Serum hepcidin is significantly associated with iron absorption from food and supplemental sources in healthy young women

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ABSTRACT

Background: Hepcidin is a key regulator of iron homeostasis, but to date no studies have examined the effect of hepcidin on iron absorption in humans.

Objective: Our objective was to assess relations between both serum hepcidin and serum prohepcidin with nonheme-iron absorption in the presence and absence of food with the use of dual stable-iron-isotope techniques.

Design: The study group included 18 healthy nonpregnant women. Women received in random order a supplemental iron source (7.6 mg FeSO₄ providing 0.9 mg ⁵⁷Fe as FeSO₄) and 6.8 mg ⁵⁷Fe ferrous sulfate tracer administered with a nonheme food source [orange-fleshed sweet potato (OFSP): 1.4 mg native Fe]. Iron absorption was determined by analyzing blood samples taken 14 d after dosing with the use of magnetic sector thermal ionization mass spectrometry. Serum hepcidin was assessed by a new competitive serum enzyme-linked immunosorbent assay (ELISA) specific for the refolded, mature 25-amino acid form, and serum prohepcidin was assessed by an ELISA specific for amino acids 28–47 of the hepcidin prohormone.

Results: In these women, iron absorption averaged 14.71 ± 10.7% from the supplemental iron compared with 3.63 ± 6.5% from the OFSP. Absorption of nonheme iron assessed in the presence of food was not significantly related to serum hepcidin compared with prohepcidin and hypothesized that iron absorption would be more strongly associated with serum hepcidin expression and iron absorption in humans has not been established. This is in part because of the small size of this protein [25 amino acids (AAs)] and the inherent difficulties associated with its measurement. This has led some researchers to measure prohepcidin, a linear 60-AA precursor to the mature, refolded, 25-AA peptide containing 4 disulfide bonds. At present, the validity of using prohepcidin as an index of hepcidin expression is questioned because of the inability to find an association between prohepcidin and iron absorption.

The objective of this study was to assess relations between serum concentrations of prohepcidin and hepcidin with iron absorption with the use of dual stable-iron-isotope techniques. A second objective was to compare the strength of these relations from a food-based matrix compared with that observed from a supplemental source of iron (ferrous sulfate). We hypothesized that iron absorption would be more strongly associated with serum hepcidin compared with prohepcidin and that the strength of these relations would be higher from a supplemental source administered in the absence of a nonheme food matrix.

INTRODUCTION

Hepcidin has recently emerged as a key regulator of iron homeostasis (1). This protein is a small cysteine-rich peptide hormone produced in the liver and is measurable in human urine (2) and plasma (3). Hepcidin is believed to affect iron metabolism by regulation of iron absorption in the gut, iron recycling from macrophages, and control of hepatic iron storage; this hormone also appears to regulate iron transfer through the placental syncytiotrophoblast during pregnancy (1). In the enterocyte, hepcidin binds to the basolateral iron export protein, ferroportin, initiating its internalization and degradation, effectively blocking iron flux from the cell and reducing iron absorption (4). Overexpression of hepcidin is associated with anemia of inflammation (5, 6) and severe iron deficiency anemia in transgenic mice (7). However, considerably reduced hepcidin expression occurs in patients with hereditary hemochromatosis (8, 9).

Although hepcidin expression was shown to be inversely associated with iron absorption and expression of iron transport proteins in rats (10), to date a significant relation between hepcidin expression and iron absorption in humans has not been established. This is in part because of the small size of this protein [25 amino acids (AAs)] and the inherent difficulties associated with its measurement. This has led some researchers to measure prohepcidin, a linear 60-AA precursor to the mature, refolded, 25-AA peptide containing 4 disulfide bonds. At present, the validity of using prohepcidin as an index of hepcidin expression is questioned because of the inability to find an association between prohepcidin and iron absorption (11, 12).

The objective of this study was to assess relations between serum concentrations of prohepcidin and hepcidin with iron absorption with the use of dual stable-iron-isotope techniques. A second objective was to compare the strength of these relations from a food-based matrix compared with that observed from a supplemental source of iron (ferrous sulfate). We hypothesized that iron absorption would be more strongly associated with serum hepcidin compared with prohepcidin and that the strength of these relations would be higher from a supplemental source administered in the absence of a nonheme food matrix.

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Subjects

Eighteen young women, aged 18-32 y, were recruited into the study beginning in the spring of 2007. Subjects were eligible for the study if they were not taking any vitamin or mineral supplements and did not plan to ingest any during the 2-wk study interval. Subjects were also questioned on their previous supplement use, and none of the subjects had taken supplements for ≥1 mo before the study. None of the subjects had a history of intestinal or malabsorption problems, blood disorders, ulcers, or joint disease, and none were taking any prescription medications known to affect iron homeostasis. Informed written consent was obtained from each subject, and the study was approved by the Institutional Review Board of Cornell University.

On the morning of the study, fasted subjects were admitted to the Human Metabolic Research Unit (HMRU) at Cornell University. On arrival, the women's height and weight were measured (in street clothes and without shoes) with the use of a stadiometer and calibrated scale, respectively. On the first day of the study women were randomly assigned to receive either ferrous sulfate alone or combined with a nonheme-iron food-based source [orange-fleshed sweet potato (OFSP)]. The OFSPs used for this study were shipped from Peru and were baked in the oven at 350°F until soft. Once cooked, the skin was removed from the potatoes; the potatoes were mixed thoroughly. Individual servings were frozen until use and briefly microwaved before serving. The nonheme-iron--based meal consisted of 240 g of potatoes; the potatoes were mixed thoroughly. Individual servings were frozen until use and briefly microwaved before serving.

The supplemental iron consisted of 7.6 mg FeSO₄ (0.9 mg of which was ²⁵⁸Fe tracer as ferrous sulfate). The supplemental iron was given with 1.5 mL of flavored syrup composed of a liquid sugar substitute (Superose, Medina, NY) flavored with 2-3 drops of orange extract (McCormick, Hunt Valley, MD) and administered orally by a syringe. The only beverage allowed during consumption of meals was water.

Laboratory analysis

Serum ferritin was measured by a commercially available enzyme immunoassay procedure (Ramco Laboratories Inc, Stafford, TX). Serum soluble transferrin receptors were measured with an enzyme-linked immunosorbent assay (ELISA; Ramco Laboratories Inc). Total body iron was calculated by the ratio of serum transferrin receptor to serum ferritin as described by Cook et al (13): body iron (in mg/kg) = -[log (serum transferrin receptor/serum ferritin)] - 2.8229/0.1207. Hemoglobin was analyzed with the use of HemoCue (HemoCue Inc, Lake Forest, CA). Serum folate, vitamin B-12, and C-reactive protein (CRP) were measured with the use of the Immulite1000 immunos assay method (Immulite, Tarrytown, NY). Serum prohepcidin was measured by a solid-phase ELISA with antibodies specific for peptides 28-47 of the hepcidin prohormone molecule (DRG International Inc, Mountainside, NJ). Blinded serum samples were analyzed by Intrinsic LifeSciences (La Jolla, CA) to measure serum hepcidin with the use of a newly developed competitive ELISA specific for the mature peptide. Detailed methods for the performance of this assay were recently published (14). With the use of this method, typical intraassay precision and interassay CVs achieved are 5-19% and 12%, respectively. Moreover, this assay was also shown to appropriately reflect alterations in iron homeostasis (14).

Isotope preparation and sample analysis

Iron isotopes (⁵⁷Fe at 88% enrichment and ⁵⁸Fe at 93% enrichment) were purchased as metal from Trace Sciences International (Richmond Hill, Canada). Both oral tracers were converted into a sterile, pyrogen-free solution of ferrous sulfate by Anazao Health Corporation (Tampa, FL). The isotopic composition of the tracer solutions was validated with the use of a ThermoQuest Triton TI Magnetic Sector Thermal Ionization Mass Spectrometer (ThermoQuest Corporation, Bremen, Germany).

Whole-blood samples (0.5 mL) were digested with 4 mL concentrated Ultrex nitric acid in a polytetrafluoroethylene beaker. Samples were then dried overnight on a hot plate at 80°C and redissolved in 7 mol/L ultrapure hydrochloric acid (HCl; Ultrex II; JT Baker, Phillipsburg, NJ). Iron was extracted with the use of a modified anion exchange chromatography method based on a previously described method (15, 16). Small plastic columns were filled with anion exchange resin (AG 1-X8; Bio-Rad Laboratories, Hercules, CA) in deionized water. The column was washed with the following sequence: twice with deionized water, once with 0.5 mol/L ultrapure HCl, twice with deionized water, and 4 times with 7 mol/L ultrapure HCl. Digested blood samples in 7 mol/L ultrapure HCl solution were then added to the column, and 7 mol/L ultrapure HCl was added dropwise to the columns before collecting the eluted iron with the dropwise addition of 0.5 mol/L ultrapure HCl. The iron samples were dried on the hot plate and were reconstituted in 50 μL 3% HNO₃.

Extracted iron samples (8 μL) were loaded onto a rhenium filament (H Cross Co, Weehawken, NY) with 4 μL of silica gel (Sigma-Aldrich Inc, St Louis, MO) and 4 μL of phosphoric acid (0.7 N). Isotopic ratios of ⁵⁷Fe to ⁵⁶Fe (⁵⁷/⁵⁶Fe) and ⁵⁸Fe to ⁵⁶Fe (⁵⁸/⁵⁶Fe) were measured, and ratios were normalized to the ratio of ⁵⁷Fe to ⁵⁶Fe (⁵⁷/⁵⁶Fe). The fractional abundance values used were 0.02317 for ⁵⁷Fe and 0.00308 for ⁵⁸Fe. Relative SDs obtained averaged 0.015% and 0.16% for ⁵₇Fe and ⁵₈Fe, respectively.

Calculations

Iron absorption was calculated with the use of previously described methods (17, 18). The quantity of ⁵⁷Fe and ⁵⁸Fe
incorporated into erythrocytes was determined by enrichment and total circulating iron, which was estimated by using a mean blood volume for women (70 mL/kg), the concentration of iron in the hemoglobin (3.47 g/kg), and the subjects' hemoglobin value (in g/L) and weight (in kg). The final calculation for iron absorption was determined based on the assumption that 80% of the absorbed isotope was incorporated into erythrocytes. An additional correction factor was also used in the analysis to take into account the small amount of $^{58}$Fe in the $^{57}$Fe tracer (18).

**Data analysis**

All statistical analyses were completed using the STATVIEW 5.0.1 software program (Abacus Concepts, Berkeley, CA). Paired t-tests were used to determine the significance of the relations between each iron source and iron absorption. Simple linear regression analysis was used to determine the relations between iron status (serum ferritin, transferrin receptor, body iron, and hemoglobin), iron absorption, serum prohepcidin, and serum hepcidin. Data distributions were viewed by examining the normal quantile plots and histograms of the data, and normality was assessed using the goodness-of-fit test (Shapiro-Wilk test) with the use of JMP 7.0 (SAS Institute Inc, Cary, NC). Normally distributed data are presented as the mean ± SD, and data not normally distributed ($^{57}$Fe absorption, age, serum ferritin, CRP, and serum hepcidin) are presented as the geometric mean ± SD. Variables that were not normally distributed (serum hepcidin, $^{57}$Fe absorption, CRP, and serum ferritin) were transformed by using a natural logarithm before analysis for statistical purposes. Results shown are the nontransformed data for interpretation purposes. Results were considered significant if $P < 0.05$.

**RESULTS**

**Subject characteristics**

General characteristics of the study subjects are shown in Table 1. All subjects had folate and vitamin B-12 statuses within normal ranges (defined as >5 ng/mL and >200 pg/mL, respectively). Two subjects had elevated CRP concentrations (15 and 12.1 mg/L). However, these subjects did not have elevated serum ferritin concentrations, and all serum ferritin concentrations were within a normal range reported for healthy young women (19). The exclusion of subjects with elevated CRP from subsequent analyses did not significantly alter study results, so these subjects were included in all remaining analyses. A limited range of iron statuses was observed among these healthy participants with the majority being iron replete, as evidenced by the finding that none had a hemoglobin concentration <11 g/dL (mean hemoglobin concentration in the group: 12.6 ± 1.3 g/dL; a concentration above the cutoff of 12 g/dL used to define anemia in nonpregnant women); and all subjects had serum transferrin receptor concentrations <$8.5$ mg/L, indicating the absence of tissue iron deficiency (20). Only 1 of the 18 subjects had a body iron concentration <0 (negative values are indicative of depleted iron reserves and tissue iron deficiency), and this subject also had a serum ferritin concentration <$12$ pg/L. There was a mean difference of $23$ pg/L between the serum ferritin concentrations of subjects with hemoglobin concentrations <$12$ g/dL compared with hemoglobin concentrations ≥$12$ g/dL ($23.9 ± 10.2(n = 7)$ compared with $46.9 ± 41.0$ g/dL, $n = 11$), although this difference was not statistically significant ($P = 0.17$). Relations among iron status indicators were examined. From these analyses the only significant relation observed was between serum transferrin receptor and the ln of serum ferritin ($R^2 = 0.458, P = 0.002$).

Serum prohepcidin ranged from $30.7$ to $77.8$ µg/L ($47.0 ± 12.9$ µg/L) and was not significantly correlated with either the ln of serum ferritin, serum soluble transferrin receptor, body iron, hemoglobin, or the ln of CRP. Moreover, no significant relations were observed between serum prohepcidin and the ln of serum hepcidin ($P = 0.93$). Serum hepcidin concentrations ranged from $1.5$ to $248.5$ µg/L ($39.95 ± 69.6$ µg/L). The ln of serum hepcidin was significantly associated with the ln of CRP concentration ($P = 0.009$). This relation held even when the 2 subjects with elevated CRP were excluded from the analysis ($P = 0.009$). The ln of serum hepcidin was not significantly correlated with body mass index (in kg/m$^2$) or measures of iron status (ln of serum ferritin, serum soluble transferrin receptor, body iron, or hemoglobin).

**Iron absorption**

Iron absorption from ferrous sulfate administered in the fasted state was significantly greater than that measured in combination with a nonheme-iron food–based iron source ($P = 0.0006$; mean difference: $8.97$%). No significant differences in iron absorption were shown as a consequence of the order of feeding OFSPs compared with supplemental iron alone. Women absorbed an average of $14.71 ± 10.7$% ($0.58-43.9$%) from the $^{58}$Fe ferrous sulfate compared with $3.63 ± 6.5$% ($1.4-22.1$%) from the OFSPs (nonheme-iron food source) labeled with $^{57}$Fe. The intrinsic iron content of the OFSP variety used in this study was $6.15$ ± $0.6$ µg/g ($n = 5$), and the calcium content of the OFSPs was $304 ± 21$ µg/g ($n = 3$). Because of environmental, genotypic, and other factors, iron concentration in OFSPs can vary widely, but the iron content of the OFSPs we used is comparable with that reported from a similar source ($9$ µg/g), using earlier data from the Food and Agriculture Organization (21).

Despite the limited number of iron-deficient subjects, inverse nonsignificant trends were observed between the ln of serum...
ferritin and absorption of ferrous sulfate \( (P = 0.08) \) and between the ln of serum ferritin and the ln of iron absorption from the OFSPs \( (P = 0.12) \). The exclusion of the 2 subjects with elevated CRP from these analyses did not change the significance of the relation \( (P = 0.09 \) and \( P = 0.13 \), respectively). The ln of serum hepcidin was significantly correlated with both the absorption of supplemental ferrous sulfate given alone \( (P = 0.0296) \) or from the ln of iron absorption when tested in the presence of the OFSPs \( (P = 0.038) \) (Figure 1). This relation also remained significant when the 2 subjects with elevated CRP were excluded from the analysis \( (P = 0.0283 \) and \( P = 0.0112 \), respectively).

In contrast, serum prohepcidin was not significantly correlated with iron absorption from the ferrous sulfate administered alone \( (P = 0.776) \) or in combination with OFSPs \( (P = 0.429; \text{tested as the ln of iron absorption from OFSPs}) \). Other iron status indicators, including body iron, serum transferrin receptor, and hemoglobin, were not significantly related to iron absorption from ferrous sulfate administered alone or in combination with OFSPs.

**DISCUSSION**

This study adds to the growing body of literature that affirms the role of hepcidin as a key iron regulatory hormone, and, to our knowledge, it is the first study to assess the relation between this 25-AA hormone and measures of iron absorption in humans. With the use of this approach, iron absorption from ferrous sulfate administered to fasted subjects alone or in combination with a nonheme-iron food source was inversely associated with the mature 25-AA form of serum hepcidin in healthy young women. In contrast, iron absorption from the food-based or supplemental iron source used in this study was unrelated to serum prohepcidin, a finding that is similar to other nonsignificant associations published in the literature (11, 12).

Hepcidin is initially synthesized as a linear 84-AA preprohepcidin protein containing a putative 24-AA signal sequence, a 35-AA proregion, and a 25-AA active hepcidin peptide (1). Research by Valore and Ganz (22) shows that the precursor protein undergoes 2 cleavages: first the signal sequence is lost and then the proregion is cleaved by the hepatic prohormone convertase furin. In addition, there appears to be no consistent ratio or relation between the refolded, mature form of hepcidin and prohepcidin concentrations, and factors that affect hepcidin such as iron and hypoxia have no response on the cleavage of prohepcidin (22).

In the present study, serum prohepcidin concentrations ranged from 30.7 to 77.8 \( \mu \text{g/L} \), which is a lower range than previously reported in healthy persons by Hadely et al (11) \( (99-376 \mu \text{g/L}; \ n = 28 \text{ women}) \) and Kukakis et al (23) \( (51.6-153.4 \mu \text{g/L}; \ n = 26 \text{ men and women}) \) with the same approach. We found no significant relations between serum prohepcidin, iron absorption, or related indicators of iron status. This is consistent with previous research by Roe et al (12) who reported no significant association between prohepcidin and iron stores, nor did these concentrations differ between pregnant women, patients with hereditary hemochromatosis, and healthy men. In a similar population of healthy nonpregnant women, no significant relations between iron absorption and serum or urinary prohepcidin were observed, leading the investigators to propose that serum prohepcidin may not reflect the refolded, active form of hepcidin responsible for regulating iron absorption (11).

Serum hepcidin in these women ranged from 1.50 to 248.5 \( \mu \text{g/L} \) and was predominantly within previously reported 5th–95th percentile serum hepcidin ranges in men \( (29-254 \mu \text{g/L}) \) and women \( (17-286 \mu \text{g/L}) \) with the use of the same serum ELISA (14). However, in other reports that used liquid chromatography tandem mass spectrometry (LC-MSMS), a much lower range of serum hepcidin was reported in healthy men \( (<1.0-19.8 \mu \text{g/L}) \) and women \( (1.5-45.6 \mu \text{g/L}) \) (24). Serum hepcidin was significantly associated with iron absorption in our study population. From limited data available at present, a 10-fold lower serum hepcidin concentration was reported in anemic than in nonanemic persons (25). With the use of our observed relation between hepcidin and iron absorption, a 10-fold decrease in the mean serum hepcidin \( (66.7 \text{ to } 6.67 \mu \text{g/L}) \) would be expected to increase iron absorption by \( \approx 2 \)-fold (ie, from 12.34\% to 23\% for ferrous sulfate alone and from 3.0\% to 7.2\% when administered with the OFSP source as in this study).

No significant relations between hepcidin and other measured indicators of iron status (serum ferritin, body iron, serum transferrin receptor, or hemoglobin) were observed in the current study. This finding may be due in part to the small sample size and the limited number of iron-deficient study participants. In other published data, serum hepcidin was significantly related to
serum ferritin but not to hemoglobin, serum iron, or serum transferrin receptor (26). In the current study, serum hepcidin was directly associated with CRP. Two subjects had elevated CRP, which may be in response to recent infection or inflammation; however, all findings remained significant with or without the inclusion of these 2 subjects.

Iron absorption from the iron supplement (14.71%) was significantly higher when given without additional nonheme iron as OFSPs (geometric mean: 3.63; 95% CI: 5.75%). Our value of iron absorption from an 8-mg dose of ferrous sulfate was similar to that reported by Olivares et al (27) in women who were studied before and after daily iron supplementation with 60 mg of ferrous sulfate (9.7% and 12.5%, after adjustment to a reference dose). Our value of iron absorption studied with the use of OFSPs is comparable with that reported in a group of iron-replete women with similar ferritin status (6.26% and 42.6 µg/L, respectively) (28). The lower iron absorption from the OFSP meal relative to iron supplement is probably due to the presence of the food matrix, because the components of the OFSPs may affect solubility, gastric pH, and absorption.

OFSPs were selected as the nonheme-iron source because of the importance of this staple crop in many areas of world. HarvestPlus has identified the sweet potato as one of the 6 target crops for biofortification because of its potential to help alleviate micronutrient deficiencies in developing countries (29). In particular, the OFSP is a rich source of β-carotene (30) and was proven effective in improving vitamin A status in children (31, 32). Although the iron content in OFSPs is relatively low compared with animal food sources, because of its widespread daily consumption it may help contribute to daily iron needs. In this study, a 240-g serving of this vegetable would provide ~0.1 mg of absorbable iron that corresponds to ~7% of the estimated average daily amount of absorbed iron required (1.5 mg) by nonpregnant woman (33). However, it is important to note that these results were obtained from a single meal composed of only OFSP and additional dietary factors may influence its bioavailability. In addition, the amount of 57Fe required to trace iron absorption from this meal is considerably higher than the iron load that would be ingested by a meal containing only OFSPs. Intrinsinc labeling techniques or the addition of multiple smaller iron tracer doses on subsequent days would be needed to more precisely assess iron bioavailability from this food source.

At present, multiple methods can be used to measure hepcidin and prohepcidin. A commercially available ELISA is available to measure prohepcidin in urine and serum samples (11, 12, 34). In addition to the competitive assay ELISA used for this study, hepcidin can also be measured by surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (25, 35), LC-MSMS (24), and a laboratory-developed immunonot assay for urinary hepcidin (5). SELDI-TOF-MS and LC-MSMS have limitations on equipment expense and availability, and SELDI-TOF-MS also has limited analytic sensitivity and ability to detect low levels of serum hepcidin (35). Interpretation of the values reported to date is confounded by the different ranges reported in these techniques and the lack of a reference material to allow for standardization of values obtained between analytic approaches and laboratories.

Iron deficiency remains the most common nutrient deficiency in the world (36). Deficiency of this mineral is increasingly recognized to have long-term and irreversible effects during key life stages, including fetal development, early childhood, and pregnancy (37). Despite the importance of this nutrient, many key aspects of its regulation are unanswered. These data, generated with the use of a new immunoassay specific for the mature, bioactive form of hepcidin, present novel findings on the inverse relation between serum hepcidin and iron absorption from nonheme iron administered as a supplement with or without the presence of a nonheme-iron food source in a group of healthy nonpregnant women. Because of the role of iron in human health, continued research on the role of hepcidin and its effect on whole-body iron kinetics of both heme and nonheme iron is needed in relation to age, physiologic state, and iron status.

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