-type, 2 ± 0.2% dose; P < 0.001) and was similar to that in TfR2 mutant and wild-type mice at 10 wk of age.

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Gene expression. Liver hepcidin mRNA expression was reduced significantly in TfR2 mutant mice compared with wild-type mice.
wild-type mice at 6, 10, and 15 wk of age (Fig. 3B) and increased significantly in wild-type mice fed an Fe-supplemented diet compared with both TfR2 mutant and wild-type mice fed a control diet at 10 wk of age. In contrast, Fe-loaded wild-type mice had elevated liver hepcidin expression and markedly reduced duodenal Fe transport gene expression and Fe absorption, and only a small percentage of the $^{59}$Fe given to the mice was deposited in the liver.

**DISCUSSION**

In this study, we demonstrated that TfR2 mutant mice have progressive hepatic Fe overload. The Y245X mutation in TfR2 led to decreased liver hepcidin levels and increased duodenal Fe transport gene expression, causing an upregulation in Fe absorption. Almost all of the absorbed Fe was deposited rapidly in the liver. This increase in hepatic Fe uptake is likely to make a major contribution to the development of liver Fe overload in HH type 3. Wild-type mice fed an Fe-supplemented diet had elevated liver Fe and plasma Fe parameters similar to those seen in TfR2 mutant mice. However, in contrast to TfR2 mutant mice, Fe-loaded wild-type mice had elevated liver hepcidin expression and markedly reduced duodenal Fe transport gene expression and Fe absorption, and only a small percentage of the $^{59}$Fe given to the mice was deposited in the liver.

**Table 2. Duodenal mRNA expression in TfR2 mutant and wild-type mice**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Wild-Type</th>
<th>TfR2 Mutant</th>
<th>Wild-Type + Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMT1</td>
<td>1.4±0.1</td>
<td>3.1±0.4*</td>
<td>1.4±0.2†</td>
</tr>
<tr>
<td>Dcytb</td>
<td>2.4±0.4</td>
<td>9.1±1.8*</td>
<td>1.1±0.2‡</td>
</tr>
<tr>
<td>Ferroportin</td>
<td>10.2±1.0</td>
<td>16.8±1.5*</td>
<td>8.4±0.4‡</td>
</tr>
</tbody>
</table>

Values are means ± SE, where n = 4–6 mice. Duodenal mRNA expression was measured in 10-wk-old mice and normalized to β-actin expression. *P<0.01, TfR2 mutant vs. wild-type mice; †P<0.01, wild-type + Fe vs. TfR2 mutant mice; ‡P<0.05, wild-type + Fe vs. wild-type mice.
leads to a downregulation of Fe absorption, and, in *TfR2* mutant mice, despite their elevated Fe status, this effect has been lost. This suggests *TfR2* mutant mice do not have the ability to sense their body Fe levels and regulate Fe absorption appropriately.

Fe absorption was elevated in the *TfR2* mutant mice with an increase in Fe uptake across the apical surface of the duodenum, which is likely to be due to the upregulation of DMT1 and Dcytb expression and an increase in Fe transfer across the basolateral membrane of the duodenum to the blood, which may be caused by an increase in ferroportin expression (Fig. 1; Table 2).

In *TfR2* mutant mice, hepatic hepcidin mRNA levels were reduced relative to wild-type mice (Fig. 3B). This confirms recent observations by other investigators using *TfR2* mutant mice (19) and is consistent with reports that urinary hepcidin peptide levels were also reduced in humans with HH type 3 (30). The mechanism by which *TfR2* regulates liver hepcidin expression is unknown.

In vitro, hepcidin has been shown to bind to ferroportin and induce its downregulation by internalization and degradation of the protein, causing a decrease in Fe release from the cells and an increase in cellular Fe levels (31). Therefore, in *TfR2* mutant mice, the low levels of hepcidin expression would be anticipated to increase the level of ferroportin protein and Fe transfer by the duodenum; low hepcidin levels may also be responsible for the observed increase in ferroportin mRNA levels by an undetermined mechanism. In addition, the mechanism responsible for the upregulation in duodenal DMT1 and Dcytb and subsequent increase in Fe uptake by the duodenum in *TfR2* mutant mice is not clear. Both DMT1 and Dcytb are upregulated by Fe deficiency (14, 29), which is also associated with low hepcidin levels and increased ferroportin expression. Therefore, in the duodenum of *TfR2* mutant mouse, increased Fe efflux mediated by ferroportin may result in an upregulation of duodenal DMT1 and Dcytb expression. However, a recent study by Gunshin et al. (15) showed that Dcytb is not essential for Fe absorption, indicating that another reductase may be involved. Others have suggested that DMT1 expression and Fe uptake by the duodenum but not ferroportin expression and Fe transfer are regulated by hepcidin (24).

Liver Fe loading in *TfR2* mutant mice is unlikely to involve transferrin receptor-mediated uptake of diferric transferrin because both TIR2 (11, 21) and TIR1 protein levels are downregulated (R. Fleming, unpublished observation). In *TfR2* mutant mice, plasma transferrin was almost completely saturated with Fe and plasma NTBI levels were increased (Table 1). Therefore, much of the recently absorbed Fe is likely to be in the plasma in the form of NTBI. It is extremely toxic, and 60–75% of the NTBI is rapidly cleared from the circulation on first pass through the liver (4). Therefore, it is likely that uptake by the liver of recently absorbed Fe in the form of NTBI plays an important role in the development of Fe overload in *TfR2* mutant mice. Similar results have been obtained in the *Hfe* knockout mouse model of HH type 1, in which plasma NTBI levels were elevated and the uptake of NTBI by hepatocytes was increased (8).

*TfR2* mutant mice progressively accumulated Fe in the liver up to at least 21 wk of age (Fig. 3A), and this was accompanied by reduced hepcidin levels (Fig. 3B) and increased duodenal Fe transport gene expression up to at least 15 wk of age (Table 2). These findings are in contrast to those reported for *TfR2* knockout mice, where at 10 wk of age the expression levels of hepcidin and duodenal Fe transport genes were similar to expression levels in wild-type mice (39), suggesting that liver Fe loading may have reached a plateau. Differences in gene expression between *TfR2* knockout mice and *TfR2* mutant mice may be due to variations in genetic background, which affects both gene expression and severity of Fe loading in *Hfe* knockout mice (9, 12). Liver Fe accumulation in *Hfe* knockout mice was rapid during the first weeks of life; however, by 10 wk of age, accumulation reached a plateau and was accompanied by a normalization of hepcidin expression and Fe absorption (1, 2). The reason for differences between *Hfe* knockout and *TfR2* mutant mice is unclear, but it may reflect a more severe clinical phenotype associated with HH type 3 compared with HH type 1 (6).

In conclusion, our findings indicate that *TfR2* mutant mice develop Fe overload in the absence of functional TIR2 as a result of an impaired ability to sense body Fe levels and to express hepcidin appropriately, leading to the upregulation of Fe absorption and increased deposition of the absorbed Fe in the liver. These findings have important implications for understanding the molecular pathogenesis of HH type 3 in humans.

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