Brief Report

Scientific Category: Red Cells, Iron and Erythropoiesis

Deletion of HIF-2α in the enterocytes decreases the severity of tissue iron loading in hepcidin knockout mice

Running head: Duodenal HIF-2α contributes to hemochromatosis

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ABSTRACT

Hereditary hemochromatosis (HH) is a highly prevalent genetic disorder characterized by excessive parenchymal iron accumulation leading to liver cirrhosis, diabetes and in some cases hepatocellular carcinoma. HH is caused by mutations in the genes encoding upstream regulators of hepcidin or more rarely in the hepcidin gene itself. A deficit in hepcidin results in intestinal iron hyperabsorption; however, the local effectors mediating the upregulation of iron absorption genes are unknown. We hypothesized that HIF-2 could mediate high iron absorption rates in HH. We generated Hepc−/− mice (a murine model of hemochromatosis) lacking HIF-2 in the intestine and showed that duodenal HIF-2 was essential for the upregulation of genes involved in intestinal iron import and the consequent iron accumulation in the liver and pancreas. This study highlights a role of HIF-2 in the dysregulation of iron absorption and chronic iron accumulation, as observed in patients with hemochromatosis.
INTRODUCTION

Hereditary hemochromatosis (HH) is a heterogeneous genetic disease characterized by excessive iron accumulation in the liver and parenchyma. Clinical manifestations include liver cirrhosis, diabetes, cardiomyopathy, arthropathy, hypermelanotic skin pigmentation and hepatocellular carcinoma. HH is typically caused by mutations in genes encoding either upstream signaling molecules involved in the induction of hepcidin expression (HFE; transferrin receptor 2, TfR2; hemojuvelin, HJV), or more rarely in the hepcidin gene itself. Iron absorption in the duodenum is the only way to control iron entry in the body and is finely regulated in response to systemic iron requirements. At the apical brush border of duodenal enterocytes, duodenal cytochrome b (DCYTB) facilitates non-heme iron uptake by divalent metal transporter 1 (DMT1), whereas ferroportin (FPN) exports iron across the basolateral membrane.

Hepcidin is the central regulatory molecule of systemic iron homeostasis and regulates cellular iron efflux by binding to FPN and inducing its internalization and subsequent degradation in the lysosome. While hepcidin is known to act at a systemic level to regulate the rate of iron absorption by controlling the amount of iron exported across the basolateral membrane by FPN, the local effectors mediating the upregulation of apical iron absorption genes in hemochromatosis are unknown. We and others have previously demonstrated that the Hypoxia-Inducible Factor-2α (HIF-2α) transcription factor, and not HIF-1α, regulates DMT1, DCYTB and FPN expression in the duodenum at basal level, iron deficiency and in conditions of increased erythropoiesis conditions. HIF-1 and HIF-2 are heterodimeric transcriptional factors and central mediators of cellular and systemic adaptation to
hypoxia. In the presence of oxygen, the HIF-α subunit is hydroxylated by oxygen- and iron- dependent prolyl hydroxylases (PHDs) and targeted to the proteasome after the binding to the von Hippel-Lindau (VHL) protein. Upon hypoxia (or iron deficiency), HIF-α is stabilized and binds to the HIF-β constitutive subunit to induce the transcription of target genes 10.

We hypothesized that HIF-2 could be a mediator of high iron absorption rates in HH and addressed this question by breeding the hepcidin knockout mice (Hepc−/−), a model of severe iron overload, with mice lacking HIF-2 in the intestinal epithelium.

METHODS

Animals

Animal studies described here were reviewed and approved (Agreement n° P2.CP.151.10.) by the “Président du Comité d'Ethique pour l'Expérimentation Animale Paris Descartes”. We intercrossed mice homozygous for germline knockout of hepcidin 11 and mice with loss of HIF-2α specifically in the intestinal epithelium HIF-2αlox/loxVillin-Cre+ 7, both in a C57BL/6J genetic background, to produce the Hepc−/−/HIF-2αlox/loxVillin-Cre+ mouse strain (referred as Hepc−/−HIF-2αΔint). Finally, we interbred Hepc−/−HIF-2αΔint and Hepc−/−/HIF-2αlox/loxVillinCre− mice (here referred as Hepc−/−). Male mice were analyzed at the age of 5 months and compared to control genotypes, including Hepc+/+ HIF-2αlox/loxVillinCre− and Hepc+− HIF-2αlox/loxVillinCre− (referred as controls, CTR).
Reverse transcription and real-time quantitative PCR

RNA extraction, reverse transcription, quantitative PCR and sequences of the primers used have been previously described \(^7\). All samples were normalized to the threshold cycle value for cyclophilin.

Western blot

Frozen whole duodenal tissue was homogenized using a pestle and smash and extraction of membrane proteins was performed as previously described \(^7\). The following antibodies were used: DMT1 antibody recognizing both DMT1-IRE and non-IRE isoforms \(^12\) (kind gift of François Cannone-Hergaux), DCYTB antibody (Alpha Diagnostic DCYTB11-A), FPN antibody (Alpha Diagnostic, MTP11-A).

Iron measurements and Immunostaining

Plasma and tissue iron were quantified colorimetrically by a previously described method \(^7\). For histology, tissues were fixed in 4\% formaldehyde and embedded in paraffin and stained with Perls’ Prussian blue and nuclear fast red counter stain.

Statistical analysis

Analysis was performed using GraphPad Prism 4.0 and the significance of experimental differences was evaluated by one-way ANOVA analysis followed by a Bonferroni posttest. Values in the figures are expressed as mean +/- SEM. Statistical significance is indicated by * symbols (*p< 0.05, **p< 0.01, ***p< 0.001).
RESULTS AND DISCUSSION

To test whether HIF-2 can mediate the upregulation of iron absorption genes in HH, we generated Hepc<sup>−/−</sup> mice deleted for HIF-2α in the duodenum (Hepc<sup>−/−</sup>HIF-2α<sup>Δint</sup> mice). These mice do not exhibit any overt phenotypic abnormalities. We previously reported that DMT1, DCYTB and FPN protein levels were increased in the duodenum of hepcidin deficient mice (the Usf2<sup>−/−</sup> mouse model<sup>13</sup>). We confirmed this result (Figure 1B) and further demonstrated that Hepc<sup>−/−</sup> mice presented high levels of DMT1, DCYTB and FPN mRNA (although to a lesser extent) in comparison to control mice (Figure 1A) suggesting that a transcriptional control of these genes takes place in the duodenum of these mice. The levels of DMT1, DCYTB and FPN transcript and protein, were fully attenuated in Hepc<sup>−/−</sup>HIF-2α<sup>Δint</sup> (Figure 1A, B) compared to Hepc<sup>−/−</sup> mice with levels not statistically different of wild type mice. The duodenal deletion of HIF-2α decreased significantly FPN protein levels, despite the lack of hepcidin, which should prevent FPN degradation by systemic regulation.

We next asked whether the decrease of genes involved in iron absorption at the apical (DMT1 and DCYTB) and the basolateral membrane (FPN) was sufficient to prevent the hyperabsorption characteristic of the Hepc<sup>−/−</sup> mice. Interestingly, the double knockout presented a significantly decreased accumulation of non-heme iron in the liver and pancreas as compared to Hepc<sup>−/−</sup> littermates. This was assessed both quantitatively (Figure 2A) and qualitatively by Perls’ blue staining (Figure 2B). Plasma ferritin levels, reflecting parenchymal iron storage, were significantly diminished in Hepc<sup>−/−</sup> mice lacking duodenal HIF-2, compared to Hepc<sup>−/−</sup> mice (Figure 2C). However, plasma iron concentrations or transferrin saturation (Figure 2C) did
not differ between the Hepc+/HIF-2αΔint and Hepc−/− littermates suggesting a contribution of the iron recycled from the spleen, an organ which is not affected by the deletion of HIF-2α14. Indeed, most circulating iron is provided by macrophage iron recycling, and this process seems not affected in the Hepc+/HIF-2αΔint mice as compared to the Hepc−/− mice, as shown by the lack of detectable iron in the macrophages of the spleen in both models (Figure 2B). Interestingly, hematological parameters, (Hemoglobin, Hematocrit, Mean Corpuscular Volume), were decreased in the Hepc+/HIF-2αΔint mice compared to the Hepc−/− mice and not statistically different of wild type mice (supplementary figure).

Altogether, our data suggest that HIF-2 contributes to the intestinal iron hyperabsorption in a mouse model of HH but may not overcome all of the negative consequences of the abnormal iron metabolism. Associations between SNPs at HIF-2α locus and blood-related phenotypes have been recently demonstrated15,16. It would be of interest to determine whether HIF-2α polymorphisms could be found associated with iron burden in hemochromatosis. Current treatments for iron overload disorders are limited to phlebotomy or, in case of severe anemia, cardiac failure, or poor tolerance, to chelation therapies2. Here, we propose that therapeutical intervention on intestinal HIF-2α activity might be beneficial to reduce the rates of iron absorption and parenchymal iron overload.

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AUTHORSHIP CONTRIBUTIONS

All the authors conceived, analyzed and interpreted the experiments. M.M, P.M, S.D. and JC.D performed experiments. M.M, S.V and C.P wrote the manuscript.

DISCLOSURE OF CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.
REFERENCES

FIGURE LEGENDS

Figure 1 : Iron absorption genes are decreased in Hepc<sup>-/-</sup>HIF-2α<sup>Δint</sup> compared to Hepc<sup>-/-</sup> mice.

(A) Relative mRNA expression of DMT1+IRE, DCYTB and FPN normalized to Cyclophilin in the duodenum of Hepc<sup>-/-</sup>HIF-2α<sup>Δint</sup> (circle; n=9) versus Hepc<sup>-/-</sup> (square; n=9) and controls, CTR (triangle; n=9) mice. (B) Western Blot of FPN, DMT1 and DCYTB on membrane extracts of whole duodenum from Hepc<sup>-/-</sup>HIF-2α<sup>Δint</sup> and Hepc<sup>-/-</sup> mice versus controls, CTR, littermates. Expression was normalized to beta actin. Results were quantified by using Image J software (available at http://rsb.info.nih.gov/ij/). All genotypes used contain the HIF-2α<sup>lox/lox</sup> allele. Statistical significance is indicated by * symbols (*p< 0.05, **p< 0.01, ***p< 0.001).

Figure 2 : Iron parameters are decreased in the Hepc<sup>-/-</sup>HIF-2α<sup>Δint</sup> mice compared to Hepc<sup>-/-</sup> mice.

(A) Quantification of liver (n=12 per group) and pancreas (n=6 per group) iron levels in Hepc<sup>-/-</sup>HIF-2α<sup>Δint</sup> (circle) and Hepc<sup>-/-</sup> (square) versus controls, CTR (triangle) mice. (B) Perls’ blue staining of the liver, pancreas and spleen of controls (CTR), Hepc<sup>-/-</sup> and Hepc<sup>-/-</sup>HIF-2α<sup>Δint</sup> mice. One representative picture of each genotype is shown. Bars = 200 μm. (Nikon E800 microscope, CDD QICAM cooled camera).
(C) Plasma ferritin, plasma iron and transferrin saturation in Hepc⁻/⁻HIF-2αΔint (triangle; n=9) versus Hepc⁻/⁻ (square; n=9) and controls, CTR (circle n=9) mice. Statistical significance is indicated by * symbols (**p < 0.01, ***p < 0.001). ns : not significant.
FIGURE 2

A

Liver iron (μg/g tissue)

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Pancreatic iron (μg/g tissue)

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B

CTR       Hepc −/−       Hepc −/− HIF-2 Δint

Liver

200 μm

Pancreas

200 μm

Spleen

200 μm

C

Plasma iron (mmol/l)

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