Erythropoietin administration in humans causes a marked and prolonged reduction in circulating hepcidin

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ABSTRACT

Expression of hepcidin, the key hormone governing iron transport, is reduced by anemia in a manner which appears dependent on increased bone marrow activity. The temporal associations between plasma hepcidin and other iron parameters were examined in healthy humans after erythropoietin administration and venesection. Profound hepcidin suppression appeared abruptly 24 hours after subcutaneous erythropoietin (P=0.003), and was near maximal at onset, with peak (mid-afternoon) levels reduced by 73.2%, gradually recovering over the following two weeks. Minor changes in circulating iron, soluble transferrin receptor and growth differentiation factor-15 were observed after the reduction in hepcidin. Similar but more gradual changes in these parameters were observed after reducing hematocrit by removal of 250 mL blood. These human studies confirm the importance of a rapidly responsive marrow–hepcidin axis in regulating iron supply *in vivo*, and suggest that this axis is regulated by factors other than circulating iron, soluble transferrin receptor or growth differentiation factor-15.

Key words: iron, hepcidin, erythropoietin, GDF15, ferritin, anemia of chronic disease.

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Introduction

The ready transition between oxidation states explains the dual importance of iron to nearly all organisms: it is both an indispensable nutrient and a potential toxin. Despite constant transport from tissue stores to sites of utilization, principally the bone marrow,[1] low levels of circulating iron must, therefore, be maintained. A consequence of this low capacity / high throughput plasma iron compartment is that iron supply must be rapidly responsive to changes in iron demand. The hormone hepcidin, which has emerged in recent years as the master regulator of iron transport, is anticipated to play a key role in this process.[2-5]

Hepcidin is a 25 amino acid peptide hormone produced in the liver which binds to the iron export protein ferroportin which is expressed by macrophages, enterocytes and hepatocytes leading to its degradation.[6] Hepcidin therefore acts to prevent iron recycling and absorption, and usually responds to iron availability[7-9] thereby preventing iron overload: a relative deficiency in humans, as has been documented in most forms of hereditary hemochromatosis, leads to progressive iron loading.[10,11] Hepcidin is suppressed by anemia, and evidence in whole animals suggests that an intact bone marrow is necessary for this response.[12] It has been suggested that hepatocytes are simply responding to the decreased iron availability due to increased iron utilization by the marrow.[13] However, the observation that urinary hepcidin is reduced in patients with thalassemia and iron overload suggests that a separate marrow derived factor mediates hepcidin suppression in some circumstances.[14,15]

Previous work has shown that erythropoietin administration is able to suppress circulating hepcidin levels in chronic kidney disease,[16] and urinary hepcidin in healthy controls,[17] both within two days of administration and accompanied by reduced circulating iron. This study examined the temporal associations between changes in circulating hepcidin and iron parameters during increased erythropoiesis in humans, and in particular, whether the hepcidin changes can be attributed to increased marrow iron utilization.

Design and Methods

This study was NHS Research Ethics Committee approved (*08/Q0707/153*) and conducted according to the principles of the

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Declaration of Helsinki. Blood samples (2.5 mL each, taken into heparinised tubes with immediate centrifugation and storage at –20°C) were taken from healthy volunteers 4 times per day during two separate weeks of study, separated by a washout period of four weeks. On the morning of the second day of each week subjects received either an erythropoietin injection (5000 units epoetin beta subcutaneously) or removal of 250 mL blood by venesection.

Plasma hepcidin-25 was measured by a competitive immunoassay described previously.[18] Ferritin and iron were measured using the Abbott Architect ci8000 system (Abbott Diagnostics, Ireland). Erythropoietin, sTFR and GDF15 were measured by sandwich ELISA (R&D Systems, UK).

Changes during each week were analyzed separately from diurnal changes by fitting a linear mixed model in which repeated measurements were assigned an autoregressive covariance structure (SPSS, Chicago, IL, USA). This prevents overestimation of effects arising from the tendency of measurements separated by short time intervals to be similar. In addition, mid-afternoon hepcidin levels were compared between days 1 and 3 using the paired *t* test. Results are expressed as mean ± standard error.

Results and Discussion

Six healthy volunteers were recruited (5 male, ages 24-38) with normal baseline blood tests (hemoglobin 12.1-15.6 g/dL, ferritin 28-168 ng/mL).

Throughout both study weeks, the expected diurnal variation in circulating hepcidin was observed,[19] with levels rising during the day ($P<0.001$ for either afternoon time point compared with 09:00) reaching a plateau in the mid-afternoon, and falling slightly, but not significantly, between 15:00 and 18:00 hours.

During the first study week, subjects received a single subcutaneous injection of erythropoietin (5000 units epoetin beta) at 09:00 on day 2. Profound suppression of hepcidin was observed starting 24 h after erythropoietin administration, continuing throughout the week, with incomplete recovery occurring over a two week period (Figure 1A, $P=0.003$, <0.001 and <0.001 for day 3, 4 and 5 levels compared with day 1). Hepcidin suppression was near maximal during day 3, with peak (mid-afternoon) levels reduced by 73.2% ($P=0.018$). Individual responses are shown in the *Online Supplementary Appendix Figure S1*.

Suppression of hepcidin expression by erythropoietin is thought to be mediated by increased bone marrow activity, since prior bone marrow suppression by cytotoxic agents or irradiation abolishes the response[13,20] – a circulating marrow-derived mediator is, therefore, anticipated. Rather than reflecting the observed pharmacokinetic profile of plasma erythropoietin (Figure 1D), the observed delay in the suppression and recovery of hepcidin levels found in this study are more in keeping with an effect mediated by a secondary factor. Erythropoietin acts via cell membrane receptors expressed in erythroid progenitor cells, increasing mitosis and survival so that the number of cells entering the precursor pool is increased.[21] The factor responsible for hepcidin suppression might be released by cells in response to erythropoietin signal, or in proportion to the number of cells at a particular stage of maturation. This delay, which was consistent in all subjects (*Online Supplementary Appendix Figure S1*) may, therefore, reflect

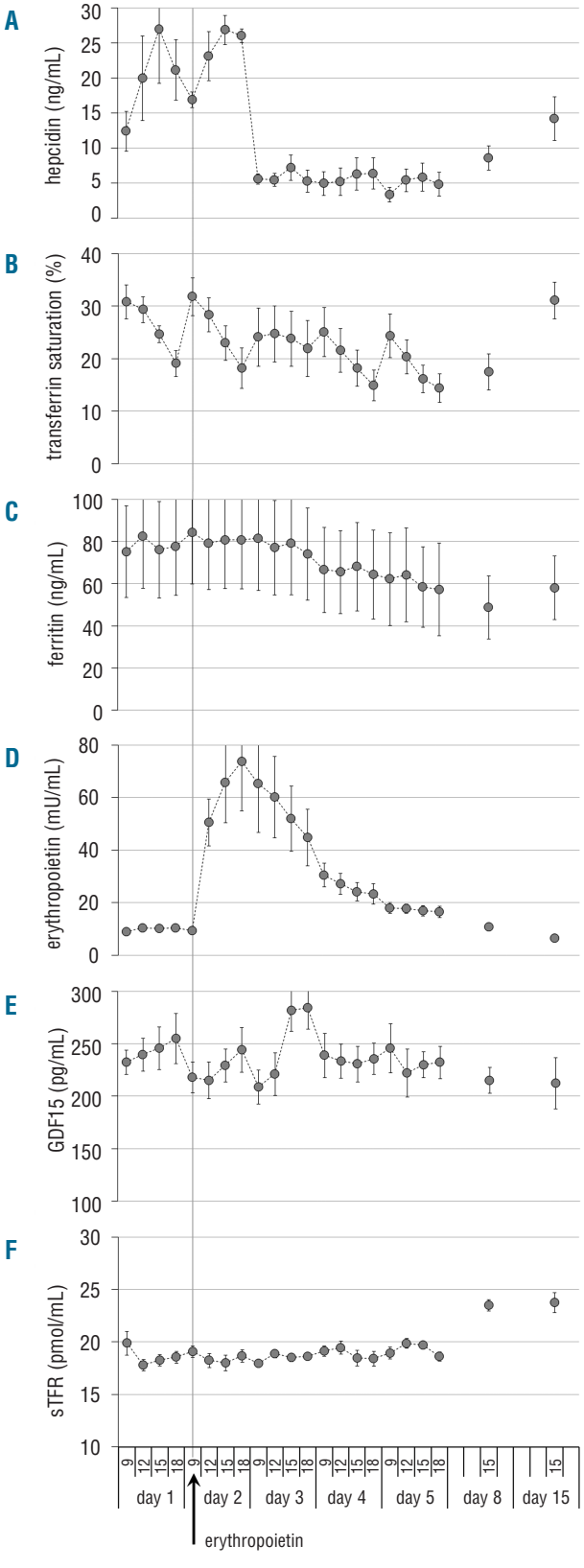

Figure 1. Effect of erythropoietin administration on plasma hepcidin and related parameters. Healthy controls (n=6) received subcutaneous erythropoietin at 09:00 on the second day. Levels of plasma hepcidin (A), transferrin saturation (B), ferritin (C), erythropoietin (D), GDF15 (E) and sTFR (F) are shown throughout the week (mean±se). GDF15: growth differentiation factor-15, sTFR: soluble transferrin receptor.

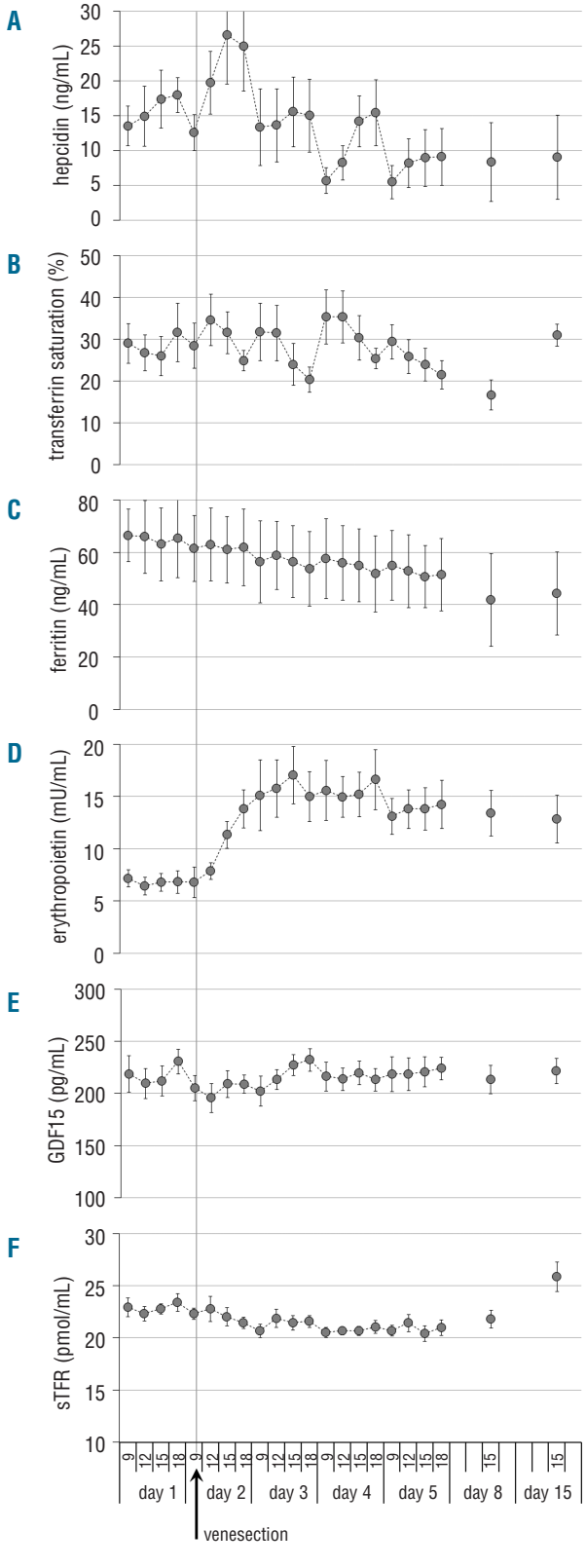

Figure 2. Effect of venesection on plasma hepcidin and related parameters. In healthy controls (n=6) hematocrit was acutely reduced by venesection (250ml) at 09:00 on the second day. Levels of plasma hepcidin (A), transferrin saturation (B), ferritin (C), erythropoietin (D), GDF15 (E) and sTFR (F) are shown throughout the week (mean±se). GDF15: growth differentiation factor-15, sTFR: soluble transferrin receptor.

the time required for this proliferative response to occur: following erythropoietin administration in humans, reticulocyte numbers begin to increase after a dose-independent delay of around 1.7 days.[22]

Three potential marrow-derived mediators of this effect were examined. Firstly, it has been suggested that rather than a novel mediator, bone marrow iron utilization in response to erythropoietin might lead directly to hepcidin suppression via a reduction in circulating transferrin saturation (TS) which would be sensed by hepatocytes. TS levels in this study demonstrated the expected diurnal pattern, with levels significantly lower at 15:00 and 18:00 than at 09:00 ($P<0.001$ for both), and levels did gradually decline during the week. During day 2 however, prior to the change in hepcidin, mean TS levels were not significantly changed from baseline, and in 4 of the 6 subjects were slightly increased (*Online Supplementary Figure S2*). The marked decrease in hepcidin seen in all subjects therefore preceded the reduction in circulating TS, suggesting that increased iron utilization *per se* is not the mechanism mediating the hepcidin suppression (Figure 1B; $P=0.028$ and 0.001 for day 4 and 5 levels compared with day 1). Total iron binding capacity was very stable during the period of observation, showing no diurnal pattern or trend over the week: changes in circulating iron therefore very closely reflected those in TS (*Online Supplementary Figures S3 and S4*). Ferritin levels similarly exhibited a gradual decline during the week (Figure 1C).

An attractive candidate for a circulating indicator of marrow iron requirement is soluble transferrin receptor (sTFR). The membrane bound form of this receptor binds holotransferrin leading to iron uptake by erythroid precursors and is subsequently shed during further red cell maturation – its circulating levels are therefore generally proportional to iron uptake,[23] and it has been suggested as a mediator of iron regulation in effective and ineffective erythropoiesis.[24] After erythropoietin administration, however, sTFR levels remained constant throughout the week, only beginning to rise from day 8 (23.5±0.6 *vs.* 18.3±0.5 pmol/mL, $P=0.002$, Figure 1F).

Specifically in the context of ineffective erythropoiesis, growth differentiation factor-15 (GDF15), a TGFb family cytokine, has been suggested as a mediator of hepcidin suppression based on two observations. Firstly, GDF15 has been shown to suppress hepcidin expression in cultured hepatocytes, and secondly, it circulates at extremely high levels in patients with beta thalassemia, potentially explaining their inappropriate hepcidin suppression in spite of iron overload.[25,26] This hypothesis was strengthened by the recent discovery that iron depletion *in vitro* induces GDF15 expression, and that infusion of the iron chelator deferoxamine increases plasma GDF15 in humans.[27] In our study, a modest but significant diurnal pattern to GDF15 was observed, with levels increasing slightly during each day, but the pattern remained constant during the week (Figure 1E; the rise towards the end of day 3, which appears to be exaggerated, is not significantly different from day 1). GDF15 therefore appears unlikely to mediate the hepcidin suppression resulting from erythropoietin administration.

During the second study week, 250 mL blood was removed from each subject at 09:00 on day 2. Hepcidin lev-

els were again subsequently reduced, although the reduction was more gradual than that seen after erythropoietin administration, with no apparent recovery even by day 15 (Figure 2A; P=0.020 and <0.001 for day 4 and 5 relative to day 1). Analysis of related parameters demonstrated a more gradual decline in circulating TS, otherwise similar to the first study week (Figure 2B; P=0.006 for day 5 compared with day 1), whilst sTFR and GDF15 were again unchanged during the week (Figure 2E and F). That these changes were similar but more gradual than those observed during the first week, may be explained by the slower onset and lower amplitude of the plasma erythropoietin profile following venesection compared to that seen after erythropoietin administration (Figure 2D).

This degree of hepcidin suppression points towards erythropoietin as a more useful therapy for the anemia of chronic disease than is currently accepted. Two key mechanisms underlying the anemia of chronic disease are an insufficient erythropoietin response to anemia, and restricted iron transport, the latter being due to increased hepcidin expression mediated by interleukin-6.[28] Both these mechanisms are addressed by therapeutic erythropoietin: in addition to augmenting insufficient endogenous hormone, the resulting suppression of circulating hepcidin levels is able to restore the delivery of iron to the marrow.

Erythropoietin administration results in profound and sustained suppression of circulating hepcidin, which is not mediated by reduced circulating iron. These data add to the rationale behind the use of erythropoietin for the anemia of chronic disease improving the disorder of iron transport as well as supplementing a diminished erythropoietin response. This study also confirms the existence of a hepcidin-based mechanism for increasing the delivery of iron at times of increased utilization; the anticipated marrow-derived mediator of this effect remains to be identified.

Authorship and Disclosures

DRA, DPG, MB, KGM, FWKT, PHM and PC designed the study, which was carried out by DRA and DPG, with help and supervision from NDD, TDC, DHT and PC. MB and RC developed and performed the hepcidin assay with KGM and SRB. DRA, DPG and MB analyzed the data and wrote the manuscript, which was edited by all authors. PHM is a scientific director of and holds equity in ReOx Ltd. PHM also received payments from Wellcome Trust and Roche Foundation for Anaemia Research SAB.

There are no other potential conflicts of interest.

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