Deficiency of heme-regulated eIF2α kinase decreases hepcidin expression and splenic iron in *HFE*$^{-/-}$ mice

Sijin Liu,[1] Rajasekhar N.V.S. Suragani,[1] Anping Han,[1] Wanting Zhao,[1] Nancy C. Andrews,[2] and Jane-Jane Chen[1]

[1]Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA; [2]Division of Hematology/Oncology, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA

ABSTRACT

Heme-regulated eIF2α kinase (HRI) is essential for regulating globin translation in iron deficiency and in β-thalassemia. We investigated the role of heme-regulated eIF2α kinase in hemoglobin and red blood cell production as well as in iron homeostasis in a mouse model of iron overload. We show that HRI deficiency does not significantly affect red cell parameters of hemochromatosis (*HFE*$^{-/-}$) mice. Importantly, heme-regulated eIF2α kinase deficiency exacerbates decreases in hepcidin expression and splenic macrophage iron in *HFE*$^{-/-}$ mice. Furthermore, the serum level of bone morphogenic protein 2, which positively regulates hepcidin, is reduced in heme-regulated eIF2α kinase deficiency, but not in HFE deficiency.

Key words: heme-regulated eIF2α kinase, hepcidin, iron deficiency.

Citation: Liu S, Suragani RNVS, Han A, Zhao W, Andrews NC, and Chen J-J. Deficiency of heme-regulated eIF2α kinase decreases hepcidin expression and splenic iron in HFE$^{-/-}$ *mice. Haematologica 2008 May;93(5):753-756. doi: 10.3324/haematol.12175*

©2008 Ferrata Storti Foundation. This is an open-access paper.

Introduction

Hepcidin, a key regulator of iron homeostasis, controls plasma iron levels by inhibiting the absorption of dietary iron from the intestine and the release of iron from macrophages.[1,2] Hepcidin exerts its function by binding to the iron exporter ferroportin and targeting ferroportin for degradation.[3] Hepcidin expression is homeostatically regulated by body iron status, inflammation and erythropoietic needs. It is enhanced by iron overload[4] and inflammation,[5-8] and is inhibited by anemia and hypoxia.[5] In addition, inappropriately low hepcidin production seems to be the common mechanism for hereditary hemochromatosis, an iron overload disease caused by mutations in *HFE*, hemojuvelin (*HFE2*), transferrin receptor 2 (*TFR2*) and hepcidin (*HAMP*).[9] Recently, bone morphogenic protein (BMP) signaling was found to positively regulate hepcidin expression through hemojuvelin, a co-receptor for BMP.[10-13]

Heme-regulated eIF2α kinase (HRI) balances heme and globin production by controlling globin protein synthesis via phosphorylation of the α-subunit of the eukaryotic translational initiation factor (eIF2α).[14] Since HRI plays a critical role during iron/heme deficiency in the production of hemoglobin and formation of red blood cells that contain nearly 70% of the total body iron, it may also be important in systemic and cellular iron homeostasis. Furthermore, HRI is activated by non-heme stresses, especially oxidative stress,[15] which occurs in iron overload. We have already shown that HRI is activated in β-thalassemia to reduce the severity of β-thalassemia including splenic and hepatic iron overload.[16] Recently, we demonstrated that HRI protein is also expressed in macrophages and is important for iron recycling and hepcidin expression.[17] In this study, we investigated the role of HRI in hemoglobin synthesis and iron homeostasis under iron overload conditions in *HFE*$^{-/-}$ mice[18] by generating compound *HRI*$^{-/-}$ *HFE*$^{-/-}$ mice.

Design and Methods

Mouse breeding and genotyping

Mouse production and experimentation were approved by the committee on Animal Care at Massachusetts Institute of Technology. *HR*$^{-/-}$ (mixed genetic backgrounds of C57BL and S129) and *HFE*$^{-/-}$ (S129) mice were generated as previously described,[18,19] and were used to generate double mutant mice. Genotyping of the HRI gene was as previously described.[19] Genotyping of HFE gene was carried out by PCR using primers 5'GTCACGAAG-TTG GGAGTGG-TGTCC-GAGTC3' and 5'GCACAGTGAGGGTTTCCTACAGAG-

Funding: this work was supported in part by grants from NIH DK016272 (JJC), DK066373 (NCA) and from Cooley's Anemia Foundation (SJL, RNVSS and APH).
Manuscript received August 30, 2007. Revised version arrived on November 13, 2007. Manuscript accepted December 10, 2007.
Correspondence: Jane-Jane Chen, E25-406A, MIT, 77 Massachusetts Avenue, Cambridge, MA 02139, USA. E-mail: j-jchen@mit.edu

GTCAC3' for the knockout allele, and primers 5'AAGAGGCAGTGAG-AGGCTGG3' and 5'TGGT-GA-AAGTGAC-TCGCCAC3' for the wild type allele.

Hematologic and pathological analyses and non-heme iron assays

Blood, liver and spleen samples were collected from 4 month-old mice. The complete blood count analyses and reticulocyte counts were performed as previously described.[19] Liver and spleen non-heme iron were assayed and stained by Prussian blue as previously described.[18]

Quantitative RT-PCR and serum BMP2

RNA samples were isolated from livers using a total RNA isolation kit (Promega). Quantitative measurements of mRNA were carried out as previously described.[17] eIF2α was used as an internal control. Serum BMP2 and TGF-β1 were measured by ELISA according to instructions from R&D systems.

Data analysis

Statistical analyses were performed by the two-tailed Student's *t* test. $p<0.05$ was considered statistically significant.

Results and Discussion

Enhanced changes in splenic and serum iron concentrations in HRI$^{-/-}$HFE$^{-/-}$ mice

We analyzed 207 mice from 23 litters produced by mating HRI$^{+/-}$*HFE*$^{+/-}$ mice. Mice of each of the nine predicted genotypes were born according to expected Mendelian ratios. Deletion of the HRI gene did not have an obvious effect on complete blood counts or erythrocyte parameters of *HFE*$^{-/-}$ mice. *HRI*$^{-/-}$*HFE*$^{-/-}$ mice did not develop anemia or polycythemia (*data not shown*). Therefore, in contrast to the essential role of HRI in iron deficiency,[19] HRI does not play a significant role in regulating hemoglobin synthesis and red blood cell production under the condition of iron overload induced by HFE deficiency. *HFE*$^{-/-}$ mice had a significant increase in the liver iron ($p<0.005$, Figure 1A). However, HRI deficiency did not affect the liver iron content of *HFE*$^{-/-}$ mice (Figure 1A). In contrast, splenic iron was further reduced in *HFE*$^{-/-}$mice ($p=0.01$, Figure 1B) compared with *HFE*$^{+/+}$*HFE*$^{-/-}$ mice. Decreased iron in *HRI*$^{-/-}$, *HFE*$^{-/-}$ and *HRI*$^{-/-}$*HFE*$^{-/-}$ mice was also evident when spleen tissue sections were stained for iron (Figure 1C). Notably, the iron was seen mainly in macrophages. Serum iron in *HFE*$^{-/-}$ mice was further increased when the mice were also deficient for HRI ($p<0.05$, Figure 1D). *HRI*$^{-/-}$*HFE*$^{+/+}$ mice also displayed decreased splenic iron and increased serum iron compared to wild type (Wt) mice ($p<0.05$, Figure 1B), similar to the original *HRI*$^{-/-}$ mice reported recently.[17] Altogether, these results demonstrate that splenic iron was reduced in both HFE deficiency and HRI deficiency. The more drastic decrease of splenic iron in combined HRI and HFE deficiencies is likely to be the result of the additive effect of the single gene deficiencies.

Decreased hepcidin expression in HRI$^{-/-}$HFE$^{-/-}$ mice

As expected, hepatic hepcidin expression in *HRI*$^{+/+}$*HFE*$^{-/-}$ mice was decreased by 64.0% compared with Wt ($p<0.005$, Figure 2A). *HRI*$^{-/-}$*HFE*$^{+/+}$ mice also had a 41.0% decrease in hepcidin expression compared with Wt ($p<0.005$, Figure 2A). Importantly, hepcidin expression was further decreased by 89.0% in *HRI*$^{-/-}$*HFE*$^{-/-}$ mice ($p<0.005$, Figure 2A). The severe reduction of hepcidin expression in *HRI*$^{-/-}$*HFE*$^{-/-}$ mice appeared to be of a magnitude expected from the effects of the single knockouts combined. The more moderate reduction of hepcidin in *HRI*$^{-/-}$ mice may help explain the lack of iron overload in *HRI*$^{-/-}$ liver in contrast to *HFE*$^{-/-}$ liver. In human patients, the decrease in urinary hepcidin levels correlated with the severity of hemochromatosis.[20,21] It is unclear why *HRI*$^{-/-}$*HFE*$^{-/-}$ mice did not develop more severe iron overload in the liver at four months. There was no significant difference in the distribution of iron in hepatocytes and macrophages between *HFE*$^{+/+}$*HFE*$^{-/-}$ and *HRI*$^{+/+}$*HFE*$^{-/-}$ mice. Most of the iron stain was in hepatocytes.

Decreased serum BMP2 in HRI deficiency

Since BMP signaling is important for hepcidin expression, we measured serum BMP2 levels of *HRI*$^{-/-}$ and *HFE*$^{-/-}$ mice. As shown in Figure 2, the serum BMP2 level was significantly decreased in *HRI*$^{-/-}$ mice ($p<0.05$) and in *HRI*$^{-/-}$*HFE*$^{-/-}$ mice ($p<0.005$), but not in *HFE*$^{-/-}$ mice. Furthermore, there was no significant difference in the serum BMP2 levels between *HRI*$^{-/-}$ *HFE*$^{+/+}$ and *HRI*$^{-/-}$*HFE*$^{-/-}$ mice. Therefore, the decrease in hepcidin expression in *HFE*$^{-/-}$ mice was independent of serum BMP2 level and is mediated by yet unknown factors. Since no ELISA kit is available for other BMP family members, we could not test whether other BMP proteins are affected in HRI deficiency. However, there was no significant difference in serum TGF-β1 levels between Wt and *HRI*$^{-/-}$ mice (*data not shown*), consistent with the previous observation that members of the TGF-β subfamily do not have as important a role as the BMPs in regulating hepcidin expression.[10,11] It is important to note that the serum BMP2 levels reported here are within the linear range of the dose response curve for hepcidin induction by BMP2.[12,13] The reduced serum BMP2 level in HRI deficiency is consistent with the reduced hepcidin expression in *HRI*$^{-/-}$ mice. The decreases in serum BMP2 and hepatic hepcidin were observed in the original *HRI*$^{-/-}$ mice (C57BL and S129 mixed genetic backgrounds, *data not shown*),[17] as well as in *HRI*$^{-/-}$*HFE*$^{+/+}$ mice (Figure 2) derived from the cross with *HFE*$^{-/-}$ mice (S129 genetic background). These results strongly suggest that genetic background doesn't have a significant role in the reduction of BMP2 and hepcidin in *HRI*$^{-/-}$ mice.

The mechanism and physiological significance of this decreased serum BMP2 in *HRI*$^{-/-}$ mice must be further investigated. It was suggested that BMP signaling for hepatic hepcidin expression might be an autocrine event.[11] However, intravenous administration of BMP2 to mice resulted in increased hepcidin production and decreased serum iron levels.[11] We found that hepatic BMP2 and BMP4 mRNA expression was not signifi-

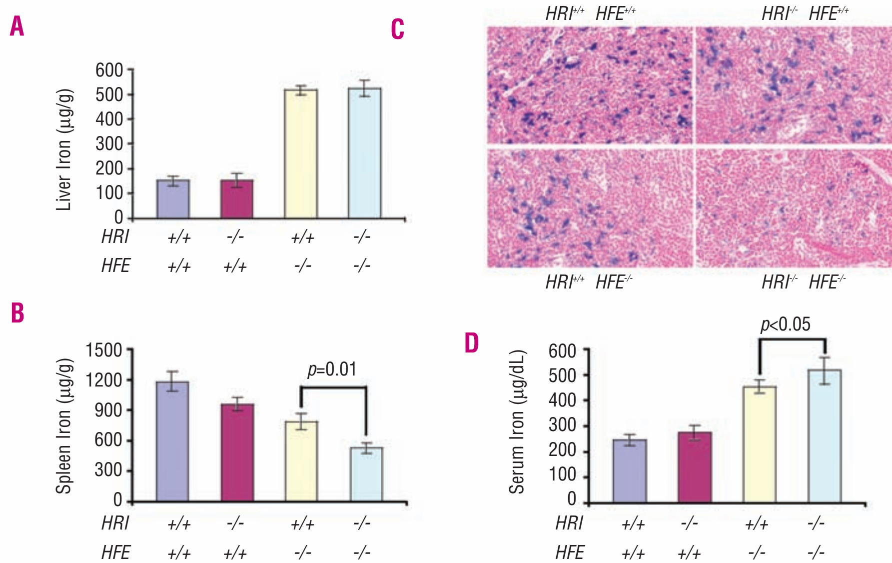

Figure 1. Effects of HRI on the tissue and serum iron contents in *HFE*$^{-/-}$ mice. (A) Hepatic iron contents, (B) Splenic iron contents, (C) Iron stain of spleen tissue sections (D) Serum iron contents. Results are presented as mean±SEM (n=7-9).

cantly different between Wt and *HRI*$^{-/-}$ mice (*data not shown*).

We showed that HRI is also expressed in macrophages, although at a lower level than in erythroid precursors.[17] HRI deficiency impaired the maturation of macrophages and *HRI*$^{-/-}$ mice showed a weaker anti-inflammatory response with reduced cytokine production upon lipopolysaccharide challenge.[17] Therefore, defective maturation of *HRI*$^{-/-}$ macrophages might also contribute to the lower hepcidin expression in *HRI*$^{-/-}$ mice through reduced expression of yet unknown macrophage-derived factors. It is possible that HRI might be expressed in BMP producing cell types such as osteoblasts and bone marrow stromal cells, and might modulate BMP production.

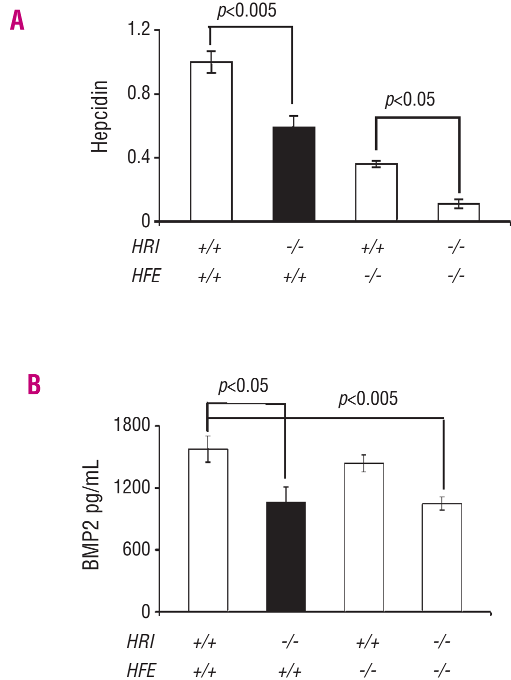

Figure 2. Effects of HRI, HFE and iron on hepatic hepcidin expression and serum BMP2 levels. (A) Hepatic hepcidin mRNA expression. (B) Serum BMP2 levels. Hepcidin mRNA expression was analyzed by qRT-PCR and normalized with eIF2α. Hepcidin expression in Wt is defined as 1. Results are presented as mean±SEM (n=7-9).

Interestingly, the serum level of growth differentiation factor (GDF),[15] which is a member of TGF-β superfamily, was found to be elevated in β-thalassemic patients.[22] Expression of GDF15 was increased during erythroid maturation and was secreted into medium of erythroblasts culture. Furthermore, hepcidin expression in primary hepatocyes was inhibited by high concentrations of GDF15.[22] Therefore, GDF15 may be one of the factors generated by increased erythropoietic activity, which is known to reduce hepcidin expression.[23,24] While *HRI*$^{-/-}$ mice are not anemic, they do exhibit mild erythrohyperplasia.[19] It is possible that some factor similar to GDF15 may be produced by *HRI*$^{-/-}$ erythroblasts to inhibit hepcidin expression. We found that GDF15 expression was not significantly altered in *HRI*$^{-/-}$ E14.5 fetal liver cells by genechip expression analysis (*Liu SJ and Chen J-J, unpublished observation*).

To summarize, this study demonstrates that HRI deficiency can affect the phenotype of *HFE*$^{-/-}$ mice by further decreasing hepcidin expression.

Authorship and Disclosures

SJL and JJC designed the research, analyzed the data and wrote the paper; SJL, RNS, APH and WTZ performed experiments. NCA provided HFE$^{-/-}$ mice and helped write the paper. The authors reported no potential conflicts of interest.

References

1. Ganz T, Nemeth E. Regulation of iron acquisition and iron distribution in mammals. Biochim Biophys Acta 2006;1763:690-9.
2. Andrews NC, Schmidt PJ. Iron homeostasis. Annu Rev Physiol 2007;69: 69-85.
3. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306:2090-3.
4. Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 2001;276: 7811-9.
5. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 2002;110:1037-44.
6. Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2003; 101:2461-3.
7. Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 2004; 113: 1271-6.
8. Lee P, Peng H, Gelbart T, Wang L, Beutler E. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. Proc Natl Acad Sci USA 2005;102:1906-10.
9. Camaschella C. Understanding iron homeostasis through genetic analysis of hemochromatosis and related disorders. Blood 2005;106:3710-7.
10. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, et al. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 2006;38:531-9.
11. Babitt JL, Huang FW, Xia Y, Sidis Y, Andrews NC, Lin HY. Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. J Clin Invest 2007;117:1933-9.
12. Truksa J, Peng H, Lee P, Beutler E. Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6. Proc Natl Acad Sci USA 2006; 103: 10289-93.
13. Lin L, Valore EV, Nemeth E, Goodnough JB, Gabayan V, Ganz T. Iron-transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. Blood 2007;110:2182-9.
14. Chen JJ. Regulation of protein synthesis by the heme-regulated eIF2alpha kinase: relevance to anemias. Blood 2007;109:2693-9.
15. Lu L, Han AP, Chen JJ. Translation initiation control by heme-regulated eukaryotic initiation factor 2alpha kinase in erythroid cells under cytoplasmic stresses. Mol Cell Biol 2001; 21:7971-80.
16. Han AP, Fleming MD, Chen JJ. Heme-regulated eIF2alpha kinase modifies the phenotypic severity of murine models of erythropoietic protoporphyria and β-thalassemia. J Clin Invest 2005;115:1562-70.
17. Liu S, Suragani RN, Wang F, Han A, Zhao W, Andrews NC, et al. The function of heme-regulated eIF2alpha kinase in murine iron homeostasis and macrophage maturation. J Clin Invest 2007;117:3296-305.
18. Levy JE, Montross LK, Cohen DE, Fleming MD, Andrews NC. The C282Y mutation causing hereditary hemochromatosis does not produce a null allele. Blood 1999;94:9-11.
19. Han AP, Yu C, Lu L, Fujiwara Y, Browne C, Chin G, et al. Heme-regulated eIF2α kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. EMBO J 2001;20:6909-18.
20. Papanikolaou G, Tzilianos M, Christakis JI, Bogdanos D, Tsimirika K, MacFarlane J, et al. Hepcidin in iron overload disorders. Blood 2005; 105: 4103-5.
21. Nemeth E, Roetto A, Garozzo G, Ganz T, Camaschella C. Hepcidin is decreased in TFR2 hemochromatosis. Blood 2005;105:1803-6.
22. Tanno T, Bhanu NV, Oneal PA, Goh SH, Staker P, Lee YT, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat Med 2007;13: 1096-101.
23. Vokurka M, Krijt J, Sulc K, Necas E. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. Physiol Res 2006;55: 667-74.
24. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. Blood 2006;108:3730-5.