Iron Regulation of Hepcidin Despite Attenuated Smad1,5,8 Signaling in Mice Without Transferrin Receptor 2 or Hfe

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BACKGROUND & AIMS: HFE and transferrin receptor 2 (TFR2) are each necessary for the normal relationship between body iron status and liver hepcidin expression. In murine *Hfe* and *Tfr2* knockout models of hereditary hemochromatosis (HH), signal transduction to hepcidin via the bone morphogenetic protein 6 (Bmp6)/Smad1,5,8 pathway is attenuated. We examined the effect of dietary iron on regulation of hepcidin expression via the Bmp6/Smad1,5,8 pathway using mice with targeted disruption of *Tfr2*, *Hfe*, or both genes. **METHODS:** Hepatic iron concentrations and messenger RNA expression of Bmp6 and hepcidin were compared with wild-type mice in each of the HH models on standard or iron-loading diets. Liver phospho-Smad (P-Smad)1,5,8 and *Id1* messenger RNA levels were measured as markers of Bmp/Smad signaling. **RESULTS:** Whereas Bmp6 expression was increased, liver hepcidin and Id1 expression were decreased in each of the HH models compared with wild-type mice. Each of the HH models also showed attenuated P-Smad1,5,8 levels relative to liver iron status. Mice with combined *Hfe/Tfr2* disruption were most affected. Dietary iron loading increased hepcidin and Id1 expression in each of the HH models. Compared with wild-type mice, HH mice demonstrated attenuated (*Hfe* knockout) or no increases in P-Smad1,5,8 levels in response to dietary iron loading. **CONCLUSIONS: These observations show that Tfr2 and Hfe are each required for normal signaling of iron status to hepcidin via the Bmp6/Smad1,5,8 pathway. Mice with combined loss of Hfe and Tfr2 up-regulate hepcidin in response to dietary iron loading without increases in liver Bmp6 messenger RNA or steady-state P-Smad1,5,8 levels.**

Keywords: Bone Morphogenetic Protein 6; Id1.

Hereditary hemochromatosis (HH) is a genetically heterogeneous hereditary disorder caused by elevated iron absorption from the diet, with consequent iron overload and tissue injury.[1,2] The most common form of HH is caused by mutation in the *HFE* gene. A much more rare form of HH (type 3) results from mutations in the gene for transferrin receptor 2 (*TFR2*).[3] It is now widely accepted that impaired regulation of hepcidin expression plays a central role in the pathogenesis of HH. Hepcidin acts to down-regulate the iron exporter ferroportin on the surface of duodenal enterocytes and macrophages, thereby inhibiting iron release from these cells.[4] Human patients and mouse models of *TFR2*-related[5,6] and *HFE*-related[7–10] HH each show inappropriately low expression of hepcidin. The mechanisms by which TFR2 and HFE influence hepcidin expression remain unclear. Several observations suggest a model in which TFR1 may participate as well. In this model, as the transferrin saturation increases, diferric transferrin displaces HFE from TFR1, thereby making HFE available to bind to TFR2.[11–13] The complex of HFE and TFR2 is then postulated to influence hepcidin expression differently than TfR2 alone.[14] Mice with inactivating mutations in both *Hfe* and *Tfr2* show a severe HH phenotype and very low hepcidin expression, raising the possibility that each may serve to regulate hepcidin expression even in the absence of the other.[15]

A bone morphogenetic protein 6 (BMP6)-dependent signaling pathway has been shown to play a key role in regulation of hepcidin expression.[16,17] BMPs bind to type I and type II serine threonine kinase receptors, which phosphorylate specific intracellular SMAD proteins (SMAD1,5,8). Phosphorylated SMAD1,5,8 (P-SMAD1,5,8) binds to the common mediator SMAD4, and the SMAD complex translocates to the nucleus to affect transcription of target genes such as *ID1*.[18,19] *HAMP* (encoding hepcidin) is transcriptionally up-regulated by BMPs.[20–23] Impaired hepatic Bmp signaling, through mutations in genes encoding either the ligand Bmp6,[16,17] the Bmp coreceptor hemojuvelin (Hjv),[24,25] or Smad4[26] leads to low hepcidin levels and iron overload in mice. Conversely, dietary iron loading increases hepatic *Bmp6* messenger RNA (mRNA) expression in mice concordantly with *Hamp1* and *Id1* mRNAs.[27] Collectively, these data show that BMP-SMAD signaling is an important regulatory pathway for hepcidin expression and thus iron metabolism. In *Hfe* knockout mice[28,29] and in patients with *HFE*-associated HH,[30,31] the induction of *Bmp6* mRNA by iron is intact, but Smad1,5,8 signaling to hepcidin is impaired.

Abbreviations used in this paper: ANOVA, analysis of variance; BMP, bone morphogenetic protein; HH, hereditary hemochromatosis; Hjv, hemojuvelin; RT-PCR, reverse-transcription polymerase chain reaction; Tfr2, transferrin receptor 2.

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Impaired Bmp6 signaling to hepcidin has also been reported in murine models of *Tfr2*-associated HH.[15,32]

The goal of this study was to investigate the Bmp6-Smad-hepcidin signaling pathway in the *Tfr2* and *Hfe* mutant mouse models of HH under standard iron diets and with dietary iron loading. We observed the expected impaired signaling to hepcidin via the Bmp6/Smad pathway in *Tfr2* and *Hfe* HH mouse models. Signaling to hepcidin via the Bmp/Smad pathway was more impaired in *Hfe/Tfr2* mice than in mice with loss of either gene product individually. Dietary iron loading increased hepcidin expression in each of the murine HH model systems. In *Tfr2* mice and *Hfe/Tfr2* mice, hepcidin up-regulation occurred without an increase in liver P-Smad1,5,8 levels. Taken together, these results indicate that Hfe and Tfr2 are each necessary for normal signaling from Bmp6 to hepcidin, that each can influence hepcidin expression independent of the other, and that mechanisms regulating hepcidin expression in response to dietary iron exist that do not require Hfe or Tfr2.

Materials and Methods

Animal Care

Hfe knockout mice[33] and *Tfr2*^{Y245X} mice[34] were bred to uniformity on an FVB background for more than 7 generations. The *Tfr2*^{Y245X} mice have no detectable Tfr2 or truncated form of the protein in hepatocellular membrane preparations and are a functional knockout.[34] These 2 mouse lines were crossed with each other and bred to homozygosity for each mutant allele. Colonies were maintained as homozygotes for each allele individually (hereafter referred to as *Hfe* mice or *Tfr2* mice) and as compound mutant homozygotes (*Hfe/Tfr2* mice). Mice were fed standard chow (Purina 5001, containing 270 ppm iron; Purina Mills, Richmond, IN) ad libitum after weaning at 21 days. Dietary iron loading was achieved by weaning mice onto a diet containing an additional 25,000 ppm of carbonyl iron. At 5 weeks of age, the mice were killed by exposure to hypercarbia followed by exsanguination, and tissues were harvested. To minimize potential variability related to sex, samples from only male mice were used in subsequent studies. Sample sizes unless otherwise indicated in figure legends were as follows: 13 wild-type on a standard diet, 4 wild-type on high iron, 3 *Hfe* knockout, 3 *Hfe* knockout on high iron, 5 *Tfr2* knockout, 3 *Tfr2* knockout on high iron, 5 *Hfe/Tfr2*, and 3 *Hfe/Tfr2* on high iron. The murine studies were performed under protocols approved by the Institutional Animal Care and Use Committee of Saint Louis University and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Liver Iron Content

Liver specimens were homogenized, and a portion was desiccated overnight at room temperature and analyzed for nonheme iron content by the method of Torrance and Bothwell.[35] Data were expressed as micrograms of iron per gram dry weight of liver.

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from mouse liver tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA), with deoxyribonuclease digestion using the RNase-Free DNase Set (Qiagen). Quantitation of murine *Bmp6*, *Hamp1*, *Id1*, and *Rpl19* messenger RNA transcripts was performed using 2-step quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) as previously described.[28] Samples were analyzed in triplicate, and expression levels were normalized to the housekeeping gene *Rpl19*. Additional quantitation of *Bmp6*, *Hamp1*, and *β-actin* mRNA transcripts were performed using One-Step Quantitative Real-Time RT-PCR (TaqMan, Applied Biosystems, Carlsbad, CA ABI7700) and the following probes and primers: Hamp1 forward CCTATCTCCATCAACAG-GTG, reverse AACAGATACCACACTGGGAA, and probe 6FAM-CCCTGCTTTCTTCCCCGTGCAAAGT-TAMRA; β-actin forward CCGTGAAAAGATGACCCAGATCATG, reverse TCTTCATGA-GGTAGTCCGTCAGGTC, and probe 6FAM-TACGAGGGCTAT-GCTCTCCCTCACGCT-TAMRA. Hamp1 expression relative to β-actin expression was compared across groups using both the ΔCt method and the method described by Pfaffl et al[36] using REST software (Qiagen). Similar results were obtained using each analytical and real-time PCR method.

Western Blot Analyses

Liver specimens were homogenized in lysis buffer (1× Tris-buffered saline, 0.1% sodium dodecyl sulfate, 10 μL/mL Triton X-100, 1 g/dL sodium deoxycholate, 2 μL/mL EDTA) containing protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail; Thermo Fisher Scientific, Pittsburgh, PA). Western blots of liver lysates for P-Smad1,5,8 protein (relative to total Smad1 protein and to β-actin) and chemiluminescence quantitation were performed as previously described.[28] P-Stat3 was quantified by Western blot using the PhosphoPlus Stat3 Antibody Kit (Cell Signaling, Danvers, MA) per the manufacturer's instructions and normalized to β-actin.

Statistical Analyses

Statistical analyses across multiple groups were performed by analysis of variance (ANOVA) with Dunnett's test comparing each experimental group with each control (wild-type) group or (in separate experiments) by ANOVA with Newman–Keuls test when comparing across each group. For iron loading studies, comparison was made within each genotype between mice on a high-iron diet or standard diet by 2-tailed Student *t* test. $P < .05$ was considered statistically significant.

Results

Elevated Bmp6 mRNA Expression is Associated With Hepatic Iron Loading in Mice With Loss of Hfe and/or Tfr2

Functional loss of Hfe or Tfr2 is known to result in inappropriately low hepatic expression of hepcidin and consequent iron overload. Several lines of evidence suggest that up-regulation of Bmp6 contributes to iron-dependent regulation of hepcidin. We measured the hepatic expression of *Bmp6* mRNA in *Hfe*, *Tfr2*, and *Hfe/Tfr2* mice to assess if the decreased hepcidin expression could be attributed to decreased hepatic Bmp6 expression. To provide a comparison group for the degree of hepatic iron loading observed in the murine HH models, wild-type mice were placed on a high-iron (25,000 ppm) diet. As seen in Figure 1, *Hfe*, *Tfr2*, and *Hfe/Tfr2* mice on a standard iron diet had the expected elevated liver iron con-

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