Disruption of Hemochromatosis Protein and Transferrin Receptor 2 Causes Iron-Induced Liver Injury in Mice

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Mutations in hemochromatosis protein (HFE) or transferrin receptor 2 (TFR2) cause hereditary hemochromatosis (HH) by impeding production of the liver iron-regulatory hormone, hepcidin (HAMP). This study examined the effects of disruption of Hfe or Tfr2, either alone or together, on liver iron loading and injury in mouse models of HH. Iron status was determined in Hfe knockout (Hfe−/−), Tfr2 Y245X mutant (Tfr2mut), and double-mutant (Hfe−/− × Tfr2mut) mice by measuring plasma and liver iron levels. Plasma alanine transaminase (ALT) activity, liver histology, and collagen deposition were evaluated to assess liver injury. Hepatic oxidative stress was assessed by measuring superoxide dismutase (SOD) activity and F2-isoprostane levels. Gene expression was measured by real-time polymerase chain reaction. Hfe−/− × Tfr2mut mice had elevated hepatic iron with a periportal distribution and increased plasma iron, transferrin saturation, and non-transferrin-bound iron, compared with Hfe−/−, Tfr2mut, and wild-type (WT) mice. Hamp1 expression was reduced to 40% (Hfe−/− and Tfr2mut) and 1% (Hfe−/− × Tfr2mut) of WT values. Hfe−/− × Tfr2mut mice had elevated plasma ALT activity and mild hepatic inflammation with scattered aggregates of infiltrating inflammatory cluster of differentiation 45 (CD45)–positive cells. Increased hepatic hydroxyproline levels as well as Sirius red and Masson’s Trichrome staining demonstrated advanced portal collagen deposition. Hfe−/− and Tfr2mut mice had less hepatic inflammation and collagen deposition. Liver F2-isoprostane levels were elevated, and copper/zinc and manganese SOD activities decreased in Hfe−/− × Tfr2mut, Tfr2mut, and Hfe−/− mice, compared with WT mice. Conclusion: Disruption of both Hfe and Tfr2 caused more severe hepatic iron overload with more advanced lipid peroxidation, inflammation, and portal fibrosis than was observed with the disruption of either gene alone. The Hfe−/− × Tfr2mut mouse model of iron-induced liver injury reflects the liver injury phenotype observed in human HH. (HEPATOLOGY 2012;56:585-593)

Primary and secondary iron overload disorders are important causes of liver disease and associated morbidity worldwide.1 The most common primary iron overload disorder is hereditary hemochromatosis (HH), which affects approximately 1 in 200 individuals of Northern European descent.2 There are four types of HH; the most common, HH type 1, is caused primarily by homozygosity for the C282Y

[Abbreviations: ALT, alanine transaminase; BMP6, bone morphogenic protein 6; CD45, cluster of differentiation 45; DAPI, 4',6-diamidino-2-phenylindole; HAMP, hepcidin; HFE, hemochromatosis protein; HH, hereditary hemochromatosis; HJV, hemojuvelin; HSCs, hepatic stellate cells; Idl, inhibitor of differentiation 1; LPO, lipid peroxidation; mRNA, messenger RNA; NTBI, non-transferrin-bound iron; ROS, reactive oxygen species; SEM, standard error of the mean; SMAD, mothers against decapentaplegic; SOD, superoxide dismutase; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2; WT, wild type.]

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mutation in the hemochromatosis protein (HFE). Iron overload disease develops in up to 30% of these individuals and can result in significant hepatic, pancreatic, cardiac, or musculoskeletal tissue damage. Juvenile, or HH type 2, is rare and is caused by mutations in hemjuvelin (HJV) or hepcidin (HAMP). HH type 3 is also rare and is caused by mutations in transferrin receptor 2 (TFR2), whereas HH type 4 is caused by mutations in ferroportin. Almost all cases of HH result in impaired HAMP synthesis. Decreased HAMP levels in HH cause increased iron absorption from the duodenum, with the excess iron being deposited mainly in the liver.

Studies have shown that when hepatic iron concentration exceeds 60 μmol/g, hepatic stellate cells (HSCs) begin to exhibit early signs of activation, an integral event in the initiation of hepatic fibrosis. As hepatic iron levels increase further, the risk of significant liver fibrosis and, ultimately, cirrhosis increases. Although the exact mechanisms of liver injury induced by iron overload have not yet been fully elucidated, it is thought that the accumulation of excess iron-catalyzed reactive oxygen species (ROS) plays a significant role. Previous studies have demonstrated decreased hepatic levels of antioxidants, such as superoxide dismutase (SOD), ascorbate, β-carotene, and vitamins E and A in iron overload conditions. Furthermore, iron increases the level of lipid peroxidation (LPO) products, such as malondialdehyde and F2-isoprostanes, which can cause mutagenesis in DNA. LPO-induced DNA lesions are increased 2- to 3-fold in the livers of HH patients and, together with the iron overload observed in HH, are associated with an approximately 20-fold increased risk of hepatocellular carcinoma. Oxidative stress has been shown to activate apoptosis and necrosis, promoting the synthesis and release of proinflammatory and fibrogenic factors that alter Kupffer cell and hepatocyte functions, triggering the activation of HSCs and fibrogenesis.

There are a number of murine models that recapitulate the disturbed iron metabolism of HH. The first HH mouse developed was an Hfe knockout (Hfe−/−) mouse model of HH type 1. Hfe and Hamp knockout mouse models effectively reflect HH type 2. There are several models of HH type 3, including the Tfr2 Y245X mutant (Tfr2mut) mouse that is orthogonal to the Y250X mutation identified in some patients with HH type 3. Knockout of ferroportin is embryonically lethal; however, the flatiron mouse, which has a missense mutation (H32R) in ferroportin, exhibits a phenotype similar to that observed in HH type 4. To date, there is no report on the induction of liver toxicity, injury, or fibrosis in any untreated genetic mouse models of HH. Tan et al., however, recently reported early signs of fibrosis in Hfe−/− mice fed a modified fat diet. In the present study, we describe iron-induced liver injury in Hfe−/− × Tfr2mut mice, where disruption of both Hfe and Tfr2 causes more severe iron loading than disruption of either Hfe or Tfr2 alone, leading to enhanced liver injury and fibrosis.

Materials and Methods

Animals. Hfe−/− mice were generated by the disruption of the Hfe gene using homologous recombination, as described by Zhou et al. Tfr2mut mice were generated with a Y245X mutation in Tfr2, as reported previously. Hfe−/− and Tfr2mut mice were backcrossed for 10 generations onto an AKR genetic background (Animal Resource Center, Murdoch, Western Australia, Australia). Hfe−/− and Tfr2mut mice were then crossed to generate Hfe−/− × Tfr2mut double-mutant mice. Hfe−/−, Tfr2mut, Hfe−/− × Tfr2mut, and wild-type (WT) mice (AKR background) were fed standard mouse chow (200 mg iron/kg diet; Specialty Feeds, Glen Forrest, Western Australia, Australia) ad libitum from 4 weeks of age. An additional group of WT mice was fed an iron-supplemented diet (20 g carboxyl iron/kg diet; Specialty Feeds) for 3 weeks from 8 weeks of age. At 11 weeks of age, after overnight fasting, blood was collected by cardiac puncture and organs were perfused in situ with isotonic saline. Livers were collected and snap-frozen in liquid nitrogen or fixed in formalin. This study was approved by The University of Western Australia (Perth, Western Australia, Australia) Animal Ethics Committee.

RNA Expression. Total RNA was isolated from liver tissue using TRI Reagent (Ambion Biosystems, Scoresby, Victoria, Australia) and reverse-transcribed using
Liver Injury. Plasma alanine aminotransferase (ALT) was measured as an indicator of liver injury using a kit according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). Liver F₂-isoprostanes, a marker of LPO, was measured by gas chromatography/mass spectrometry using a deuterium-labeled internal standard, as previously described. The antioxidant, butylated hydroxytoluene, was added to liver tissue to scavenge any ROS generated during tissue storage and processing. Activities of antioxidant enzymes copper/zinc and manganese SOD were measured in the liver as an index of oxidative stress using a kit according to the manufacturer’s instructions (Cayman Chemical, Sydney, New South Wales, Australia). Liver hydroxyproline content was measured as a biochemical marker of liver collagen using a kit according to the manufacturer’s instructions (QuickZyme Biosciences, Leiden, Netherlands).

Statistical Analysis. Results are expressed as mean ± standard error of the mean (SEM), where n = 5-15 mice per group. Differences between groups were analyzed using analysis of variance with Tukey’s multiple comparison post-test or an unpaired Student’s t test (GraphPad Prism; GraphPad Software, Inc., La Jolla, CA). Differences between groups were defined as statistically significant for P < 0.05.

Results

Liver Gene Expression. Expression of Hfe, Tfᵣ1, Tfᵣ2, Bmp6, Id1, and Hamp1 genes is shown in Table 1. Hfe expression in Tfr₂⁻/⁻ and WT mice was similar and undetectable in Hfe⁻/⁻ and Hfe⁻/⁻ × Tfr₂⁻/⁻ mice (P < 0.001). Tfᵣ2 mRNA expression in Tfr₂⁻/⁻ and Hfe⁻/⁻ × Tfr₂⁻/⁻ mice was decreased by approximately 65%, compared with non-iron-loaded WT mice (P < 0.001). Tfᵣ2 mRNA expression in Hfe⁻/⁻ and iron-loaded WT mice was also lower than non-iron-loaded WT mice (P < 0.05). Tfᵣ1 mRNA expression was reduced in all types of HH mice and iron-loaded WT mice, compared with non-loaded WT mice (P < 0.05), consistent with liver iron loading. Expression of the gene encoding the iron-regulatory hormone, Hamp1, was decreased to approximately 40% in Hfe⁻/⁻ and Tfᵣ₂⁻/⁻ mice, compared with non-iron-loaded WT mice. In Hfe⁻/⁻ × Tfr₂⁻/⁻ mice, Hamp1 expression was almost abolished, being further reduced to approximately 1% or 3% of that observed in non-iron-loaded WT mice (P < 0.01) or Hfe⁻/⁻ and Tfᵣ₂⁻/⁻ mice (P < 0.05), respectively. Hamp1 expression, as expected, was increased in iron-loaded WT mice, compared with non-iron-loaded WT mice (P < 0.05) and HH mice (P < 0.001). Bmp6 expression was approximately 40% in Tfᵣ₁⁻/⁻, Tfᵣ₂⁻/⁻, Bmp6⁻/⁻, Id1⁻/⁻, and Hamp1⁻/⁻ mice, compared with non-iron-loaded WT mice (P < 0.05), consistent with liver iron loading. Expression of the gene encoding the iron-regulatory hormone, Hamp1, was decreased to approximately 40% in Hfe⁻/⁻ and Tfᵣ₂⁻/⁻ mice, compared with non-iron-loaded WT mice. In Hfe⁻/⁻ × Tfr₂⁻/⁻ mice, Hamp1 expression was almost abolished, being further reduced to approximately 1% or 3% of that observed in non-iron-loaded WT mice (P < 0.01) or Hfe⁻/⁻ and Tfᵣ₂⁻/⁻ mice (P < 0.05), respectively. 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expression was increased in all HH and iron-loaded WT mice (\(p < 0.05\)), compared with non-iron-loaded WT mice, consistent with iron-dependent regulation of Bmp6. However, phosphorylated mothers against decapentaplegic (Smad)1/5/8 protein levels were decreased significantly in \(Hfe^{-/-}\times Tfr2\text{mut}\) mice, compared with all other types of mice (\(p < 0.05\)), and inhibited in \(Hfe^{-/-}\) and \(Tfr2\text{mut}\) mice, compared with iron-loaded WT mice (\(p < 0.05\); Supporting Fig. 1). \(Id1\) (Bmp6/ pSmad1/5/8 target), as with \(Hamp1\) expression, was decreased in all HH mice, compared with non-iron-loaded WT mice (\(p < 0.05\)). This is consistent with impaired pSmad1/5/8 signaling in HH mice.

**Plasma Iron Parameters.** Plasma iron concentration and transferrin saturation were higher in \(Hfe^{-/-}\times Tfr2\text{mut}\), \(Tfr2\text{mut}\), \(Hfe^{-/-}\), and iron-loaded WT mice, compared with non-iron-loaded WT mice (\(p < 0.05\); Fig. 1A,B). Iron concentration and transferrin saturation were greatest in \(Hfe^{-/-}\times Tfr2\text{mut}\) mice (\(p < 0.05\); Fig. 1A,B). Plasma iron concentration in \(Tfr2\text{mut}\) mice was increased, compared to \(Hfe^{-/-}\) mice (\(p < 0.05\)). Plasma NTBI concentration was also elevated in all iron-loaded mice (\(p < 0.05\)). In \(Hfe^{-/-}\times Tfr2\text{mut}\) mice, NTBI levels were 7-fold higher than non-iron-loaded WT mice and more than 2-fold higher than \(Hfe^{-/-}\), \(Tfr2\text{mut}\), and iron-loaded WT mice (\(p < 0.001\); Fig. 1C).

**HIC.** HIC was elevated in all iron-loaded mice, compared with non-iron-loaded mice. HIC in \(Hfe^{-/-}\), \(Tfr2\text{mut}\), and iron-loaded WT mice was similar and approximately 3-fold higher than non-loaded WT mice (\(p < 0.001\); Fig. 2A). HIC was greater in \(Hfe^{-/-}\times Tfr2\text{mut}\) mice, compared with either \(Hfe^{-/-}\) or \(Tfr2\text{mut}\) mice (\(p < 0.01\); Fig. 2A) and approximately 5-fold that of the non-iron-loaded WT mice. Perl’s Prussian blue staining of liver sections from \(Hfe^{-/-}\times Tfr2\text{mut}\) mice demonstrated a periportal distribution of iron, similar to that observed in \(Hfe^{-/-}\), \(Tfr2\text{mut}\), and iron-loaded WT mice. However, the intensity of iron staining was greater in \(Hfe^{-/-}\times Tfr2\text{mut}\) than in the other types of mice (Fig. 2B-D). These results indicate an increased iron burden in \(Hfe^{-/-}\times Tfr2\text{mut}\) mice.

**Liver Injury.** H&E-stained liver sections from \(Hfe^{-/-}\times Tfr2\text{mut}\) mice demonstrated mild inflammation with evidence of scattered foci of infiltrating inflammatory cells throughout the liver parenchyma (Fig. 3). Immunofluorescent detection of the pan leukocyte marker, CD45, revealed that the cell aggregates consisted mainly of CD45⁺ inflammatory cells (Fig. 3A,E) that colocalized predominately, but not exclusively, with the iron storage protein, ferritin, in periportal regions of the liver (Supporting Fig. 2). The number of CD45⁺ inflammatory cells was significantly

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Table 1. mRNA Expression of Iron Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>WT+Fe</th>
<th>(Hfe^{-/-})</th>
<th>(Tfr2\text{mut})</th>
<th>(Hfe^{-/-}\times Tfr2\text{mut})</th>
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| \(Hfe\) | 33 ± 2 | 29 ± 1 | -*
|\(\dagger\) | 34 ± 1
|\(\dagger\) | 34 ± 1
|\(\dagger\)
|\(\dagger\) |

Results are expressed as mean ± SEM (n = 5-15) mRNA copy number of the gene of interest relative to ß-actin.

*\(P < 0.05\) versus WT.
\(\dagger\)\(P < 0.05\) versus WT+Fe.
\(\ddagger\)\(P < 0.05\) versus \(Tfr2\text{mut}\) denote significance between groups.

\(\S\)\(P < 0.05\) versus \(Tfr2\text{mut}\) denote significance between groups.
increased in the livers from \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mice, compared with the other groups of mice \((P < 0.05)\), whereas the number of CD45+ cells in \( \text{Hfe}^{-/-}, \text{Tfr2}^{\text{mut}} \), and iron-loaded WT mice was not significantly different from those in non-iron-loaded WT mice (Fig. 3F). Another unique feature of \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mice was the evidence of inflammatory sideronecrosis of hepatocytes, which was not observed in any other group of mice (Fig. 3E).

Liver injury was assessed by examining plasma ALT as well as hepatic SOD and F\(_2\)-isoprostane levels. Plasma ALT activity was increased in \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mice by at least 1.8-fold, compared with all other types of mice \((P < 0.001); \) Fig. 4A). Both hepatic copper/zinc \((\text{cytosolic})\) and manganese \((\text{mitochondrial})\) SOD activities were significantly decreased in all HH mice. In \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mice copper/zinc SOD levels were similar, whereas manganese SOD levels were significantly lower than \( \text{Hfe}^{-/-} \) and \( \text{Tfr2}^{\text{mut}} \) mice \((P < 0.01); \) Fig. 4B). Liver F\(_2\)-isoprostanes were elevated in all groups of HH mice, compared with non-iron-loaded WT mice \((P < 0.01)\), with \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mice having similar liver F\(_2\)-isoprostane levels to iron-loaded WT mice and significantly higher levels than either \( \text{Hfe}^{-/-} \) or \( \text{Tfr2}^{\text{mut}} \) mice \((P < 0.01); \) Fig. 4C.

**Collagen Deposition.** Hepatic collagen deposition, a marker of fibrosis, was examined by histology using Sirius red and Masson’s trichrome staining and by biochemical measurement of hydroxyproline levels. Hydroxyproline levels were increased in all iron-loaded mice. In \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mice, hydroxyproline levels were significantly increased, compared with \( \text{Tfr2}^{\text{mut}} \) mice, and both were elevated, compared with \( \text{Hfe}^{-/-} \) and iron-loaded WT mice (Fig. 4D; \( P < 0.05 \)). Likewise, \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mice had significantly increased Sirius red staining, compared with \( \text{Hfe}^{-/-}, \text{Tfr2}^{\text{mut}} \), and iron-loaded WT mice \((P < 0.05)\), which, in turn, exhibited greater collagen deposition than non-iron-loaded WT mice \((P < 0.01); \) Fig. 5A-F). Sirius red staining revealed portal tract thickening and periportal fibrosis in \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mice. In addition, there was evidence of portal tract bridging in \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mice, which was not evident in other groups. Quantification of Sirius red staining correlated with HIC \((r^2 = 0.98; P = 0.001)\), plasma NTBI \((r^2 = 0.82; P = 0.033)\), as well as hydroxyproline \((r^2 = 0.89; P = 0.015)\) and F\(_2\)-isoprostane levels \((r^2 = 0.77; P = 0.048)\) in HH mice. This suggests that the collagen levels measured by biochemical assay were consistent with histological observations using Sirius red staining and were dependent on both plasma NTBI and HIC in HH mice. Furthermore, the intensity of trichrome staining, a commonly used, but less sensitive, marker of fibrosis, was also significantly enhanced in \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) and \( \text{Tfr2}^{\text{mut}} \) mice (Fig. 6F), with evidence of collagen thickening in the periportal region of the liver (Fig. 6A-E).

**Discussion**

In this study, we have used \( \text{Hfe}^{-/-} \) and \( \text{Tfr2}^{\text{mut}} \) mouse models of HH types 1 and 3, respectively, and a \( \text{Hfe}^{-/-} \times \text{Tfr2}^{\text{mut}} \) mouse model to examine the effects of disruption of \( \text{Hfe} \) and \( \text{Tfr2} \), either alone or in combination, on liver iron loading and iron-induced liver injury. We describe, to our knowledge, the first report of a genetic HH mouse model of iron-induced liver injury. We describe, to our knowledge, the first report of a genetic HH mouse model of iron-induced liver injury. We describe, to our knowledge, the first report of a genetic HH mouse model of iron-induced liver injury.
reduced hepcidin levels in $Hfe^{-/-}$, $Tfr2^{mut}$, and $Hfe^{-/-} \times Tfr2^{mut}$ mice would lead to increased iron absorption and hepatic iron deposition.\(^8\) In association with increased liver iron loading, there was a pronounced elevation of plasma ALT activity, a marker of liver injury, in $Hfe^{-/-} \times Tfr2^{mut}$ mice. There was also mild hepatic inflammatory cell infiltration with scattered foci of CD45\(^+\) leukocytes and some evidence of hepatocyte sideronecrosis in $Hfe^{-/-} \times Tfr2^{mut}$ mice. Elevated hydroxyproline levels as well as Sirius red and tri-chrome staining showing marked portal tract collagen deposition and portal bridging in $Hfe^{-/-} \times Tfr2^{mut}$ mice clearly demonstrates the presence of liver fibrosis in areas of greatest iron accumulation. In comparison, $Hfe^{-/-}$ and $Tfr2^{mut}$ mice had less collagen deposition and inflammation. Histological evidence of a more pronounced liver damage in $Hfe^{-/-} \times Tfr2^{mut}$ mice was corroborated by decreased SOD activity and enhanced LPO in the liver, indicating elevated hepatic oxidative stress.

The iron-dependent regulation of HAMP is controlled by HFE and TFR2, as well as BMP6/SMAD cell-signaling pathways.\(^{22,23,28}\) It has been demonstrated that HFE can interact with TFR1 and TFR2 to form a complex that is hypothesized to sense plasma transferrin saturation and modulate hepcidin synthesis accordingly.\(^{1,8}\) However, the nature of this mechanism is yet to be fully elucidated. Our findings support previous studies that suggest there is cross-talk between HFE/TFR2- and BMP6/SMAD-signaling pathways, because the absence of functional HFE and/or TFR2 attenuated iron-induced phosphorylation of SMAD1/5/8 and hepcidin expression.\(^{23,28}\)

Mice with deletions in both $Hfe$ and $Tfr2$ have been generated on other genetic backgrounds.\(^{23,28}\) These mice, as with our HH murine model, exhibited...
elevated plasma and liver iron levels, compared with mice with the appropriate deletion of Hfe or Tfr2, as well as a marked reduction in Hamp1 expression, consistent with increased liver iron accumulation. However, the degree of regulation of Hamp1 observed in Hfe−/− and Tfr2mut mice, and its near abrogation induced by the disruption of both Hfe and Tfr2, suggests that the formation of a Hfe and Tfr2 complex is not a prerequisite for the initiation of iron-dependent HAMP synthesis. The degree of iron overload, however, varies between strains, which is consistent with previous observations that iron metabolism is modified by genetic background. Our HH mice were generated on an AKR background and have relatively high plasma and liver iron levels, compared with other strains of mice. Colocalization of a more marked fibrotic process in areas of greatest iron deposition in the hepatic periportal regions in our Hfe−/−×Tfr2mut mice provides further evidence of the importance of genetic background and phenotypic expression of iron overload in the pathogenesis of liver injury in HH.

Rodents are generally relatively resistant to iron-induced liver injury. Dietary carbonyl iron loading of rats for 3 months produced iron loading in hepatocytes, similar to the levels observed in the Hfe−/−×Tfr2mut mice in the present study, but demonstrated only early signs of liver injury, including increased LPO and collagen gene expression. Long-term iron loading was required for up to 12 months before morphological evidence of fibrosis was observed. Dietary iron supplementation in combination with hepatotoxins, such as ethanol and carbon tetrachloride, was...
required to accelerate liver injury.\textsuperscript{32,33} In the present study, the degree of liver fibrosis observed in \textit{Hfe}\textsubscript{−−}\textit{C0/C0/C0/C2 Tfr2mut} mice at 3 months of age was similar to that observed after dietary loading of rodents for 12 months.\textsuperscript{30,31} In our \textit{Hfe}\textsubscript{−−}\textit{C0/C0/C0/C2 Tfr2mut} mice, hepatic inflammation, fibrosis, and LPO occurred in the presence of marked elevation of both plasma NTBI and hepatic iron levels, similar to those observed in human HFE-related HH.\textsuperscript{34,35} Furthermore, the degree of fibrosis observed in the HH mice was dependent on both HIC and NTBI levels.

The observation that \textit{Hfe}\textsuperscript{−−}\textit{× Tfr2mut} mice have increased plasma ALT levels is consistent with previous observations in HH patients, where the majority of patients had mildly elevated ALT levels.\textsuperscript{36} Levels of the antioxidant enzymes, cytosolic copper/zinc and mitochondrial manganese SOD, were both decreased in \textit{Hfe}\textsuperscript{−−}\textit{× Tfr2mut} mice consistent with increased oxidative stress. Earlier studies have also reported decreased copper/zinc SOD in dietary iron-overloaded animals, whereas manganese SOD was decreased in \textit{Hfe} knockout and increased in iron-loaded rodents.\textsuperscript{11,20,37} Furthermore, LPO was increased in HH mice. Unexpectedly, the level of \textit{F2}-isoprostanes in dietary iron-loaded mice was greater than in HH mice with similar HIC. This may be the result of differences between dietary iron (i.e., high HAMP) and genetic HH (i.e., low HAMP) models of liver iron overload where variation in cellular iron distribution between parenchymal and Kupffer cells occurs, despite similar total HIC.

Mild liver inflammation was observed only in \textit{Hfe}\textsuperscript{−−}\textit{× Tfr2mut} mice, suggesting that there was an iron-concentration threshold effect. Mild inflammation has been documented in human HH studies during the development of fibrosis and cirrhosis.\textsuperscript{38} Deugnier et al. reported inflammatory infiltrates in approximately 50% of liver biopsies from HH patients.\textsuperscript{39} Inflammation was predominantly present in portal and periportal regions and correlated with histological iron scores, sideronecrotic changes in hepatocytes, and hepatic fibrosis. Another study showed that approximately 25% of liver biopsies from untreated HH patients displayed moderate inflammatory infiltration.\textsuperscript{40} Bridle et al. also reported that 60% of liver biopsies from HH patients showed mild inflammation consisting of scattered inflammatory foci. Furthermore, patients with hepatic inflammation had a higher incidence of hepatic fibrosis.\textsuperscript{41} Iron-loaded and apoptotic/necrotic hepatocytes are purported to induce the activation of HSCs by various signaling mechanisms, resulting in enhanced production of proinflammatory and -fibrogenic cytokines as well as the recruitment of inflammatory cells.\textsuperscript{8} Our study provides further support for the direct hepatotoxic effects of iron overload, which results from the disruption of \textit{Hfe} and \textit{Tfr2}, manifesting as inflammation and increased collagen deposition, suggesting the activation of HSCs.

Iron plays an important part in the progression of hepatic injury, and it does this through its ability to catalyze the formation of highly reactive, damaging ROS. ROS induce tissue injury by promoting LPO as well as protein and DNA modification, leading, ultimately, to apoptosis and necrosis. Further investigation into the molecular mechanisms of iron toxicity and how it causes liver injury will provide a better understanding of the role iron plays in the progression of liver disease. The \textit{Hfe}\textsuperscript{−−}\textit{× Tfr2mut} mouse represents a model of the genetic iron overload disorder, HH, that mimics both iron overload and consequent liver injury observed in humans with HH.

\textbf{References}


