Research Article

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Iron uptake from plasma transferrin by a transferrin receptor 2 mutant mouse model of haemochromatosis

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Background & Aims: Hereditary haemochromatosis type 3 is caused by mutations in transferrin receptor (TFR) 2. TFR2 has been shown to mediate iron transport *in vitro* and regulate iron homeostasis. The aim of this study was to determine the role of Tfr2 in iron transport *in vivo* using a *Tfr2* mutant mouse.

Methods: *Tfr2* mutant and wild-type mice were injected intravenously with ^{59}Fe-transferrin and tissue ^{59}Fe uptake was measured. Tfr1, Tfr2 and ferroportin expression was measured by real-time PCR and Western blot. Cellular localisation of ferroportin was determined by immunohistochemistry.

Results: Transferrin-bound iron uptake by the liver and spleen in *Tfr2* mutant mice was reduced by 20% and 65%, respectively, whilst duodenal and renal uptake was unchanged compared with iron-loaded wild-type mice. In *Tfr2* mutant mice, liver Tfr2 protein was absent, whilst ferroportin protein was increased in non-parenchymal cells and there was a low level of expression in hepatocytes. Tfr1 expression was unchanged compared with iron-loaded wild-type mice. Splenic Tfr2 protein expression was absent whilst Tfr1 and ferroportin protein expression was increased in *Tfr2* mutant mice compared with iron-loaded wild-type mice.

Conclusions: A small reduction in hepatic transferrin-bound iron uptake in *Tfr2* mutant mice suggests that Tfr2 plays a minor role in liver iron transport and its primary role is to regulate iron metabolism. Increased ferroportin expression due to decreased hepcidin mRNA levels is likely to be responsible for impaired splenic iron uptake in *Tfr2* mutant mice.

Keywords: Transferrin receptor 2; Iron transport; Haemochromatosis; Iron overload; Liver.

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Abbreviations: TFR, transferrin receptor; IRE, iron regulatory element; HJV, haemojuvelin; BMP, bone morphogenetic protein; FPN, ferroportin; HH, hereditary haemochromatosis; Hamp, hepcidin; IRP, iron regulatory protein.

Introduction

Iron is an essential trace metal required for cellular growth and metabolism. Iron is normally transported in the plasma bound to transferrin and binds to transferrin receptors (TFR) expressed at the cell surface. There are two types of TFRs; TFR1 is ubiquitously expressed whilst TFR2 is highly expressed in hepatocytes and to a lesser extent in erythrocytes and duodenum [1]. The main function of TFR1 is to deliver transferrin-bound iron to cells via receptor-mediated endocytosis [2]. TFR2 has a lower binding affinity for diferric transferrin than TFR1 [3,4]. When TFR2 is over-expressed in Chinese Hamster Ovary cells, it mediates the endocytosis and recycling of transferrin [2] and uptake of transferrin-bound iron [1,2]. Whether this is the case in the liver, erythrocytes and duodenum, where TFR2 is expressed is yet to be determined. Recent evidence suggests that TFR2, like TFR1, also binds to HFE. Conversely, HFE does not compete with transferrin for TFR2 binding and binds TFR2 at a site that is distinct from the site of HFE-TFR1 interaction [5]. HFE has also been shown to increase the affinity of TFR2 for transferrin and increase transferrin-bound iron uptake *in vitro* [6].

TFR1, but not TFR2, is inversely regulated by intracellular iron levels by a post-transcriptional mechanism involving iron responsive elements (IRE). Instead, TFR2 is regulated by extracellular diferric transferrin levels by a post-translational mechanism. Diferric transferrin binds to TFR2 and increases its stability by redirecting TFR2 from a degradative pathway to a recycling pathway inside the cell, thereby increasing the half-life of the protein [7]. The regulation of TFR2 by transferrin saturation controls the expression of the iron regulatory peptide, hepcidin, by an unknown mechanism. The interaction of HFE and TFR2 regulates hepcidin expression [8] which may involve haemojuvelin/bone morphogenetic protein (HJV/BMP) signalling in hepatocytes [9]. Hepcidin is highly expressed by hepatocytes and is secreted into the circulation to regulate systemic body iron levels. It binds

to the iron export protein, ferroportin (FPN), which is highly expressed in macrophages and enterocytes and is also expressed in hepatocytes. Upon binding, hepcidin induces the internalisation and degradation of FPN resulting in decreased iron release [10].

Mutations in TFR2 results in the iron overload disorder, hereditary haemochromatosis (HH) type 3. A *Tfr2* mutant mouse model of HH type 3 has been generated with a Y245X mutation in the *Tfr2* gene which is orthologous to the Y250X mutation identified in humans [11]. These mice have similar characteristics of the iron overload observed in subjects with HH type 3 [11,12]. The Y245X mutation in *Tfr2* results in decreased hepcidin mRNA expression leading to increased iron absorption and the rapid deposition of the absorbed iron in the liver resulting in hepatic iron overload [12]. Iron overload that results from liver specific deletion of the *Tfr2* gene is comparable to the complete *Tfr2* knockout mice [13], indicating the central role of the liver in the regulation of iron metabolism.

In the present study, the role of Tfr2 in transferrin-bound iron uptake *in vivo* was investigated using a *Tfr2* mutant mouse model of HH type 3. We provide evidence that TFR2 has a minor role in iron transport and hepatic iron-loading *in vivo*. In the spleen, a decrease in iron uptake in *Tfr2* mutant mice is likely to be due to increased Fpn-mediated iron export as a result of a down-regulation of hepcidin expression.

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 5 generations onto an AKR background and homozygous mutant and wild-type mice were derived from *Tfr2* (Y245X) heterozygous mice (Animal Resource Centre, Australia). Female *Tfr2* mutant and wild-type mice were fed either a control diet (70 mg iron/kg) or an iron-supplemented diet (20 g carbonyl iron/kg; Specialty Feeds, Australia) for 3 weeks from 7 to 10 weeks of age. All mice were studied between 10 and 14 weeks of age. This study was approved by The University of Western Australia Animal Ethics Committee.

Non-haem iron measurements

Liver and spleen non-haem iron levels were measured using the method of Kaldor [14].

Plasma iron clearance

Tfr2 mutant and wild-type mice were injected with 150 μg of ^{59}Fe–^{125}I-transferrin and 150 μg ^{131}I-albumin intravenously into the ventral tail vein. Blood samples were collected at 2, 30, 60 and 90 min after injection and blood, liver, spleen, kidney and duodenum were collected 120 min after injection and counted for radioactivity. Tissue uptake of transferrin-bound iron and the rate of plasma iron turnover were determined as described previously [15]. See supplementary information for further details.

Western blot analysis

Tfr1, Tfr2, Fpn and actin protein expression were determined in liver and spleen tissue from *Tfr2* mutant and non-iron and iron-loaded wild-type mice as described previously [16,17]. Tfr1, Tfr2 and Fpn protein expression were normalised to actin expression and expressed relative to non-iron-loaded wild-type mice.

RNA expression

Total RNA was isolated from liver and spleen tissue and reverse transcribed as described previously [16,17]. *Tfr1*, *Tfr2*, *Fpn*, *Hamp1* and *β-actin* mRNA transcripts were measured by real-time polymerase chain reaction (PCR) in a Rotorgene (Corbett Research, Australia) using primers listed in Table 1 and quantified using standard curves generated from serial dilutions of known copy number of plasmids containing cDNA of the gene of interest. *Tfr1*, *Tfr2*, *Fpn* and *Hamp1* mRNA expression were normalised against *β-actin* mRNA expression.

Table 1. Primers for real-time PCR.

Gene	Sequence (5′–3′)	Genbank number
Tfr2	Fwd:TTCCTACATCATCTCGCTTAT Rev:TGGCGACACATACTGGGGACAG	NM 015799
Tfr1	Fwd:TTCCTACATCATCTCGCTTAT Rev:CATAGTGTTCATCTCGCCAGA	NM 011638
Fpn	Fwd:TTGCAGGAGTCATTGCTGCTA Rev:TGGAGTTCTGCACACCATT	NM 016917
Hamp1	Fwd:TTGCGATACCAATGCAGAAGA Rev:GATGTGGCTCTAGGCTATGTTTTG	NM 032541
β-actin	Fwd:CTGGCACCACACCTTCTA Rev:GGGCACAGTGTGGGTGAC	NM 007393

Tfr1, transferrin receptor 1; *Tfr2*, transferrin receptor 2; *Fpn*, ferroportin; *Hamp1*, hepcidin1.

Immunohistochemistry

Frozen liver tissue was fixed with methanol/acetone (1:1), permeabilised with 0.02% Tween® 20 in PBS and blocked with 5% goat serum in PBS. Tissue sections were incubated with primary antibodies, rabbit anti-Fpn (Alpha Diagnostic, USA) and/or rat anti-F4/80, a macrophage marker (kind gift from Professor Ruth Ganss, Western Australian Institute for Medical Research) at 1:150 overnight at 4 °C in REAL™ antibody diluent (DAKO, Denmark). Proteins were detected with secondary antibodies, goat anti-rabbit Alexa Fluor® 488 and/or goat anti-rat Alexa Fluor® 594 (Invitrogen Australia) at 1:200 in antibody diluent for 1 h in the dark. Sections were washed in PBS and mounted with ProLong Gold® antifade reagent with DAPI (Invitrogen) to counterstain the nuclei.

Statistics

Results are expressed as mean ± SEM where $n = 4$–8 mice per group. Differences between group means were analysed using ANOVA with Tukey's multiple comparison tests (GraphPad PRISM, USA) and were statistically significant for $p < 0.05$.

Results

Non-haem iron

Non-haem iron levels in the livers of *Tfr2* mutant, non-iron-loaded and iron-loaded wild-type mice were measured to confirm the iron status of the mice. Liver non-haem iron concentration in *Tfr2* mutant and iron-loaded wild-type mice was similar and 5-fold greater than non-iron-loaded wild-type mice (Fig. 1A). Similarly, plasma iron and transferrin saturation levels were increased in *Tfr2* mutant and iron-loaded wild-type mice compared to non-iron-loaded mice as shown previously [11,12]. In the spleen, non-haem iron concentration in *Tfr2* mutant mice was reduced by 70% and 80% compared with non-iron-loaded and iron-loaded wild-type mice, respectively (Fig. 1B). Liver and spleen ferritin protein expression correlated with non-haem iron concentrations (data not shown).

Transferrin-bound iron uptake

Liver uptake of transferrin-bound iron by *Tfr2* mutant mice was not significantly different from iron uptake by non-iron-loaded wild-type mice and was reduced by 20% compared with iron-