Distinct requirements for Hfe in basal and induced hepcidin levels in iron overload and inflammation

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Abstract

Hepcidin is a negative regulator of iron absorption produced mainly by the liver in response to changes in iron stores and inflammation, and its levels have been shown to regulate the intestinal basolateral iron transporter ferroportin1 (*Fp1*). Hereditary hemochromatosis patients and Hfe-deficient mice show inappropriate expression of hepcidin but, in apparent contradiction, still retain the ability to regulate iron absorption in response to alterations of iron metabolism. To further understand the molecular relationships among *Hfe*, *hepcidin*, and *Fp1*, we investigated *hepcidin* and *Fp1* regulation in Hfe-deficient mice (*Hfe*$^{-/-}$ and *β2m*$^{-/-}$) in response to iron deprivation, iron loading, and acute inflammation. We found that whereas basal *hepcidin* levels were manifestly dependent on the presence of Hfe and on the mouse background, all Hfe-deficient mice were still able to regulate *hepcidin* in situations of altered iron homeostasis. In the liver, *Fp1* was modulated in opposite directions by iron and LPS, and its regulation in Hfe-deficient mice was similar to that observed in wild-type mice. In addition, we found that iron-deprived mice were able to mount a robust response after LPS challenge and that Toll-like receptor 4 (TLR-4)-deficient mice fail to regulate *hepcidin* expression in response to LPS. In conclusion, these results suggest that although Hfe is necessary for the establishment of hepcidin basal levels, it is dispensable for hepcidin regulation through both the iron-sensing and inflammatory pathways, and hepatic *Fp1* regulation is largely independent of hepcidin and Hfe. The inflammatory pathway overrides the iron-sensing pathway and is TLR-4 dependent.

Keywords

ferroportin 1; Toll-like receptor 4; β2m; hereditary hemochromatosis; lipopolysaccharide

The maintenance of body iron balance is imposed by the fact that total iron deficiency is incompatible with life, whereas iron excess is dangerously toxic. In mammals, iron metabolism is characterized by a limited external exchange and by efficient reutilization of

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iron from internal sources. Because the capacity to excrete iron is very limited, total body iron is regulated mainly by controlling the level of absorption from the diet, via intestinal cells (3), such that sufficient iron is provided to keep stores replete, but not excessive, and erythroid demands are met. When the regulatory function of intestinal iron absorption is altered and the amount of iron entering the body exceeds the amount lost over a sustained period of time, iron overload arises (41). Progressive iron deposition eventually leads to tissue damage and fibrosis and ultimately to organ failure.

Hereditary hemochromatosis (HH), a common genetic disorder in Caucasian populations, is characterized by high levels of iron absorption from the diet, leading to iron accumulation in the liver and other organs (41). The gene associated with HH type 1 has been identified as *HFE*, coding for a major histo-compatibility complex class I (MHC-I)-like molecule (11). As with other MHC-I molecules, HFE needs to associate with β_2-microglobulin (β2m) for appropriate expression at the cell surface (12). Accordingly, mice lacking HFE or β2m recapitulate HH in humans and develop iron overload primarily in the liver (25, 45, 49) due to hyperabsorption of iron in the duodenum (4, 45).

Recently, a small, cysteine-rich peptide called hepcidin emerged as a major regulator of iron absorption (20, 40, 42). Hepcidin is cleaved from a larger precursor expressed predominantly in the liver and its mRNA levels are regulated by iron, anemia, hypoxia, and inflammation (38). Mice lacking hepcidin develop iron overload (36), and conversely, mice overexpressing hepcidin incur severe anemia (37). In addition, hepcidin injection in mice has been shown to downregulate iron absorption (21). Collectively, these data offer strong support for the role of hepcidin as a negative regulator of intestinal iron absorption.

The mechanisms by which hepcidin exerts its effects on intestinal iron absorption include transcriptional and posttranslational regulation of ferroportin 1 (Fp1), the only iron exporter in mammals (9). In fact, *Fp1* mRNA expression in the gut has been shown to be downregulated by high *hepcidin* levels (14, 48), and in the human embryonic kidney cell line model HEK-293, hepcidin was shown to bind to Fp1, resulting in internalization and degradation of Fp1 and leading to decreased export of cellular iron (35). The regulation of Fp1 by hepcidin is proposed to complete a homeostatic loop through which iron regulates the secretion of hepcidin by liver cells, which in turn controls the expression and the concentration of Fp1 on the cell surface of gut enterocytes (35).

In iron-loaded HH patients and in Hfe-deficient mice due to the lack of Hfe or β2m, basal hepatic *hepcidin* mRNA levels have been reported to be either lower than (2, 5, 28, 31) or not significantly different (15, 17, 28, 32) from those in the controls. Because hepcidin levels in normal mice rise in response to iron overload (38, 42), this has been interpreted as the lack of iron sensing and the inability to regulate *hepcidin* expression in the absence of Hfe, which is in line with its proposed role as a negative regulator of intestinal iron absorption (16). This, however, contradicts other studies that demonstrated that HH patients (29) and both $\beta2m^{-/-}$ (44) and $Hfe^{-/-}$ (4, 22) mice, in fact, retain the ability to regulate iron absorption levels in response to altered iron metabolism. These observations raise the question of whether Hfe is absolutely required for the regulation of *hepcidin* and downstream *Fp1* levels in response to altered iron metabolism.

In this study, we set out to evaluate the capacity of Hfe-deficient mice ($Hfe^{-/-}$ and $\beta 2m^{-/-}$) to regulate *hepcidin* expression and subsequent downstream responses, namely, *Fp1* expression, via the iron-sensing and inflammation pathways. Because hepcidin levels in mice have been shown to depend on their genetic background (8, 10, 13), we investigated Hfe-knockout mice from two distinct backgrounds, C57BL/6 (B6.$Hfe^{-/-}$) and 129/SvEvTac (129Sv.$Hfe^{-/-}$), by comparing their responses to corresponding wild-type (Wt) mice, B6.Wt and 129Sv.Wt. In addition, we further studied the involvement of upstream elements, namely, Toll-like receptor 4 (TLR-4), in the iron-sensing and inflammatory pathways.

MATERIALS AND METHODS

Animals

All procedures were performed in accordance with Canadian Council on Animal Care guidelines after approval by the institutional animal care committee of the Centre Hospitalier de l'Université de Montréal (CHUM). $Hfe^{-/-}$ mice were kindly provided by Dr. N. C. Andrews [Howard Hughes Medical Institute and Harvard Medical School, Children's Hospital, Boston, MA (24)] in the 129/SvEvTac background and were backcrossed onto the C57BL/6 (B6) background for 10 generations. B6, $\beta 2m^{-/-}$, C3H/HeJ, and C3H/HeOuJ mice were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All animals were permanently housed under specific pathogen-free conditions.

Animal treatments

Control mice were given a commercial diet (Teklad Global 18% protein rodent diet; Harlan Teklad, Madison, WI). Dietary iron overload was produced by giving 8-wk-old mice the same commercial diet supplemented with 2.5% (wt/wt) carbonyl iron (Sigma-Aldrich, St. Louis, MO) for 2 wk. Iron deprivation was induced by feeding mice the same commercial diet deficient in iron for 2 wk.

Acute inflammation was produced by a single dose of LPS (*Escherichia coli* serotype 055:B5, 5 mg/kg ip, Sigma-Aldrich). Control mice were similarly injected with an equivalent volume of sterile saline solution (0.09% NaCl). The animals were killed 6 h after the injection.

Quantitative RT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, Burlington, ON, Canada), and RT was performed with the Thermoscript RT-PCR system (Invitrogen). *Hepcidin*, *Fp1*, and *β-actin* mRNA levels were measured by real-time PCR in a Rotor Gene 3000 real-time DNA detection system (Montreal Biotech, Kirkland, QC, Canada) with the QuantiTect SYBRGreen I PCR kit (Qiagen, Mississauga, ON, Canada), as described (28). The primers employed were: *β-actin*, 5′-TGTTACCAACTGGGACGACA-3′ and 5′-GGTGTTGAAGGTCTCAAA-3′; *hepcidin*, 5′AGAGCTGCAGC-CTTTGCAC3′ and 5′GAAGATGCAGATGGGGAAGT3′; and *Fp1* 5′CCCATCCCCATAGTCTCTGT3′ and 5′CTTGCAGCAACTGT-GTCACC3′. Expression levels were normalized to the housekeeping gene *β-actin*.

Hematological measurements and transferrin saturation

Red blood cell (RBC) count, Hb, hematocrit, and mean corpuscular volume were measured with an automated cell counter calibrated for murine samples (ABC *vet* counter; ABX hématologie, Montpellier, France). Serum iron, total iron-binding capacity, and transferrin saturation were assessed by colorimetric assay (30) with the Kodak Ektachem DT60 system (Johnson & Johnson, Ortho Clinical Diagnostics, Mississauga, ON, Canada).

Measurement of tissue iron concentration

Liver and spleen iron concentrations were assessed by acid digestion of tissue samples, followed by iron quantification with atomic absorption spectroscopy (30).

Hepatocyte isolation and treatments

Hepatocytes were isolated from adult mouse livers according to a procedure already described (34). Isolated cells were resuspended in Williams' E media with 5% FBS and seeded onto collagen-coated plates at a density of 2.4×10^5 cells per well. After 3 h, unattached cells were removed, and the medium was replaced by fresh medium. Twenty-two hours after isolation, the medium was changed and replaced by fresh medium alone or medium containing IL-6 (20 ng/ml), TNF-α (20 ng/ml) for 24 h, or ferric ammonium citrate (FAC; 10 μM, Sigma-Aldrich) for 16 h.

Statistical analysis

All values in the figures are expressed as means ± SD. Multiple comparisons were statistically evaluated by one-way ANOVA.

RESULTS

Iron restriction

To evaluate the ability of Hfe-deficient mice to regulate *hepcidin* mRNA levels in response to lowered iron stores, mice were placed for 2 wk on an iron-deficient diet. As reported previously (28), iron parameters, including serum iron, transferrin saturation, and liver iron concentrations in mice kept on the standard diet, were significantly higher in Hfe-deficient animals compared with Wt controls from the same background, and liver iron stores were more elevated in the 129Sv background (322 ± 50 in 129Sv.Wt vs. $1,675 \pm 249$ μg iron/g dry weight in 129.*Hfe*$^{-/-}$; *P* < 0.0001) compared with B6 (221 ± 20 in B6.Wt vs. 772 ± 202 in B6.*Hfe*$^{-/-}$ and 671 ± 67 μg iron/g dry weight in B6.*β2m*$^{-/-}$; *P* < 0.001). Iron restriction resulted in a significant decrease of liver iron stores in all mouse strains, whereas erythroid parameters such as Hb and RBC count remained unaffected, indicating that the treatment did not lead to anemia (data not shown).

As seen in Figure 1*A*, there were no significant differences in *hepcidin* mRNA basal levels among B6.Wt, B6.*Hfe*$^{-/-}$ and B6.*β2m*$^{-/-}$ mice fed a standard diet. After an iron-deficient diet, all strains responded by significantly decreasing *hepcidin* mRNA levels.

A similar response was observed in the 129Sv background (Fig. 1*B*), with five- and fourfold decreases in *hepcidin* levels in 129Sv.Wt and 129.*Hfe*$^{-/-}$ mice fed the iron-deficient diet,

respectively. However, and as reported previously (28), in this mouse background, basal hepcidin levels were already significantly lower in 129Sv.*Hfe*$^{-/-}$ mice fed the standard diet compared with 129Sv.Wt mice (*P*< 0.001) on the same diet.

These results indicate that Hfe-deficient mice retain the ability to lower hepatic hepcidin levels in situations of iron deprivation.

Iron loading

To evaluate hepcidin regulation in response to increased iron stores, mice were placed for 2 wk on a carbonyl iron-supplemented diet (2.5% wt/wt). This treatment resulted in a two- to threefold increase of liver iron stores in all mouse strains tested (772 ± 202 in B6.Wt, 1,571 ± 251 in B6.*Hfe*$^{-/-}$, and 1,317 ± 305 µg iron/g dry weight in B6. *β2m*$^{-/-}$; 1,230 ± 227 in 129Sv.Wt and 3,686 ± 989 µg iron/g dry weight in 129.*Hfe*$^{-/-}$; *P*< 0.001 compared with mice on the standard diet). We found a 2.4-fold elevation of *hepcidin* mRNA levels in B6.Wt mice and a 1.9-fold increment in B6.*Hfe*$^{-/-}$ and B6. *β2m*$^{-/-}$ mice fed the iron-enriched diet compared with corresponding controls kept on the standard diet (Fig. 2*A*). When comparing dietary iron-loaded B6.Wt mice with B6.*Hfe*$^{-/-}$ and B6. *β2m*$^{-/-}$ mice, *hepcidin* levels were significantly higher (*P*< 0.005) in B6.Wt mice.

In the 129Sv background, 129Sv.Wt and 129Sv.*Hfe*$^{-/-}$ mice showed 14- and 15-fold increases in *hepcidin* levels, respectively, compared with controls fed the standard diet. Although the response observed was similar in Wt and Hfe-deficient mice in terms of fold increase, because basal levels were lower in 129Sv.*Hfe*$^{-/-}$, *hepcidin* remained significantly lower than in 129Sv.Wt (*P*< 0.001).

Thus Hfe-deficient mice from the two different backgrounds tested were still able to increase hepcidin in response to iron loading, although the absolute levels reached were lower than in iron-loaded Wt mice.

Acute systemic inflammation

Besides iron stores, hepcidin has been shown to also be modulated by inflammation (38). To evaluate changes in iron metabolism and hepatic hepcidin expression by inflammation, we challenged mice with LPS and measured iron parameters as well as hepcidin mRNA levels in the liver 6 h after treatment. The results for the B6 background are presented in Fig. 3. *Hepcidin* levels rose significantly in all mouse strains (Fig. 3*A*) and were even higher in LPS-treated B6.*Hfe*$^{-/-}$ and B6. *β2m*$^{-/-}$ (3.4- and 3.6-fold increase, respectively) compared with B6.Wt (2-fold increment; *P*< 0.05). Hypoferremia, evidenced by a two- to fourfold lowering of serum iron and transferrin saturation, was noted in all mouse strains tested (Fig. 3, *B* and *C*). In addition, we found a small but significant elevation of liver iron concentrations in all mice challenged by LPS (Fig. 3*D*), possibly as a result of reduced iron export from liver cells.

In the 129Sv background, *hepcidin* levels were also elevated by LPS in 129Sv.Wt (2-fold increase) as well as in 129Sv.*Hfe*$^{-/-}$ (8.7-fold increase; Fig. 4*A*). However, in this mouse background, because basal *hepcidin* levels in *Hfe*$^{-/-}$ mice were considerably lower than in Wt controls, *hepcidin* remained significantly lower than in Wt (*P*< 0.01) after LPS

treatment, as it occurred with the response to iron loading. In addition, hypoferremia also occurred in both LPS-treated mouse strains, with a two- to threefold lowering of serum iron and transferrin saturation (Fig. 4, *B* and *C*), but no changes took place in liver iron concentrations (Fig. 4*D*).

These results show that Hfe-deficient mice, irrespective of the background, can still mount a robust response by elevating hepcidin expression and developing hypoferremia in the systemic inflammation setting induced by LPS treatment.

Fp1 mRNA regulation

Next, to assess whether changes in *Fp1* mRNA expression associated with upregulation of *hepcidin* expression are appropriately elicited in Hfe-deficient mice, we measured *Fp1* mRNA expression in two distinct situations leading to augmented *hepcidin* levels: iron loading and inflammation. We found that, despite the fact that both challenges induced *hepcidin* mRNA levels, *Fp1* mRNA expression in the liver was distinctly regulated. It increased significantly in mice fed the iron-enriched diet but declined in mice treated with LPS (Fig. 5*A*). The response to iron loading in the liver was similar in all mouse strains tested, i.e., Wt and Hfe-deficient strains. Importantly, basal *Fp1* mRNA levels in the liver of *Hfe*$^{-/-}$ and *β2m*$^{-/-}$ mice were significantly higher than those in Wt controls ($P < 0.001$). Furthermore, the difference persisted in mice fed the iron-enriched diet as well as in mice treated with LPS ($P < 0.001$). This shows that the capacity to regulate *Fp1* in the liver in response to disrupted iron homeostasis remains intact in Hfe-deficient mice.

The regulation of *Fp1* mRNA levels was different in the duodenum. First, the distinct regulation observed in the liver of iron-loaded mice compared with LPS-treated mice did not occur, because both treatments resulted in reduced duodenal *Fp1* mRNA levels in B6.Wt controls (Fig. 5*B*). Second, we also found that, as reported by others (31), basal duodenal *Fp1* mRNA levels were significantly reduced in both B6.*Hfe*$^{-/-}$ and B6. *β2m*$^{-/-}$ mice compared with B6.Wt mice ($P < 0.001$), which may explain why no further changes were seen in Hfe-deficient mice after the treatments.

To further investigate whether *Fp1* can be directly regulated by iron and cytokines in hepatocytes, isolated hepatocytes from B6 mice were treated with FAC, LPS, IL-6, and TNF-α and *Fp1* expression was measured. As illustrated in Fig. 5*C*, we found that iron treatment induced *Fp1* mRNA expression in hepatocytes, whereas LPS, TNF-α, and IL-6 treatments suppressed it.

Taken together, these results show that *1*) *Fp1* mRNA is oppositely regulated in the liver by iron and inflammation, and *2*) Hfe-deficient mice retain the ability to regulate hepatic *Fp1* mRNA levels through both the iron-sensing and inflammatory pathways.

Iron-sensing versus inflammatory pathway

Because iron deficiency and inflammation elicit opposite responses in hepcidin regulation, we questioned how *hepcidin* would be modulated by inflammation in iron-deficient mice. B6.Wt mice were placed on the iron-deficient diet and challenged after 2 wk by LPS. Compared with untreated controls, hypoferremia revealed by lower serum iron levels and

transferrin saturation was evident both in mice treated only with LPS and in iron-deficient mice challenged with LPS (Fig. 6, *A* and *B*). Liver iron levels were decreased slightly in iron-deficient mice and augmented in LPS-treated mice. Combined treatment resulted in decreased liver iron concentrations (Fig. 6*C*).

We found that *hepcidin* mRNA levels in the liver rose significantly with combined treatment (Fig. 6*D*). Conversely, *Fp1* mRNA levels decreased in these mice in both the liver and duodenum (Fig. 6, *E* and *F*), as observed in mice treated only with LPS, whereas in iron-deficient mice, *Fp1* mRNA levels did not change in the liver but were augmented in the duodenum.

Taken together, these results indicate that the inflammatory pathway overcomes *hepcidin* and *Fp1* regulation by iron stores in both the liver and duodenum.

TLR-4 pathway

Transduction of the LPS signal across the cell membrane is mediated by TLR-4, which, in turn, activates transcription factor NF-kB, resulting in the production of various proinflammatory cytokines, including IL-6 and TNF-α (18). To gain further insight into the possible upstream pathways involved in *hepcidin* regulation by inflammation, we tested mice deficient in TLR-4 (C3H/HeJ) and Wt mice from the same background (C3H/HeOuJ). As shown in Fig. 7, we found that *hepcidin* activation by iron levels remained intact in TLR-4-deficient mice but was suppressed when the animals were challenged by LPS. Thus the pathway involved in *hepcidin* regulation during inflammation is TLR-4 dependent, whereas *hepcidin* regulation by iron is not dependent on TLR-4.

DISCUSSION

Hfe-independent regulation of hepcidin levels by iron and inflammation

In this study, we showed that Hfe-deficient mice from two different backgrounds retain the ability to regulate hepcidin levels in response to altered iron stores. In fact, Hfe-deficient mice were similar to Wt mice in terms of fold change of *hepcidin* regulation. However, because basal *hepcidin* levels, which are considerably affected by background, are inappropriately low in these mice, *hepcidin* mRNA levels in iron-loaded, Hfe-deficient mice did not reach the values in Wt animals. Similar responses were observed in LPS-treated mice, with the development of hypoferremia in all strains. This suggests that total *hepcidin* expression levels on iron loading and inflammation are additive to baseline levels. The changes in *hepcidin* levels after these treatments are presumably sufficient to significantly affect iron absorption levels and thus reconcile the observation that Hfe-deficient mice can regulate iron absorption in response to alterations of iron metabolism (4, 22, 44) with the proposed role for hepcidin as a negative regulator of iron absorption (16). Possible explanations for discrepancies between studies reporting the absence of *hepcidin* regulation in $Hfe^{-/-}$ mice after iron loading or acute inflammation (2, 43) may include differences in experimental design, i.e., animal background (mixed, C57BL/6 × 129SvTavEv background), different treatments (parenteral vs. dietary iron loading), doses, and treatment schedules. Previous investigations of the response to acute inflammation were performed 1.5 h after

LPS injection (43), earlier than studies reporting *hepcidin* regulation, 6 h in our experiments and in those of Lee et al. (23) and 16 h after Freund's complete adjuvant injection (15). It is possible that Hfe is required for the initial response and is dispensable later during the inflammatory response. In terms of relevance to the anemia of chronic disease (ACD), long-term responses may be more pertinent, because ACD is associated with chronic inflammation and diseases.

Taken together, these observations suggest that Hfe has an essential role in setting basal *hepcidin* levels and, hence, basal iron absorption levels but that further regulation of *hepcidin* by iron stores and inflammation is largely Hfe independent.

The cumulative effect of background in setting *hepcidin* basal levels may be related to differences in MHC-I molecules. Several lines of evidence indicate that MHC-I molecules other than Hfe may influence the degree of iron loading. For example, *Hfeβ2m*$^{-/-}$ compound mutants develop more severe iron loading than single knockout mice (24), and MHC-deficient mice incur iron overload (6).

Regulation of Fp1 mRNA in the liver is largely independent of hepcidin levels and Hfe

We showed that the regulation of *Fp1* mRNA in the liver was in the opposite direction during acute inflammation and in iron overload. Because both treatments lead to increase hepcidin production, this indicates that the regulation of *Fp1* mRNA levels in the liver is independent of hepcidin levels. In contrast, in the duodenum, both treatments lead to the same response, i.e., the suppression of *Fp1* mRNA expression. Possibly, *Fp1* in the liver is regulated locally by iron, most likely via the iron responsive elements/iron regulatory proteins (IRE/IRP)-dependent system (26, 27), in both macrophages (19) and hepatocytes (current study). However, direct regulation of *Fp1* by iron via the IRE/IRP system seems to be suppressed in the duodenum, because *Fp1* mRNA is downregulated in the duodenum of iron-loaded Wt mice. The opposite direction of regulation for *Fp1* in the gut is also found in sex-linked anemia mice, where *Fp1* mRNA and Fp1 protein levels remain elevated, even though enterocyte iron and ferritin levels are significantly higher than in iron-deficient Wt mice (7). Presumably, in the gut, *Fp1* mRNA and Fp1 protein levels (1) are regulated by systemic factors, such as hepcidin. Ultimately, depending on which pathway is activated, the iron-sensing or the inflammatory pathways, there seem to be multiple mechanisms for *Fp1* regulation in the liver and the gut.

We found that basal hepatic *Fp1* mRNA levels were higher in Hfe-deficient mice than in Wt mice. Similarly, hepatic *Fp1* mRNA expression is increased in mice that develop iron overloading due to a deficiency in hemojuvelin (*Hjv*), an upstream regulator of hepcidin expression in the iron-sensing pathway (39), as well as in hepcidin-deficient mice (*Usf2*$^{-/-}$) (47). Solely on the basis of the response of *Fp1* to iron loading in the gut, this could be interpreted as an inappropriate response to downregulate *Fp1* in the liver due to the lack of basal *hepcidin* expression. However, the fact that the normal response to iron loading in the liver is an increase of *Fp1* mRNA and Fp1 protein (1) expression suggests that Hfe-deficient, *Hjv*$^{-/-}$ and *Usf2*$^{-/-}$ mice may be appropriately responding to higher iron levels in the liver and thus further indicating that local regulation of *Fp1* mRNA by iron in the liver is independent of hepcidin levels.

Inflammatory pathway overrides the iron-sensing pathway for hepcidin and Fp1 regulation

We observed that iron-deprived mice, despite significantly reduced iron stores, still mount a robust response to acute inflammation by increasing hepatic *hepcidin* mRNA and reducing *Fp1* levels in both the liver and duodenum. This suggests that the iron-sensing pathway for hepcidin regulation is overruled during inflammation, a finding that may have significant implications in ACD. In fact, sustained hepcidin expression and *Fp1* reduction in the duodenum may result in decreased iron absorption and thus in the development of true iron deficiency in patients with chronic inflammation and cancer. Iron deficiency in these situations would be insufficient to trigger an appropriate response to restore body iron stores and would thus aggravate and perpetuate the anemic state. This is particularly pertinent in cancer settings, where anemia is associated with reductions in the quality of life and survival in cancer patients (46). In fact, anemia causes tissue hypoxia, which has been shown to promote tumor growth and to enhance the resistance of tumor cells to radiotherapy and chemotherapy (46).

Inflammatory pathway for hepcidin regulation is TLR-4 dependent

TLRs are an essential component of signaling receptors expressed on the cell surface that recognize pathogen-associated molecular patterns (PAMP) produced by micro-organisms. LPS is a PAMP that is recognized by TLR-4 and the CD14 coreceptor, leading to subsequent activation of NF-κB and resulting in the production of various proinflammatory cytokines, including IL-6 and TNF-α (33). We found that hepcidin regulation in TLR-4-deficient mice was completely abrogated after LPS treatment but not by iron loading. These results suggest that the inflammatory pathway, but not the iron-sensing pathway of hepcidin regulation, is TLR-4 dependent.

In conclusion, we showed that Hfe is necessary for the establishment of basal hepcidin levels but dispensable for hepcidin regulation through both the iron-sensing and inflammatory pathways. In addition, *Fp1* regulation in the liver is independent of hepcidin levels and of Hfe. Finally, the inflammatory pathway overrides the iron-sensing pathway and is TLR-4 dependent.

Acknowledgments

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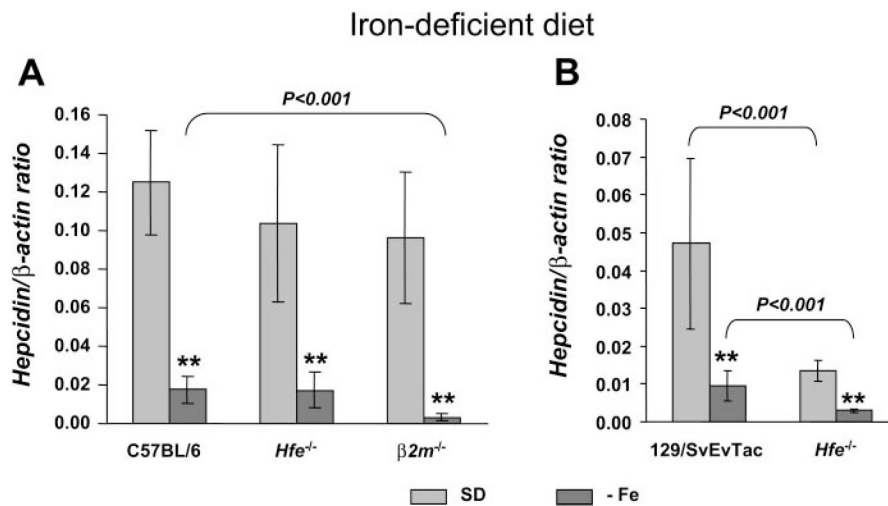
Fig. 1.
Hepcidin is downregulated in the liver of Hfe-deficient mice in response to iron-deprivation. *Hepcidin* gene expression in the liver of mice fed a standard diet (SD; light gray) or an iron-deficient diet (−Fe; dark gray) from the C57BL/6 (*A*) and 129/SvEvTac background (*B*). *Hepcidin* mRNA expression was quantified by quantitative RT-PCR (qRT-PCR) and normalized to *β-actin*. The *hepcidin*/*β-actin* ratio is shown. The results are presented as means ± SD (n = 3–6 mice/group). Statistical analysis was performed by 1-way ANOVA; **P < 0.001 for comparison with appropriate control mice (SD diet).
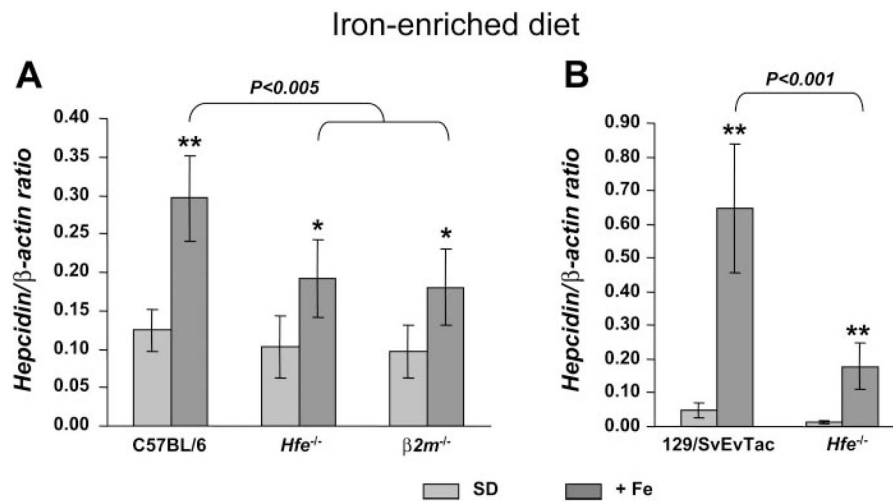
Fig. 2.
Hepcidin is up-regulated in the liver of Hfe-deficient mice in response to iron-loading.
Hepcidin gene expression in the liver of mice fed a standard diet (SD; light gray) or an iron-enriched diet (+Fe; dark gray) from the C57BL/6 (*A*) and 129/SvEvTac background (*B*).
Hepcidin mRNA expression was quantified by qRT-PCR and normalized to *β-actin*. The
hepcidin/β-actin ratio is shown. The results are presented as means ± SD (*n* = 5–7 mice/
group). Statistical analysis was performed by 1-way ANOVA; *P< 0.01, and **P< 0.001
for comparison with relevant saline-treated mice (SD diet).
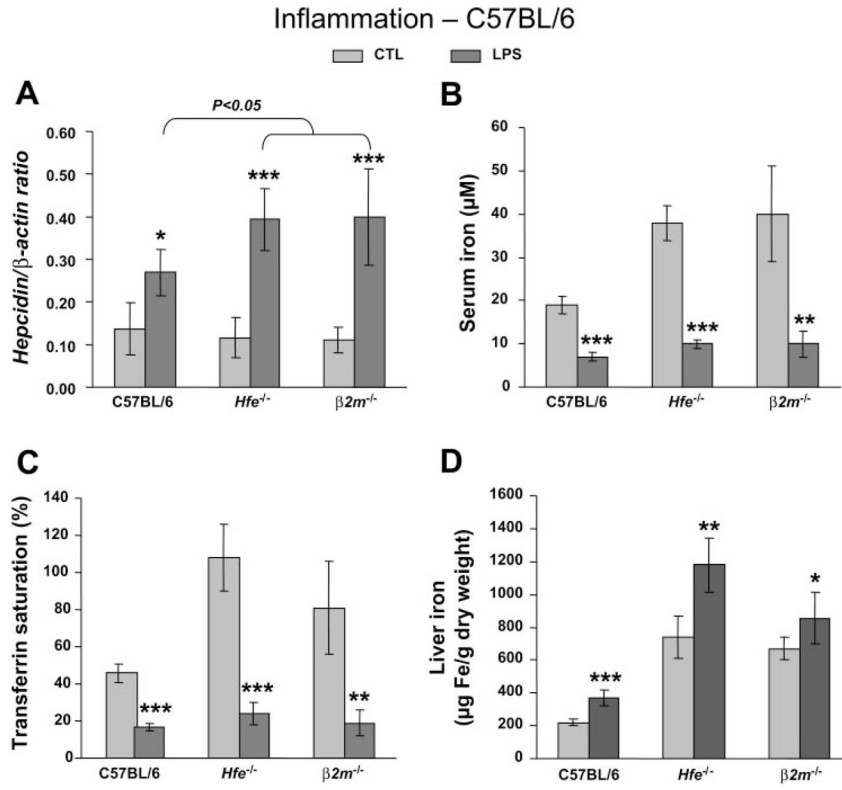
Fig. 3.
Hypoferremia and hepcidin induction by inflammation in Hfe-deficient mice from the C57BL/6 background. *Hepcidin* gene expression (*A*), serum iron (*B*), transferrin saturation (*C*), and iron concentration in the liver (*D*) in saline-treated control mice (CTL; light gray) and LPS-treated mice (dark gray). *Hepcidin* mRNA expression was quantified by qRT-PCR and normalized to *β-actin*. The *hepcidin/β-actin* ratio is shown. The results are presented as means ± SD (*n* = 6 mice/group). Statistical analysis was performed by 1-way ANOVA; *$P <$ 0.05, **$P <$ 0.01, and ***$P <$ 0.001 for comparison with relevant control mice.
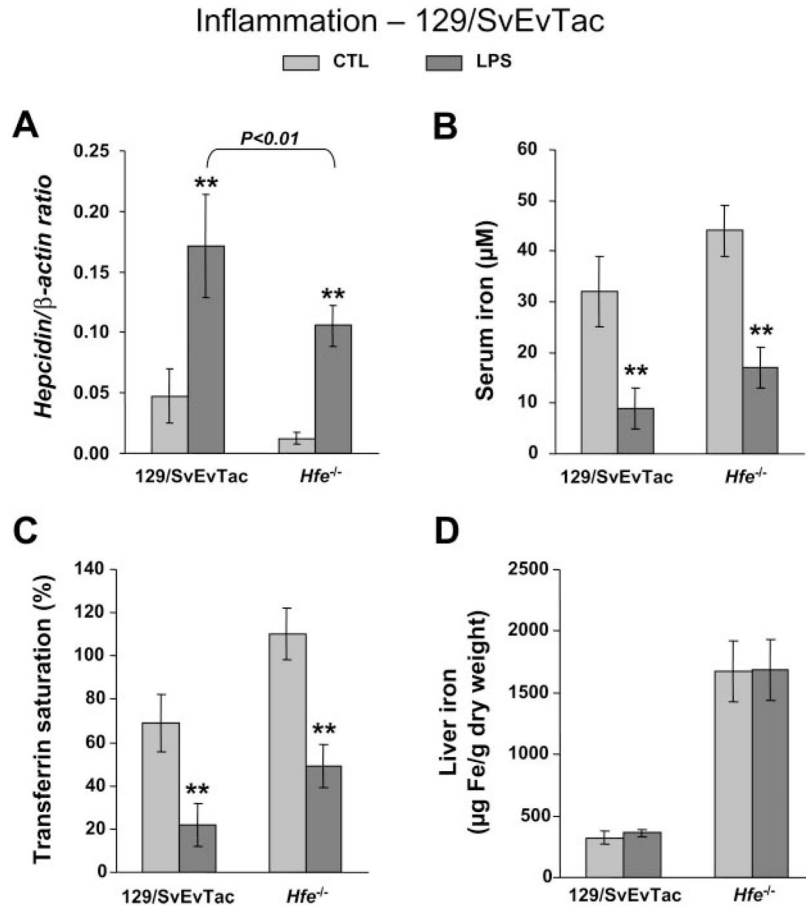
Fig. 4.
Hypoferremia and hepcidin induction by inflammation in Hfe-deficient mice from the 129/SvEvTac background. *Hepcidin* gene expression (*A*), serum iron (*B*), transferrin saturation (*C*), and iron concentration in the liver (*D*) in saline-treated control mice (CTL; light gray) and LPS-treated mice (dark gray). *Hepcidin* mRNA expression was quantified by qRT-PCR and normalized to β-actin. The *hepcidin/β-actin* ratio is shown. The results are presented as means ± SD (n = 6 mice/group). Statistical analysis was performed by 1-way ANOVA; **P < 0.001 for comparison with respective control mice.
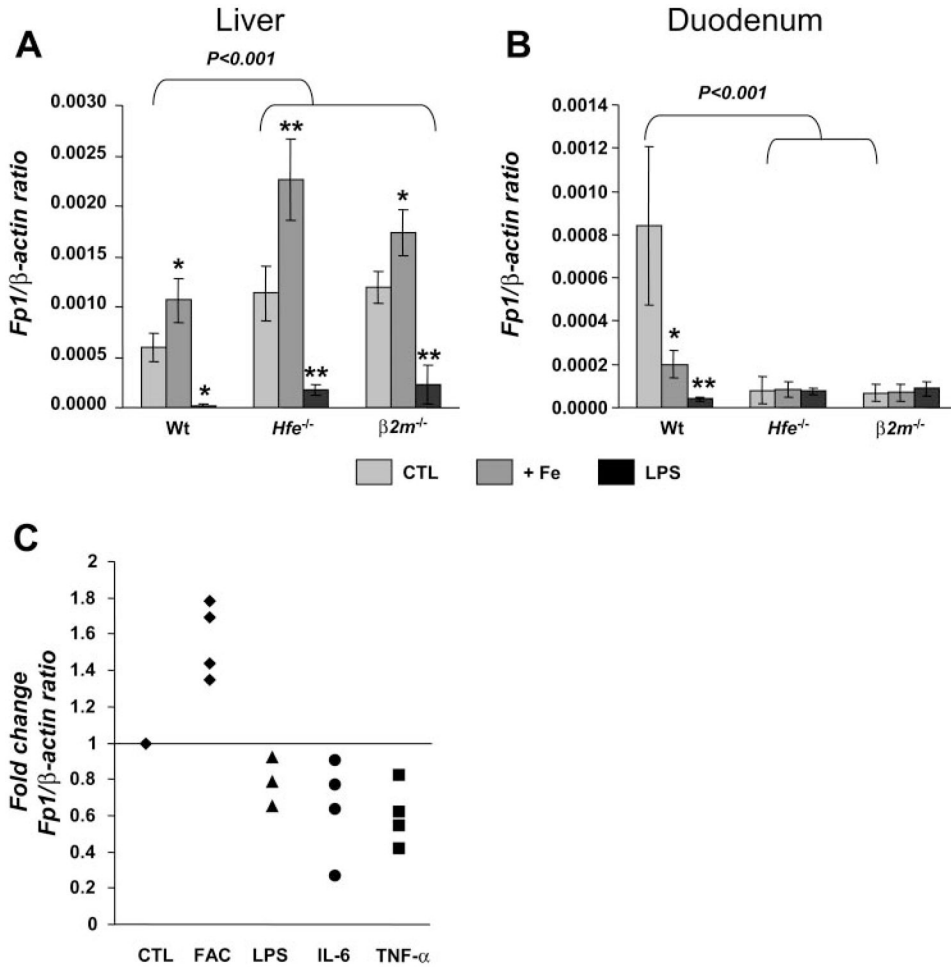
Fig. 5.

Hepatic Fp1 regulation by iron is independent of hepcidin levels. *Fp1* mRNA levels in the liver (*A*) and duodenum (*B*) in control (CTL; light gray), iron-loaded (+Fe; dark gray), and LPS-treated mice (black). The results are presented as means ± SD ($n = 6$ mice/group). Statistical analysis was performed by 1-way ANOVA; $*P < 0.01$, and $**P < 0.001$ for comparison with relevant control mice. *Fp1* mRNA expression was quantified by qRT-PCR and normalized to *β-actin*. The *Fp1/β-actin* ratio is shown. *C*: effect of iron, LPS, IL-6, and TNF-α on *Fp1* expression by primary murine hepatocytes. Each symbol represents 1 experiment in which 3 samples of primary murine hepatocytes were incubated with culture medium, FAC, LPS, or the cytokines indicated. The results are expressed as fold changes of *Fp1* mRNA normalized to *β-actin* of treatments compared with CTL.
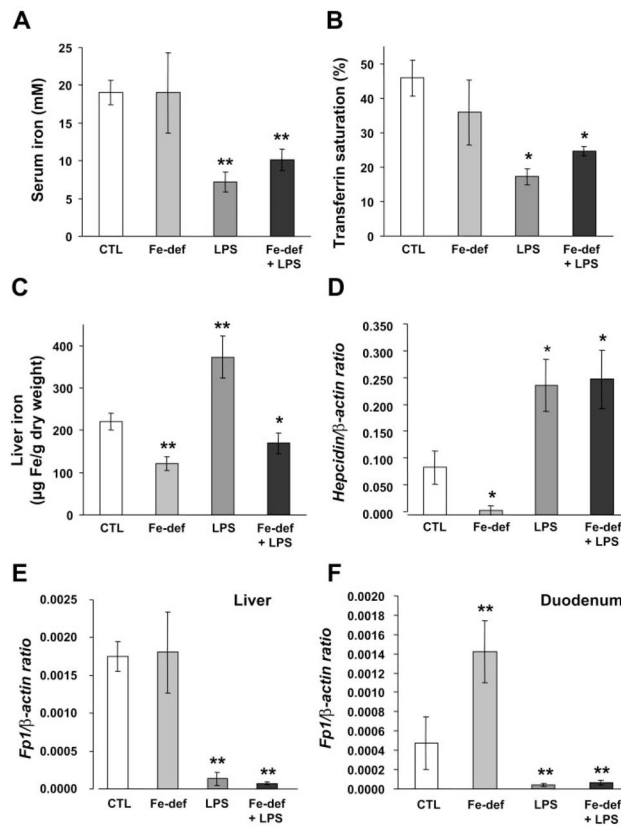
Fig. 6.
The inflammatory pathway overrides the iron-sensing pathway. Serum iron (*A*), transferrin saturation (*B*), and iron concentration in the liver (*C*) in control saline-treated mice (CTL), iron-deficient (Fe-def), LPS-treated, and mice with combined treatments (Fe-def + LPS). Hepatic *hepcidin* (*D*) and *Fp1* gene expression in the liver (*E*) and duodenum (*F*). mRNA expression was quantified by qRT-PCR and normalized to β-*actin*. The *hepcidin/β-actin* and *Fp1/β-actin* ratios are shown. The results are presented as means ± SD (*n* = 6 mice/group). Statistical analysis was performed by 1-way ANOVA; *P < 0.01, and **P < 0.001 for comparison with control mice.

Tlr4-deficient mice
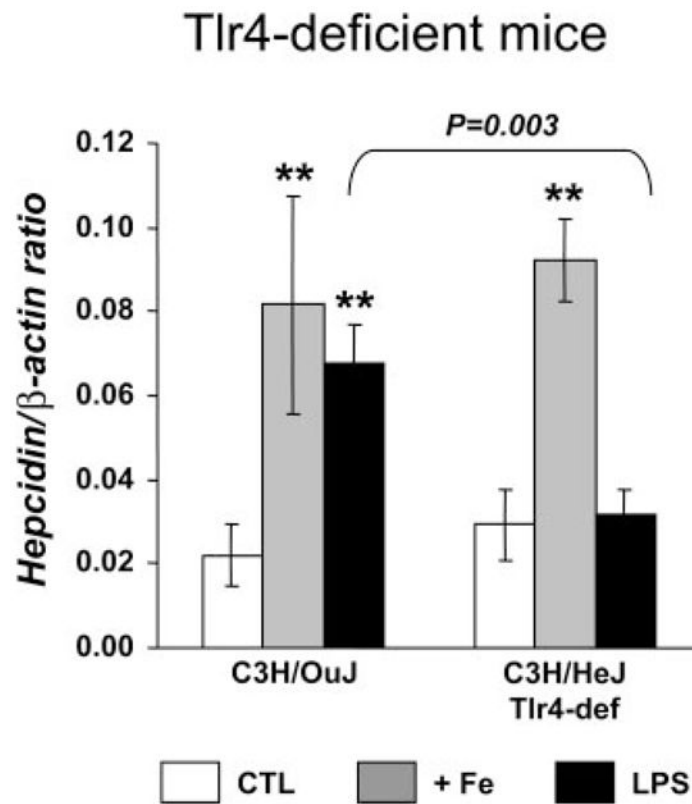
Fig. 7.
The inflammatory pathway for hepcidin regulation is Toll-like receptor 4 (TLR-4) dependent. Hepatic *hepcidin* mRNA expression was quantified by qRT-PCR and normalized to *β-actin*. The *hepcidin/β-actin* ratio is shown. The results are presented as means ± SD ($n = $ 4–6 mice/group). Statistical analysis was performed by 1-way ANOVA; $**P < 0.001$ for comparison with relevant control mice.