Brief report

Deregulation of proteins involved in iron metabolism in hepcidin-deficient mice

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Evidence is accumulating that hepcidin, a liver regulatory peptide, could be the common pathogenetic denominator of all forms of iron overload syndromes including HFE-related hemochromatosis, the most prevalent genetic disorder characterized by inappropriate iron absorption. To understand the mechanisms whereby hepcidin controls iron homeostasis in vivo, we have analyzed the level of iron-related proteins by Western blot and immunohistochemistry in hepcidin-deficient mice, a mouse model of severe hemochromatosis. These mice showed important increased levels of duodenal cytochrome b (Dcytb), divalent metal transporter 1 (DMT1), and ferroportin compared with control mice. Interestingly, the level of ferroportin was coordinately up-regulated in the duodenum, the spleen, and the liver (predominantly in the Kupffer cells). Finally, we also evidenced a decrease of ceruloplasmin in the liver of hepcidin-deficient mice. We hypothesized that the deregulation of these proteins might be central in the pathogenesis of iron overload, providing key therapeutic targets for iron disorders. (Blood. 2005;105:4861-4864)

Introduction

Tight regulation of iron absorption is critical for the organism, as both iron deficiency and iron excess are associated with cellular dysfunction. Dietary iron is absorbed by duodenal enterocytes. Briefly, Fe^{3+} is thought to be reduced at the apical side of the enterocyte to Fe^{2+} by the reductase duodenal cytochrome b (Dcytb) and transported through the plasma membrane by the divalent metal transporter 1 (DMT1). Once in the cytoplasm, iron is either stored in ferritin or transferred to the plasma by the basolateral exporter ferroportin. Then, Fe^{2+} is oxidized by the ferroxidase hephaestin and enters the circulation bound to transferrin (for review, see Miret et al[1]). Iron absorption represents only 10% of the iron needs, and to meet the demand for heme production necessary for erythropoiesis, iron is recycled from senescent red blood cells by the macrophages of the reticuloendothelial system.

Recently, hepcidin, a peptide secreted by the liver in response to iron loading and inflammation, has been proposed to regulate plasma iron by limiting duodenal iron absorption and iron release by macrophages (for review, see Ganz[2] and Nicolas et al[3]). Conversely, hepcidin production was found inhibited by anemia and hypoxia, allowing for increased iron absorption.[4] There were 2 mouse models that helped in establishing the regulatory role of hepcidin. First, the *Usf2* knock-out mice[5] that completely lack hepcidin, which demonstrated massive iron overload resembling the hemochromatosis phenotype (ie, multivisceral iron overload with sparing of the reticuloendothelial system), and, second, the transgenic mouse model constitutively expressing hepcidin, which developed severe hyposideremia and anemia.[6] Still, little is known about the mode of action of hepcidin in vivo. To address this question and to understand the mechanisms of the deregulation of iron absorption in hepcidin-deficient mice, the level of iron-related proteins was studied in the 3 organs implicated in iron homeostasis, namely duodenum, spleen, and liver, the major site of iron storage.

Study design

Animals and treatment

Usf2 knock-out mice were previously described.[5] Mice were maintained on a standard laboratory mouse chow (AO3; Usine d'Alimentation Rationnelle, Epinay-sur-Orge, France). Iron overload was produced by 3% carbonyl iron supplemented in the diet for 14 days.

Western blot analysis

Microsomal and cytosolic fractions from frozen tissues of 2- to 5-month-old mice were prepared as previously described.[7] Rabbit polyclonal antimouse DMT1 and antimouse ferroportin antibodies were described (Canonne-Hergaux et al[7]; and F.C-H., Adriana Donova, C. Delaby, H.-J. Wang, N.C. Andrews, P. Gros, "Coordinated upregulation of duodenal and macrophage ferroportin proteins in response to iron restricted erythropoiesis," submitted April 2005). Rabbit polyclonal antimouse Dcytb was a kind gift from A. McKie.[8] Mouse monoclonal antimouse ceruloplasmin antibody was purchased

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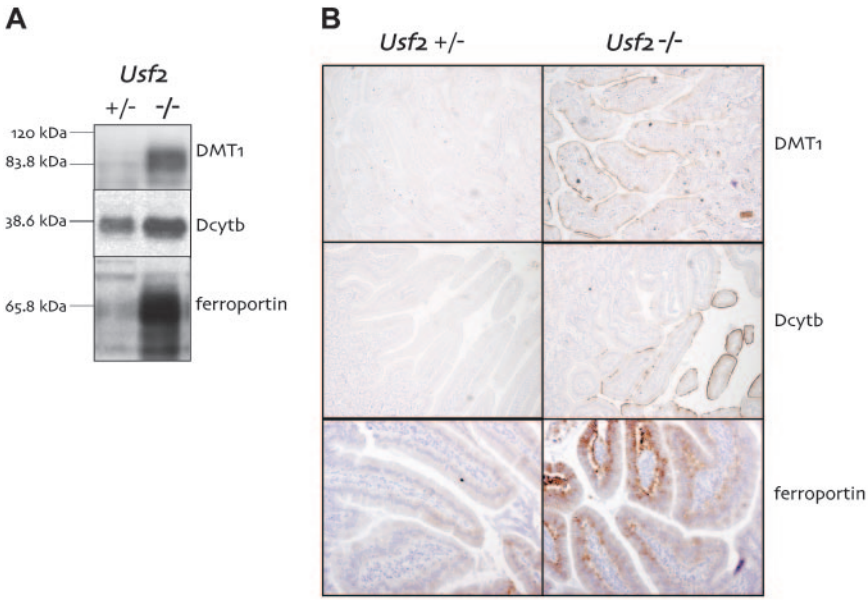

Figure 1. Protein levels of iron-related genes in the duodenum of hepcidin-deficient mice. (A) Western blot analysis of DMT1, Dcytb, and ferroportin (on 80 μg crude membrane preparation) from the duodenum of *Usf2*$^{-/-}$ and *Usf2*$^{+/-}$ mice. Molecular weight markers are indicated on the left. A typical experiment, representative of at least 3 independent experiments, is shown. (B) Immunohistochemistry using antimouse DMT1, Dcytb, and ferroportin antibodies on the duodenum of *Usf2*$^{-/-}$ and *Usf2*$^{+/-}$ mice. Original magnification, ×10.

from BD Biosciences (San Jose, CA). Finally, rabbit polyclonal antimouse L-ferritin antibody was a kind gift from P. Santambrogio and S. Levi.[9]

Immunohistochemistry

Tissues from 9- to 10-month-old mice were fixed in 4% formaldehyde. Sections in paraffin were subjected to microwave and then processed for immunohistochemistry using the EnVision+ System–horseradish peroxidase (HRP) (diaminobenzidine [DAB]) kit (DAKO, Trappes, France) according to the manufacturer's methods. Primary antibodies against DMT1, ferroportin, and Dcytb were diluted at 1:1500, 1:75, and 1:50 respectively, before use. All microscopy was performed using a Nikon Eclipse E800 microscope equipped with 4×/0.10 NA, 20×/0.75 NA, and 60×/1.40 oil objective lenses. Digital images were captured with a Nikon Dxm1200 camera and Nikon ACT-1 v2.63 software (Nikon France, Champigny-sur-Marne, France).

Results and discussion

The level of proteins implicated in iron absorption was assessed in the duodenum of hepcidin-deficient mice by Western blot and immunohistochemistry (Figure 1A-B). DMT1, Dcytb, and ferroportin were found to be dramatically increased in *Usf2*$^{-/-}$ mice compared with their littermate controls. This increase was observed at the apical border of *Usf2*$^{-/-}$ enterocytes for DMT1 and Dcytb, and at the basolateral side for ferroportin. We hypothesized that the increase of proteins responsible for iron absorption would be one mechanism accounting for massive iron overload in hepcidin-deficient mice. In good agreement with our results, the lab of G. Anderson has provided strong indirect inverse correlative data between hepcidin expression and the duodenal level of DMT1, Dcytb, and ferroportin proteins in various pathophysiologic situations including iron-deficient diet (Frazer et al[10]), pregnancy (Millard et al[11]), and phenylhydrazine treatment (Frazer et al[12]). In contrast, synthetic hepcidin in Caco2 cells was recently demonstrated to act only at the apical side by decreasing iron uptake through down-regulation of DMT1 protein and without any modification of the iron efflux and ferroportin expression.[13] The reason for this discrepancy is difficult to explain but might be related to the in vitro system, and the nature and concentration of the synthetic hepcidin used in these experiments.

In agreement with the liver iron accumulation in the *Usf2*$^{-/-}$ mice,[5] an increased content of hepatic L-ferritin was observed (Figure 2A). Interestingly, hepcidin-deficient mice showed increased hepatic ferroportin levels, mainly due to an up-regulation of ferroportin in the Kupffer cells, a well-known site of ferroportin expression[14,15] (Figure 2A and 2B). However, while hepatocytes of control mice were negative for the presence of ferroportin, a faint staining was evidenced in the hepatocytes of hepcidin-deficient mice. Although it is established that the ferroportin gene is expressed in hepatocytes and hepatoma cell lines, the presence and the subcellular localization of the protein in these cells have remained elusive.[14-17] However, detection of ferroportin in the liver of hepcidin-deficient mice favors a role of hepcidin in hepatocytic and macrophagic expression of ferroportin.

Ceruloplasmin, a soluble ferroxidase produced by the liver, plays a key role in iron homeostasis by favoring cellular iron release and iron incorporation into transferrin (for review, see Hellman and Gitlin[18]). Surprisingly, while the ferroportin exporter was up-regulated in the liver of hepcidin-deficient mice, ceruloplasmin was found to be down-regulated (Figure 2A). This down-regulation of ceruloplasmin was confirmed by immunohistochemistry (data not shown). The increased ferroportin and decreased ceruloplasmin levels are expected to be the consequence of hepcidin deficiency rather than liver iron overload since in experimentally iron-loaded mice, the level of hepatic ferroportin was unaltered, while ceruloplasmin levels were increased (data not shown). The decrease of hepatic ceruloplasmin in *Usf2*$^{-/-}$ mice was quite unexpected and deserves further investigation. However, in agreement with the liver iron overload associated to a marked impairment in hepatocyte iron efflux observed in ceruloplasmin knock-out mice,[19] we speculate that ceruloplasmin deficiency could contribute to the iron overload in *Usf2*$^{-/-}$ mice.

Splenic macrophages are important for iron recycling from senescent red blood cells. In hepcidin-deficient mice, we previously described the apparent iron deficiency of the spleen,[5] an observation further illustrated here by the decreased expression of

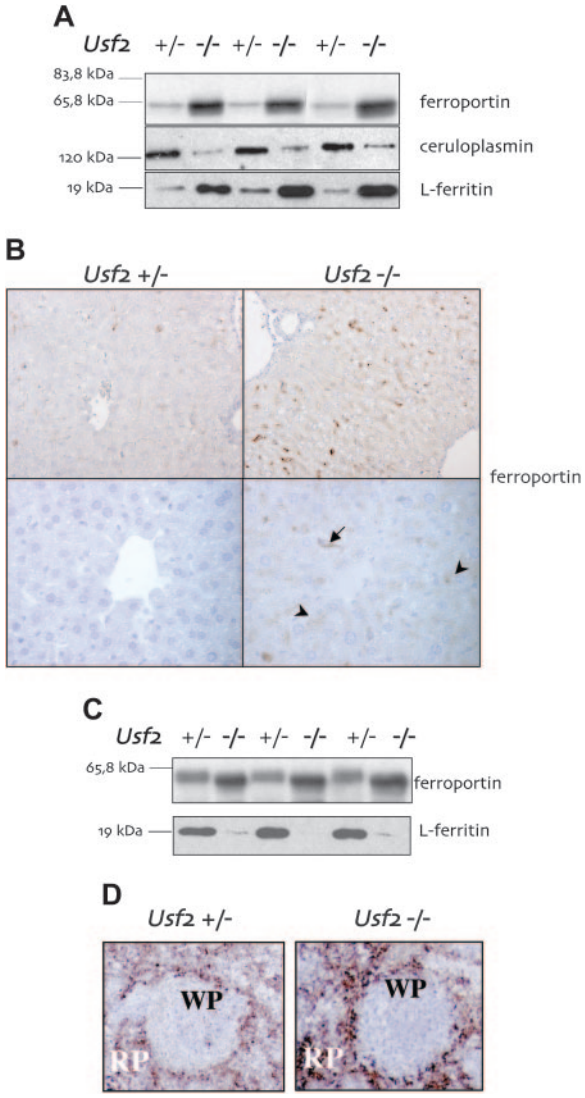

Figure 2. Protein levels of iron-related genes in the liver and the spleen of hepcidin-deficient mice. Western blot analysis of ferroportin (on 80 μg crude membrane preparation), and of ceruloplasmin and L-ferritin (on 20 μg cytosolic preparation) from the liver (A) and the spleen (C) of *Usf2*$^{-/-}$ and *Usf2*$^{+/-}$ mice. Molecular weight markers are indicated on the left. Immunohistochemistry using antimouse ferroportin antibody on the liver (B) and on the spleen (D) of *Usf2*$^{-/-}$ and *Usf2*$^{+/-}$ mice. Original magnification, ×20 (top row, panel B), ×60 (bottom, and ×4, panel D). Kupffer cell (black arrow) and hepatocyte (black arrowheads) staining are pointed. RP and WP indicate red pulp and white pulp, respectively.

splenic L-ferritin (Figure 2C). Interestingly, an increase of ferroportin was also observed in the spleen of *Usf2*$^{-/-}$ mice together with a slight change in the molecular size of the protein, most likely due to posttranslational modification. The increase of ferroportin was specific to the macrophages of the red pulp as assessed by immunohistochemistry (Figure 2D). The splenic ferroportin up-regulation could explain the iron deficiency in the spleen of these mice.

In conclusion, hepcidin deficiency is leading to an up-regulation of DMT1, Dcytb, and ferroportin in the duodenum; a decrease of ceruloplasmin in the liver; and a coordinate up-regulation of ferroportin levels in duodenum, liver, and spleen. This latter result is in agreement with the data of Canonne-Hergaux et al (F.C-H., Adriana Donova, C. Delaby, H.-J. Wang, N.C. Andrews, P. Gros, "Coordinated upregulation of duodenal and macrophage ferroportin proteins in response to iron restricted erythropoiesis," submitted April 2005), demonstrating similar up-regulation of ferroportin in situations of pathophysiologic hepcidin deficiency due to iron-restricted erythropoiesis. In addition, the same correlation was found in heterozygous polycythemic *Pcm* mice where down-regulation of hepcidin during postnatal development correlates with profound increases in ferroportin protein levels in duodenum and liver.[20] Very interestingly, while this paper was in preparation, Nemeth et al[21] reported that, in cultured cells, ferroportin levels were regulated by direct interaction with hepcidin, resulting in the internalization and degradation of ferroportin. In absence of hepcidin, this hepcidin-induced degradation of ferroportin would be abrogated, leading to ferroportin accumulation at the cell surface. From these results, it remains to be established whether the duodenal increases of DMT1 and Dcytb are secondary responses to local enterocyte iron deficiency. Further experiments are now needed to determine whether the same hepcidin/ferroportin pathway is used in all 3 tissues to regulate iron transport activity and to determine the impact of ferroportin regulation on the other iron-related genes in the duodenum.

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