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Dietary Iron Regulates Hepatic Hepcidin 1 and 2 mRNAs in Mice

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Recently discovered peptide-hepcidin (Hepc) may be a central player in the communication of iron body stores to the intestinal absorptive cells and thus involved in the maintenance of iron homeostasis. The aim of this study was to determine the effects of the level of dietary iron on Hepc gene expression in the liver. OF1 male mice were fed for 3 weeks either control diet (35 mg iron/kg diet), low-iron diet (1 mg iron/kg diet), or high-iron diet (500 mg iron/kg diet), and Hepc 1 and 2 mRNA abundance in the liver was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR). Results clearly showed that Hepc gene expression is upregulated by high dietary iron and downregulated when the dietary iron level is low. Both Hepc 1 and Hepc 2 expression responds coordinately to dietary iron. This work provides additional evidence of the key role of Hepc in the regulation of iron homeostasis.

RECENTLY a novel hepatic antimicrobial peptide was discovered in both human blood and urine named liver expressed antimicrobial peptide (LEAP-1) or hepcidin (Hepc, hepatic bactericidal protein).[1,2] It has since been reported that Hepc expression in the liver increased in mouse, rat, and bass during acute phase response.[3-5] Recent data indicate a role of Hepc in iron metabolism.[3,6] It was suggested that the lack of Hepc expression was responsible for iron overload[6,7] and inversely, early liver expression of Hepc gene in transgenic mice resulted in phenotypic traits of iron deficiency.[8] Hepc was also identified as a gene, which is induced in mice liver by an excess of iron.[3,9] More recently it was shown that constitutive Hepc expression prevents iron overload in a mouse model of hemochromatosis.[10] Thus, from these data, it was proposed that Hepc acts as a signaling molecule involved in the maintenance of iron homeostasis.[6] It also has been shown that Hepc gene expression decreases under experimentally induced anemia and hypoxia.[11] This suggests that Hepc, probably by controlling the release of iron from cells, may be a central player in the communication of body stores to the intestinal absorptive cells.[7] This role of Hepc was confirmed in humans. A refractory anemia was observed in patients with large hepatic adenomas overexpressing Hepc,[12] but mutations in the Hepc gene were reported in families with severe form of hemochromatosis.[13]

It was shown that Hepc has predominant hepatic expression and that the Hepc gene is likely one of the most highly expressed genes in adult liver.[14] However, at present, little is known about the regulation of Hepc gene expression. As discussed by Fleming and Sly,[7] no iron-responsive elements were identified in the Hepc transcript, so the mechanism for the change in mRNA content with change in iron status is unclear. Several binding sites for liver enriched transcription factors CCAAT/enhancer-binding protein (C/EBP) and hepatocyte nuclear factor (HNF4) were identified by sequence analysis of the 5′ flanking region of human and mouse Hepc genes.[9] Recently Courselaud et al[14] have shown that the liver-enriched nuclear factor, C/EBPα, a critical regulator of several hepatic metabolic processes, likely plays an essential role in the control of Hepc gene expression. This suggests possible interaction between iron metabolism and other metabolic pathways.

To investigate further the relationship between Hepc and dietary iron, we have examined Hepc gene expression in the liver from mice fed low- or high-iron diets.

MATERIALS AND METHODS

Animals and Experimental Diets

Eighteen male OF1 (CAW) mice aged 4 weeks and weighing about 21 g were purchased from IFFA-CREDO (L'Arblesle, France). They

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Table 1. Body and Liver Weight and Hepatic Iron Concentration in Mice Fed for 3 Weeks Either Control, Low-Iron, or High-Iron Diet

	Control Diet	Low-Iron Diet	High-Iron Diet	ANOVA
Body weight (g)	35.3 ± 1.0[a]	32.0 ± 0.8[b]	37.0 ± 0.6[a]	$P < .002$
Relative liver weight (g/100 g body weight)	4.5 ± 0.3	4.5 ± 0.1	4.5 ± 0.3	NS
Hepatic iron (mg/g wet weight)	67 ± 8[a]	21 ± 2[b]	197 ± 14[c]	$P < .001$

NOTE. Values are means ± SEM (n = 6). Means in a row without common superscript letters are significantly different ($P < .05$) as determined by Student Newman Keuls test.

Abbreviation: NS, not significant.

were randomly divided into 3 groups of 6 mice per group. The groups consumed one of the following diets for 3 weeks: a semipurified diet containing 35 mg iron/kg diet (control diet); 1 mg iron/kg diet (low-iron diet); 500 mg iron/kg diet (high-iron diet). Iron was provided as iron citrate. The semipurified diets contained the following (gram/kilogram [g/kg]): casein (200), starch (650), corn oil (50), alphacel (cellulose) (50), DL-methionine (3), choline bitartrate (2), AIN-76A vitamin mix (10) (ICN Biomedicals, Orsay, France), calcium (Ca) (5.2), phosphorus (P) (4), sodium (Na) (1.02), potassium (K) (3.6), magnesium (Mg) (0.5), manganese (Mn) (0.054), copper (Cu) (0.006), zinc (Zn) (0.03), I (0.0002), selenium (Se) (0.0001), chromium (Cr) (0.002), chloride (Cl) (1.56), sulfate (SO_4) (1). Iron level in the 3 experimental diets was verified by flame atomic absorption spectrometric analysis (Perkin-Elmer 560, St Quentin en Yvelines, France). All mice had free access to distilled water and food. At the end of the 3-week feeding period, mice were anesthetized with pentobarbital (40 mg/kg body weight). Blood was then withdrawn via heart puncture and the liver was removed, blotted, and weighed. Portions of the liver were frozen in liquid nitrogen and stored in −80°C for further analyses. The institution's guidelines for the care and use of laboratory animals were followed.

Iron determination in the liver. Samples of liver were dry-ashed (10 hours at 500°C) and then extracted at 130°C in HNO_3/H_2O_2 (2/1, Merck, Suprapur, Darmstadt, Germany) until decoloration; final dilution was made in 2% HNO_3. Iron concentration was determined by atomic absorption spectrometry (Perkin-Elmer 560) in an acetylene-air flame at 248 nm. Corresponding controls were analyzed with each set of measurements.

Reverse Transcriptase-Polymerase Chain Reaction Analysis of Hepc1 and 2 mRNA Abundance

Total RNA was isolated from mouse liver using RNeasy Mini Kit (Qiagen, Chatsworth, CA). Concentrations and purity of the RNA were assessed by A260/A280 absorption, and RNA samples were stored at −70°C for further analysis. The changes in Hepc 1 and 2 mRNA abundance were monitored by reverse transcriptase-polymerase chain reaction (RT-PCR). For this purpose, 3 μg total RNA converted into first strand cDNA using Ready-To-Go You-Prime First-Strand Beads Kit (Amersham, Piscataway, NJ) and 1 μL (0.5 mg/mL) oligo (dT) Primer (Promega, Lyon, France) were incubated at 37°C for 60 minutes. PCR was performed as described by Nicolas et al[6] except that Hepc 2 PCR conditions were 30 cycles. After PCR, the amplified products, 171 bp for Hepc 1 and Hepc 2, and 250 bp for β-actin used as control, were separated by electrophoresis on 1.5% agarose gel and visualized under ultraviolet (UV) light. Sequences of the primers were as follows: Hepc 1, 5′-CCTATCTCCATCAACAGATG-3′ (forward) and 5′-AACAGATACCACACTGGGAA-3′(reverse); Hepc 2: 5′-CCTATCTCCAGCAACAGATG-3′ (forward) and 5′AACAGATACCACAGGAGGGT-3′ (reverse); β-actin, 5′-AGCCATGTACGTAGCCATCC-3′ (forward) and 5′-TTTGATGTCACGCACGATTT-3′ (reverse). The design and specificity of these primers were described previously.[6,9] Real-time RT-PCR was performed using LightCycler RNA Amplification kit SYBR Green I in a fluorescence temperature cycler (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany). The amplification of PCR products was verified using the melting curve option. Control diet-fed mouse liver mRNA was used to construct standard curve for Hepc 1, Hepc 2, and β-actin. PCR data analysis and relative quantification were performed using LightCycler Software Version 3.5. Results are expressed as the ratio of Hepc 1 or 2/β-actin mRNA abundance.

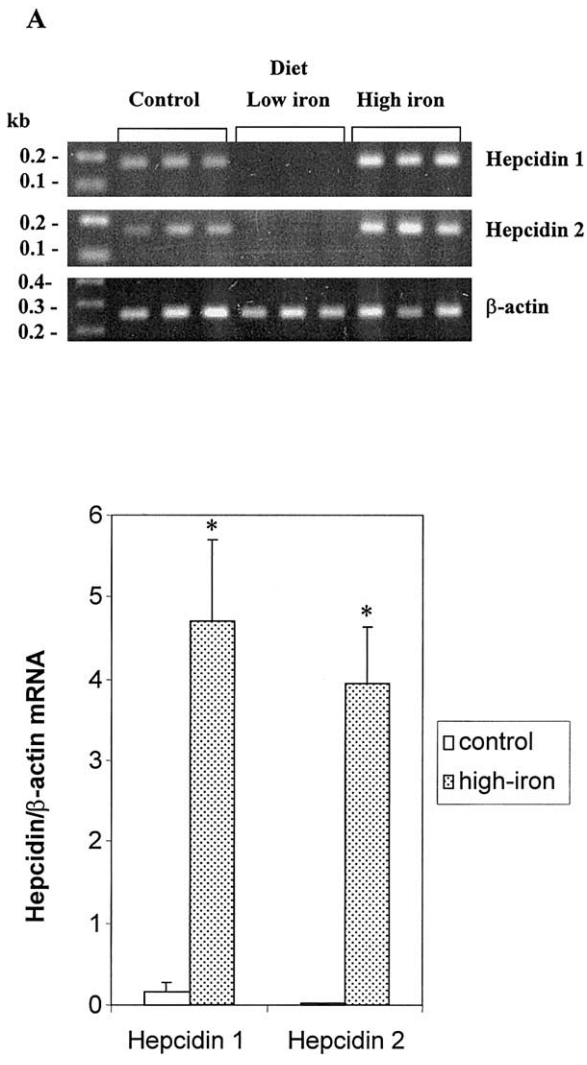

Fig 1. RT-PCR analysis of the expression of Hepc 1 and 2 in the liver of control, low-iron, or high-iron diet-fed mice. (A) Representative electrophoretic analysis of PCR products from groups studied. β-actin was used as control. (B) Quantification of Hepc 1 and 2 mRNA abundance in the liver of control and high-iron diet-fed mice. β-actin was used as control, and results are expressed as the ratio Hepc/β-actin abundance (means ± SEM; n = 5); *$P < .01$.

Statistical Analysis

Results for each group are expressed as the mean ± SEM. Levels of significance of the difference between groups were determined by 1-way analysis of variance (ANOVA) with post hoc Student Newman Keuls test or by Student *t* test (Instat software, GraphPad Software, San Diego, CA). Differences at $P < .05$ were considered significant.

RESULTS

The effect of dietary iron content on animals is presented in Table 1. It was observed that mice fed the low-iron diet for 3 weeks had lower body weight than controls. High-iron diet, as compared with the control diet, did not affect mice growth. The relative liver weight was not affected by iron content in the diet (Table 1). After 3 weeks of feeding experimental diets, there was a significant effect on the iron content in the liver (Table 1). The animals fed a low-iron diet had low iron concentrations in the liver (about 3-fold less than controls) and those fed a high-iron diet had an increased concentration of iron in the liver (about 3-fold more than control).

As shown in Fig 1A, low-iron diet-fed mice presented low hepatic Hepc 1 and 2 mRNA abundance as compared with controls. In contrast, a high-iron diet led to higher Hepc 1 and 2 mRNA abundance in the liver. The quantitative RT-PCR for both Hepcs confirmed the results obtained after electrophoresis of PCR products. A marked increase of Hepc 1 and 2 mRNAs in the liver of high-iron diet-fed mice as compared with controls was observed (Fig 1B). The expression of Hepcs in low-iron diet-fed mice was too low to be evaluated by quantitative RT-PCR.

DISCUSSION

The present study was undertaken to determine the interactions between dietary iron and Hepc mRNA level in the liver. Hepatic gene expression of Hepc 1 and 2 in mice receiving control, low-iron, or high-iron diets was studied. It is clearly shown that the level of iron in the diet markedly affects Hepc gene expression. Our results are in agreement with recently published data on the relationship between iron and Hepc gene expression. In fact, other investigators have shown that expression of Hepc was strongly upregulated by iron excess obtained by experimental carbonyl iron and iron-dextran overload.[3,9] From a recent study[15] on rats switched from an iron-replete diet to an iron-deficient diet, it appears that Hepc expression negatively correlated with iron absorption. Two highly related Hepc genes, Hepc1 and Hepc 2, were identified in the mouse.[3,6] However, in previous studies the specific expression of both Hepcs was poorly studied. Recently, Ilyin et al[9] have reported a comparative analysis of sequence, genomic structure, expression, and iron regulation. It was suggested that both Hepc genes are involved in iron metabolism, but could exhibit different activities and/or play distinct roles. In the present study, we have shown that when modifying dietary iron, both Hepcs 1 and 2 react in similar ways. In humans, as discussed by Nicolas et al[6] there is probably one Hepc gene or cDNA that corresponds to Hepc 1. It was suggested by these investigators that the presence of only one Hepc gene in the human genome indicates the probable redundancy of Hepc 1 and Hepc 2 gene functions in the mouse. It was proposed that inactivation of only one of these genes would not have been sufficient to lead to the development of an iron overload phenotype. Our results support the coordinate response of both Hepcs to iron.

In conclusion, this study found experimental evidence of an effect of dietary iron on Hepc gene expression. It appears that Hepc gene expression is upregulated by high dietary iron and downregulated when iron availability is low. This work provides an additional clue to the key role of Hepc as a signaling molecule regulating iron absorption and thus as an important regulator of iron homeostasis.

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