Circulating iron levels influence the regulation of hepcidin following stimulated erythropoiesis

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ABSTRACT

The stimulation of erythrocyte formation increases the demand for iron by the bone marrow and this in turn may affect the levels of circulating diferric transferrin. As this molecule influences the production of the iron regulatory hormone hepcidin, we hypothesized that erythropoiesis-driven changes in diferric transferrin levels could contribute to the decrease in hepcidin observed following the administration of erythropoietin. To examine this, we treated mice with erythropoietin and examined diferric transferrin at various time points up to 18 hours. We also investigated the effect of altering diferric transferrin levels on erythropoietin-induced inhibition of *Hamp1*, the gene encoding hepcidin. We detected a decrease in diferric transferrin levels 5 hours after erythropoietin injection and prior to any inhibition of the hepatic *Hamp1* message. Diferric transferrin returned to control levels 12 hours after erythropoietin injection and had increased beyond control levels by 18 hours. Increasing diferric transferrin levels via intravenous iron injection prevented the inhibition of *Hamp1* expression by erythropoietin without altering hepatic iron concentration or the expression of *Erfe*, the gene encoding erythroferrone. These results suggest that diferric transferrin likely contributes to the inhibition of hepcidin production in the period shortly after injection of erythropoietin and that, under the conditions examined, increasing diferric transferrin levels can overcome the inhibitory effect of erythroferrone on hepcidin production. They also imply that the decrease in *Hamp1* expression in response to an erythropoietic stimulus is likely to be mediated by multiple signals.

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Introduction

Hepcidin is a key regulator of body iron homeostasis. This 25-amino acid peptide is produced predominantly by hepatocytes and is secreted into the circulation where it binds to the iron export protein ferroportin on the surface of body cells.[1] This interaction causes the ferroportin/hepcidin complex to be internalized and degraded, inhibiting iron release and allowing hepcidin to regulate critical processes such as dietary iron absorption and the recycling of erythrocyte iron by macrophages.

The production of hepcidin is tightly regulated. The main stimuli triggering changes in hepcidin synthesis are the level of iron in the body, referred to as the stores regulator, and the adequacy of iron supply to the erythroid marrow, termed the erythroid regulator.[2,3] The molecular basis of the stores regulator has been relatively well characterized. As body iron stores increase, non-parenchymal cells in the liver secrete bone morphogenetic protein 6 (BMP6), which binds to the BMP receptor complex on the surface of hepatocytes and stimulates hepcidin expression by activating the SMAD signaling pathway.[4-6] This increase in hepcidin production inhibits dietary iron absorption, limiting further increases in body iron stores, and tissue iron release, and resulting in the storage of excess iron within macrophages and hepatocytes. Various other molecules, such as hemojuvelin and transmem-

brane protease, serine 6 modulate BMP/SMAD signaling and refine hepcidin production, allowing the precise regulation necessary to maintain iron homeostasis.[4,7-9]

The influence of the erythroid regulator is readily apparent in conditions such as β-thalassemia, in which the increased iron demands of the expanded erythroid marrow signal a decrease in hepcidin production and subsequent iron loading.[10] However, the molecular mechanism by which developing erythrocytes signal their iron needs to hepatocytes remains poorly understood. One signaling molecule that has recently been identified is erythroferrone, a member of the tumor necrosis factor superfamily of cytokines which is encoded by the *ERFE* gene.[11] Erythroferrone is produced by erythroblasts[11] and is detectable in the circulation following stimulated erythropoiesis.[12] Importantly, recombinant erythroferrone has been shown to inhibit hepcidin production in both primary hepatocytes and in mice.[11] In addition, the inhibition of hepcidin by stimulated erythropoiesis is blunted in *Erfe* knockout mice and hepcidin expression normalizes in β-thalassemic animals lacking erythroferrone,[11] providing strong evidence supporting a role for erythroferrone in hepcidin regulation.

There is clear evidence that erythroferrone can influence hepcidin expression in response to stimulated erythropoiesis, but the level of iron in the circulation has also been proposed to be involved.[13-15] Iron in the plasma is predominantly bound to the protein transferrin.[16] As each transferrin molecule can bind two iron atoms and physiological levels of circulating iron are not high enough to saturate all transferrin binding sites, circulating transferrin can exist in four different states: apo-transferrin; two forms of monoferric transferrin; and diferric transferrin.[17] Iron bound to transferrin is taken up by cells via receptor-mediated endocytosis following the binding of transferrin to cell surface transferrin receptor 1 (TFR1).[16] By far the largest sink for transferrin-bound iron in the circulation is the developing erythrocytes of the bone marrow[10] and, as TFR1 has the greatest affinity for the diferric isoform,[16] increases in the erythropoietic rate would be expected to preferentially reduce circulating diferric transferrin levels. Indeed, we have previously demonstrated such a change following treatment with the hemolytic agent phenylhydrazine in rats.[18] As diferric transferrin has been implicated in the regulation of hepcidin expression,[13,19] changes in diferric transferrin levels following a stimulus to increase erythropoiesis could augment the effect of erythroferrone and play a role in the inhibition of hepcidin production. Such a mechanism has been suggested by Nai *et al.* to explain the reduction in hepcidin expression following the administration of erythropoietin to mice.[14] While Kautz *et al.* saw no change in serum iron levels in mice injected with erythropoietin, they did not specifically examine diferric transferrin.[11] In order to determine whether diferric transferrin might contribute to the decrease in hepcidin expression that occurs following stimulated erythropoiesis, we examined its level in erythropoietin-injected mice. We also examined the effect of altering diferric transferrin levels in mice following stimulated erythropoiesis.

Methods

Animals

Six-week old male C57BL/6 mice obtained from the Animal Resources Centre (Perth, Australia) were used for all experiments and were maintained on a standard rodent chow (120 mg/kg iron, Norco Stockfeed, Lismore, Australia). To stimulate erythropoiesis, mice were intravenously administered 10 U/g body weight of human erythropoietin (Epoetin alfa, Eprex, Janssen, Macquarie Park, Australia). The mice were euthanized 0, 5, 9, 12, 15 or 18 h later. Prior to euthanasia, mice were anesthetized (200 mg/kg ketamine, 10 mg/kg xylazine) and blood was withdrawn by cardiac puncture. Blood for diferric transferrin quantitation was collected in heparin-coated tubes, briefly centrifuged, and the plasma stored at -80°C. Blood for serum iron determination was allowed to clot and the serum stored at -80°C. Following euthanasia, the liver, spleen and bone marrow were removed and snap frozen for subsequent analysis.

To study the effects of altering serum iron on erythropoietin-induced inhibition of hepcidin expression, four groups of mice were used. Two groups were intravenously injected with erythropoietin (10 U/g body weight) 9 h prior to euthanasia, while the remaining two groups served as uninjected controls. Of the two groups injected with erythropoietin, one group was administered iron intravenously (2.5 μg/g body weight ferric citrate monohydrate in 5 mM citrate buffer, pH 7.0) while the other group was injected with sodium citrate (equimolar with the citrate in the ferric citrate solution) in 5 mM citrate buffer as a control. Similar injections were administered to the two groups not given erythropoietin. All ferric citrate or sodium citrate injections were given 4 h prior to euthanasia. To confirm that the injected iron led to an increase in circulating diferric transferrin, additional groups of mice were euthanized 5 min after the iron or citrate injections and blood was collected for analysis.

To control for circadian variations in serum iron levels and the expression of *Hamp1*, the gene encoding hepcidin, all experimental animals were euthanized between 10:00 am and 12:30 pm local time. We did not observe any consistent alterations in hepatic *Hamp1* expression during this period.

Male mice were used in all studies as differences in the absolute levels of some iron parameters have been reported in males and females.[20] However, the mechanisms regulating iron homeostasis and hepcidin expression are thought to be similar in both genders. All animal experiments were approved by the QIMR Berghofer Animal Ethics Committee.

Analysis of blood and tissue samples

Details of the analysis of serum iron levels, liver iron concentration and gene and protein expression are included in the *Online Supplementary Methods*.

Statistics

All results are expressed as mean ± standard error of the mean (SEM). Statistical differences between groups were calculated using ANOVA followed by either Tukey *post hoc* testing for samples with equal variance or Games-Howell *post hoc* testing for samples with unequal variance. IBM SPSS Statistics version 22 software (IBM Australia, St Leonards, Australia) was used. A *P* value of less than 0.05 was considered statistically significant.

Results

Reduced hepatic *Hamp1* expression is associated with increased splenic and bone marrow *Erfe* production following injection of erythropoietin

Previous studies in mice have implicated erythroferrone in the inhibition of hepcidin production following administration of erythropoietin.[11,12] Other factors, such as the level of diferric transferrin in the circulation, might also

play a role. To examine this, we established a model of erythropoietin-induced hepcidin inhibition. In this model, we ensured that all mice were euthanized within a narrow window of time to ensure that known circadian variations in both hepatic *Hamp1* expression and serum iron levels[21,22] did not influence the results.

In agreement with findings of previous studies,[11,14] hepatic *Hamp1* levels progressively decreased following erythropoietin administration, with significant inhibition first observed after 9 h (Figure 1A). By 18 h, *Hamp1* expression was only 12% of the control value. Only minor changes in spleen weight were observed (Figure 1B), possibly because the time points examined were too soon after erythropoietin injection for significant increases in the number of erythroid precursors to have occurred. Despite this, a large increase in *Erfe* expression (>86-fold) was seen in both the spleen (Figure 1C, D) and bone marrow (Figure 1E, F) of injected animals at the 5 h time point. This increase in *Erfe* expression was observed at the 5, 9, 12 and 15 h time points, but splenic levels had reduced by 18 h after erythropoietin injection. Normalizing *Erfe* expression to the general housekeeper *hypoxanthine guanine phosphoribosyl transferase (Hprt)* or the erythroid precursor specific marker *glycophorin A (Gypa)* did not alter the expression pattern seen. Our results agree with those of previous studies[11,14] and confirm that our model can be used to examine the potential role of diferric transferrin in erythropoietin-induced hepcidin inhibition.

Erythropoietin administration increases *Tfr1* expression in the bone marrow and spleen and causes a transient decrease in circulating diferric transferrin levels

Developing erythrocytes are the major sink for circulating iron and this iron demand is increased when erythropoiesis is stimulated.[10] To determine how rapidly this increase in demand occurs, we examined the expression of the *Tfr1* gene, the product of which is central to the main cellular iron import pathway in developing erythrocytes.[10] In the bone marrow, *Tfr1* expression increased rapidly after erythropoietin administration, showing maximal expression at 5 h when normalized to either *Hprt* (Figure 2A), or the erythroid-specific housekeeper, *Gypa* (Figure 2B). The increase in splenic *Tfr1* was not as pronounced, with significant increases only observed when its levels were normalized to those of *Gypa* (Figure 2C,D). These results indicate that there is a very rapid increase in the iron demands of developing erythroid cells following erythropoietin administration, and imply that iron is removed from the circulation more rapidly than normal,

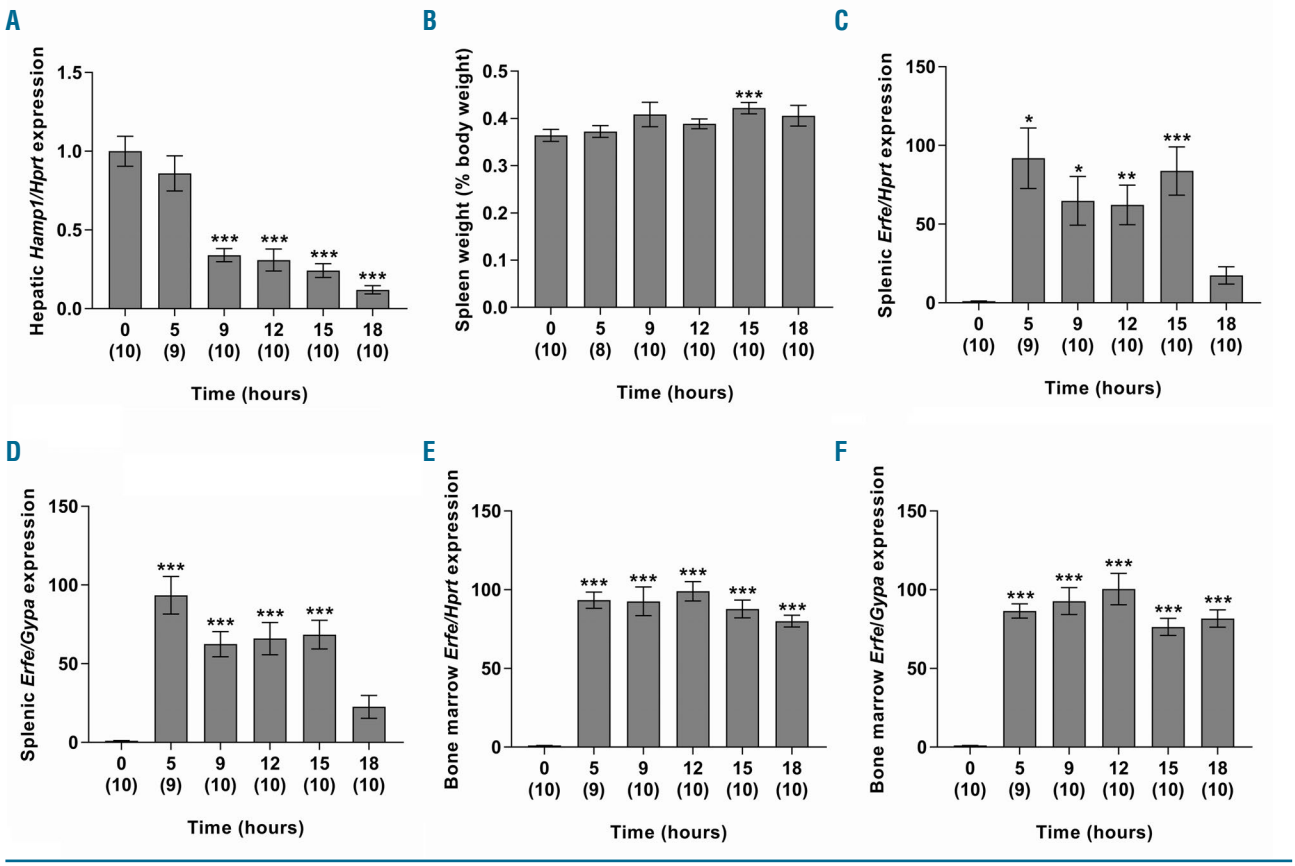

Figure 1. *Hamp1* and *Erfe* expression following erythropoietin injection in mice Six-week old male C57BL/6 mice were euthanized 0, 5, 9, 12, 15 or 18 h following the intravenous injection of 10 U/g body weight human erythropoietin and tissues were taken for analysis. Hepatic *Hamp1* expression (A), spleen weight (B), splenic *Erfe* expression (C, D) and bone marrow *Erfe* expression (E, F) were determined for each time point. Gene expression levels were calculated relative to either the general housekeeping gene *Hprt* or the erythroid specific marker *Gypa*, and are expressed as a proportion of the values at 0 h. The data represent the mean ± SEM with the number of mice in each group indicated in parentheses along the x-axis. Statistical significance is shown relative to the 0 h group. **P*<0.05; ***P*<0.01; ****P*<0.005.

possibly leading to reduced diferric transferrin levels.

To determine whether circulating iron levels were indeed reduced by the increase in erythroid *Tfr1* expression following erythropoietin injection, we examined total serum iron and transferrin saturation at each time point. While there was a small decrease in both serum iron (Figure 3A) and transferrin saturation (Figure 3B) at 5 h, these changes were not significantly different from control values. However, TFR1 preferentially takes up diferric transferrin,[16] and so any increase in TFR1-mediated iron uptake due to enhanced erythropoiesis would preferentially affect diferric transferrin levels. We hypothesized that the small changes in circulating iron levels seen (Figures 3A, B) might reflect larger changes in the level of circulating diferric transferrin. To test this hypothesis, we examined the various transferrin species by western blotting. Diferric transferrin is expressed as a proportion of total transferrin, so we first confirmed that there were no significant changes in total transferrin by examining total iron binding capacity (and hence total transferrin levels) in the samples analyzed (Figure 3C). We then demonstrated that there was a significant reduction in diferric transferrin levels at the 5 h time point (51% of the control value) (Figure 3D). This decrease was transient, with diferric transferrin returning to control levels after 12 h. As diferric transferrin has been implicated in the regulation of hepcidin production, these results suggest that reductions in diferric transferrin levels might contribute to the decrease in *Hamp1* expression following erythropoietin injection. Indeed, the decrease in diferric transferrin levels preceded the decrease in *Hamp1* expression (*Online Supplementary Figure S1*). The significant increases in serum iron, transferrin saturation and diferric transferrin that were observed at the latter time points likely reflect the increase in iron release that would occur following a decrease in hepcidin production.

Intravenous injection of iron raises diferric transferrin levels without increasing hepatic iron stores

Having established that a transient reduction in diferric transferrin occurs following injection of erythropoietin, we sought to determine the consequences of increasing diferric transferrin levels during this period. To achieve this, mice were intravenously injected with a dose of ferric citrate estimated to be twice the amount required to fully saturate circulating transferrin, ensuring that transferrin was as close to saturated as possible, with any remaining non-transferrin bound iron at such low levels that it would not significantly alter tissue iron stores once removed from the circulation. Control mice were injected with an equimolar amount of citrate as sodium citrate. The iron was injected 5 h after erythropoietin administration as, at this time point, diferric transferrin was reduced whereas *Hamp1* expression had not yet been affected (Figures 1A and 3D), allowing us to determine whether *Hamp1* levels would decrease if diferric transferrin was elevated during the treatment period.

In order to check that the injected iron bound to circulating transferrin, a cohort of mice was euthanized 5 min after the iron injections. As non-transferrin bound iron is rapidly cleared from the circulation with a half-life of 30 s,[23] any iron remaining in the bloodstream after 5 min should be bound to transferrin. Total serum iron (Figure 4A) and transferrin saturation (Figure 4B) were significantly elevated in mice injected with iron compared to those injected with sodium citrate, regardless of whether they had received a prior injection of erythropoietin, with transferrin saturations above 80% being achieved. The

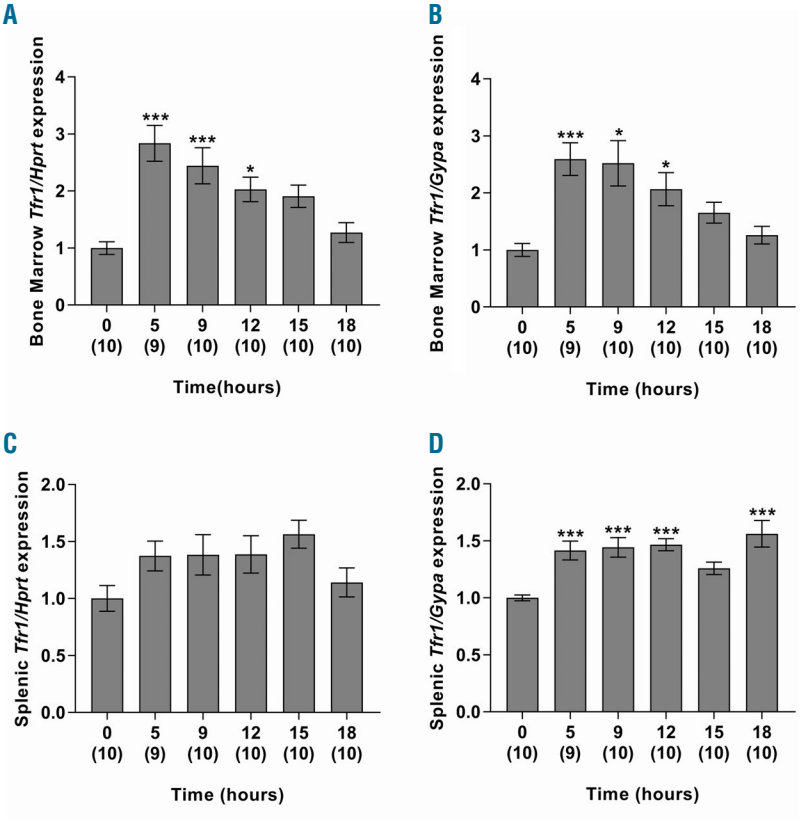

Figure 2. *Tfr1* expression following erythropoietin injection in mice. Six-week old male C57BL/6 mice were euthanized 0, 5, 9, 12, 15 or 18 h following the intravenous injection of 10 U/g body weight human erythropoietin and tissues were taken for analysis. Bone marrow *Tfr1* expression (A, B) and splenic *Tfr1* expression (C, D) were determined for each time point. Gene expression levels were calculated relative to either the general housekeeping gene *Hprt* or the erythroid-specific marker Gypa, and are expressed as a proportion of the values at 0 h. The data represent the mean ± SEM with the number of mice in each group indicated in parentheses along the x-axis. Statistical significance is shown relative to the 0 h group. **P*<0.05; ****P*<0.005.

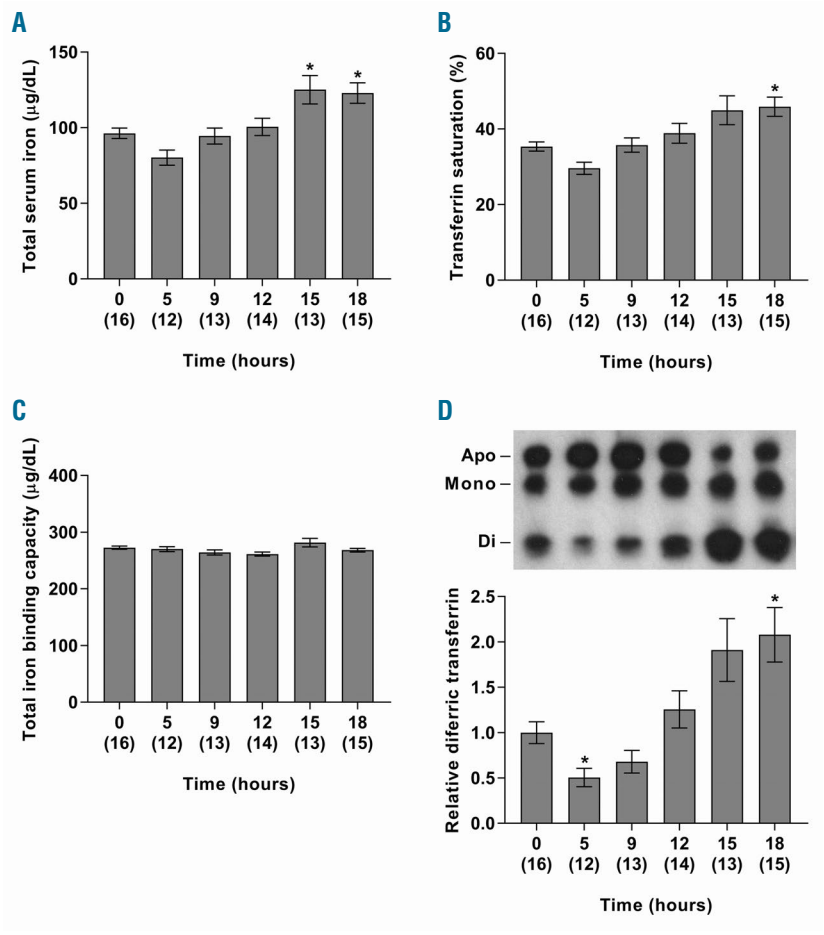

Figure 3. Circulating iron parameters following erythropoietin injection in mice. Six-week old male C57BL/6 mice were euthanized 0, 5, 9, 12, 15 or 18 h following the intravenous injection of 10 U/g body weight human erythropoietin and blood was taken for analysis. Total serum iron (A), transferrin saturation (B), total iron binding capacity (C) and relative diferric transferrin levels (D) were determined for each time point. The relative diferric transferrin levels represent the percentage of transferrin in the diferric form expressed as a proportion of the values at 0 h. The data represent the mean ± SEM with the number of mice in each group indicated in parentheses along the x-axis. Apo: apotransferrin; Mono: monoferric transferrin; Di: diferric transferrin. Statistical significance is shown relative to the 0 h group. *P<0.05.

iron injections had no effect on total iron binding capacity, a measure of total transferrin levels (Figure 4C). The analysis of transferrin species showed that transferrin was almost fully saturated 5 min after iron injection, with no apo-transferrin detectable (Figure 4D). In agreement with our earlier observations (Figure 3D), a decrease in diferric transferrin was detected in mice injected with erythropoietin and sodium citrate when compared to those injected with sodium citrate alone.

A second cohort of mice was euthanized 4 h after iron injection (9 h after erythropoietin injection). Total serum iron and transferrin saturation in these iron-injected mice were lower than those observed at the 5 min time point (Figure 4A,B), with significant differences seen only in the groups injected with erythropoietin (Figure 5A,B). Once again, the injections had no influence on total iron binding capacity (i.e. total transferrin levels) (Figure 5C). Diferric transferrin levels remained significantly elevated 4 h after iron injection (Figure 5D), although the levels were reduced when compared with those 5 min after injection (Figure 4D). An analysis of liver tissue showed no change in hepatic iron levels in the groups treated with iron (Figure 5E), indicating that, although iron had been removed from the circulation in the 4 h since the iron injection, the amounts were not enough to influence storage iron levels.

Increasing serum iron levels can overcome the inhibitory effect of erythropoietin injection on *Hamp1* expression without altering *Erfe* production

The effect of increasing serum iron levels on *Hamp1* expression was examined in our erythropoietin-treated mice sacrificed 4 h after iron injection. We found that increasing serum iron was able to overcome the inhibitory effect of stimulated erythropoiesis, with *Hamp1* expression in mice injected with both erythropoietin and iron remaining at levels similar to those in mice not injected with erythropoietin (Figure 6A). The level of phosphorylated SMAD1/5/8 was decreased in the erythropoietin-injected mice and increased with subsequent iron injection (Figure 6B); this finding supports those of previous studies indicating that the SMAD pathway is involved in the regulation of *Hamp1* expression by both circulating erythroferrone and iron levels.[14,15,24]

Despite *Hamp1* expression returning to normal following iron injection, splenic and bone marrow *Erfe* levels remained elevated in response to erythropoietin (Figure 7A-D). We also observed a significant increase in spleen weight in the erythropoietin-injected group receiving iron, possibly indicating an increase in erythroblast proliferation and implying that erythropoiesis is iron restricted in the hours following an erythropoietic stimulus (Figure 7E). Consistent with this, we detected an increase in the expression of *Tfr1* (normalized to *Hprt*) (Figure 8A) in the spleen of mice treated with both erythropoietin and iron when compared to those treated with erythropoietin alone, although the increase failed to reach statistical significance (P=0.089). A similar non-significant increase in splenic *Erfe/Hprt* was also seen (Figure 7A). In both cases, the difference was lost when gene expression was normalized to *Gypa* (Figures 7B and 8B), indicating that the changes were due to an increase in erythroblast number rather than individual cell expression. In contrast, we detected a decrease in *Tfr1* expression in the bone marrow

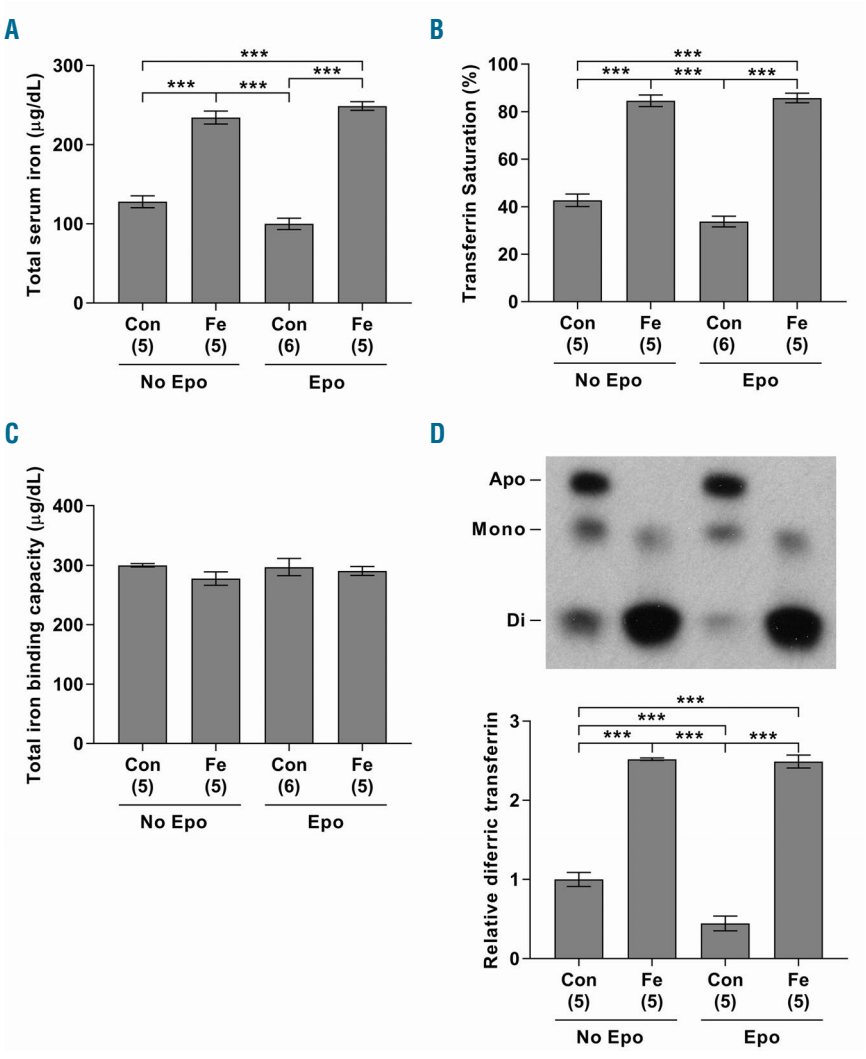

Figure 4. Circulating iron parameters immediately after iron injection of erythropoietin-treated mice. Six-week old male C57BL/6 mice were injected intravenously with 10 U/g body weight human erythropoietin. Five hours later, mice were intravenously injected with either 2.5 µg/g body weight ferric citrate or an equimolar amount of citrate as sodium citrate. Mice were euthanized 5 min after this final injection and blood taken for analysis. Total serum iron (A), transferrin saturation (B), total iron binding capacity (C) and relative diferric transferrin levels (D) were determined for each group. The relative diferric transferrin levels represent the percentage of transferrin in the diferric form expressed as a proportion of the values in mice injected with sodium citrate but not with erythropoietin. The data represent the mean ± SEM with the number of mice in each group indicated in parentheses along the x-axis. Con: control mice that were injected with sodium citrate; Fe: mice that were injected with ferric citrate; No Epo: mice that were not injected with erythropoietin; Epo: mice that were injected with erythropoietin; Apo: apotransferrin; Mono: monoferric transferrin; Di: diferric transferrin. ***P<0.005.

of mice treated with both erythropoietin and iron when compared with those treated with erythropoietin alone (Figure 8C). Interestingly, the decrease remained when *Tfr1* levels were normalized to *Gypa* (Figure 8D), indicating lower *Tfr1* expression in individual erythroblasts and a reduction in iron demand following iron injection.

Discussion

Developing erythrocytes are by far the largest sink for iron in the body,[10] so it is not surprising that erythropoiesis is a major regulator of hepcidin production. Our understanding of how the erythroid mass in the bone marrow and spleen signal changes in *HAMP* expression in hepatocytes has been advanced considerably with the recent discovery of erythroferrone, a protein produced by developing erythrocytes and secreted into the circulation, which inhibits hepatic hepcidin production,[11,12] As a result, iron absorption and storage iron release are increased, providing more iron for erythrocyte development. However, other factors are also known to regulate hepcidin expression. Of these, serum iron levels, particularly diferric transferrin, may be relevant to hepcidin expression following stimulated erythropoiesis. We have previously shown that diferric transferrin correlates with inhibited *Hamp* expression in rats following treatment with the hemolytic agent phenylhydrazine,[18] and have suggested that the level of diferric transferrin in the circulation might signal the iron demands of the erythroid marrow to the liver and influence hepcidin production.[13,19]

In the current study we have demonstrated for the first time that the rapid decrease in *Hamp1* expression that occurs following erythropoietin injection in mice is preceded by a transient decrease in circulating diferric transferrin. The evidence in favor of serum iron and, therefore, diferric transferrin, influencing hepcidin production is strong. We have previously shown that reductions in serum iron levels correlate closely with the inhibition of *Hamp* expression in rats switched to an iron-deficient diet[25] and following phenylhydrazine treatment.[18] We and others have also demonstrated that increased serum iron levels stimulate hepcidin expression in mice.[15,19] In addition, a mechanism by which hepatocytes might detect the levels of circulating diferric transferrin has been described. It has been proposed that the hemochromatosis protein HFE on the surface of hepatocytes competes with diferric transferrin for binding to TFR1, with the HFE/TFR1 complex being favored when diferric transferrin levels are low.[13,26] Thus, diferric transferrin would not be expected to

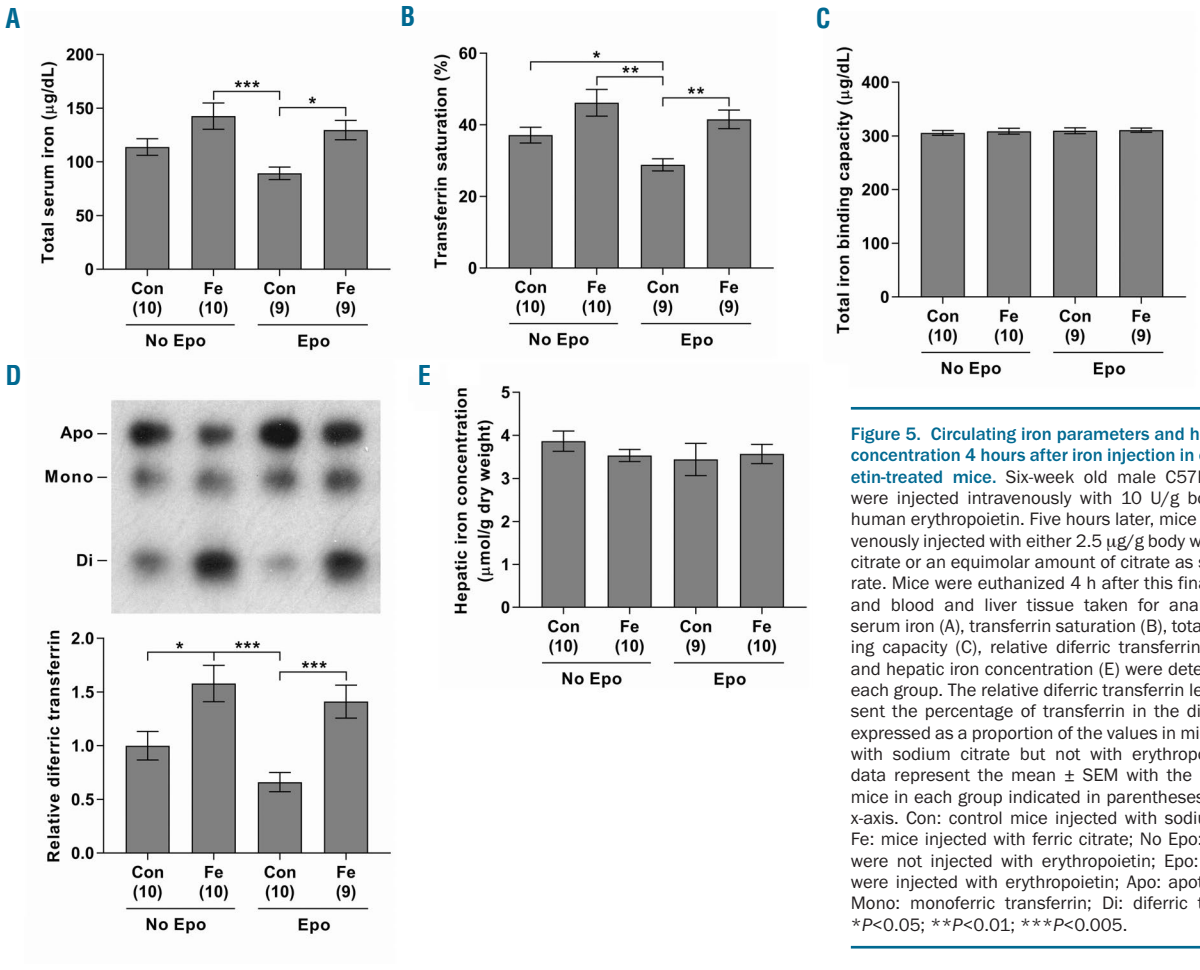

Figure 5. Circulating iron parameters and hepatic iron concentration 4 hours after iron injection in erythropoietin-treated mice. Six-week old male C57BL/6 mice were injected intravenously with 10 U/g body weight human erythropoietin. Five hours later, mice were intravenously injected with either 2.5 µg/g body weight ferric citrate or an equimolar amount of citrate as sodium citrate. Mice were euthanized 4 h after this final injection and blood and liver tissue taken for analysis. Total serum iron (A), transferrin saturation (B), total iron binding capacity (C), relative diferric transferrin levels (D) and hepatic iron concentration (E) were determined for each group. The relative diferric transferrin levels represent the percentage of transferrin in the diferric form expressed as a proportion of the values in mice injected with sodium citrate but not with erythropoietin. The data represent the mean ± SEM with the number of mice in each group indicated in parentheses along the x-axis. Con: control mice injected with sodium citrate; Fe: mice injected with ferric citrate; No Epo: mice that were not injected with erythropoietin; Epo: mice that were injected with erythropoietin; Apo: apotransferrin; Mono: monoferric transferrin; Di: diferric transferrin. *P<0.05; **P<0.01; ***P<0.005.

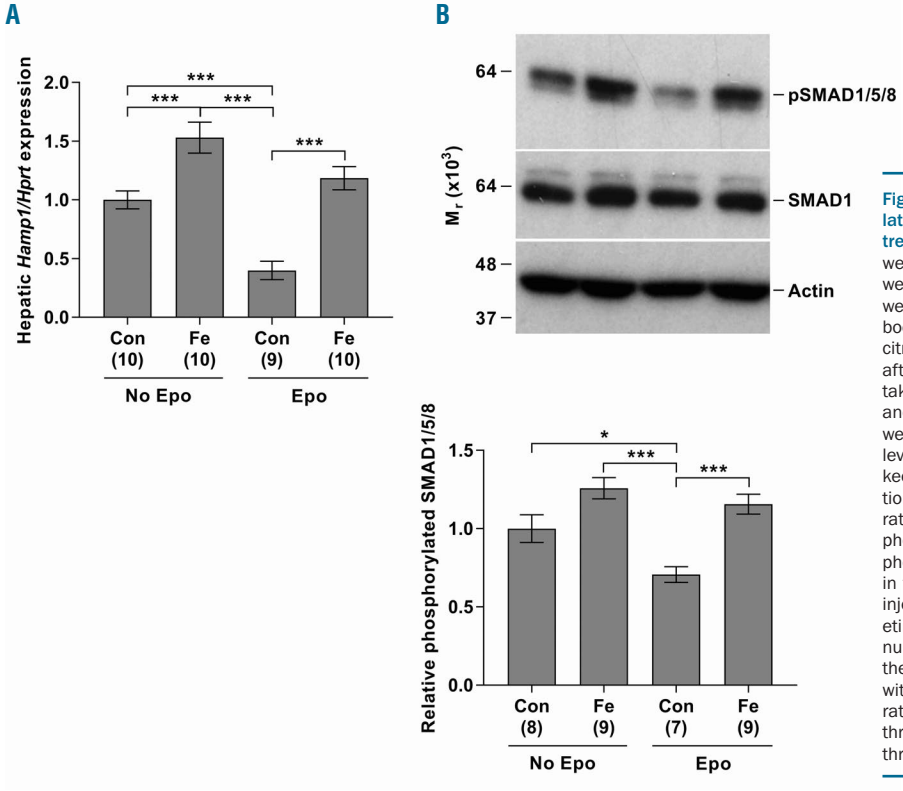

Figure 6. *Hamp1* expression and SMAD phosphorylation 4 hours after iron injection in erythropoietin-treated mice. Six-week old male C57BL/6 mice were injected intravenously with 10 U/g body weight human erythropoietin. Five hours later, mice were intravenously injected with either 2.5 µg/g body weight ferric citrate or an equimolar amount of citrate as sodium citrate. Mice were euthanized 4 h after this final injection and various tissues were taken for analysis. Hepatic *Hamp1* expression (A) and the amount of phosphorylated SMAD1/5/8 (B) were determined for each group. Gene expression levels were calculated relative to the general housekeeping gene *Hprt* and are expressed as a proportion of the values in mice injected with sodium citrate but not with erythropoietin. The relative phosphorylated SMAD1/5/8 levels are expressed as phosphoprotein/total SMAD1/Actin and presented in the graph as a proportion of the values in mice injected with sodium citrate but not with erythropoietin. The data represent the mean ± SEM with the number of mice in each group indicated in parentheses along the x-axis. Con: control mice injected with sodium citrate; Fe: mice injected with ferric citrate; No Epo: mice that were not injected with erythropoietin; Epo: mice that were injected with erythropoietin. *P<0.05; ***P<0.005.

be able to regulate *HAMP* expression in an individual with HFE-related hemochromatosis, although it could still be regulated through other pathways. An additional detection mechanism involving TFR2, a TFR1 homolog, has also been described.[13] Although the precise pathways by which these molecules signal changes in hepcidin production remains unclear, their involvement in detecting diferric transferrin levels is widely accepted.[3,10,27,28] While showing a correlation only, when viewed in conjunction with the evidence supporting diferric transferrin as a regulator of hepcidin, the current study suggests that diferric transferrin levels are likely to contribute to the initial reduction in *Hamp1* expression seen following an erythropoietic stimulus. However, whether the decrease in diferric transferrin levels is required for the inhibition of hepcidin is unclear. *Erfe* expression was also increased at the 5 h time point, although the lack of a reliable commercial assay for mouse erythroferrone made it difficult to determine the amount of functional erythroferrone at this time. Further research is required to determine whether both stimuli are required or whether there is a degree of redundancy in the pathways inhibiting hepcidin at these early time points. In contrast, serum iron is clearly not involved in reducing *Hamp1* expression towards the end of the time course, as serum iron parameters quickly returned to normal levels and even exceeded control values at these later time points.

Our results also show that increasing serum iron levels soon after erythropoietin injection can overcome the inhibitory effect of stimulated erythropoiesis and prevent any decrease in *Hamp1* expression. While our data suggest that changes in diferric transferrin levels are the likely cause, a role for non-transferrin-bound iron (NTBI) cannot be excluded, as this is the form of iron injected in our studies. However, there are several reasons why a major role for NTBI in hepcidin regulation is unlikely. Firstly, studies using the hypotransferrinemic mouse suggest that it is the transferrin-bound iron in the circulation that is important for hepcidin regulation. These mice have greatly reduced transferrin levels and, as a consequence, high levels of NTBI.[29,30] With limited transferrin, iron supply to the marrow is compromised and the resulting anemia reduces hepcidin production. So hepcidin levels are low, despite very high levels of NTBI.[29] Even in hypotransferrinemic mice subjected to myeloablation to remove the effect of stimulated erythropoiesis, hepcidin expression could only be increased if transferrin was administered, making it unlikely that NTBI has a major effect on hepcidin produc-

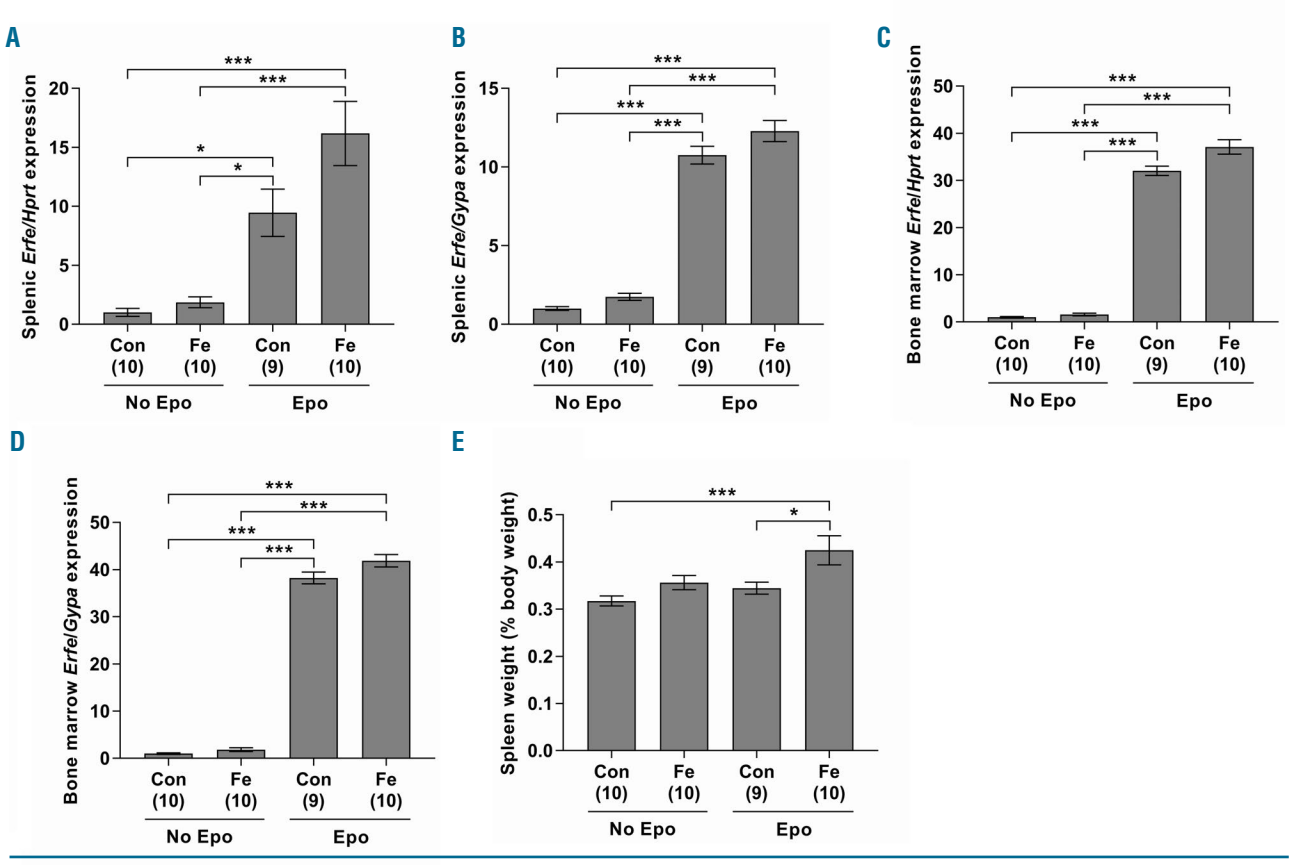

Figure 7. *Erfe* expression and spleen weight 4 hours after iron injection in erythropoietin-treated mice. Six-week old male C57BL/6 mice were injected intravenously with 10 U/g body weight human erythropoietin. Five hours later, mice were intravenously injected with either 2.5 μg/g body weight ferric citrate or an equimolar amount of citrate as sodium citrate. Mice were euthanized 4 h after this final injection and various tissues were taken for analysis. Splenic *Erfe* expression (A, B), bone marrow *Erfe* expression (C, D) and spleen weight (E) were determined for each group. Gene expression levels were calculated relative to either the general housekeeping gene *Hprt* or the erythroid-specific marker *Gypa* and are expressed as a proportion of the values in mice injected with sodium citrate but not with erythropoietin. The data represent the mean ± SEM with the number of mice in each group indicated in parentheses along the x-axis. Con: control mice injected with sodium citrate; Fe: mice injected with ferric citrate; No Epo: mice that were not injected with erythropoietin; Epo: mice that were injected with erythropoietin. **P*<0.05; ****P*<0.005.

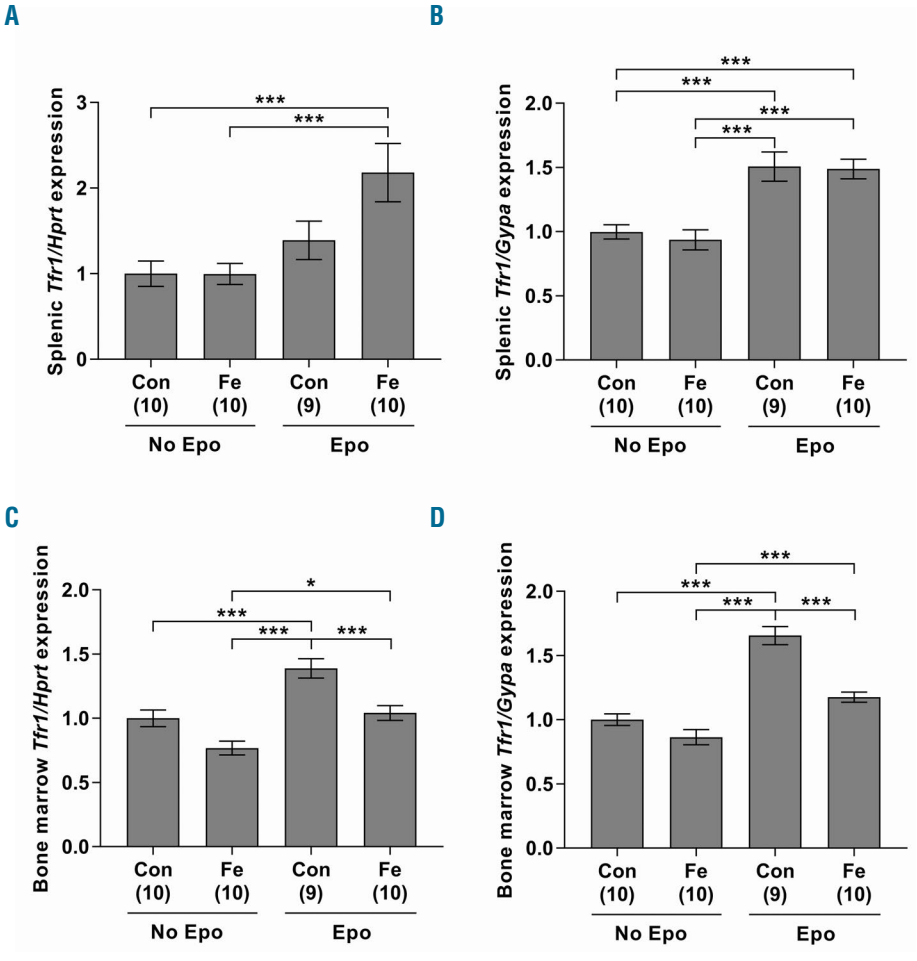

Figure 8. *Tfr1* expression 4 hours after iron injection in erythropoietin-treated mice. Six-week old male C57BL/6 mice were injected intravenously with 10 U/g body weight human erythropoietin. Five hours later, mice were intravenously injected with either 2.5 mg/g body weight ferric citrate or an equimolar amount of citrate as sodium citrate. Mice were euthanized 4 h after this final injection and tissues were taken for analysis. Splenic *Tfr1* expression (A, B) and bone marrow *Tfr1* expression (C, D) were determined for each group. Gene expression levels were calculated relative to either the general housekeeping gene *Hprt* or the erythroid-specific marker *Gypa* and are expressed as a proportion of the values in mice injected with sodium citrate but not with erythropoietin. The data represent the mean ± SEM with the number of mice in each group indicated in parentheses along the x-axis. Con: control mice injected with sodium citrate; Fe: mice injected with ferric citrate; No Epo: mice that were not injected with erythropoietin; Epo: mice that were injected with erythropoietin. *P<0.05; ***P<0.005.

tion.[31] This latter finding is consistent with that of an earlier study (carried out before hepcidin was identified) showing that injected transferrin was able to suppress iron absorption in hypotransferrinemic mice in which erythropoiesis had been normalized.[32] However, as transferrin levels subsequently declined, iron absorption increased. Secondly, although NTBI in the form of ferric citrate was administered to the mice in the current study, it had previously been demonstrated that any NTBI formed in this way is removed very rapidly from the circulation with a half-life of <30 s.[23] In contrast, we have shown that diferric transferrin levels remain elevated 4 h after iron injection (Figure 5D). Thirdly, as mentioned previously, a mechanism for the detection of diferric transferrin levels in the circulation and the subsequent regulation of *Hamp1* expression has been proposed and is supported by *in vivo* evidence,[33,34] whereas no such mechanism has been described for NTBI. Finally, these results are consistent with multiple cell culture studies showing that treatment of cells with iron salts does not directly stimulate hepcidin production.[35,36] In fact, *Hamp* expression could only be stimulated when transferrin-bound iron was supplied.[36] Therefore, despite not being able to definitively exclude a role for NTBI, it is far more likely that diferric transferrin is the major stimulator of *Hamp1* expression in our studies.

The increase in *Hamp1* expression occurred despite significant increases in *Erfe* expression in the bone marrow or spleen. In fact, *Erfe* expression in both tissues was higher in mice that received both erythropoietin and iron compared to those that received erythropoietin only, although the changes were not significant. However, as spleen size was also increased, it is highly likely that circulating erythroferrone levels are higher in erythropoietin-treated animals injected with iron, implying that increased serum iron levels can overcome the effect of erythroferrrone, at least in the early stages of erythroid stimulation. However, this does not always occur. For example, we observed a significant increase in diferric transferrin levels 18 h after erythropoietin injection despite *Hamp1* expression being at its lowest level at this time. While the reason for this is unclear, it is possible that circulating erythroferrone levels increased over the time course. While no such change was observed in *Erfe* message levels, a previous study showed that *Erfe* mRNA expression does not always correspond to serum erythroferrone levels.[12] Another possible explanation is that the increase in diferric transferrin seen later in our time course had not had sufficient time to influence *Hamp1* expression, as changes in serum iron levels can take several hours to exert an effect on hepcidin production.[15]

Certain mouse models of β-thalassemia exhibit high transferrin saturation yet continue to load with iron due to low hepcidin levels.[37] It is likely that, in these instances, the expansion of the erythroid marrow due to chronic

anemia would result in a much larger increase in serum erythroferrone than would occur following a single injection of erythropoietin, allowing the erythroid regulator to overcome the effect of increases in diferric transferrin. In contrast, the current study involves the acute stimulation of erythropoiesis and, as such, any expansion of the erythroid marrow would be minimal, allowing the stimulating effect of increased diferric transferrin to overcome the inhibitory effect of erythroferrone and prevent any reduction in *Hamp1* expression. Such competition between pathways influencing hepcidin production has been reported previously[12,38,39] and these studies indicate that it is the strength of each signal, rather than its origin, that dictates *HAMP* expression. It is also possible that, in β-thalassemia, the preferential uptake of diferric transferrin by the greatly expanded erythroid precursor mass causes circulating diferric transferrin levels to be relatively low despite higher than normal transferrin saturation, and that this might contribute to the observed decrease in hepcidin.

The increase in spleen size following iron injection also suggests that erythropoiesis is iron-restricted shortly after stimulation with erythropoietin, with the additional iron allowing further expansion of the erythroid marrow. This is supported by the observation of decreased *Tfr1* expression in the bone marrow in iron-injected animals. Interestingly, a recent study has suggested that there is an erythroferrone-independent role for erythroid cell TFR1 in the regulation of hepcidin production.[40] The authors propose that TFR1 on erythroid precursor cells regulates the production of an unknown soluble factor that influences hepcidin expression in the liver. We would suggest that a novel factor is unnecessary, as alterations in erythroid TFR1 production will rapidly and preferentially influence the levels of circulating diferric transferrin, which will, in turn, affect hepcidin production. Our results also indicate that such a change in diferric transferrin would not necessarily result in a significant difference in serum iron levels, and thus could remain undetected in many studies examining the regulation of hepcidin.

In conclusion, we have demonstrated that diferric transferrin levels are transiently decreased following the stimulation of erythropoiesis by erythropoietin injection and suggest that this molecule may contribute to the initial inhibition of hepcidin expression that occurs. We also show that, in certain situations, diferric transferrin can overcome the inhibitory effect of erythroferrone to prevent hepcidin inhibition. However, this is likely to depend on the strength of the individual signals, with the effect of erythroferrone clearly overcoming increases in diferric transferrin levels at later time points. As the changes in diferric transferrin levels are not always detected by measuring serum iron or transferrin saturation, we suggest that studies investigating the regulation of hepcidin would benefit from direct measurements of diferric transferrin.

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