Hepcidin Decreases Iron Transporter Expression in Vivo in Mouse Duodenum and Spleen and in Vitro in THP-1 Macrophages and Intestinal Caco-2 Cells

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Abstract

Hepcidin is thought to control iron metabolism by interacting with the iron efflux transporter ferroportin. In macrophages, there is compelling evidence that hepcidin directly regulates ferroportin protein expression. However, the effects of hepcidin on intestinal ferroportin levels are less conclusive. In this study, we compared the effects of hepcidin on iron transporter expression in the spleen and duodenum of mice treated with hepcidin over a 24- to 72-h period and observed a marked decrease in the expression of ferroportin in both duodenal enterocytes and splenic macrophages following treatment. Changes in transporter protein expression were associated with significant decreases in duodenal iron transport and serum iron. In THP-1 macrophages, ferroportin protein levels were decreased by 300 and 1000 nmol/L hepcidin. In contrast, ferroportin protein expression was unaltered in intestinal Caco-2 cells following exposure to hepcidin. However, iron efflux from Caco-2 cells was significantly inhibited in the presence of hepcidin, suggesting that the peptide could block ferroportin function in these cells. We conclude that hepcidin regulates the release of iron from both enterocytes and macrophages. However, taken together with our previous work, it is apparent that macrophages are more sensitive than enterocytes to a hepcidin challenge.

Introduction

Body iron homeostasis is maintained by a balance between iron recycling from senescent erythrocytes within the reticuloendothelial system (formed largely of splenic macrophages and hepatic Kupffer cells) and iron absorption from the diet by the duodenal enterocytes. Hepcidin, a 25-amino acid peptide released by hepatocytes into the circulation, is thought to exert a major regulatory influence on both of these homeostatic pathways [reviewed in (1)]. A number of in vitro studies have indicated that ferroportin, the iron efflux transporter highly expressed in a number of tissues including enterocytes, macrophages, and hepatocytes (2–4), acts as the cellular receptor for hepcidin (5). Upon hepcidin binding, the ferroportin transporter is internalized, ubiquitinated, and targeted for lysosomal degradation (6). As a consequence, in the presence of hepcidin, iron efflux from ferroportin-expressing cells is greatly diminished, leading to hypoferremia (7,8).

Methods and Materials

1 Supported by a grant from the Biotechnology and Biological Sciences Research Council (D015458).
3 Supplemental Table 1 is available with the online posting of this paper at jn.nutrition.org.
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current UK Home Office legislation. Male C57BL/6 mice aged 4 wk were housed in the Comparative Biology Unit at University College London (Hampstead campus) and were fed a diet containing 44 mg Fe/kg diet (Iron Deficient Diet, Harlan Teklad, supplemented with FeSO₄) and allowed free access to water for 3 wk prior to experimentation. Animals were given daily injections of hepcidin-25 [10 μg/mouse, intraperitoneal (i.p.)]²; Peptides International] or an equivalent volume of saline for either 24 or 72 h. This peptide has been used successfully in previous in vivo (8) and in vitro studies (12,13) investigating the effects of hepcidin on ferroportin expression. Published work (7) together with our preliminary data (S. K. Srai, unpublished data) demonstrates that following i.p. injection, hepcidin is completely cleared from the circulation within 24 h. Therefore, to ensure that hepcidin was present, a final injection was administered 4 h prior to experimentation.

Blood was collected by cardiac puncture from all mice of the end of the experimental period to measure serum iron using a commercially available assay (Total Iron Binding Capacity kit, Randox Laboratories).

In vivo iron uptake. The procedure for the measurement of duodenal ⁵⁹Fe uptake in vivo has been described in detail previously (8). Briefly, in anesthetized mice (pentobarbitone sodium, 60 mg/kg, i.p.), tied-off duodenal segments were flushed with saline, followed by air, filled with HEPES-buffered saline (pH 6.5) containing 0.2 mmol/L ⁵⁹Fe complexed with 4 mmol/L ascorbate, and incubated for 10 min. At the end of the exposure period, the amount of ⁵⁹Fe in the duodenal mucosa and the mouse carcass were determined by gamma counting. The total amount of ⁵⁹Fe within the mice (duodenum plus carcass) at the end of the experiment was designated total mucosal uptake (TMU). Iron remaining in the duodenal tissue was defined as mucosal retention (MR). The amount of iron transferred from the mucosa to the mouse carcass, mucosal transfer (MT), was calculated by subtracting MR from TMU.

Immunofluorescence. These procedures have been described in detail elsewhere (8). Briefly, hepcidin- or saline-treated mice were anesthetized and their tissues perfusion-fixed in situ. The duodenum and spleen from control mice and their tissues perfusion-fixed in situ. The duodenum and spleen from control mice and saline-injected mice were sectioned at 8 μm thick and mounted onto poly-L-lysine–coated slides. Following blocking, sections were incubated with anti-ferroportin (MTP11A) or anti-divalent metal transporter 1 (DMT1) (NRAMP24A), which recognizes all DMT1 isoforms) antibodies (1:100 dilution, Alpha Diagnostics) overnight at 4°C. Immunoreactivity was visualized using a FITC-conjugated swine anti-rabbit IgG (Dako) (1:100 dilution) and Vectashield mounting medium that contained propidium iodide (Vector Laboratories). To determine localization of the ferroportin-positive cell type in the spleen, sections of spleen from saline-injected mice were incubated with an antibody to the macrophage marker F4/80 (1:100 dilution, AbD Serotec). Immunoreactivity was visualized using a TRITC-conjugated swine anti-rat IgG (1:100 dilution, Open Biosystems). Images were captured using a Leica TCS laser scanning confocal microscope and the manufacturer’s dedicated software.

Cell culture. Caco-2 cells were grown in DMEM containing 10% fetal bovine serum for 21 d on Transwell inserts (Corning-Costar). THP-1 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum in 6-well plates and were differentiated into macrophages by the addition of 50 nmol/L phorbol myristate acetate. The day prior to serum in 6-well plates and were differentiated into macrophages by the addition of 50 nmol/L phorbol myristate acetate. The day prior to serum in 6-well plates and were differentiated into macrophages by the addition of 50 nmol/L phorbol myristate acetate. The day prior to exposure to THP-1 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 1.458 Chung et al.

Statistics. Data are presented as means ± SEM. Differences (P<0.05) between multiple groups were calculated using the SPSS statistical package by employing a 1-way ANOVA followed by Tukey’s post hoc test. Duodenal iron transport data were rank-transformed prior to ANOVA to account for unequal variances between groups. In studies containing 2 experimental groups, Student’s unpaired t test was used to compare means (P<0.05).

Results

Serum iron. We showed previously that a rapid decrease in ferroportin in mouse spleen after 4-h hepcidin treatment was associated with hypoferremia (8). Here, following hepcidin treatment serum iron was also less than in the saline-injected controls (40.0 ± 3.1 μmol/L) at both 24 h (25.6 ± 3.0 μmol/L) and 72 h (26.0 ± 2.4 μmol/L) (P<0.01).

Duodenal iron transport. In parallel studies, the effect of hepcidin on duodenal iron transport was measured in vivo (Fig. 1). In hepcidin-treated mice, significantly less iron was transferred (MT) from the duodenal mucosa to the circulation in the hepcidin-treated mice. As a result, the total amount of iron absorbed (TMU) was significantly less in both the 24- and 72-h hepcidin treatment groups compared with the saline-injected control mice.

Iron transporter expression in mouse spleen and duodenum. The effects of hepcidin on the expression of ferroportin protein in the spleen of hepcidin-treated and control

６ Abbreviations used: DMT1, divalent metal transporter 1; i.p., intraperitoneal; MR, mucosal retention; MT, mucosal transfer; TMU, total mucosal uptake.
mice were measured by immunofluorescence (Fig. 2). Ferroportin protein colocalized with the macrophage marker F4/80 in the spleen of saline-injected mice (Fig. 2A). Ferroportin immunofluorescence was not observed in the splenic white pulp. In the 24-h hepcidin treatment group, ferroportin levels were markedly less than in saline-injected mice (Fig. 2B). Whereas there was some recovery of ferroportin protein in the 72-h hepcidin treatment group, the fluorescence intensity remained less than in the untreated controls. At the mRNA level, splenic ferroportin expression was not altered following hepcidin treatment at either time point (data not shown).

Duodenal ferroportin protein localized to the basolateral membranes of enterocytes (Fig. 2C). However, following exposure to hepcidin for 24 or 72 h, ferroportin immunofluorescence was greatly diminished in the epithelial layer. DMT1 localized to the brush border membrane of upper- and mid-villus enterocytes of saline-injected mice, consistent with its essential role in intestinal iron absorption (Fig. 2D). In both groups of hepcidin-treated mice, DMT1 expression was also markedly less than in the saline-injected controls. Despite these dramatic changes in protein expression, hepcidin did not affect either ferroportin or DMT1 mRNA expression in mouse duodenum (data not shown).
In vitro iron transport. Transport of $^{59}$Fe across the Caco-2 cell monolayer was significantly decreased following exposure to hepcidin (Fig. 3A). Interestingly, cellular iron accumulation was significantly increased over the experimental period in the hepcidin-treated cells (Fig. 3B) and hepcidin treatment was also associated with a significant increase in cell ferritin levels (Fig. 3C).

In vitro effects of hepcidin on iron transporter expression. In THP-1 macrophages, ferroportin protein levels were reduced in those exposed to 300 and 1000 nmol/L hepcidin compared with untreated cells (Fig. 4). Consistent with the in vivo data, hepcidin (1 μmol/L) did not affect ferroportin mRNA expression (data not shown).

In Caco-2 cells treated with 1 and 3 μmol/L hepcidin, DMT1 protein expression was significantly less than in the untreated controls (Fig. 5). Interestingly, and in contrast to the in vivo data, treatment with hepcidin did not alter Caco-2 cell ferroportin protein levels (Fig. 5). Following hepcidin (1 μmol/L) treatment DMT1 mRNA expression was significantly less than in untreated control cells ($P < 0.01$); however, hepcidin did not affect ferroportin mRNA expression (data not shown).

Discussion

Hepcidin is the major circulating regulator of body iron metabolism. A number of studies using in vitro models over-expressing ferroportin have permitted the development of a robust model for the actions of hepcidin whereby it binds directly to the iron export protein ferroportin impairing the cellular efflux of iron (1). Despite this wealth of data, the in vivo effects of hepcidin in ferroportin-expressing tissues still need to be fully validated. The best studied cell type is the macrophage, where the evidence supporting direct inhibitory effects of hepcidin on ferroportin levels is compelling (8,9,12,19). We (8) and others (7) have demonstrated that injection of hepcidin into mice induces rapid hypoferremia. Furthermore, we have demonstrated that this decrease in serum iron is associated with a dramatic reduction in ferroportin protein levels in the macrophage-rich red pulp of the spleen (8). Our studies further support the link between elevated hepcidin, the regulation of ferroportin expression, and hypoferremia and indicate that serum iron remains low in the face of extended periods of high circulating hepcidin levels.

In the in vitro THP-1 macrophage cell model, hepcidin significantly decreased ferroportin protein levels with an $IC_{50}$ of the order of 100–300 nmol/L. This agrees with the $IC_{50}$ from transfected cell studies (5) and is comparable with circulating hepcidin levels in humans reported in a number of recent studies (20–22). Together with our in vivo data above, our findings confirm that macrophages are a major target for the actions of hepcidin.

In contrast to studies in macrophages, the mechanisms by which hepcidin controls intestinal iron homeostasis is less clear. A number of investigations employing a diverse range of experimental treatments have permitted an indirect assessment of the association between hepatic hepcidin mRNA levels and intestinal ferroportin expression. However, these studies have yielded conflicting results. In some studies where hepcidin levels increased, there was a corresponding decrease in ferroportin expression (23–25). However, in other reports, ferroportin levels were either unchanged or even increased despite elevated hepcidin expression (26–28). Similarly, in situations where hepcidin expression was decreased, several studies reported an increase in ferroportin levels (29–34), whereas in other published works, low hepcidin levels were associated with either no change or a decrease in ferroportin expression (23,35,36). In most instances, these data were produced using either knockout mice or models of chronic disease and therefore a number of other factors may account for the lack of correlation between hepcidin and ferroportin expression in these studies. Nonetheless, these conflicting data highlight the need for studies such as our own designed to investigate directly the effects of hepcidin on intestinal iron transport.

Hepcidin inhibits intestinal iron transport. We demonstrated previously that mice injected with very high levels of hepcidin daily (50–100 μg hepcidin/mouse) over a period of 3 d exhibited a significant decrease in in vivo duodenal iron transport. Interestingly, however, the mucosal uptake step seemed to be the main pathway affected by hepcidin (10). These findings have recently been confirmed by Nuñez et al. (9) following exposure of isolated ex vivo rat duodenum to hepcidin-containing medium. In vitro data from studies with Caco-2 cells further
support a major effect of hepcidin on the apical iron uptake pathway (9,11), although, importantly, in our previous work we also noted a significant decrease in iron efflux (11). In the present study, we also observed a significant decrease in iron absorption in both mouse duodenum and in Caco-2 cells following hepcidin treatment. However, and in contrast to our previous observations (10), the changes in iron transport in mice could not be explained simply in terms of a decrease in apical iron uptake. In these studies, it was apparent that basolateral iron transfer was also significantly downregulated by hepcidin treatment. The reasons for these differences are unclear but may reflect differences in the mouse strains [CD1 mice in (10); C57BL/6 in the present study] and the iron concentration of the diets employed [180 mg/kg in (10); 44 mg/kg in the present study]. A number of strain-specific differences have been observed with respect to the expression of duodenal iron transport genes (16). In addition, dietary iron levels have been shown to be an important determinant of the hepcidin response. For example, 1 study reported that hepcidin levels were not altered in response to a proinflammatory insult in mice fed an iron-rich diet (37).

To investigate the molecular basis for the changes in iron absorption, we determined the effects of hepcidin on intestinal iron transporter expression. Previous studies have reported decreased expression of DMT1 (no change was observed for ferroportin) (9), ferroportin (DMT1 expression was not measured in this study) (38), or both transporters (39) following direct exposure to hepcidin in rodent models. In agreement with the latter study, we observed a decrease in duodenal DMT1 and ferroportin protein levels in mice treated with hepcidin for 24 or 72 h. Importantly, these findings are consistent with the inhibition of duodenal iron transport by hepcidin in the in vivo transport assay.

DMT1 protein levels were also decreased in hepcidin-treated Caco-2 cells. However, despite the decrease in iron efflux from these cells following hepcidin treatment, hepcidin did not affect ferroportin protein levels. It is possible that the differential effects of hepcidin on ferroportin expression in mouse duodenum and Caco-2 cells may reflect variations in the rate of protein turnover between the in vivo and in vitro models. This is consistent with previous biotinylation studies in Caco-2 cells that indicate that ferroportin does not traffic away from the plasma membrane in this cell line following exposure to hepcidin (9). Taken together, these data suggest that in Caco-2 cells, hepcidin might bind to ferroportin and block its efflux function without inducing the trafficking and subsequent degradation of the protein that has been observed in other studies (5,6).

The data from our present studies may provide insight into the mechanism by which hepcidin regulates duodenal iron transport. Evidence from the in vivo uptake studies suggests that the primary effect of hepcidin is to downregulate iron efflux across the basolateral membrane of enterocytes. In Caco-2 cells, it is also evident that decreased iron export and the consequent increase in cellular iron accumulation appear to precede both the increase in ferritin protein expression and the decrease in DMT1 expression. This supports a sequence of events whereby hepcidin is released into the circulation, e.g. following a proinflammatory stimulus, binds to ferroportin on the basolateral surface of enterocytes, and blocks iron efflux. As a result, iron builds up inside the cells and leads to an increase in ferritin protein to store the excess iron and a decrease in DMT1 to limit iron uptake form the diet. It is not known as this stage whether these changes involve regulation through iron responsive element/iron regulatory protein interactions, but this remains a possibility.

In conclusion, we have shown that hepcidin regulates iron metabolism in both macrophages and intestinal enterocytes. Taken together with our previous work (8), it is evident that macrophages respond rapidly to hepcidin by decreasing ferroportin protein levels and thereby reducing the release of iron into the serum. Importantly, this response is sustained in the presence of hepcidin. Iron transport across enterocytes from the intestinal lumen to the circulation requires 2 transporters, namely DMT1 and ferroportin (40). We have shown that both of these transport pathways are inhibited by hepcidin in vivo and in vitro. By controlling iron transporter expression in macrophages and enterocytes, hepcidin fulfills its role as the master regulator of body iron homeostasis.
Literature Cited


