Research Article

JOURNAL OF HEPATOLOGY

Iron overload in hereditary tyrosinemia type 1 induces liver injury through the Sp1/Tfr2/hepcidin axis

Wen-Dai Bao[1], Yao Fan[1], Yue-Zhen Deng[1], Ling-Yun Long[1], Jing-jing Wang[1], Dong-Xian Guan[1], Zhen-Yu Qian[1], Peng An[1], Yuan-Yuan Feng[1], Zhi-Ying He[2], Xiao-Fan Wang[3], H. Phillip Koeffler[4,5], Ronggui Hu[6], Jianshe Wang[9], Xin Wang[7], Fudi Wang[8], Jing-Jing Li[1], Dong Xie[1,*]

[1]*Institute of Nutrition Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China;* [2]*Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, 225 Changhai Road, Shanghai 200438, China;* [3]*Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, USA;* [4]*Cancer Science Institute of Singapore, National University of Singapore, 14 Medical Drive, 12-01, Singapore 117599, Singapore;* [5]*Cedars-Sinai Medical Center, Division of Hematology/Oncology, UCLA School of Medicine, Los Angeles, CA 90048, USA;* [6]*State Key Laboratory of Molecular Biology, Chinese Academy of Sciences, 320 Yue-yang Road, Shanghai 200031, China;* [7]*Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota, MN 55455, USA;* [8]*Department of Nutrition, School of Public Health, Zhejiang University, Hangzhou, Zhejiang 310058, PR China;* [9]*Center for Pediatric Liver Diseases, Children's Hospital of Fudan University, 399 Wanyuan Road, Minhang District, Shanghai 201102, PR China*

Background & Aims: Iron is an essential metal for fundamental metabolic processes, but little is known regarding the involvement of iron in other nutritional disorders. In the present study, we investigated disordered iron metabolism in a murine model of hereditary tyrosinemia type I (HT1), a disease of the tyrosine degradation pathway.

Methods: We analysed the status of iron accumulation following NTBC withdrawal from *Fah*$^{-/-}$ mice, a murine model for HT1. Liver histology and serum parameters were used to assess the extent of liver injury and iron deposition. To determine the physiological significance of iron accumulation, mice were subjected to a low-iron food intake to reduce the iron accumulation. Mechanistic studies were performed on tissues and cells using immunoblotting, qRT-PCR, adenovirus transfection and other assays.

Results: Severe iron overload was observed in the murine model of HT1 with dramatically elevated hepatic and serum iron levels. Mechanistic studies revealed that downregulation and dysfunction of Tfr2 decreased hepcidin, leading to iron overload. The *Fah*$^{-/-}$ hepatocytes lost the ability of transferrin-sensitive induction of hepcidin. Forced expression of Tfr2 in the murine liver reduced the iron accumulation. Moreover, transcription factor Sp1 was downregulated and identified as a new regulator of *Tfr2* here. Additionally, low-iron food intake effectively reduced the iron deposits, protected the liver and prolonged the survival in these mice.

Conclusions: Iron was severely overloaded in the HT1 mice via the Sp1/Tfr2/Hepcidin axis. The iron overload induced liver injury in the HT1 mice, and reduction of the iron accumulation ameliorated liver injury.

Lay summary: Primary and secondary iron overload is an abnormal status affecting millions of people worldwide. Here, we reported severe iron overload in a murine model of HT1, a disease of the tyrosine degradation pathway, and elucidated the mechanistic basis and the physiological significance of iron overload in HT1. These studies are of general interest not only with respect to secondary iron-induced liver injury in HT1 but also are important to elucidate the crosstalk between the two metabolic pathways.

Keywords: Iron overload; Hereditary tyrosinemia; Tfr2/Hepcidin; Liver injury; Low-iron food.

Received 8 July 2015; received in revised form 25 February 2016; accepted 10 March 2016; available online 22 March 2016

* Corresponding author. Address: Institute of Nutrition Science, 320 Yueyang Road, Shanghai 200031, PR China. Tel.: +86 21 5492 0918.

E-mail address: dxie@sibs.ac.cn (D. Xie).

Abbreviations: HTI, hereditary tyrosinemia type I; Fah, fumarylacetoacetate hydrolase; Tfr2, transferrin receptor 2; HH, hereditary hemochromatosis; Hjv, hemojuvelin; NTBC, 2-(2-nitro-4-trifluoromethylbe -nzoyl)-1,3-cyclohexanedione; Fpn, ferroportin; Tfr1, transferrin receptor 1; ROS, reactive oxygen species; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ALT, alanine aminotransferase; AST, aspartate transaminase; ALB, albumin; TBIL, total bilirubin; CR, crea; BUN, blood urea nitrogen; TS, transferrin saturation; TIBC, total iron binding capacity; PCT, porphyria cutanea tarda; URO, uroporphyrin; UIBC, unsaturated iron binding capacity.

Introduction

Iron is essential for fundamental metabolic processes in cells. Iron is also toxic in excessive amounts, especially to the iron storage organ, the liver. Abnormal iron accumulation leads to oxidative stress, which is detrimental to cell membranes, proteins, nucleic acids, and can be potentially lethal [1]. Hereditary hemochromatosis (HH) is an autosomal recessive disorder that disrupts the body's regulation of iron, which is manifested as increased intestinal iron absorption and liver iron overload. This disease is caused by mutations in a variety of proteins involved in iron homeostasis, including Hfe [2], Hjv [3], hepcidin [4], transferrin receptor 2 (Tfr2) [5,6], or ferroportin (Fpn) [7]. HH patients

develop liver injury, hepatic cirrhosis and a high risk of hepatocellular carcinoma (HCC). Secondary iron overload also occurs in certain other liver diseases, such as hepatitis C virus (HCV) infection, HCC, hepatic porphyria and non-alcohol fatty liver disease [8,9]. The secondary iron has been shown to be a contributory factor in the development or progression of these diseases [10]. For example, excess hepatic iron is known to enhance both porphyria cutanea tarda (PCT) and experimental uroporphyria [11]. Previous studies have demonstrated that a modest depletion of hepatic iron was sufficient to prevent uroporphyrin accumulation and could be an effective therapy for PCT [12]. The liver has a wide range of other functions, including the synthesis and catabolism of various metabolites and nutritional elements. However, the involvement of iron in the metabolism of other nutritional disorders remains largely unknown.

Hereditary tyrosinemia type I (HT1) is the most severe disease of the tyrosine degradation pathway [13]. HT1 is caused by inactivation mutations of the gene fumarylacetoacetate hydrolase (*Fah*), which encodes the last enzyme of the tyrosine catabolism pathway. This gene is mainly expressed in hepatocytes. The disease is characterized by severe, progressive liver diseases, including liver failure and chronic liver damage with a high incidence of hepatoma [14]. The accumulation of the metabolites upstream of Fah, are thought to be the cause of liver failure. However, details of the molecular mechanisms of this disease are still unclear, and how this disorder of tyrosine metabolism results in severe liver injury remains a mystery. There is evidence to implicate iron disorders in HT. A previous study reported a case of iron overload in an infant with supposed hereditary tyrosinemia [15], and iron mobilization may be affected by succinylacetone methyl ester in rats as a model for hereditary tyrosinemia [16]. However, the metabolic status of iron and the physiological function of iron metabolism in this disease are still unknown.

The drug NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione), which blocks the pathway upstream of Fah, is currently used to treat patients with HT1. However, patients who persistently take NTBC still have mild liver injury and a higher risk of HCC [17]. Therefore, studies concerning the mechanism of liver injury in this disease will improve therapies to protect HT1 patient from liver damage. *Fah* knockout mice are used as a murine model of HT1. Mice receiving NTBC treatment demonstrated prolonged liver injury-free survival. In contrast, the mice developed phenotype and biochemical manifestations of HT1 after the removal of NTBC.

In this study, we detected severe iron overload in the acute HT1 murine model, and identified the significance of aberrant iron metabolism in the pathogenesis of HT1. Our study also suggests that low-iron food rescued the iron overload, thereby protecting the $Fah^{-/-}$ mice from liver injury, and prolonging their survival. Moreover, we identified Sp1/Tfr2/Hepcidin signaling as the molecular mechanism underlying the iron disorders in HT1.

Materials and methods

Mice and human samples

The mice were kept on a 129/SvEvTac background. $Fah^{-/-}$ mutant animals were treated with NTBC-containing water at a concentration of 7.5 mg/L unless otherwise indicated. All of the mice were maintained in specific pathogen-free husbandry and fed a standard rodent diet unless indicated. Food with low-iron had the same nutritional and caloric content as the control food except less iron. All of the murine chows were bought from SLAC Laboratory Animal Company (Shanghai, China). Eight- to ten-week old mice were used in all of the experiments, and sacrificed at different ages as called for by the different experimental designs. All of the human samples were collected from the Centre for Pediatric Liver Diseases, Children's Hospital of Fudan University (Shanghai, China). Patient diagnoses was made according to the diagnostic criteria of the International Organization for Rare Disorders (NORD). All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Sciences (Shanghai, China).

Measurement of iron indices and serum parameters

Quantitative measurement of tissue non-heme iron was performed using the method of Torrance and Bothwelld [18]. Serum iron and total iron binding capacity (TIBC) were measured with the iron and iron binding–capacity kit (Thermo Scientific).Transferrin saturation was directly calculated from the serum iron and TIBC. Total glutathione content was measured with Tissue Total Glutathione Kit (Genmed Scientifics inc. USA). Serum parameters of liver and kidney functions, including alanine aminotransferase (ALT), aspartate transaminase (AST), albumin (ALB), total bilirubin (TBIL), crea (CR) and blood urea nitrogen (BUN) were measured by automatic biochemical analyser at the Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University (Shanghai, China).

Histology

Formalin-fixed tissues were embedded in paraffin, sectioned, and stained. Hematoxylin and eosin (H&E) staining was performed using a standard protocol. TUNEL staining for the evaluation of apoptotic cell death was performed using the Dead-End™ Fluorometric TUNEL System kit (Promega) according to the manufacturer's instructions. Non-heme iron staining was performed as previously described [19].

Quantitative RT-PCR

RNA extraction and real-time polymerase chain reaction analysis were performed as described previously [20]. Real-time PCR measurements of murine samples were normalized to 18S rRNA. The primer sequences are detailed in the Supplementary material.

Western blotting

Cells and tissues were lysed in RIPA buffer and the protein concentrations were determined using Bradford reagents (Sigma, St Louis, MO). Proteins were probed with antibodies against Tfr1 (Invitrogen), Slc40A1 (Fpn1, Novus Biologicals, CO), Tfr2, Gapdh and c-myc (Santa cruz).

Statistical analysis

All data are presented as means and standard errors of the mean. The Student's *t* test was used for the comparison of measurable variants of two groups. The survival curves were calculated using the Kaplan-Meier method, and the differences were assessed by a log-rank test. A p value of less than 0.05 was considered statistically significant.

Further details of materials and methods are available in the Supplementary materials.

Results

Iron is accumulated in $Fah^{-/-}$ mice after NTBC withdrawal

To determine the status of iron deposition in the HT1 murine model, $Fah^{-/-}$ mice were anesthetized six weeks after NTBC withdrawal and their blood and tissues were collected for analysis. Compared to the control mice with NTBC (NTBC+), the iron levels in the plasma and liver were both significantly elevated in all of the mice without NTBC in their drinking water (NTBC−) (Fig. 1A, Table 1). The non-heme hepatic iron concentration in NTBC− mice was approximately five-fold higher than that of NTBC+ mice