Strain and gender modulate hepatic hepcidin 1 and 2 mRNA expression in mice

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Abstract

Hepcidin (HEPC) plays a key role in iron homeostasis and an abnormally low level of hepcidin mRNA has been reported in HFE-1 genetic hemochromatosis. Considering the well-known phenotypic variability of this disease, especially between men and women, it is important to define factors susceptible to modulate hepatic hepcidin expression and, consequently, to influence the development of iron overload in HFE-1 hemochromatosis. Therefore, our aim was to analyze the effects of strain and gender on hepatic hepcidin expression in the mouse. C57BL/6 and DBA/2 wild-type mice were included in this study. Liver and splenic iron contents were measured. Specific hepatic Hepc1 and Hepc2 mRNA levels were quantified using real-time reverse transcription polymerase chain reaction (RT-PCR). C57BL/6 mice expressed predominantly Hepc1 mRNA, whereas Hepc2 mRNA was the main form in DBA/2 mice. In both strains, females had higher levels of iron stores and Hepc mRNAs compared to males. Our results demonstrate that the expression of both hepcidin mRNAs varies according to strain and gender. They suggest that sex and genetic background, which are regulators of hepcidin expression, could play a role in the phenotypic expression of genetic hemochromatosis.

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Introduction

Hepcidin (HEPC, HAMP; OMIM 606464), also named LEAP-1 [1], originally described as having an antimicrobial activity, is mainly produced by the liver, and its mature form (20–25 amino acids) is found in the plasma ultrafiltrate [1] and urine [2]. Humans and rats have a single identified hepcidin gene [3], whereas two functional genes, Hepc1 (hepcidin 1) and Hepc2 (hepcidin 2), which have only 68% identity at the protein mature form level, are present in the mouse genome [3–5].

A link between hepcidin and iron metabolism in the mouse was first highlighted by Pigeon et al. [3], who pointed out a relationship between iron stores and hepatic hepcidin mRNA expression in induced or β2-microglobulin knockout (β2m−/−) iron overload models. Later on, Nicolas et al. [4] found that Usf2−/− mice developed an iron overload phenotype similar to that observed in Hfe−/− mice, and that the hepcidin 1 and 2 genes, which are located close to Usf2 genes, were also totally silent in these animals. They hypothesized from these observations that hepcidin could be an iron metabolism regulating hormone acting on enterocytes and macrophages [4,6]. This key role of hepcidin in iron metabolism has been further supported by the iron deficiency phenotype observed in transgenic mice hyperexpressing Hepc1 [7] and by the association of severe juvenile hemochromatosis with a mutation or a deletion in the hepcidin gene [8]. From the two hepcidin genes identified in the mouse, only hepcidin 1 has been clearly implicated in iron metabolism. The role of Hepc2 remains to be characterized.
HFE-1 genetic hemochromatosis (HGH) is a frequent inherited disease mainly related to the C282Y mutation of the HFE gene in populations of northern European origin [9]. Patients who are homozygous for the mutation classically develop iron overload, which is at least partly related to intestinal iron hyperabsorption. In the liver, early stages of iron overload involve parenchymal cells, whereas Kupffer cells (i.e., resident macrophages) are belatedly overloaded during the disease, conversely to secondary iron overload [10]. Therefore, both enterocytes and macrophages have an altered iron metabolism leading to the specific phenotype of HGH. Classically, HGE leads to visceral complications involving predominantly the liver, pancreas, and heart, but also joints, skin, and endocrine system [11,12]. However, the spectrum of the disease is variable and the penetrance incomplete [13–15], as recently strengthened by large epidemiological studies [16,17]. Such a variability in the phenotypic expression of homozygotes for the HFE C282Y mutation might be at least partly related to genetic factors. The role of these factors is indirectly supported by studies performed in wild type [18,19] and in genetically modified iron overloaded mice, including β2-microglobulin deficient mice and Hfe−/− mice [20–23]. Thus, multiple genes could play a role in the modulation of iron overload phenotype related to HFE mutation.

The key role of hepcidin in the development of iron overload during HGH and its murine models has recently been emphasized. Indeed, the hepatic Hepc mRNA level was found to be abnormally low in young Hfe−/− mice [24]. In humans, Bridle et al. [25] reported a decrease of the hepatic hepcidin mRNA level in C282Y homozygous patients with clinical manifestations of the disease and Gehrke et al. [26] found an absence of liver hepcidin mRNA increase in HFE-1 genetic hemochromatotic patients, despite their marked iron overload. All these data suggest that an inadequate regulation of hepcidin mRNA expression may contribute to the development of iron overload in HGH patients. This hypothesis was reinforced by the correction of the iron overload phenotype of Hfe−/− animals by a constitutive expression of Hepc1 mRNA [27].

Taken together, these observations suggest that the level of hepcidin expression could be one of the critical factors accounting for the phenotypic expression of iron overload during HGH. Therefore, our aim was to evaluate the influence of the genetic background, including strain and gender, on the expression of the two hepcidin genes in the mouse liver.

**Material and methods**

**Animals**

Eleven week-old C57BL/6 and DBA/2 mice were obtained from CERJ (France) and housed in the IFR30 animal facility in Toulouse. Twenty animals (five per group) were included in this study. All animals were maintained in accordance to French law and regulations in a temperature- and light-controlled environment. They were given free access to tap water and food. Mice liver and spleen samples were frozen in liquid nitrogen and kept at −80°C until further processing.

**Iron load measures**

Liver (LIC) and splenic (SIC) iron concentrations were evaluated biochemically [28] on frozen specimens.

**Total RNA isolation and reverse transcription (RT)**

Total RNA was extracted from livers of C57BL/6 and DBA/2 mice using the SV total RNA isolation system (Promega, Charbonnières, France). cDNA first-strand synthesis was performed with the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA), using 1 μg of total RNA and oligo dT primers. Reverse transcription products were diluted fivefold in pure sterile water before use for real-time PCR (polymerase chain reaction) assays.

**cDNA probes**

Mouse hepcidin cDNA probe was obtained by cloning in pCRII-TOPO vector (TOPO™ TA Cloning® kit, Invitrogen Life Technologies, Cergy Pontoise, France), the iron-induced hepcidin cDNA isolated by suppressive subtractive hybridization as described previously [3]. A 232-bp hepcidin cDNA fragment was then released by BamH1 and XhoI digestion and used as probe for Northern blot analysis.

**Northern blot analysis**

For all templates, 10 μg of total RNA were loaded on a denaturing 1.2% agarose gel and transferred onto Hybond N+ filters (Amersham, Buckinghamshire, England). Filters were then hybridized with the mouse hepcidin cDNA probe labeled with [α32P] dCTP (3000Ci/mmol) (Amersham and Random Primers DNA Labelling System™, Invitrogen Life Technologies). Equal mRNA loading was checked by methylene blue staining (not shown).

To assess hepcidin mRNA expression level, we exposed Northern blot membranes to overnight autoradiography and then performed densitometry measures with the assistance of Densylab v2.0.5 software (Microvision Instruments, Evry, France).

**Real-time RT-PCR analysis**

Primers validated in previous studies [4,23] have been used to amplify Hepc1 (5′-CCTATTCCTCATCAACAGAA-TG-3′ and 5′-AACAGATACCACACTGGGAA-3′), Hepc2 (5′-CTATTCCTCAACACAGATG-3′ and 5′-AACAGATACCAGGAGGT-3′), and β-actin (5′-GACGGCCA-
AGTCATCAGCTATTG-3' and 5'-CCACAGGATTCCATACCCACAGGATTCCATAC-3'). These three primer pairs lead to 171-, 171-, and 88-bp amplification products, respectively. To avoid coamplification of genomic DNA, the forward Hepc1 and Hepc2 primers have been designed to overlap exons I and II and the reverse primer was positioned on exon III.

TaqMan® probes for real-time PCR analysis were designed with the Primer Express Software (PE Biosystems) and purchased from Eurogentec (Seraing, Belgium). Probe 5'-FAM-CCCTGCTTTCTTCCCCGTGCAAAG-TAMRA-3' was used to detect Hepc1 and Hepc2 and probe 5'-FAM-TCCGATGCCCTGAGGCTCTTTTCC-TAMRA-3' to detect β-actin [5].

All quantitative real-time PCR assays were performed in triplicate in 96-well microplates using the qPCR™ Core reagent kit (Eurogentec) and an ABI PRISM 7700 Sequence Detection System (PE Biosystems). Each microplate included the target cDNAs, a negative no-template control, and two standard curves prepared from normal adult mouse liver cDNA. For each cDNA sample, the difference between the threshold cycle for Hepc1 or for Hepc2 amplification and the threshold cycle for β-actin has been calculated. This allows normalization of the amount of target to the endogenous reference, β-actin. To compare the expression of the two hepcidin messengers according to strain or gender, we expressed the amount of targets relative to a calibrator Hepc1 mRNA level, which is specified on each figure.

Statistical analysis

Results were expressed as mean ± SD. Nonparametric Mann–Whitney tests were performed to test that the distributions of Hepc1 or Hepc2 mRNA levels were the same in the two strains, or the two genders, or that the distributions of Hepc1 and Hepc2 were the same within each strain/gender group. A significance probability less than 0.05 was considered as an indication of a significant mRNA expression difference between the compared groups.

Results

Strain and gender modulate hepatic and splenic iron concentrations in mice

Hepatic and splenic iron loads were evaluated biochemically and analyzed according to the mouse strain, that is,
C57BL/6 or DBA/2, and gender. As can be seen in Fig. 1A, females of both strains had significantly higher LIC than the corresponding males. In addition, LIC of DBA/2 males were significantly higher than those found in C57BL/6 males.

Similarly, as can be seen in Fig. 1B, females of either the C57BL/6 or the DBA/2 strain exhibited higher SIC than the corresponding males. However, SIC were significantly lower in DBA/2 females than in C57BL/6 females.

Total hepatic hepcidin mRNA level is modulated by gender and strain

Northern blot analysis (Fig. 2) indicated that females of both strains expressed higher \( \text{Hepc} \) mRNA levels than males. In addition, DBA/2 females expressed higher levels of hepcidin mRNA than the corresponding C57BL/6 group, and clearly were the mice with the highest hepcidin mRNA level.
Whereas the role of hepcidin 1 gene product on iron metabolism strongly modulate iron absorption and recycling [4]. In mice, suggested that elements regulating its expression could be modulated [4,7]. Indeed, although both hepcidin 1 and 2 mRNAs are expressed in the mouse, the effects of genetic factors, including strain and gender, on the expression of the two hepcidin genes in the mouse was studied. In mice [20], it has indeed been suggested that strain and gender may influence the iron overload phenotype [22]. Moreover, Dupic et al. [23] pointed out that strain may have a significant impact on the development of iron overload in Hepc−/− mice, through an effect on the expression of duodenal genes involved in iron absorption. Overall, our results, together with the finding of abnormally low levels of hepcidin mRNA in HFE-1 genetic hemochromatosis, suggest that variations in the basal hepcidin levels of hepcidin could modulate the impact of the HFE C282Y mutation on the iron overload phenotype.

Table 1

Schematic representation of Hepc1 and Hepc2 mRNA expression in C57BL/6 and DBA/2 mice

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
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<tr>
<td></td>
<td>Males</td>
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<td>Hepc1 mRNA</td>
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<tr>
<td>Hepc2 mRNA</td>
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Expression levels of Hepc1 and Hepc2 according to each strain and gender are symbolized by crosses. A growing number of crosses indicates an increase in the mRNA expression level.

expression among the different strain/gender combinations studied.

Since Northern blot analysis reflects global expression of both hepcidin 1 and hepcidin 2 mRNAs, we investigated the expression level of each mRNA separately, using real-time RT-PCR with probes specific of each mRNA to discriminate the two messengers.

Hepatic hepcidin 1 mRNA expression is modulated by gender

Males of the two C57BL/6 and DBA/2 strains expressed Hepc1 mRNA at similar levels. The same held true for females of the two strains (Fig. 3). However, in both strains, Hepc1 mRNA levels were significantly higher in females (Fig. 4).

Hepatic hepcidin 1 and hepcidin 2 mRNAs are differentially expressed

Whatever the gender, the predominant hepatic hepcidin mRNA was Hepc1 in C57BL/6 and Hepc2 in DBA/2 mice. Moreover, we found that DBA/2 males and females expressed Hepc2 mRNA at very high levels compared to C57BL/6 males and females, respectively (Fig. 3). For both strains, Hepc2 mRNA levels were significantly higher in females than in males (Fig. 4). Modulations of Hepc1 and Hepc2 mRNA expression by gender and strain are schematically summarized in Table 1.

Discussion

The role of hepcidin in iron metabolism has been demonstrated in mice [4,7] and in humans [8]. Therefore, it is suggested that elements regulating its expression could strongly modulate iron absorption and recycling [4]. In mice, two closely related hepcidin genes have been identified. Whereas the role of hepcidin 1 gene product on iron metabolism is established [4,7], biological functions of hepcidin 2 remain to be fully characterized. Our goal was to evaluate the effects of genetic factors, including strain and gender, on the expression of the two hepcidin genes in the mouse.

We found that C57BL/6 and DBA/2 strains differentially expressed hepcidin 1 and 2 mRNAs. Indeed, although both strains expressed Hepc1 mRNA at similar levels, we found that Hepc1 mRNA was the predominant messenger in C57BL/6 strain, whereas Hepc2 mRNA was the main species in DBA/2 strain. These data demonstrate that despite strong homologies in the 5' flanking region of the two hepcidin genes [5,29], there are important regulatory differences between the two genes, likely related to the genetic background. Such a differential regulation of Hepc1 and Hepc2 mRNAs has also been recently reported in mice between the liver and the pancreas [5]. It must be pointed out that, to date, there is no data depicting that the two polypeptides, which exhibit the eight cysteine motif but have only 68% homology, exert the same biological activities. Therefore, the impact of this differential expression between hepcidin genes on iron metabolism remains to be evaluated.

Females of both strains expressed both hepatic hepcidin mRNAs at higher levels than males, suggesting that there are gender-linked factors that are involved in the hepcidin 1 and 2 mRNA expression control. This increase of both mRNAs in females did not change the predominance of Hepc1 and Hepc2 mRNAs in the C57BL/6 and DBA/2 strains, respectively.

The high levels of global hepcidin mRNA in females were associated with higher LIC and SIC values compared to males, suggesting that a relationship between iron stores and hepcidin expression does exist. Such a link was previously reported [3,27,30]. However, in some cases, including anemic anemiaemic mice, whereas livers exhibited significant iron overload, hepatic hepcidin mRNA expression was paradoxically decreased [30], suggesting a role of other factors, independent of iron stores, on hepcidin mRNA expression regulation. Accordingly, the impact of lipopolysaccharides [3], bacterial challenge [31], inflammatory cytokines [32], and hepatocyte phenotype [29] on hepcidin mRNA expression has been described, as well as the decreasing effect of hypoxia on its expression, likely partly mediated via erythropoietin pathway [33,34]. Whether the high level of hepcidin mRNA in females is a consequence of higher iron stores remains to be established.

In mice, the role of genetic background, including strain and gender, in the control of iron metabolism is supported by studies performed in wild type [18] as well as in β2-microglobulin deficient mice and Hfe+/− mice. In β2m−/− mice, in which absence of Hfe expression at the cell surface leads to an iron overload similar to that observed in Hfe−/− mice [20], it has indeed been suggested that strain and gender may influence the iron overload phenotype [22]. Moreover, Dupic et al. [23] pointed out that strain may have a significant impact on the development of iron overload in Hepc−/− mice, through an effect on the expression of duodenal genes involved in iron absorption. Overall, our results, together with the finding of abnormally low levels of hepcidin mRNA in HFE-1 genetic hemochromatosis, suggest that variations in the basal hepcidin levels of hepcidin could modulate the impact of the HFE C282Y mutation on iron metabolism.
iron metabolism and thus participate in the incomplete penetrance of the disease.

In summary, our results (i) demonstrate that hepcidin mRNAs in the mouse are differentially expressed according to the strain and the gender; (ii) suggest that these genetic factors may play a noticeable role in the phenotypic expression of genetic hemochromatosis. Identification of both hepcidin expression regulators and molecular partners will provide key elements to improve our understanding of HFE-1 hemochromatosis and iron metabolism related diseases.

Note added in proof


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References


