Parenteral vs. oral iron: influence on hepcidin signaling pathways through analysis of Hfe/Tfr2-null mice

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Iron supplementation is a common treatment for iron deficiency anemia, with the route being either dietary or parenteral, depending on the cause of anemia (3). Following absorption, dietary iron binds circulating transferrin, with excess iron sequestered in hepatocytes. In contrast, injected iron-poly saccharide complexes, such as iron-dextran, are taken up by macrophages where iron is either stored as ferritin or released into the circulation.

Hepcidin, the central iron-regulatory hormone, encoded by the HAMP gene in humans, controls iron levels through an interaction with the mammalian cellular iron exporter ferroportin. This interaction leads to ferroportin internalization and degradation, reducing cellular iron efflux, dietary iron absorption, and the recycling of iron through the reticuloendothelial system (18). Systemically effective levels of hepcidin are produced within the liver, where the bone morphogenetic protein (BMP)/Mothers against decapentaplegic (SMAD) interaction that modulates signaling of the BMP/SMAD pathway has emerged as the likely canonical mechanism regulating its expression. From this large family of growth factors, BMP6 has been identified as the major ligand responsible for upregulating hepcidin in vivo; mice lacking Bmp6 develop severe iron overload due to reduced Smad pathway activation and subsequent hepcidin deficiency (1, 17). BMP6 expression is upregulated by an increase in iron status, with the signal transduced through the phosphorylation of Smad1/5/8 (pSMAD1/5/8), which in complex with SMAD4, directly increases transcription of HAMP (14).

Although the role of the BMP6/SMAD pathway in regulating HAMP is relatively well defined, the mechanisms underlying the regulation of BMP6 in response to iron, as well as the specificity of BMP6-induced pSMAD1/5/8 signaling for hepcidin, are less so. The hereditary hemochromatosis (HH)-associated molecules hemochromatosis protein (HFE) and transferrin receptor 2 (TFR2) are required for this regulatory pathway; however, their precise role remains a key question within the field. One theory proposes that body iron status is sensed by the formation of a complex between HFE and TFR2, which then modulates signaling of the BMP/SMAD pathway. The proposed HFE/TFR2 “iron-sensing complex” is able to form when holo-transferrin competitively binds to transferrin receptor (TFRC), displacing it from a default complex with HFE. This then frees HFE to form a new complex with TFR2. This concept has been proposed primarily on the basis of in vitro studies in cell lines overexpressing HFE and TFR2 proteins (10, 11). More recent studies, however, suggest that the formation of a complex between HFE and TFR2 is not necessary for hepcidin regulation. We have previously shown that the deletion of both Hfe and Tfr2 in mice results in a more severe iron overload phenotype and reduced hepcidin expression compared with deletion of Hfe or Tfr2 alone, suggesting that there is residual functionality of Hfe and Tfr2 in the absence of the other (26). This has also been supported by the observation that HH patients with mutations in both HFE and TFR2 have a more severe iron overload phenotype than HFE- or TFR2-related HH cases alone (19). Schmidt and Fleming (22) recently showed that transgenic overexpression of Hfe in mouse liver could regulate hepcidin in the absence of functional Tfr2. The actual links between the Hfe and Tfr2 proteins, iron sensing, and Bmp6 regulation still remain to be fully elucidated.

Hepatocytes, the parenchymal cells of the liver, are thought to be the major hepcidin-expressing cells, and as a result, Hamp regulation in response to iron is generally considered to occur within these cells (28). The liver is also populated by nonparenchymal cells, including sinusoidal endothelial cells, hepatic stellate cells, and Kupffer cells that are also capable of significant iron storage. Recently, Bmp6 mRNA expression and
Bmp6 protein have been localized to a variety of these cell types within the liver, with localization appearing to vary with circumstance (2, 8, 12, 13, 21, 27). Bmp6 mRNA was detected in isolated nonparenchymal cells in mice under iron-deficient conditions caused by a low-iron diet and under iron overload, resulting from Hemojuvelin (Hjv) deletion (8, 27). In subjects with iron overload due to HFE mutations and in mice on a high-iron diet, Bmp6 protein was detected by immunohistochemistry predominantly within hepatocytes (12, 13, 21). This difference in the cellular location of Bmp6 suggests that iron localization may lead to a difference in the cell type in which Bmp6 is expressed. Studies in which acute iron loading occurs in Kupffer cells, following parenteral administration of Fe-dextran, have also demonstrated a Bmp6 and pSmad1/5/8 response (5, 7, 20).

Here, we have assessed wild-type and Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> mice at either 4 days after injection with Fe-dextran or after 2 wk fed on a high-iron diet. We have compared how the liver handles these different forms of iron and the regulatory mechanism established in response to these different iron challenges. We also investigated this regulatory pathway in single-null Hfe<sup>−/−</sup> and Tfr2<sup>−/−</sup> mice. This study demonstrates a role for the Hfe and Tfr2 proteins in BMP-mediated signaling beyond any function that they may play in an iron-sensing complex and suggests a separation of the pathway to hepcidin induction that is dependent on the cell type in which iron is deposited.

METHODS

Animals. Animal studies were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee. Mice had free access to water and food under standard conditions and received humane care. The Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup>, and Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> mice on a C57BL/6 background have been previously described (25). Male mice, weaned at 21 days, were maintained either on standard laboratory chow (iron content 120.1 mg/kg; n = 4), control iron diet (75 mg/kg iron; n = 5 or 6), or a high-iron diet (20 g/kg; 2% carboxyl iron; Specialty Feeds, Glenn Forest, Australia; n = 5) ad libitum. For parental studies, mice fed on standard laboratory chow were injected intraperitoneally with either saline or Fe-dextran (0.3 mg/g Fe-dextran; Sigma Aldrich, Castle Hill, Australia) 4 days prior to death at 5 wk. Perls’ staining of liver sections from WT mice in both control diet and saline-injected groups showed no identifiable hepatic iron staining, while Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> animals showed iron accumulation exclusively in hepatocytes (Fig. 1A). All mice on the high-iron diet showed significant iron deposition exclusively in hepatocytes, producing a classical perilportal pattern, which was substantially more pronounced in the Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> mice. Conversely, in livers of Fe-dextran-treated animals, iron was retained primarily in the nonparenchymal cells (Fig. 1A). The hepatocyte iron seen in Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> control mice was also visible in Fe-dextran-treated mice at comparable levels, which was minimal compared with the nonparenchymal iron.

To gain a quantitative measure of tissue and serum iron status, HIC and serum TS were measured (Fig. 1, B–E). Both iron diet and intraperitoneal Fe-dextran resulted in significant increases in the HIC of WT and Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> mice (P < 0.05 for all comparisons; Fig. 1, B and C). The high-iron diet produced a higher HIC in Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> mice compared with wild-type mice (P < 0.0001, Fig. 1B), whereas Fe-dextran injection led to a higher HIC in wild-type mice compared with Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> mice (P < 0.05, Fig. 1C). Wild-type mice injected with Fe-dextran had a modest increase in TS compared with saline-injected control mice, but this did not reach statistical significance (Fig. 1E). Wild-type mice fed a high-iron diet, however, had a highly significant increase in TS compared with control diet-fed mice (P < 0.0001; Fig. 1D), where the TS reached close to 100%. In Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> mice without iron supplementation, TS was already close to 100%, and thus, no difference was observed following either iron treatment.

Cellular iron localization significantly affects the dependence of Bmp6 regulation and pSmad1/5/8 signaling on Hfe and Tfr2 proteins. To investigate the contribution of Hfe and Tfr2 to iron homeostasis and the influence of cellular iron localization, the expression of iron regulatory genes was examined by quantitative RT-PCR. In Hfe<sup>−/−</sup>/Tfr2<sup>−/−</sup> mice, a high-iron diet induced a statistically significant upregulation of Hamp (P < 0.05; Fig. 2A), although this response was significantly less than that seen in the WT mice (P < 0.0001). Injection with Fe-dextran also induced a significant upregulation of Hamp in WT mice (P < 0.05; Fig. 2B); however, in...
Hfe\textsuperscript{+/−}/Tfr2\textsuperscript{−/−} mice, there was effectively no Hamp response, with no statistical difference to saline-injected mice.

Next, we considered the regulation of Bmp6, a key upstream regulator of hepcidin. In WT mice, the high-iron diet induced a statistically significant increase in Bmp6 mRNA expression ($P < 0.01$; Fig. 2C); however, in Hfe\textsuperscript{−/−}/Tfr2\textsuperscript{−/−} mice, there was only a modest increase in Bmp6 that did not reach statistical significance (Fig. 2C). In contrast to the deficient
upregulation of Hamp following Fe-dextran treatment in Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice, Bmp6 showed a significant upregulation (P < 0.05; Fig. 2D) comparable to that of WT mice.

The contrasting response of Hamp and Bmp6 between treatments in Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice then led us to investigate the signaling pathway linking the two. Given that the accepted mechanism of Bmp6 signaling is through phosphorylation of Smad1/5/8, we next examined the levels of pSmad1/5/8 in the livers of mice by Western blot analysis (Fig. 3A). As can be seen in Fig. 3A, the high-iron diet led to an increase in the levels of phospho-Smad1/5/8 that is significantly higher compared with both Gapdh and total-Smad1 levels (P < 0.05; Fig. 3, B and C). In the Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice, however, the high-iron diet did not lead to an increase in phospho-Smad1/5/8, and the levels were significantly lower than the WT mice on the high-iron diet (P < 0.01; Fig. 3, A–C). The lack of increase in Bmp6 expression in Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice on the high-iron diet (Fig. 2C) may explain why there is no increase in phospho-Smad1/5/8 and point to a role for Hfe and/or Tfr2 in the regulation of Bmp6 in response to hepatocyte iron. Levels of phospho-Smad1/5/8 were more variable in the mice injected with saline or iron-dextran, and there were no statistically significant differences between the groups (Fig. 3, D and E).

The increase in Bmp6 following iron-dextran treatment in the Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice (Fig. 2D) does not appear to have led to an increase in phospho-Smad1/5/8, suggesting a breakdown in transmission of the Bmp6 signal through the Smad pathway to hepcidin in the absence of Hfe and Tfr2.

Tfr2, but not Hfe, is required for Bmp6 regulation in parenchymal cells. Following the finding that Bmp6 regulation is differentially affected in Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice depending on iron localization, and combined with previous published results (23, 25, 26) showing differences in the phenotypes of Hfe\(^{-/-}\), Tfr2\(^{-/-}\) and Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice, we compared the effects of single-gene knockout. The expression of Bmp6 mRNA was significantly elevated in the saline-injected Hfe\(^{-/-}\) mice, but not in Tfr2\(^{-/-}\) and Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice (Fig. 4C). When analyzed relative to the HIC, both Tfr2\(^{-/-}\) and Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice had a significant reduction in Bmp6 levels in a similar way to WT and Hfe\(^{-/-}\) mice. Hamp mRNA expression was also measured in these mice (Fig. 4A). Basal Hamp levels were variable between the four genotypes. However, when injected with iron-dextran, both the Tfr2\(^{-/-}\) and Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice had significantly lower Hamp expression than WT mice, suggesting that there is a deficiency in the regulation of Hamp in response to nonparenchymal iron in these mice. This deficiency in Hamp regulation is also reflected in the Hamp to HIC ratio (Fig. 4D). We have previously shown (25) a consistently decreasing gradient in the Hamp to HIC ratio in these mice in which WT > Hfe\(^{-/-}\) > Tfr2\(^{-/-}\) > Hfe\(^{-/-}\)/Tfr2\(^{-/-}\), and this same pattern was seen again here (Fig. 4B).

**DISCUSSION**

In our study, we examined the influence of hepatic cellular iron localization on the iron regulatory pathways in wild-type, Hfe\(^{-/-}\), Tfr2\(^{-/-}\), and Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice. We show that Tfr2, but not Hfe, is required for normal Bmp6 regulation in response to parenchymal iron. We also show that neither Hfe nor Tfr2 are required for Bmp6 regulation in response to nonparenchymal iron, but that Tfr2 is required for hepcidin regulation in response to parenchymal iron. Our studies show that both Hfe and Tfr2 have separate roles in signal transduction to increase hepcidin expression and that these roles go beyond their perceived positions as iron sensors. It also confirms the presence of different signaling pathways between parenchymal and nonparenchymal cells of the liver.
Fig. 3. Phosphorylation of Smad1/5/8 is increased in WT but not Hfe<sup>−/−</sup> /Tfr2<sup>−/−</sup> mice fed a high-iron diet. A: levels of phospho-Smad1/5/8 were determined by Western blot analysis. Densitometry was used to determine the amount of phospho-Smad1/5/8 relative to Gapdh (B and D) or to Smad1 (C and E). Quantitation of phospho-Smad1/5/8 levels are shown for the mice fed a control or high-iron diet (B and C) or mice injected with saline or Fe-dextran (D and E). Gray bars represent wild-type mice, while solid bars represent Hfe<sup>−/−</sup> /Tfr2<sup>−/−</sup> mice. Results are expressed as means ± SE. Statistical significance was assessed by two-way ANOVA with Tukey’s multiple-comparison post hoc tests. aP < 0.05, Hfe<sup>−/−</sup> /Tfr2<sup>−/−</sup> compared with WT on the same treatment. bP < 0.05, iron treatment compared with control of the same genotype.
Both routes of iron loading utilized in this study achieved similar levels of HIC and TS in wild-type and $Hfe^{-/-}/Tfr2^{-/-}$ mice. In spite of this similarity, a distinct separation in the pattern of iron accumulation was observed, with dietary iron accumulation occurring within parenchymal cells and Fe-dextran accumulation occurring predominantly in nonparenchymal cells. It is recognized that the $Hfe^{-/-}/Tfr2^{-/-}$ mice treated with Fe-dextran did show some hepatocyte iron loading, which likely accumulated because of the hepatocyte iron-loading phenotype of these mice; however, the amount was negligible, compared with the amount deposited in nonparenchymal cells, and it was consistent between treated and nontreated groups. This difference in the iron localization then served as the model to investigate the influence of the iron-accumulating cell type on the regulatory response and subsequent signaling cascade. Recently, Daba et al. (7) reported a study in wild-type mice utilizing a comparable iron-loading approach. These authors reported a similar pattern of iron localization to our study and also reported differences in the regulatory response similar to those observed in our wild-type animals, providing support for the separation of the signaling pathways seen in our study. However, the extended analysis of these pathways across our animal models of hemochromatosis revealed a significant difference in the dependence of regulatory responses on Hfe and Tfr2. Further, Daba et al. (7) concluded that hepcidin is only regulated in response to hepatocyte iron and not macrophage iron; however, we demonstrate that regulation of hepcidin does occur in response to macrophage iron and that Tfr2 is required for this to occur. In light of these results, we suggest that the cellular signaling that leads to hepcidin regulation should be considered in the context of cell type.

Following dietary iron loading, we observed a blunted, but still statistically significant, hepcidin response in $Hfe^{-/-}/Tfr2^{-/-}$ mice, as has been described previously (6). This observation has led to the suggestion that this regulation of hepcidin may be occurring through an alternative pathway (9). To investigate this, we compared other components in the hepcidin regulatory pathway. The response of $Bmp6$ to dietary iron was also blunted in the $Hfe^{-/-}/Tfr2^{-/-}$ mice, with no statistical difference compared with controls. In addition, the
increase in phospho-Smad1/5/8 levels in the high-iron diet fed wild-type mice was not observed in the Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice. These combined data suggest that in the absence of Hfe and Tfr2, there is a reduction in the Bmp6-Smad1/5/8 pathway regulating hepcidin. This observation suggests that the induction of hepcidin seen in Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice is not via an alternative pathway of hepcidin regulation, but an incomplete loss of the canonical pathway. It also provides further evidence that Hfe and/or Tfr2 are required for hepcidin regulation both upstream and downstream of Bmp6.

Analysis of the single-null Hfe\(^{-/-}\) and Tfr2\(^{-/-}\) mice alongside the wild-type and Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice clearly demonstrated that in these models, the loss of Hfe does not limit Bmp6 regulation relative to parenchymal iron. Corradini et al. (4) and Kautz et al. (13) have both also previously analyzed these sequential steps of the signaling pathway in Hfe\(^{-/-}\) mice and also observed a normal level of Bmp6 relative to iron. While Corradini et al. (4) identified a small reduction in the phosphorylation of Smad1/5/8 relative to Bmp6 (\(P = 0.046\)) (4), Kautz et al. (13) reported no difference. We demonstrate, for the first time, that the loss of Tfr2 has a significant effect on the regulation of Bmp6. Together, these results raise further questions about the formation of a complex of Hfe with Tfr2. Rather, they demonstrate that Tfr2 acts independently of Hfe in either the sensing of iron status or the transduction of the iron status signal to Bmp6.

In the Fe-dextran model of iron loading, leading to non-parenchymal iron accumulation, the effect of Hfe and Tfr2 ablation on the hepcidin regulatory pathway was significantly different to that observed with dietary iron loading. The up-regulation of Bmp6 relative to HIC in both the Tfr2\(^{-/-}\) and Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice was equal to that achieved in the wild-type and Hfe\(^{-/-}\) mice, suggesting for the first time that iron sensing and Bmp6 induction occurs independently of Hfe and Tfr2 in nonparenchymal cells of the liver. In spite of the seemingly normal response of Bmp6 to nonparenchymal iron, there was only limited upregulation of Hamp in the Tfr2\(^{-/-}\) mice, and an even lower response in Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice. This suggests that the induction of hepcidin, which is believed to occur within hepatocytes, in response to nonparenchymal iron accumulation is dependent on Tfr2. Interestingly, normal levels of Hamp were induced in Hfe\(^{-/-}\) mice injected with Fe-dextran. Thus, we suggest two possible explanations: 1) Bmp6 is induced in nonparenchymal cells in a Hfe- and Tfr2-independent manner. This Bmp6 can then induce Hamp expression in nonparenchymal cells in a Hfe-independent manner, which is significantly less efficient than in hepatocytes or 2) the transduction of iron response signals from nonparenchymal to parenchymal cells utilizes, but is not critically dependent on Hfe.

In conclusion, this study demonstrates a requirement for both Hfe and Tfr2 proteins in iron homeostasis beyond any role they may play as part of an iron-sensing complex. When iron is localized in hepatocytes Tfr2 is required for normal Bmp6 induction, whereas Hfe appears to impart its regulatory influence on hepcidin transcription downstream of Bmp6. We also show that the hepcidin induction seen in Hfe\(^{-/-}\)/Tfr2\(^{-/-}\) mice on a high-iron diet can be accounted for by the incomplete loss of the canonical signaling pathway. When iron is localized in nonparenchymal cells, neither Hfe nor Tfr2 proteins are required for Bmp6 induction, but Tfr2 is required for hepcidin induction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


