Iron overload induces BMP6 expression in the liver but not in the duodenum

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

The bone morphogenetic protein BMP6 regulates hepcidin production by the liver. However, it is not yet known whether BMP6 derives from the liver itself or from other sources such as the small intestine, as has been recently suggested. This study was aimed at investigating the source of BMP6 further.

Design and Methods

We used three different strains of mice (C57BL/6, DBA/2, and 129/Sv) with iron overload induced either by an iron-enriched diet or by inactivation of the *Hfe* gene. We examined Bmp6 expression at both the mRNA (by quantitative PCR) and protein (by immunohistochemistry and Western blotting analyses) levels.

Results

We showed that iron overload induces *Bmp6* mRNA expression in the liver but not in the duodenum of these mice. Bmp6 is also detected by immunohistochemistry in liver tissue sections of mice with iron overload induced either by an iron-enriched diet or by inactivation of the *Hfe* gene, but not in liver tissue sections from iron-loaded *Bmp6*-deficient mice. Bmp6 in the duodenum was below immunodetection threshold, thus confirming quantitative PCR data. Lack of specificity of available antibodies together with slight heterogeneity between 129 substrains may account for the differences with previously published data.

Conclusions

Our data strongly support the importance of liver BMP6 for regulation of iron metabolism. Indeed, they demonstrate that intestinal Bmp6 expression is modulated by iron neither at the mRNA nor at the protein level.

Key words: iron overload, BMPb expression, liver, duodenum.

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Introduction

Hepcidin, a small peptide secreted by the liver, has a key role in coordinating the use and storage of iron with iron acquisition.[1] It acts by binding to ferroportin, an iron exporter present on the surface of enterocytes and macrophages, and induces its internalization and lysosomal degradation.[2] The loss of ferroportin from the cell surface prevents iron efflux from intestinal enterocytes and recycling of iron from senescent erythrocytes by macrophages. Hepcidin expression is normally enhanced by dietary or parenteral iron loading,[3] thus providing a feed-back mechanism to limit intestinal iron absorption. By contrast, hepcidin production is inhibited by iron deficiency anemia and tissue hypoxia,[4] which increases iron availability for use in erythropoiesis.

Hepcidin expression was recently shown to be regulated by the bone morphogenetic protein (BMP) signaling pathway,[5] and our previously published data indicated that *Bmp6* mRNA levels, but not those of other *Bmps*, were concordant with changes in hepcidin mRNA concentrations, i.e. increased by iron overload and decreased by iron deficiency, suggesting that BMP6 could be the endogenous regulator of hepcidin.[6] This was confirmed by the observation that targeted disruption of *Bmp6* in mice produces a massive iron overload in the liver and other organs.[7,8] Although other Bmp molecules are functional in the severely iron-overloaded *Bmp6*-deficient mice, they do not compensate for the absence of Bmp6, demonstrating that this iron-regulatory function is unique to Bmp6 among the BMP family. However, although Bmp6 mRNA and protein expression are induced by iron overload in the mouse liver,[9,10] it is not yet known whether BMP6 only derives from the liver or also from other sources.

Recently, Arndt *et al.*[11] suggested that the small intestine was the main source of iron-induced Bmp6 expression and proposed a model of hepcidin regulation in which iron absorption in the small intestine would induce BMP6 secretion by the enterocytes. BMP6 would then be delivered to the liver via the portal circulation and activate the signaling pathway leading to hepcidin transcription. To investigate this model further, we examined Bmp6 expression in the liver and the duodenum of three different strains of mice with iron overload induced either by an iron-enriched diet or by inactivation of the *Hfe* gene. Our data show that, irrespective of the genetic background of the mice, iron overload up-regulates Bmp6 expression in the liver but not in the duodenum.

Design and Methods

Mice

Male mice of the C57BL/6 and DBA/2 backgrounds were purchased from the Centre d'Elevage Robert Janvier (Le Genest St-Isle, France). Those of the 129S2/SvPasCrIf background were purchased from Charles River (St. Germain sur l'Arbresle, France). All were maintained at the IRF150 animal facility. Nine control animals of each strain received an iron-balanced diet (200 mg iron/kg; SAFE, Augy, France) and demineralized water. Dietary iron overload was induced by feeding 5 4-week old animals of each strain a diet enriched with carbonyl iron (8.5 g/kg) for three weeks. *Hfe*-deficient mice on the C57BL/6, DBA/2, and 129/Sv backgrounds (5-6 mice per group) were derived locally as previously described for 2 of them.[12] Briefly, the disrupted *Hfe* allele, initially on a mixed background (129/Ola x C57BL/6), was bred by successive crosses for at least 10 generations onto the C57BL/6, DBA/2 or 129/Sv backgrounds, respectively. Mice heterozygous for the disrupted allele were then mated to produce *Hfe*-null (*Hfe*$^{-/-}$) mice. All mice were analyzed at seven weeks and fasted for 14 h before sacrifice. Bmp6-deficient mice[8] were used as negative controls in immunohistochemistry and Western blot experiments. Experimental protocols were approved by the Midi-Pyrénées Animal Ethics Committee.

Tissue iron measurement

Quantitative measurement of hepatic non-heme iron was performed by a colorimetric assay as described previously.[13]

RNA preparation and real-time quantitative PCR

Liver and duodenum samples were dissected for RNA isolation, rapidly frozen, and stored in liquid nitrogen. Total RNA was extracted and purified using the RNeasy Lipid Tissue kit (Qiagen, Courtaboeuf, France). Primers for *Bmp6* and the reference gene *Hprt*[6,8] were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City). Real-time quantitative PCR (Q-PCR) reactions were prepared with M-MLV reverse transcriptase (Promega, Charbonnières-les-Bains, France) and LightCycler® 480 DNA SYBR Green I Master reaction mix (Roche Diagnostics, Mannheim, Germany) and run in duplicate on a LightCycler® 480 Instrument (Roche Diagnostics).

Immunohistochemistry

Four-micrometer sections of paraffin-embedded tissues were mounted on glass slides. Antigen retrieval was performed by incubating tissue sections with trypsin (1 mg/mL) for 6 min at 37°C. Endogenous peroxidase activity was quenched by incubating specimens with Dako REAL Peroxidase Blocking Solution (Dako, Trappes, France). Tissue sections were then blocked with normal horse blocking serum (Vector Laboratories, Burlingame, CA, USA) and incubated for 30 min at RT with the primary anti-BMP6 S-20 or N-19 antibodies (1/50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in PBS-1% BSA and 1% FCS. Immunohistochemical staining was performed using the ImmPRESS Reagent (ImmPRESS Anti-Goat Ig peroxidase Kit; Vector Laboratories) according to the manufacturer's instructions. Sections were counterstained with hematoxylin. Tissue sections from *Bmp6*-deficient mice were used to check antibody specificity.

Western blots

Protein lysates were prepared as by Arndt *et al.*,[11] and 40 μg of the lysates were separated on a 10% SDS-PAGE. Western blotting was performed applying antibodies against BMP6 (S-20 or N-19; 1:500; Santa Cruz Biotechnology, USA) or β-actin (1:20000; Sigma-Aldrich) for loading control. Following incubation with a donkey anti-goat IgG antibody (1:50,000; Jackson Immuno Research Laboratories, Baltimore, USA) or an anti-mouse IgG antibody (1:5,000; Cell Signaling Technology) conjugated to horseradish peroxidase, enzyme activity was visualized by an ECL-based detection system (Amersham Pharmacia Biotech, Orsay, France). Protein lysates from *Bmp6*-deficient mice were used to check antibody specificity.

Statistical analyses

Mean liver iron contents and mean ΔCt (Ct *Hamp* –Ct *Hprt*) between the different groups of mice were compared by Student's t-tests. The 95% confidence intervals for the differences (ΔΔCt) were obtained with SAS version 9.1. Point estimations of fold changes were obtained by raising 2 to the power -ΔΔCt ($2^{-\Delta\Delta Ct}$) and

the confidence intervals for the fold changes were derived from the confidence intervals of ΔΔCt.[14]

Results

Bmp6 mRNA expression is induced in the liver but not in the duodenum of mice with experimental iron overload

Bmp6 mRNA expression is approximately 2-fold higher in the liver than in the duodenum of wild-type mice fed an iron-standard diet (expression ratio = 1.97; 95% confidence interval: 1.75 - 2.21). As shown in Figure 1A, we observed the previously described[10] induction of *Bmp6* mRNA expression in the liver of mice of three genetic backgrounds (C57BL/6, DBA/2, and 129/Sv) fed an iron-enriched diet (8.5 g/kg carbonyl iron) for three weeks. Conversely, no significant induction of *Bmp6* mRNA was detected in the duodenum of these mice. We used another model of iron overload, i.e. mice deficient for the hemochromatosis *Hfe* gene, to confirm these data. Like mice with secondary iron overload, *Hfe*-deficient mice of the three genetic backgrounds (C57BL/6, DBA/2, and 129/Sv) develop hepatic iron overload, leading to the upregulation of *Bmp6* expression in the liver but not in the duodenum (Figure 1B).

Induction of Bmp6 mRNA expression in the liver is correlated with Bmp6 protein detection by immunohistochemistry

To confirm these data at the protein level, we stained liver and duodenum tissue sections with the anti-BMP6 antibody used by Arndt *et al.*[11] (S-20; Santa Cruz Biotechnology). Examples of Bmp6 staining obtained with mice of the different genetic backgrounds and with different levels of iron overload are shown in Figure 2. As expected, *Bmp6*-deficient mice have the highest liver iron accumulation (Figure 2 A1) but no Bmp6 staining in either the liver (Figure 2 A2) or the duodenum (Figure 2 A3). Bmp6 was below detection threshold both in the liver and the duodenum of control mice of the three genetic backgrounds (*data not shown*). C57BL/6 mice with secondary iron overload induced by an iron-enriched diet and DBA/2 mice with iron overload due to *Hfe*-deficiency have similar liver iron accumulation as visualized by Perls' staining (Figure 2 B1 and C1, respectively). Whereas both present with a centrilobular pattern of Bmp6 staining in the liver (Figure 2 B2 and C2), no staining is seen in the duodenum, consistent with the lack of messenger induction (Figure 2 B3 and C3). *Hfe*-deficient mice of the 129/Sv background have lower liver iron content than the previous mice (Figure 2 D1). Interestingly, Bmp6 staining, although detectable in the liver (Figure 2 D2), is also lower in these mice. Similar data were obtained with the Santa Cruz N-19 antibody (*data not shown*).

Commercial anti-BMP6 antibodies are not suitable for Western blot analyses

To complete this study, we measured the expression of Bmp6 by Western blotting in the liver and the duodenum of the mice with the highest amount of liver iron accumulation (C57BL/6 mice fed an iron-enriched diet) using the Santa-Cruz S-20 antibody and *Bmp6*-deficient mice as negative controls. As shown in *Online Supplementary Figure S1A*, we were unable to detect Bmp6 in the duodenum of any of the mice tested. A faint 23 kD band was detected in the liver of C57BL/6 mice fed the iron-enriched diet (*Online Supplementary Figure S1B*). However, this band was also observed in *Bmp6*-deficient mice and, noticeably, its intensity increases with liver iron accumulation. The Santa Cruz S-20 anti-BMP6 antibody, therefore, is not specific for mouse Bmp6 in Western blot experiments. Similar results were obtained with the Santa Cruz N-19 anti-BMP6 antibody (*data not shown*).

Discussion

Although Bmp6 mRNA and protein expression are induced by iron overload in the mouse liver,[9,10] other sources of BMP6 may exist and small intestine has been recently proposed as the predominant site of Bmp6 production upon iron loading. In this study, we used mice of three genetic backgrounds (C57BL/6, DBA/2, and 129/Sv) in which iron accumulation was induced by either an iron-enriched diet or disruption of the hemochromatosis *Hfe* gene. Whereas we observed the increase in liver *Bmp6* mRNA initially detected by DNA microarrays,[6] there was no detectable variation in *Bmp6* mRNA expression in the duodenum. Similar data were recently obtained with hemojuvelin-deficient mice on a 129/SvEvTac background.[15] Hemojuvelin

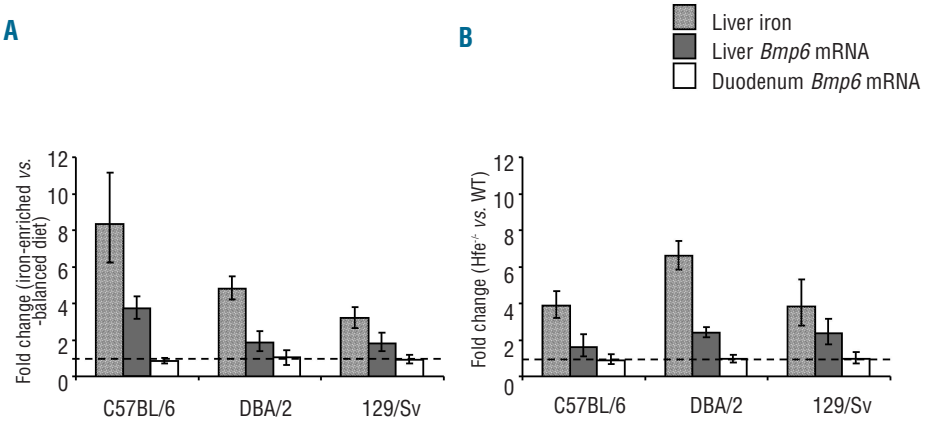

Figure 1. Effect of dietary iron-enrichment or *Hfe*-deficiency on hepatic iron concentrations and *Bmp6* gene expression in the liver and the duodenum of 7-week old C57BL/6, DBA/2, and 129/Sv mice. (A) Increase in hepatic iron concentrations and expression ratios of Bmp6 transcripts normalized to the *Hprt* reference gene mRNA (and 95% confidence intervals) in wild-type animals fed an iron-enriched diet *versus* animals fed an iron-balanced diet (5 mice per group). (B) Increase in hepatic iron concentrations and expression ratios of *Bmp6* transcripts normalized to the *Hprt* reference gene mRNA (and 95% confidence intervals) in *Hfe*-deficient *versus* wild-type mice (5-6 mice per group).

(HJV) is a GPI-linked membrane protein that acts as a BMP6 coreceptor in the liver[7] and is a critical upstream regulator of hepcidin transcription. Homozygous or compound heterozygous mutations of HJV in humans and disruption of both *Hjv* alleles in mice markedly reduce hepatic hepcidin expression and cause severe iron overload.[16-19] Interestingly, whereas *Hjv*-deficient mice have *Bmp6* mRNA levels 4 times higher than wild-type mice in their liver, no significant difference in *Bmp6* mRNA expression was found in their small intestine.[15]

Immunodetection was performed with two Santa Cruz anti-BMP6 antibodies, S-20 as in Arndt *et al.*[11] and N-19 as in Kautz *et al.*[10] A centrilobular pattern of Bmp6 staining was observed in the liver of iron overloaded mice. Noticeably, the centrilobular Bmp6 staining in these mice does not coincide with the periportal location of iron visualized by Perls' coloration, which excludes iron interference with immunodetection. As shown in Figure 2, there seems to be an iron threshold below which Bmp6 immunodetection is problematic. Immunostaining is, for instance, much lower in the moderately iron-loaded 129/Sv mice than in the highly loaded C57BL/6 mice fed an iron-enriched diet or DBA/2 *Hfe*-deficient mice. This suggests that basal levels of BMP6 (not detected by immunohistochemistry) in the liver are probably sufficient for physiological modulation of hepcidin outside of massive iron.

Noticeably, no staining was seen in the duodenum, consistent with the lack of messenger induction in the two experimental models of iron overload we studied, either dietary or due to *Hfe*-deficiency. Some aspects of responsiveness to iron overload are strain-specific[13,20] and we wanted to exclude the possibility that Bmp6 induction by iron in the small intestine would be observed in some strains and not in others. We, therefore, assessed Bmp6 expression in response to iron overload in mice of three different genetic backgrounds, C57BL/6, DBA/2, and 129/Sv, but Bmp6 expression in the duodenum was similar between iron-loaded and strain-matched controls, regardless of genetic background. There is some degree of genetic variation among 129 substrains[21] and, although the 129 mice we used in this study (129S2/SvPasCrlf) and the 129 mice used by Arndt *et al.*[11] (129Sv/Ev) both derive from the same congenic strain, residual heterozygosity may be responsible for slight heterogeneity between the two substrains and account for the differences between the two studies. However, in none of the backgrounds studied so far, other than 129Sv/Ev, was experimental iron overload able to induce Bmp6 expression either at the mRNA or at the protein level in the duodenum, whatever the mechanism used to generate iron accumulation (iron-enriched diet, *Hfe*-deficiency, or *Hjv*-deficiency).

In the current study, mice were fasted for 14 h before examination of small intestine and this was not specified as being carried out by Arndt *et al.*[11] There is a possibility that the Bmp6 signal in response to iron loading is only transient. Although it was not specified whether *Hjv*-deficient mice have been fasted,[15] we wanted to eliminate this potential bias and repeated the study on a group of 5 iron-

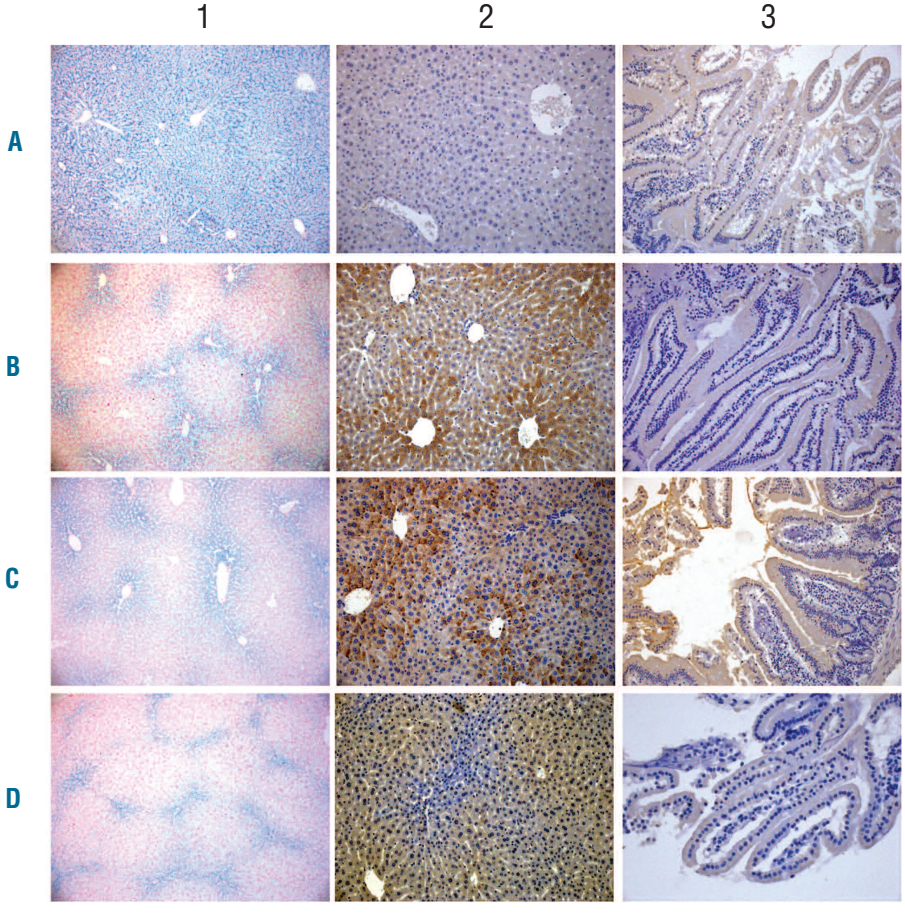

Figure 2. Cellular localization of iron and BMP6 in the liver and the duodenum of mice with iron overload secondary to an iron-enriched diet or due to Hfe-deficiency. Iron deposits are vizualized by Perls' staining (1). BMP6 expression was detected by immunohistochemistry in the liver (2) and the duodenum (3). As expected, although *Bmp6*-deficient mice (A) have the highest iron accumulation, no Bmp6 was detected. C57BL/6 mice with secondary iron overload (B) and DBA/2 *Hfe*-deficient mice (C) have significant amounts of liver iron and Bmp6 in their liver but not in their duodenum. 129/Sv mice with secondary iron overload (D) have the lowest amount of liver iron and this correlates with *Bmp6* staining in the liver. Original magnification x100 (1) or x200 (2 and 3).

loaded 129/Sv mice and 5 strain-matched controls that were not fasted before they were killed. We were not able to detect any increase in Bmp6 expression in these conditions either (*data not shown*). The way the iron loading signal would be relayed to the enterocyte is not yet entirely clear and, noticeably, does not necessarily involve direct iron sensing by the enterocytes. Indeed, Arndt *et al.*[11] observed a strong induction of Bmp6 in intestinal epithelial cells not only after feeding the mice an iron-supplemented diet but also 4 or 8 h after injection of iron-dextran into the retro-orbital plexus. Once in the blood circulation, the iron-dextran complex is not excreted from the body but taken up by the liver cells and the reticuloendothelial tissues, from which iron is made available for hemoglobin formation.[22] Iron, therefore, does not transit through the potential iron-sensing enterocytes and the signal that would command enterocytes to produce BMP6 in this situation needs to be clearly identified.

Despite the lack of immunodetection with the two anti-BMP6 antibodies in the duodenum, we performed Western blot experiments as in Arndt *et al.*[11] As expected, Bmp6 protein was below detection in the duodenum of mice fed with an iron-enriched diet but a 23 kD band was detected in the liver of the iron-loaded C57BL/6 mice fed an iron-enriched diet. Surprisingly, the same 23 kD band was also observed in the liver of *Bmp6*-deficient mice and, as shown in *Online Supplementary Figure S1*, the Santa Cruz antibodies cross-react with an unknown protein whose expression correlates with iron. This specificity control was unfortunately not shown in the previous study.[11] Noticeably, these findings do not challenge our above-described immunohistochemistry data. Indeed, the respective locations of iron and Bmp6 in the liver are not overlapping and there is no Bmp6 staining in the liver of *Bmp6*-deficient mice.

In conclusion, we have confirmed by quantitative PCR and immunohistochemistry that Bmp6 mRNA and protein expression is increased in the liver of iron-overloaded mice of three genetic backgrounds. No Bmp6 staining was observed in the liver of Bmp6-deficient mice used as negative controls. We have also shown that the Santa Cruz anti-BMP6 antibodies (N-19 and S-20) are not suitable for Western blots. This emphasizes the need for controlling antibody specificity, particularly in studies aimed at assessing BMP6 circulating in the serum in order to monitor activation of the pathway leading to hepcidin production. Our data and the recently published observations on *Hjv*-deficient mice strongly support liver but not duodenum as the source of BMP6 for regulation of iron metabolism. However, production and phenotypic analysis of mice with tissue-specific Bmp6 ablation would allow definitive conclusions to be drawn.

Authorship and Disclosures

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