Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Hepcidin knockout mice fed with iron-rich diet develop chronic liver injury and liver fibrosis due to lysosomal iron overload

Mariia Lunova[1], Claudia Goehring[1], Deniz Kuscuoglu[5], Katrin Mueller[1], Yu Chen[1], Paul Walther[2], Jean-Christophe Deschemin[3], Sophie Vaulont[3], Johannes Haybaeck[4], Carolin Lackner[4], Christian Trautwein[5], Pavel Strnad[1,5,*]

[1]*Department of Internal Medicine I, University Hospital Ulm, Ulm, Germany;* [2]*Central Electron Microscopy Facility, Ulm University, Ulm, Germany;* [3]*Institut Cochin, INSERM U1016, Université Paris Descartes, Paris, France;* [4]*Institute of Pathology, Medical University of Graz, Graz, Austria;* [5]*Department of Medicine III and IZKF, University Hospital Aachen, Aachen, Germany*

Background & Aims: Hepcidin is the central regulator of iron homeostasis and altered hepcidin signalling results in both hereditary and acquired iron overload. While the association between iron overload and development of end-stage liver disease is well established, the underlying mechanisms are largely unknown. To improve that, we analysed hepcidin knockout (KO) mice as a model of iron overload-associated liver disease.

Methods: Hepcidin wild type (WT) and KO mice fed with 3% carbonyl iron-containing diet starting at one month of age were compared to age-matched animals kept on standard chow. Liver histology and serum parameters were used to assess the extent of liver injury and fibrosis. Iron distribution was determined by subcellular fractionation and electron microscopy.

Results: Among mice kept on iron-rich diet, 6 months old hepcidin KO mice (*vs.* WT) displayed profound hepatic iron overload (3186 ± 411 *vs.* 1045 ± 159 μg/mg tissue, p <0.005), elevated liver enzymes (ALT: KO 128 ± 6, WT 56 ± 5 IU/L, p <0.05), mild hepatic inflammation and hepatocellular apoptosis. Twelve, but not six months old KO mice fed with iron-rich diet developed moderate liver fibrosis. The liver injury was accompanied by a marked lysosomal iron overload and lysosomal fragility with release of cathepsin B into the cytoplasm. Increased p62 levels and autofluorescent iron complexes suggested impaired protein degradation. As a mechanism leading to lysosomal iron overload, the autophagy (lysosomal influx) was increased.

Conclusions: Hepcidin KO mice represent a novel model of iron overload-related liver diseases and implicate lysosomal injury as a crucial event in iron toxicity.

Keywords: Hemochromatosis; Inflammation; Autophagy.
Received 11 December 2013; received in revised form 25 March 2014; accepted 22 April 2014; available online 6 May 2014

* Corresponding author. Address: Department of Internal Medicine III and IZKF, RWTH Aachen, Pauwelsstraße 30, D-52074 Aachen, Germany. Tel.: +49 (241) 80 35324; fax: +49 (241) 80 82455.
E-mail address: pstrnad@ukaachen.de (P. Strnad).

Introduction

Iron is an essential element which becomes toxic at higher levels and therefore its metabolism is tightly controlled [1,2]. Hepcidin plays an essential role as the central iron regulator which induces internalisation and degradation of ferroportin, the only known cellular iron exporter [2,3]. Thereby, it diminishes iron uptake from the intestine, as well as the release of iron from macrophages [2,3]. Macrophages constitute the major iron recyclers that transfer iron from senescent red blood cells back to erythropoiesis [4]. Consequently, impaired hepcidin activity results in iron overload, while elevated hepcidin levels lead to iron retention within macrophages and thus to anemia [2,3].

The liver is of crucial importance for iron metabolism given that hepatocytes are the predominant source of hepcidin production and the major storage place of parenchymal iron [2,3]. In agreement, multiple diseases lead to a marked hepatic iron accumulation, which contributes to development of liver fibrosis and/or hepatocellular carcinoma [1,5]. These disorders include inherited mutations in the hepcidin regulatory system termed as hereditary hemochromatosis (HHs), disorders with inefficient erythropoiesis (such as thalassemia) as well as various chronic liver diseases such as hepatitis C or alcoholic liver disease, which go along with inappropriately low hepcidin levels [1,5,6].

Despite the accepted detrimental role of hepatic iron overload, the exact mechanism how iron accumulation contributes to liver disease is complex and far from being understood. For example, HH patients display only a moderate correlation between the extent of iron accumulation and liver disease development and iron toxicity depends on the amount of the "free" iron rather than the "total" iron load [6,7]. To that end, iron is typically bound to transferrin in the blood stream while it is stored in the form of ferritin in the tissue [2] and the amount of iron which is not bound to the professional storage/transport proteins (termed as non-transferrin bound iron [NTBI]) correlates with disease development [8]. Another important feature affecting iron toxicity is its subcellular distribution [2]. Iron enters the cell via receptor-mediated endocytosis but it can also be imported into mitochondria and via autophagy into the lysosome [2,9]. Most of the cytosolic iron is stored in form of ferritin which

is transformed to hemosiderin in situations of massive iron overload [2]. Importantly, a disruption in the subcellular iron homeostasis with mitochondrial iron overload was shown to result in iron toxicity [2,6,9].

While both subcellular iron distribution and iron storage (such as ferritin-iron, NTBI, transferrin-bound etc.) play a crucial role in development of liver disease, multiple cellular events are implicated in this process as well. For example, excess free iron leads to the formation of reactive oxygen species [6]. Increased hepatocellular apoptosis represents another frequently observed feature and can be due to lysosomal/mitochondrial injury or chromosomal damage [6,9]. Moreover, recruitment of macrophages was shown in HH subjects and likely modulates liver disease development [10,11].

Although iron overload has been demonstrated to have a myriad of downstream effects, our limited understanding of the pathogenesis of iron overload-related liver disease is due to a lack of animal models faithfully reflecting this human condition. For example, mice lacking HFE that mimic the most frequent HH form, display a marked hepatic iron overload, but no liver injury even after a feeding with iron-rich diet [12]. A combined disruption of HFE and transferrin receptor 2 resulted in liver fibrosis development, but the underlying mechanisms were not studied in detail [13]. Therefore and since hepcidin mutations lead to a particularly severe hemochromatosis form in humans [5], we analysed hepcidin KO mice as a model of iron overload-associated liver disease. Our data show that hepcidin KOs fed with iron-rich diet display mild chronic hepatitis which ultimately leads to liver fibrosis development. As a potential mechanism, we detected lysosomal iron accumulation, lysosomal fragility and impaired protein degradation. Therefore, hepcidin KOs represent a novel model of iron overload-related liver disease and offer a crucial insight into its pathogenesis.

Material and methods

To study the consequences of chronic iron overload, previously described hepcidin KO mice on C57BL/6N background were used [14]. For details, see Supplementary materials and methods. The results were presented as means ± standard error of mean (SEM). One-way ANOVA with the post-hoc LSD test was used for multiple group comparisons. Differences were considered statistically significant at $p < 0.05$.

Results

Six months old hepcidin KO mice fed iron-rich diet develop chronic liver injury and hepatocellular apoptosis

To study the role of iron overload in development of liver disease, hepcidin WT and KO mice were fed with an iron-rich or a respective control diet. At six months of age, hepcidin KOs kept on iron-rich diet exhibited a moderate hepatomegaly and mildly elevated transaminases which were not observed in the other groups (Fig. 1A, Table 1).

Histological examination with morphometric quantification (Fig. 1B and C) revealed a moderate inflammation in hepcidin KOs subjected to iron-rich diet but not in other subgroups. This finding was evidenced by immunohistochemical staining with CD45 (leukocyte marker, Fig. 1D) and F4/80 (monocyte/macrophage marker, Fig. 1E). Furthermore, hepcidin KO mice fed with iron-rich diet displayed significantly elevated mRNA levels of multiple monocyte/macrophage-related inflammatory genes such as *CCL3*, *CCL5*, *CXCL10*, *LYZ2*, *CD68*, *CD11C*, *MMP12*, and *MCP1* (Table 2), which were either unaltered or substantially less elevated in the other experimental groups.

In addition to chronic inflammation, the histological evaluation suggested increased apoptosis in hepcidin KOs kept on iron–rich diet (Supplementary Fig. 1A). This finding was confirmed by immunohistochemical and TUNEL staining as well as immunoblotting against the epithelial-specific apoptotic keratin 18 fragment D237 [15]. Increased D237 labelling and TUNEL signal was seen in hepcidin KOs subjected to iron-rich diet, but not so much in the other experimental groups (Supplementary Figs. 1 and 2).

Six months old iron-fed hepcidin KO mice display a marked iron overload and hepatic stellate cell activation after iron feeding

To investigate the impact of the described experiments on iron distribution, we performed Prussian Blue staining (Fig. 1F) and an analysis of iron distribution pattern with Deugnier score (Table 3). As expected, hepcidin WT mice fed standard diet showed no obvious iron deposition, whereas WTs on iron-rich diet developed periportal iron accumulation (Table 3). On the other hand and in agreement with previous reports [14], hepcidin KOs displayed a rather ubiquitous iron storage with many large iron deposits. Massive iron accumulation in hepcidin KOs on iron-rich diet was reflected in all iron parameters examined, i.e., serum ferritin, serum iron as well as liver iron (Table 1). Apart from the differences in hepatocellular iron distribution, both subgroups fed iron-rich diet displayed similar iron distribution pattern including presence of iron-overloaded macrophages (Table 3; Supplementary Fig. 3). Hepcidin KOs kept on standard diet and WTs fed with iron-rich diet showed no significant difference in parameters of iron overload (Table 1).

Given the unexpected macrophage iron accumulation in hepcidin KOs on iron-rich diet, we performed additional analyses of iron metabolism. Irrespectively of their feeding status, hepcidin KOs displayed lower splenic iron content than non-transgenic animals (Supplementary Fig. 4A). This suggests that the observed macrophage iron overload is restricted to the liver and is most likely due to the ongoing tissue injury. As anticipated, hepatic ferritin levels paralleled the hepatic iron content while ferroportin levels were increased in hepcidin KOs irrespectively of their treatment (Supplementary Fig. 4B and C).

Next, we tested if chronic liver injury found in six months old hepcidin KOs was sufficient to induce liver fibrosis. We thus performed RT-PCR for collagen, Sirius red staining, immunohistochemical staining for alpha smooth muscle actin (α-SMA) and hydroxyproline assay (Supplementary Figs. 5 and 6). While increased collagen mRNA expression and α-SMA labelling in hepcidin KOs on iron-rich diet suggested that hepatic stellate cell activation took place, no obvious signs of liver fibrosis were detected.

12 months old hepcidin KO mice kept on iron-rich diet develop significant liver fibrosis

As 6 months old hepcidin KOs kept on iron-rich diet displayed increased collagen expression, we investigated the effect on liver fibrosis in 12 months old animals. Moderate hepatomegaly was