Severe iron deficiency blunts the response of the iron regulatory gene *Hamp* and pro-inflammatory cytokines to lipopolysaccharide

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ABSTRACT

Background
Expression of the key iron regulatory hormone hepcidin is increased by some stimuli (iron loading, inflammation) but decreased by others (increased erythropoiesis, iron deficiency). We investigated the response of hepcidin to increased erythropoiesis and iron deficiency in the presence of an acute inflammation to assess the relative strengths of these stimuli.

Design and Methods
Sprague-Dawley rats were maintained on control or iron-deficient diets and treated with lipopolysaccharide to induce inflammation or phenylhydrazine to stimulate erythropoiesis. The levels of *Hamp*, *IL-6* and *α2m* mRNA were determined by qualitative real-time polymerase chain reaction and those of serum interleukin-6 and tumor necrosis factor-α were measured by enzyme-linked immunosorbent assay. Cultured RAW264.7 and HuH7 cells were used in associated studies.

Results
The increase in hepatic hepcidin levels induced by lipopolysaccharide was not affected by phenylhydrazine treatment but was blunted by iron deficiency. Lipopolysaccharide-treated iron-deficient animals also showed lower liver *α2m* mRNA and reduced serum interleukin-6 and tumor necrosis factor-α, suggesting a more generalized effect of iron deficiency. Similarly, RAW 264.7 cells treated with iron chelators and then stimulated with lipopolysaccharide showed lower *IL-6* mRNA than cells treated with lipopolysaccharide alone. Huh7 cells treated with an iron chelator showed a blunted hepcidin response to interleukin-6, suggesting that the response of hepatic parenchymal cells to inflammatory cytokines may also be iron-dependent.

Conclusions
In any one physiological situation, net hepcidin levels are determined by the relative strengths of competing stimuli. The ability of severe iron deficiency to blunt the response to lipopolysaccharide of both hepcidin and other markers of inflammation suggests that adequate iron levels are necessary for a full acute phase response.

Key words: hepcidin, α2m, erythropoiesis, iron, inflammation.

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Introduction

Hepcidin is a liver-secreted peptide hormone that has emerged as the central regulator of body iron homeostasis. Circulating hepcidin binds to the iron export protein ferroportin, which is located on the surface of enterocytes, macrophages and other cells, and causes its internalization and degradation.[1] This in turn leads to reduced iron mobilization from these cells and, in the case of the small intestine, decreased iron absorption.[2]

Hepatic hepcidin expression is influenced by changes in body iron stores, the rate of erythropoiesis, systemic inflammation, and other stimuli.[2-4] Under normal conditions, hepcidin levels are inversely related to body iron stores, increasing in response to iron loading and decreasing with iron deficiency.[2] This feedback mechanism allows hepcidin to regulate body iron levels, increasing absorption when body iron demand is high and limiting absorption when the body is iron replete. An increase in the rate of erythropoiesis leads to a decrease in hepcidin levels, thereby increasing iron release from storage sites and iron absorption to supply the iron required for the synthesis of new red blood cells.[4-6] The regulation of hepcidin in response to changes in the rate of erythropoiesis is likely to be multifactorial, reflecting iron supply, hypoxia and possibly erythroid-specific factors. Candidates for the latter include growth differentiation factor 15 (GDF15)[7] and twisted gastrulation (TWSG1).[8] Inflammation and infection stimulate hepcidin production and this leads to iron sequestration in macrophages and a subsequent decrease in plasma iron levels.[3,9] If inflammation persists, anemia may result and this condition is called the anemia of chronic disease or the anemia of inflammation.[9]

How these various stimuli alter hepcidin expression is now at least partly understood. Bone morphogenetic proteins (BMP) appear to play a particularly important role in this process. Several members of this protein family, such as BMP 2, 4, 6 and 9, have been shown to stimulate hepcidin expression *in vitro*,[10,11] but recent studies indicate that the iron-regulated BMP6 is likely to be the most important BMP in regulating hepcidin *in vivo*.[12] The BMP act by binding to BMP receptors I and II and the co-receptor hemojuvelin (HJV) on the plasma membrane to initiate a signaling cascade that leads to phosphorylation and activation of SMAD 1/5/8.[13] These activated SMAD proteins further bind to the co-SMAD, SMAD4, and the complex translocates to the nucleus to enhance hepcidin expression.[13] A soluble form of HJV and inhibitory members of the BMP group, such as GDF15, decrease hepcidin levels, likely by inhibiting various aspects of the BMP/SMAD pathway.[7,14] Other proteins known to be involved in hepcidin regulation are the transmembrane serine protease, matriptase-2 (TMPRSS6), HFE and TfR2. TMPRSS6 inhibits hepcidin expression by cleaving HJV.[15-18] HFE and TfR2 are able to stimulate hepcidin expression, but how they do so has yet to be resolved. Mutations in HFE, TfR2, HJV and hepcidin lead to body iron loading in humans, whereas TMPRSS6 mutations lead to iron deficiency anemia.[16,17,19-21]

Inflammatory cytokines such as interleukin 6 (IL-6) stimulate hepcidin expression by activating the JAK/STAT pathway. Ligation of the IL-6 receptor leads to phosphorylation of STAT3, which then dimerizes and moves to the nucleus where it can activate *HAMP* transcription.[22,23] The finding that the BMP-SMAD and JAK-STAT pathways are interconnected in the regulation of hepcidin is particularly interesting. *Hamp* expression in SMAD4 knockout mice does not respond to IL-6 stimulation and it has been demonstrated that a functional BMP-response element in the *HAMP* promoter is required for the gene to respond to IL-6.[24,25] Taken together, these data suggest that there is cross-talk between the JAK/STAT and BMP/SMAD pathways. They also imply that the regulation of hepcidin by one stimulus may be influenced by other stimuli acting in a different manner. Moreover, in an intact organism, multiple stimuli are likely to be affecting hepcidin expression at any one time. These factors may reinforce each other or they may be antagonistic.

In the present study we examined the response of the *Hamp* gene to simultaneous stimuli.

Design and Methods

Animals, diets, treatments and tissue collection

Sprague Dawley rats were obtained from the Animal Resources Centre, Western Australia. All animals investigated were of the same sex for a given set of experiments and were 9-10 weeks of age. Both male and female rats responded similarly to the same stimuli, although the baseline iron levels of males and females differ (being higher in females) as previously described.[26,27] To induce iron deficiency, animals were maintained on a semi-synthetic iron-deficient diet (3 mg/kg wet weight) for 6 weeks before the investigations, as previously described.[2] Control rats were maintained on an iron-replete diet (159 mg/kg wet weight). Inflammation was induced by the injection of lipopolysaccharide (LPS) (0.1 mg/kg, i.p., Sigma-Aldrich, Sydney, Australia) and the rats were processed for analysis 6 hours later. This time point was chosen based on preliminary studies showing that hepcidin induction is maximal at this time. Others have reported a similar time course of the hepcidin response to LPS.[28] Erythropoiesis was stimulated by treating animals with a single dose of the hemolytic agent phenylhydrazine hydrochloride (PHZ) (100 mg/kg; i.p. injection, ICN Biomedicals Inc, Ohio, USA) 4 days before analysis.[4] Prior to analysis, animals were anesthetized (44 mg/kg ketamine and 8 mg/kg xylazine) and blood was withdrawn from the abdominal aorta for hematologic analysis and for measurement of plasma iron indices. Duodenal enterocytes were isolated as previously described[2] and snap-frozen in liquid nitrogen, as was liver tissue. All experiments described in this study were approved by the Queensland Institute of Medical Research Animal Ethics Committee.

Assessment of hematologic parameters and hepatic iron levels

Hematocrits were measured by the microcapillary method. Serum iron and transferrin saturation were measured using an Iron and Iron Binding Capacity Kit (Sigma-Aldrich). To measure hepatic iron concentration, a small amount of liver from each animal was dried overnight at 110°C, and tissue non-heme iron content determined colorimetrically as previously described.[2]

Cell culture and treatments

HuH7 and RAW 264.7 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum. Primary hepatic macrophages (Küpffer cells) were a gift from Dr Richard Ruddell (QIMR) and were isolated as previously described.[29] In some experiments, cells were pretreated for 6 h with either 20 μM desferrioxamine, 25 μM salicylaldehyde isonicotinoyl hydrazone (SIH; kindly supplied by Professor Paul Bernhardt, University of Queensland) or medium alone, before overnight incubation with IL-6 (20 ng/mL, Sigma-Aldrich) or LPS (200 ng/mL, Sigma-Aldrich).

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from tissue samples or cultured cells using TRIzol reagent (Invitrogen, Melbourne, Australia) according to the manufacturer's instructions. Total RNA (1-2 μg) was reverse transcribed with Superscript III (Invitrogen, Melbourne, Australia) to synthesize cDNA. Real-time polymerase chain reaction (PCR) was performed on this cDNA using SYBR green mix (ABI, Melbourne, Australia) in a RotorGene 3000 (Corbett Life Science, Sydney, Australia) real-time PCR machine. The data were analyzed by calculating the concentration of a sample from its C_t value using a standard curve on the RotorGene software. The primers used for *Hamp* were GCTGCCTGTCTCCTGCTTCT and CTGCAGAGCCGTAGTCTGTCTCGTC; while those for the inflammatory marker α2-macroglobulin (α2m) were TTGCTGACGTGAAGATGGTG and GCTGAACCGTGA AGGACAAG. The expression data were normalized to the expression of basic transcription factor 3 (BTF3) (primers TGGCAGCAAACACCTTCACC and AGCTTC AGCCAGTCTCCTCAAAC) for rats and to GAPDH for the cell lines (primers GAAGATGGTGATGGGATTTC and GAAGGTGAAGGTCGGAGTC).

Western blot analysis

The cells were washed with 1x phosphate-buffered saline and then lysed in 1x SDS sample buffer [62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue] and western blotting was carried out according to the manufacturer's recommendations. A rabbit polyclonal antibody to phospho-NFκB p65 (1 in 1000 dilution; Cell Signaling Technology) was used to assess NFκB activation. Total NFκB p65 (1 in 1000 dilution; Cell Signaling Technology) was used to assess the proportion of activated p65.

Interleukin-6 and tumor necrosis factor-α measurements

Enzyme-linked immunosorbent assays (ELISA) for IL-6 and TNFα (R&D Systems, Minneapolis, USA) were performed on serum according to the manufacturer's instructions.

Statistical analysis

The results were analyzed using ANOVA with Tukey's post-hoc test. *P* values below 0.05 were considered to be statistically significant. The results are presented as mean ± standard error of mean (SEM).

Results

Responses of Hamp and α2m to an inflammatory stimulus are not affected by stimulated erythropoiesis

Enhanced erythropoiesis secondary to hemolysis has been shown to repress hepcidin expression[4] whereas inflammation is known to induce it.[3] We first examined the net effect of increased erythropoiesis (following PHZ-induced hemolysis) and inflammation on hepcidin expression in rats. Data from female rats are shown. Four days after PHZ treatment, animals demonstrated lower hematocrits than vehicle-treated controls (Table 1) and a profound reticulocytosis (*data not shown*). Serum iron levels could not be determined reliably in heavily hemolysed samples. The hepatic iron concentration of PHZ-treated animals was significantly higher than that of control rats ($P<0.001$) (Table 1), consistent with hepatic scavenging of hemoglobin and heme-derived iron following hemolysis.[4] LPS treatment had no significant effect on hematocrit or hepatic iron concentration (Table 1).

Hepatic *Hamp* mRNA levels were 7-fold lower in PHZ-treated animals than in saline-treated control rats, whereas animals injected with LPS alone showed a 4.2-fold increase in *Hamp* expression ($P<0.001$) (Figure 1A). α2m, a marker of inflammation, showed a 60-fold increase following LPS treatment ($P<0.001$), but was unchanged after PHZ administration (Figure 1B). Rats treated with PHZ to induce hematopoiesis, then 4 days later treated with LPS for 6 h, showed significantly higher *Hamp* levels (4.2 fold; $P<0.001$) than controls, and this was not significantly different from the level reached in rats treated with LPS alone ($P=0.339$) (Figure 1A). Similarly, the induction of *α2m* mRNA expression in animals treated with PHZ and LPS was not significantly different from that of rats treated with LPS alone (Figure 1B). These data suggest that hepcidin response to LPS is not inhibited by induction of erythropoiesis by a relatively high dose of PHZ.

Iron deficiency blunts the Hamp and α2m responses to lipopolysaccharide

Chronic iron deficiency leads to a decrease in hepcidin levels whereas an inflammatory stimulus stimulates hepcidin expression. We, therefore, investigated the effects of an inflammatory stimulus (LPS injection) on *Hamp* expression in the setting of chronic iron deficiency in rats. Data from male rats are shown. After being maintained on an iron-deficient diet for 6 weeks, rats had significantly lower hematocrits ($P<0.001$), serum iron levels ($P<0.001$) and hepatic iron concentrations ($P<0.001$) than animals on an iron-replete diet (Table 2). LPS treatment caused a significant decrease in serum iron in animals on the control diet ($P<0.01$), however, there was no additional effect of LPS in iron-deficient rats as these animals already had very low serum iron levels. There was no significant effect of LPS administration on hepatic iron concentration (Table 2). Although the difference was not significant, serum and liver iron levels were slightly higher in LPS-treated iron-deficient animals than in iron-deficient animals alone. It has previously been shown that LPS can predispose erythrocytes to hemolysis[30] and it is possible that such an effect is being observed here as hemolysis would be expected to raise both serum and hepatic iron.

In rats maintained on an iron-deficient diet for 6 weeks, *Hamp* levels were virtually undetectable and were at least 1000-fold lower than those in animals on a control diet (Figure 2A). Treatment of rats on an iron-replete diet with LPS for 6 h led to a 5.5-fold increase in *Hamp* relative to the level in saline-treated animals ($P<0.001$). When rats that had been maintained on an iron-deficient diet for 6 weeks were treated with LPS, *Hamp* expression also increased significantly (a precise fold induction is difficult to give as basal hepcidin levels were so low), but the level reached remained significantly lower than that achieved in animals on a control diet (Figure 2A). These data suggest that an adequate iron level is essential for *Hamp* to respond fully to LPS. To investigate this further, we demonstrated that the mRNA encoding the inflammatory marker α2m also showed a less robust induction in response to LPS in iron-deficient animals than in control animals ($P<0.001$) (Figure 2B). Iron deficiency alone did not alter *α2m* mRNA levels (Figure 2B). This latter result suggests that the observed effects on *Hamp* do not simply represent the superimposition of the iron deficiency and LPS treatments and that there is a specific role for iron in the response to LPS.

Iron deficiency inhibits lipopolysaccharide-induced TLR signaling

LPS is a ligand for TLR4 and initiates signaling cascades that lead to an increase in the secretion of pro-inflammatory cytokines such as IL-6 and TNFα by macrophages and other cells. To examine the effects of iron deficiency on the action of LPS further, we measured serum IL-6 and TNFα levels by ELISA in each of our treatment groups. The levels of the two cytokines did not change significantly in animals on an iron-deficient diet, relative to the levels in controls; however, following LPS treatment, the concentrations of both IL-6 and TNFα were significantly lower in animals on an iron-deficient diet than in those on a control diet (Figure 3A, B). Consistent with this result, we found that RAW264.7 cells (a macrophage cell line) treated with iron chelators showed a blunted *IL-6* mRNA response following LPS treatment relative to cells treated with LPS alone (Figure 4).

The production of IL-6 and TNFα in response to LPS has been shown to be mediated by activation of the NFκB pathway.[31,32] In primary hepatic macrophages stimulated with LPS, we found impaired activation of NFκB (as assessed by determining the level of phosphorylated p65) after prior treatment with the iron chelator desferrioxamine (Figure 5). These results are consistent with previously published data showing that hepatic macrophages with increased iron content had increased NFκB activation and enhanced expression of pro-inflammatory genes such as *IL6*,[33,34] and also with reports that iron chelation reduces the DNA binding activity of NFκB.[32] Taken together, these findings suggest that animals and cells deficient of iron have reduced TLR4 signaling and a blunted inflammatory response to LPS that indirectly contributes to lower hepcidin levels.

Iron deficiency ameliorates interleukin-6-dependent stimulation of Hamp *expression in liver-derived Huh7 cells*

The data presented above demonstrate that one mechanism by which iron deficiency ameliorates the *Hamp* response to LPS is through reduced expression of pro-inflammatory cytokines such as IL-6. It is also feasible that iron depletion has additional roles and reduces the response of hepatic parenchymal cells to IL-6. We investigated this in HuH7 cells, a human hepatoma cell line. IL-6 has been shown to signal via the JAK/STAT pathway to increase hepcidin levels in hepatocytes. Consistent with published data, *Hamp* mRNA levels were increased in IL-6-treated Huh7 cells (Figure 6A). However, in the presence of the iron chelator desferrioxamine, the *Hamp* response to IL-6 was reduced (Figure 6A). LPS itself did not have a direct stimulatory effect on hepcidin expression in these cells, whereas desferrioxamine treatment alone reduced hepcidin expression (Figure 6A). To ensure that desferrioxamine was depleting iron in these cells, we measured the expression of *TfR1* mRNA and found it to be increased as expected (Figure 6B). These data suggest that iron deficiency could also contribute directly to lower hepcidin levels in the hepatocytes.

Discussion

In this study we investigated the interactions between various systemic stimuli that regulate hepcidin. Different

Table 1. Hematocrit and liver iron levels in 9-10 week old female rats treated with a single dose of PHZ (100 mg/kg i.p.) and/or LPS (0.1 mg/kg i.p) 4 days and 6 h respectively, prior to euthanasia.

	Control	LPS	PHZ	PHZ + LPS
Hematocrit	0.408±0.004 (n=11)	0.428±0.008 (n=11)	0.206±0.010 (n=10)	0.183±0.005 (n=7)
Liver iron (μg/g dry wt)	865.32±46.84 (n=5)	930.16±61.87 (n=5)	1334.60±57.00 (n=4)	1300.52±39.16 (n=4)

Data are presented as mean ± SEM and group sizes varied from 4-11 animals as indicated.

Table 2. Hematocrit, serum iron and liver iron levels in 9-10 week old male rats on control or iron-deficient diets treated with a single dose of LPS (0.1 mg/kg i.p) 6 h prior to euthanasia.

	Control	LPS	Def	Def+LPS
Hematocrit	0.449±0.010 (n=13)	0.448±0.006 (n=14)	0.231±0.012 (n=12)	0.243±0.013 (n=12)
Serum iron (μmol/L)	30.42±2.02 (n=14)	17.40±3.15 (n=14)	4.43±0.52 (n=15)	10.82±2.36 (n=15)
Liver iron (μg/g dry wt)	432.42±26.89 (n=14)	420.14±21.41 (n=14)	118.90±7.39 (n=13)	161.54±14.29 (n=14)

Data are presented as mean ± SEM and group sizes varied from 12-15 animals as indicated.

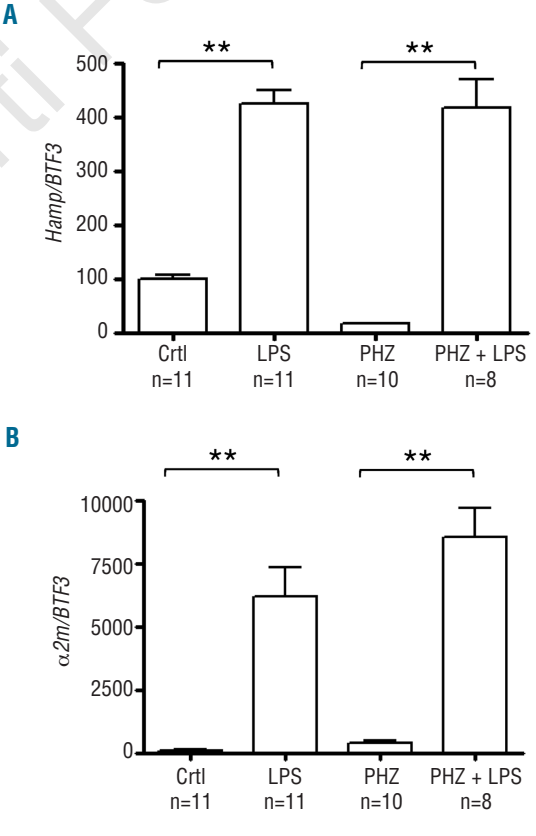

Figure 1. Hepcidin and α2m response to LPS is not affected by an increase in the rate of erythropoiesis. Expression of hepcidin (A) and α2m (B) mRNA was measured by quantitative real-time PCR in the livers of 9-10 week old female rats. Animals were treated with either LPS (0.1 mg/kg i.p.) or PHZ (100 mg/kg i.p.) or both 4 days and 6 h, respectively, prior to euthanasia. Data are presented as mean ± SEM and group sizes varied from 8-11 animals as indicated. ***P*<0.001.

stimuli modulate hepcidin expression in different ways, and the final hepcidin level is determined by a combination of various stimuli acting simultaneously. We demonstrated that acute inflammation can induce hepcidin expression even in the presence of increased erythropoiesis, indicating that the inflammatory regulator is able to override the erythropoiesis regulator. In contrast, chronic iron deficiency was able to ameliorate the inflammatory response and reduce hepcidin expression. This effect appears to be mediated, at least in part, through iron depletion interfering with the NFκB signaling pathway.

The major stimuli that alter hepcidin expression are now well delineated. Increased body iron stores and inflammation can increase hepcidin levels, while iron deficiency, increased erythropoiesis and hypoxia can reduce levels of this iron regulatory hormone.[2-4] Understanding how these factors interact to influence the net hepcidin response requires knowledge of the molecular pathways by which they affect *HAMP* gene transcription. There have been significant advances in this area in recent years, and the BMP/SMAD and JAK/STAT signaling pathways have emerged as being particularly important in controlling hepcidin expression.[13,22,23] The former appears to be required for the response of hepcidin to changes in iron stores and erythropoiesis, while the latter is necessary for hepcidin to respond to inflammatory stimuli.

The erythropoietic response of hepcidin is multifactorial, reflecting hypoxia, limitations in iron supply and erythroid-specific factors, but the overall control mechanism likely depends on BMP/SMAD signaling. In contrast, inflammatory stimuli increase cytokine IL-6 expression and this in turn activates the JAK/STAT pathway to induce *HAMP* transcript levels.[22,23] However, in SMAD4 knockout mice, IL-6 has little effect on hepcidin expression, indicating that the BMP/SMAD pathway is also required for an inflammatory response.[25] The interaction between the JAK/STAT and the BMP/SMAD pathways is also evident from the demonstration that soluble HJV, which interacts with BMP receptors to displace membrane-bound HJV and thereby inhibit SMAD activation, inhibits IL-6 stimulated hepcidin expression.[14] Similarly, the BMP inhibitor protein noggin can inhibit IL-6-induced expression of hepcidin in hepatocytes,[13] and dorsomorphin, a specific SMAD1/5/8 inhibitor, has also been reported to abolish the hepcidin response to IL-6.[35] Not surprisingly, mutation of the BMP-response element in the hepcidin promoter impairs hepcidin activation in response to IL-6, indicating that an intact BMP/SMAD signaling pathway is required for hepcidin to respond appropriately to inflammation.[24]

Inflammation is a very strong inducer of hepcidin

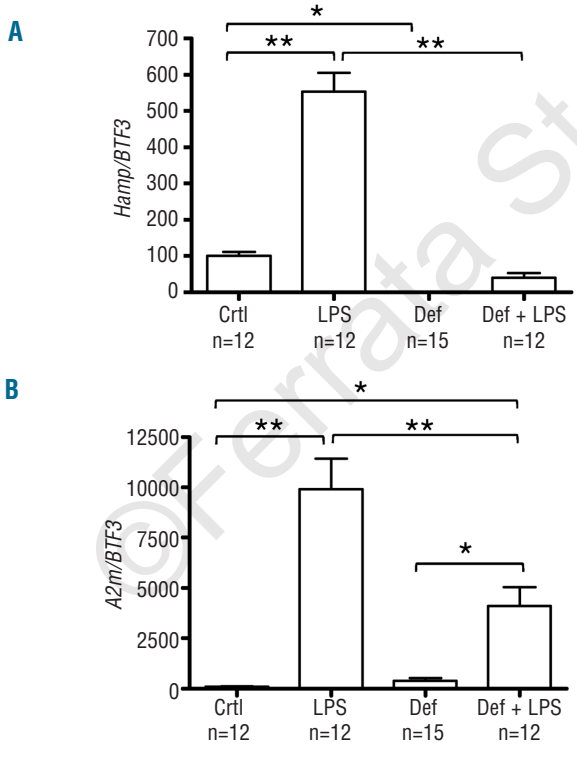

Figure 2. Iron deficiency blunts the hepcidin and α2m response to LPS. Expression of hepcidin (A) and α2m (B) mRNA was measured by quantitative real-time PCR in the livers of 9- to 10-week old male rats. Animals were fed for 6 weeks with either an iron-replete diet (159 mg/kg) or iron-deficient diet (2-3 mg/kg)(to make them iron deficient) and then treated with LPS (0.1 mg/kg i.p.) 6 h prior to euthanasia. Data are presented as mean ± SEM and group sizes varied from 12-15 animals as indicated. *P<0.05 and **P<0.001.

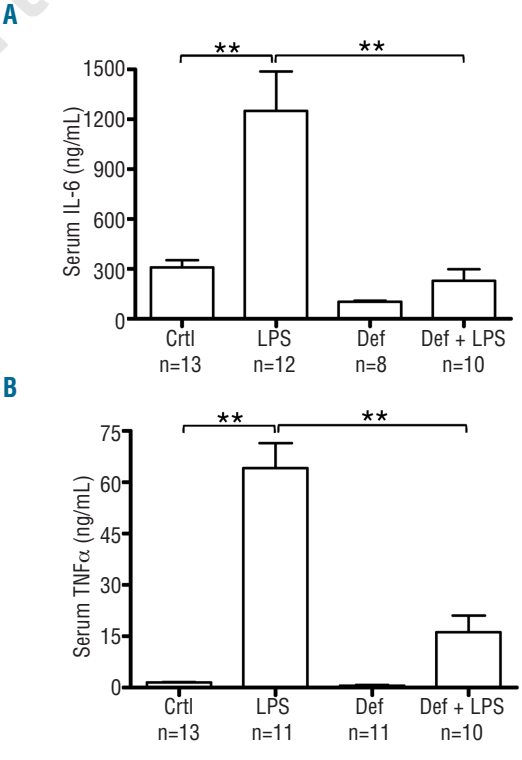

Figure 3. Iron deficiency reduces cytokine levels in response to LPS treatment in rats. Serum IL-6 (A) and TNF-α (B) levels were determined by ELISA in male rats that were fed for 6 weeks with either an iron-replete diet (159 mg/kg) or iron-deficient diet (2-3 mg/kg) and then treated with LPS (0.1 mg/kg i.p.) 6 h prior to euthanasia. Data are presented as mean ± SEM and group sizes varied from 8-13 animals as indicated. **P<0.001.

expression. In our studies, we found that the increase in hepcidin expression following LPS treatment was not affected by quite a strong erythropoietic stimulus, the response to PHZ-induced hemolysis. The hematologic changes induced by this agent were of a similar magnitude to those observed in humans with severe anemia. Hemolysis alone was able to depress *Hamp* mRNA levels significantly in our study. These data suggest that despite the negative influence of enhanced erythropoiesis, the BMP/SMAD pathway remained sufficiently active to allow normal LPS-dependent JAK/STAT signaling. In related studies in mice, Santos *et al.* similarly showed that the increase in erythropoiesis accompanying hypoxia was insufficient to block an LPS-induced increase in hepcidin expression, even though hypoxia alone significantly reduced hepcidin levels.[36] In contrast, when erythropoiesis was increased by four consecutive days of treatment with supraphysiological doses of erythropoietin, the hepcidin response to LPS was blunted, although not abolished.[36] The same group also found that the capacity of erythropoietin to reduce the LPS stimulation of hepcidin was dose-dependent, with smaller doses having relatively little effect.[36] Overall these studies suggest that if the erythropoietic stimulus is strong enough and prolonged, the effect of a concomitant inflammatory response can be blunted. This makes sense physiologically. In acute inflammatory situations the primary response is one of high hepcidin and iron retention by the tissues. This serves to reduce the amount of circulating iron that could act as a virulence factor for invading pathogens or lead to tissue injury by promoting oxidative damage. However, in chronic inflammatory situations the impaired iron supply can lead to iron-restricted erythropoiesis. Since iron is essential for the production of new red blood cells, when the erythropoietic demand increases sufficiently the influence of the inflammatory stimulus will be at least partially overridden to ensure some iron supply to the bone marrow.

A somewhat different outcome was observed when we examined the effects of chronic iron deficiency on the response of hepcidin to inflammation. In this case, systemic iron deficiency ameliorated the acute inflammatory induction of hepcidin in response to LPS. LPS still had a strong effect on hepcidin expression, but the maximum level achieved remained significantly lower in iron-deficient rats than in those on an iron-replete diet. Constante *et al.* have also examined this situation and, in contrast to us, they observed similar levels of hepcidin in LPS-treated control and iron-deficient animals.[37] There are several possible explanations for this apparent discrepancy, but the most likely is the relative strengths of the different stimuli involved. The dose of LPS used in the study by Constante *et al.* was very high (5 mg/kg) compared to the much lower dose (0.1 mg/kg) used in our study. The level of LPS we used is similar to levels achieved in human patients with septicemia and thus more closely approximates the levels found *in vivo*.[38] In addition, the rats we studied were more iron-deficient than the mice investigated by Constante *et al.* Thus the relative strength of the signal to reduce hepcidin expression was much greater in our investigations.

The iron dependency of the LPS response appears to manifest itself at two levels. The first is the response of macrophages to LPS. LPS activates the NFκB pathway after binding to its receptor, TLR4, on monocytes and macrophages.[39] The presence of iron is critical for NFκB activation and it has been shown previously that iron supplementation accentuates activation of NFκB and induction of NFκB responsive genes such as those for IL-6 and TNFα.[32-34] Consistent with these studies, we found that serum IL-6 and TNFα levels were hypo-responsive to LPS treatment in iron-deficient animals, and earlier investigations by others have reported low levels of pro-inflammatory cytokines in iron-deficient individuals.[40] Similarly, macrophages treated with iron chelators demonstrated

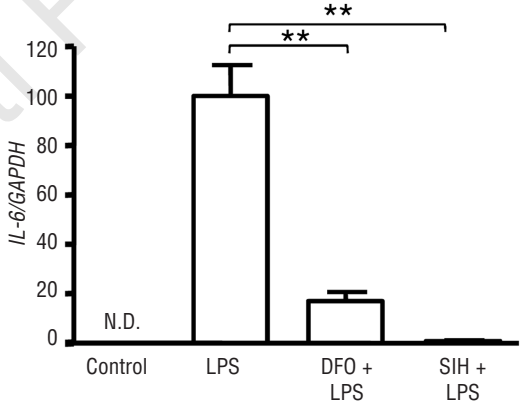

Figure 4. Iron chelation decreases the IL-6 level in cells in response to LPS treatment. *IL-6* mRNA levels in RAW 264.7 cells were determined by quantitative real-time PCR after pretreatment with 20 μM desferrioxamine (DFO), 25 μM salicylaldehyde isonicotinoyl hydrazone (SIH) or medium alone for 6 h followed by overnight incubation with LPS (200 ng/mL). Treatments were carried out in triplicate and data are presented as mean ± SEM. ***P*<0.001. ND= none detected.

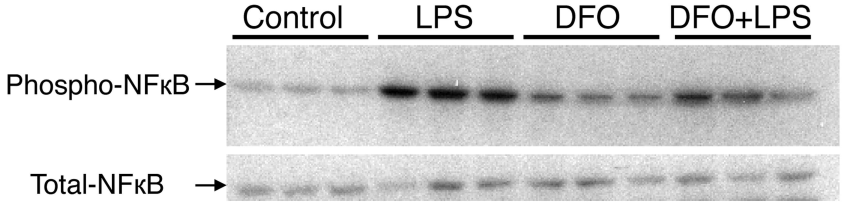

Figure 5. Iron chelation reduces NFκB activation in primary hepatic macrophages. Freshly isolated rat primary hepatic macrophages (Küpffer cells) were treated for 6 h with 20 μM desferrioxamine (DFO) or medium alone then incubated overnight with LPS (200 ng/mL). Cell extracts were prepared as indicated in the *Design and Methods* section, subjected to polyacrylamide gel electrophoresis and western blotted for phospho-NFκB p65 and total NFκB p65. Triplicate samples are shown.

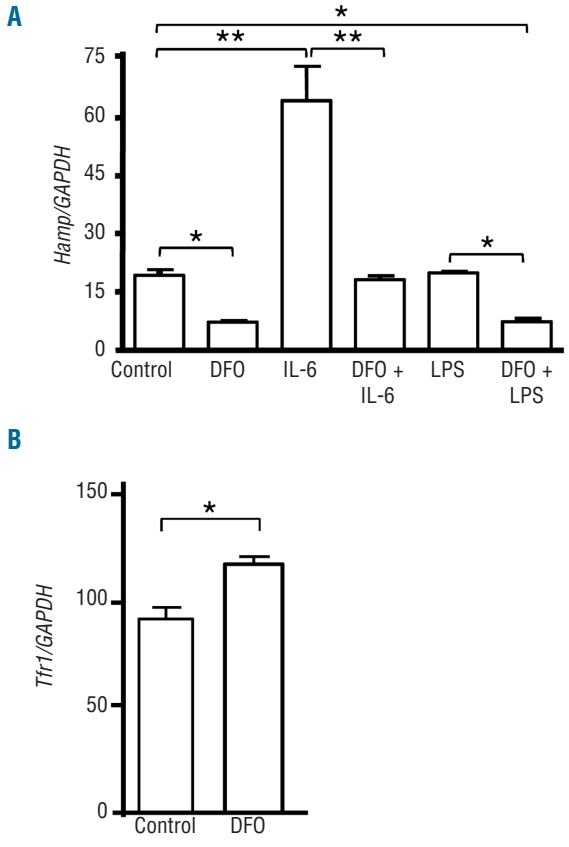

Figure 6. Iron deficiency lowers IL-6 activity and increases TfR1 levels in liver-derived Huh7 cells. (A) Hepcidin mRNA levels were measured in Huh7 cells by quantitative real-time PCR. The cells were left untreated or treated with IL-6 (20 ng/mL) or LPS (200 ng/mL) for 6 h prior to harvest and analysis. Some cells were pretreated with desferrioxamine (DFO; 20 μM) for 6 h prior to IL-6 or LPS treatment. (B) Cells treated with DFO also showed an increase in TfR1 levels due to relative iron depletion. Treatments were carried out in triplicate and data are presented as mean ± SEM. *P<0.05 and **P<0.001.

lower IL-6 transcript levels and lower NFκB activation when challenged with LPS compared to cells treated with LPS alone.[33] These data indicate that iron deficiency leads to a decrease in the general acute inflammatory response *in vivo*, so it is not surprising that the general inflammatory marker *α2m* also responded less robustly in iron-deficient animals treated with LPS. Overall, these data suggest that the reduced response of hepcidin to LPS in iron-deficient animals is in part due to iron deficiency in reticuloendothelial cells that leads to decreased TLR signaling and decreased production of inflammatory cytokines. A recent study by Wang *et al.* on the relative susceptibility of *Hfe* knockout mice to infection reached a similar conclusion. These mice have relatively low iron levels in their macrophages and the cells showed an impaired TLR4, but not TLR3, response.[41]

The other site where iron deficiency could affect hepcidin expression is in hepatocytes. We demonstrated that pre-treatment of the hepatoma cell line HuH7 with the iron chelator desferrioxamine led to decreased hepcidin levels in response to IL-6 compared to cells treated with IL-6 only. That desferrioxamine treatment reduced cellular iron levels was demonstrated by an increase in *TfR1* mRNA. These results need to be interpreted with caution as studies to investigate the regulation of hepcidin by iron *in vitro* have met with limited success; however, the lowering of *Hamp* expression with iron depletion is entirely consistent with what is observed *in vivo*. Thus our results are consistent with iron-dependency of the IL-6 response. The signaling pathways by which iron is sensed by the hepcidin regulatory machinery have not yet been completely elucidated, but current thinking suggests BMP6 plays a key role in this pathway.[12] Our data are entirely consistent with the proposal that iron does indeed signal through the BMP/SMAD system, and that an intact BMP/SMAD pathway is required for a response to IL-6, but further studies are required to investigate the mechanism by which desferrioxamine exerts its effects in this system. Since the expression of BMP6 is iron dependent,[42] it is possible that iron deficiency could exert its effects, at least partially, through a reduction in BMP6 levels.

Taken together, the data in this study and those from the literature indicate that the relative strengths of the various stimuli that alter hepcidin expression and the directions of these regulatory events combine to determine the net expression of this important regulatory peptide. Inflammation is a very strong inducer of hepcidin, but even its effects can be counteracted to some degree by iron deficiency and enhanced erythropoiesis. In the experimental setting the stimuli that are applied to alter hepcidin levels are relatively strong (e.g. a strong inflammatory stimulus or severe iron deficiency), so *in vivo* the interplay of milder interactions is likely to be very important. An example of such competing stimuli *in vivo* comes from the analysis of patients suffering from β-thalassemia (or mice with this condition). Such individuals initially have low hepcidin levels due to a high rate of erythropoiesis, but as iron stores accumulate with time, hepcidin levels increase and iron absorption drops in spite of the continued high rate of erythropoiesis.[43] As our understanding of the molecular basis of hepcidin regulation becomes clearer, so will our understanding of what determines the final hepcidin concentration in such complex *in vivo* situations.

Authorship and Disclosures

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