Iron absorption by healthy women is not associated with either serum or urinary prohepcidin\(^\text{1–4}\)

Kevin B Hadley, LuAnn K Johnson, and Janet R Hunt

**ABSTRACT**

**Background:** Although hepcidin is proposed as a regulator of iron absorption, this has not been assessed in humans.

**Objective:** Our objective was to assess the relation between serum or urinary prohepcidin and iron absorption in healthy premenopausal women.

**Design:** The subjects were 28 healthy women aged 22–51 y with normal hemoglobin concentrations (120–150 g/L). Absorption of 0.5 mg Fe with 0.2 \(\mu\)Ci \(^{59}\)Fe tracer, both as FeSO\(_4\), was measured by whole-body scintillation counting 13 d after oral administration. Fasting blood and urine samples were collected the day of and 16 wk after the absorption measurement. Serum and urinary prohepcidin concentrations were measured by an enzyme-linked immunosorbent assay by using an antibody against amino acid residues 28–47 of the proregion.

**Results:** Mean (±SD) iron absorption was 36 ± 19% (range: 4–81%), and serum ferritin (geometric \(x\) was 27 µg/L (range: 4–122 µg/L), as commonly observed in healthy premenopausal women. Serum prohepcidin was 196 µg/L (range: 99–376 µg/L) and, in contrast with urinary prohepcidin, was relatively consistent for the women between 0 and 16 wk. Serum prohepcidin correlated directly with serum ferritin (\(R^2 = 0.28, P < 0.01\)) but was unrelated to \(^{59}\)Fe absorption, in contrast to serum ferritin (\(R^2 = 0.33, P < 0.01\)).

**Conclusions:** Serum prohepcidin concentrations were relatively stable within subjects and correlated with serum ferritin. However, unlike serum ferritin, neither serum nor urinary prohepcidin concentrations were related to iron absorption in healthy women. *Am J Clin Nutr* 2006;84:150–5.

**KEY WORDS** Iron absorption, hepcidin, prohepcidin, iron status, serum ferritin, postmenopausal women

**INTRODUCTION**

Hepcidin has been proposed to function as a key regulator of iron absorption in response to body iron stores (1–4) and requirements for erythropoiesis (2, 3, 5). The inverse relation between the expression of hepcidin and solute carriers affecting intestinal iron transport capacity (6–9) provides support for a central role for hepcidin in iron homeostasis. A relation between hepcidin expression and iron absorption has not been shown in healthy humans.

Human hepcidin is a member of the B-defensin family of antimicrobial peptides (10, 11) and is expressed as an 84-amino acid (AA) pre-propeptide in hepatocytes (12, 13), renal epithelial cells (14), and several other tissues. Cleavage of the 24-AA signal peptide produces a 60-AA residue prohormone, which is detectable in serum and urine with the use of antibodies that target the proregion (13, 14). Additional processing of the 34-AA proregion results in 25-, 22-, and 20-AA peptides that are also detectable in serum and urine (10, 11). Commercial availability of a polyclonal antibody for the proregion of hepcidin provides a convenient means for estimating prohepcidin in serum and urine. Here, we report the first measurements of prohepcidin together with nonheme iron absorption in humans to test the hypotheses that prohepcidin concentrations in serum and urine correlate with dietary iron absorption and body iron concentrations in healthy premenopausal women.

**SUBJECTS AND METHODS**

**Subjects**

Twenty-eight healthy, premenopausal, nonanemic women were recruited by public advertising. Premenopausal women were studied because this was part of a project to evaluate iron bioavailability of iron sources used in food fortification. To meet selection criteria the women could not be anemic (ie, hemoglobin concentration was >120 g hemoglobin/L blood), breastfeeding, or pregnant (which was confirmed by the absence of human chorionic gonadotropin hormone in urine). The mean (±SD) age of the women was 38 ± 9 y, and their body mass index (in kg/m\(^2\)) was 24 ± 3.

The methods and protocols used in the present study were reviewed and approved by the University of North Dakota Institutional Review Board and Radioactive Drug Research Committee, and the US Department of Agriculture Radiological Safety Committee.

\(1\) From the US Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND (KBH and JRH) and the University of North Dakota, Grand Forks, ND (LJK).

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Office. Volunteers provided written informed consent before the initiation of the study.

Measurement of iron absorption
Iron absorption of each subject was measured by administering 0.2 μCi 59FeSO₄ together with 0.5 mg bakery-grade ferrous sulfate (FeSO₄ · H₂O; 32% iron by weight) in gelatin capsules containing sucrose as filler. The capsules were ingested with water. Otherwise, the subjects fasted for ≥10 h before and 4 h after ingestion of the capsules. Retention of the γ-emitting 59Fe was measured by whole-body scintillation counting before (background) and 1–4 h (initial dose) and 13 d (absorption) after ingestion, with correction for background and physical decay. The custom-made whole-body counter was described elsewhere (15).

Clinical chemistry
Fasting blood samples were collected on the morning the iron isotope was ingested and again after 16 wk (at the beginning and end of another 7 iron absorption measurements, varying in iron source and dose, that are not further described here). Hemoglobin concentration, hematocrit, and mean corpuscular volume were measured by using a CELL-DYN 3500 System (Abbott Diagnostic Division, Abbott Park, IL). Serum iron concentrations were measured by colorimetric assay with the use of a Coba-Fara Chemistry Analyzer (Hoffman–LaRoche Inc, Nutley, NJ) and commercially available chromagen (Ference, Raichem Division of Hemagen Diagnostics, San Diego, CA). Total iron-binding capacity in the presence of excess ferrous iron under alkaline conditions was measured similarly to serum iron. Transferrin saturation was measured from serum iron and total iron-binding capacity. Soluble transferrin receptor concentrations were measured by enzyme-linked immunosorbent assay (Quantikin Human Transferrin Receptor Immunoassay; R&D Systems Inc, Minneapolis, MN). Serum ferritin was measured by immunoassay (Immulett ferritin; Diagnostic Products Corp, Los Angeles, CA). Body iron was calculated from the ratio of serum transferrin receptor to serum ferritin (16) because commercial sources of transferrin receptor measurements have not been standardized. This required a calculated conversion from the values of R&D Systems to those of Ramco (Houston, TX) for transferrin receptor; these values are directly correlated (R² = 0.86) (17). C-reactive protein was measured by nephelometry (Behring Diagnostics Inc, Westwood, MA) and used as an indicator of inflammation. No ferritin values were eliminated according to the agnostics Inc, Westwood, MA) and used as an indicator of inflammation. No ferritin values were eliminated according to the

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td>38 ± 9 (22–51)²</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 ± 3 (20–34)</td>
</tr>
<tr>
<td>Iron absorption (%)</td>
<td>36 ± 19 (4–81)</td>
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<tr>
<td>Hematocrit (%)</td>
<td>41 ± 2 (36–45)</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>137 ± 8 (120–152)</td>
</tr>
<tr>
<td>Mean cell volume (FL)</td>
<td>91 ± 3 (84–98)</td>
</tr>
<tr>
<td>In Serum ferritin (µg/L)</td>
<td>3.3 ± 0.9 (1.4–4.8)</td>
</tr>
<tr>
<td>Serum ferritin (µg/L)</td>
<td>27 (4–122)¹</td>
</tr>
<tr>
<td>In Serum prohepcidin (µg/L)</td>
<td>5.3 ± 0.4 (4.6–5.9)</td>
</tr>
<tr>
<td>Serum prohepcidin (µg/L)</td>
<td>196 (99–376)</td>
</tr>
<tr>
<td>In Urinary prohepcidin (µg/g creatinine)</td>
<td>5.2 ± 0.4 (4.4–6.3)</td>
</tr>
<tr>
<td>Urinary prohepcidin (µg/g creatinine)</td>
<td>186 (83–553)</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>32 ± 18 (7–80)</td>
</tr>
<tr>
<td>Transferrin receptor (nmol/L)</td>
<td>19 ± 4 (12–27)</td>
</tr>
<tr>
<td>Body iron (mg/kg body wt)</td>
<td>4.1 ± 3.2 (2.6–9.5)</td>
</tr>
<tr>
<td>In C-reactive protein (mg/L)</td>
<td>0.6 ± 1.3 (1–9.2–2.6)</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>1.8 (0.2–14)</td>
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¹ n = 28.
² ± SD; range in parentheses (all such values).
³ Geometric ±; range in parentheses (all such values).

Data analysis
All statistical tests were done with the use of PC-SAS version 9.1 [SAS Institute Inc, Cary, NC (19)]. The intersubject and intraindividual CVs of the logarithmically transformed values were 6.6% and 2.8%, respectively, indicating that the prohepcidin concentrations of the women remained relatively constant between 0 and 16 wk. Consistent with this, the 2 serum measurements were highly correlated (R² = 0.74; Figure 1). In contrast, the urinary prohepcidin measurements were skewed, with greater intraindividual than intersubject variation, and the urinary results did not significantly correlate between 0 and 16 wk (NS; Figure 1). Serum prohepcidin did not correlate with urinary prohepcidin concentrations (measurements not shown).

The women absorbed from 4% to 81% (x ± SD: 36 ± 19%) of the relatively small dose (0.5 mg) of orally administered radio-labeled iron (Table 1). The blood measurements confirmed that the subjects were not anemic (by design, hemoglobin concentrations were ≥120 g/L) and showed broad ranges of serum ferritin (4–122 µg/L) and body iron stores (2.6–9.5 mg/kg body weight) from very low to moderately high for premenopausal women (Table 1).

Serum prohepcidin concentrations on the morning of the iron absorption measurement ranged from 99 to 376 µg/L (geometric mean: 196 µg/L; Table 1). On the basis of both blood samples, the interindividual and intraindividual CVs of the logarithmically transformed values were 6.6% and 2.8%, respectively, indicating that the prohepcidin concentrations of the women remained relatively constant between 0 and 16 wk. Consistent with this, the 2 serum measurements were highly correlated (R² = 0.74; Figure 1). In contrast, the urinary prohepcidin measurements were skewed, with greater intraindividual than intersubject variation, and the urinary results did not significantly correlate between 0 and 16 wk (NS; Figure 1). Serum prohepcidin did not correlate with urinary prohepcidin concentrations (measurements not shown).

The serum prohepcidin values, which varied by nearly 4 times between the subjects, along with iron absorption measurements of variance. Relations between the variables were assessed by linear regression analysis, except that power curves best fit the relation between iron absorption and serum ferritin (this relation is linear when data for both variables are logarithmically transformed). Multiple regression analysis was used to test whether iron absorption was predicted by both serum ferritin and prohepcidin. Unless otherwise noted, most regression analyses of relations between the variables occurred at week 0, when the iron absorption measurements were conducted. A probability of < 0.05 was considered statistically significant.

RESULTS
The women absorbed from 4% to 81% (x ± SD: 36 ± 19%) of the relatively small dose (0.5 mg) of orally administered radio-labeled iron (Table 1). The blood measurements confirmed that the subjects were not anemic (by design, hemoglobin concentrations were ≥120 g/L) and showed broad ranges of serum ferritin (4–122 µg/L) and body iron stores (2.6–9.5 mg/kg body weight) from very low to moderately high for premenopausal women (Table 1).

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from 4% to 81% and serum ferritin from 4 to 122 μg/L provided data (at week 0) to assess the relation between serum prohepcidin and iron absorption under basal conditions (ie, without iron stimulation of prohepcidin) in healthy women. Serum prohepcidin was positively correlated with ferritin ($R^2 = 0.28, P < 0.01$; Figure 2). However, iron absorption was inversely associated with serum ferritin ($R^2 = 0.33, P < 0.01$; Figure 2) and was not significantly associated with serum prohepcidin ($P = 0.8$; Figure 2).
2). Serum prohepcidin in combination with ferritin was not appreciably more useful than ferritin alone in predicting iron absorption. With the use of stepwise multiple regression, the prediction of iron absorption by serum ferritin was only slightly improved (from $R^2 = 0.33$, $P < 0.01$) by adding serum prohepcidin (overall $R^2 = 0.39$, $P < 0.01$; serum prohepcidin partial $R^2 = 0.06$, $P = 0.12$). Single measurements of transferrin saturation were more variable than the other measured iron indexes, and transferrin saturation was not significantly associated with serum prohepcidin (Figure 2). The relation of serum prohepcidin with iron absorption was not improved by evaluating body iron stores (by using serum transferrin receptor:serum ferritin; data not shown) rather than by using serum ferritin alone, probably because the iron status of these women was not sufficiently low to cause anemia.

In contrast to a hypothesized in inverse correlation, urinary prohepcidin was directly correlated with iron absorption ($R^2 = 0.28$, $P < 0.01$). Because a weak inverse correlation between ln ferritin and ln urinary prohepcidin ($R^2 = 0.16$, $P < 0.05$) at week 0 was also opposite of the expected relation, and because no significant correlation was observed between these same variables at week 16 ($R^2 = 0.05$, NS), we concluded that urinary prohepcidin was not significantly associated with serum ferritin. In a multiple regression analysis, inclusion of urinary prohepcidin did not significantly improve the prediction of iron absorption by serum ferritin.

**DISCUSSION**

The present study marks the first clinical report of the relation between intestinal iron absorption and serum prohepcidin concentrations in healthy human subjects. Serum prohepcidin concentrations ranged from 99 to 376 µg/L for these premenopausal women and were highly consistent when sampled 16 wk apart. Kulaksiz et al (13) reported a somewhat lower range of prohepcidin values, from 52 to 153 µg/L for healthy male ($n = 13$) and female ($n = 13$) subjects. Here, the percentage of iron absorption ranged from 4% to 81% of the administered dose, consistent with relatively high iron absorption from a 0.5-mg Fe dose (20, 21), and indicated a broad range of iron absorption potentials within the subject group. The observed range of serum ferritin (4−122 µg/L) was similar to the 5th and 95th percentiles for serum ferritin of US women aged 19−30 y (22). Together, these results indicate a wide range of body iron status and intestinal iron transport capacities for premenopausal women.

Because hepcidin was proposed as a key regulator of iron absorption (1−5), the relation between prohepcidin and intestinal iron absorption was of particular interest in this investigation. Remarkably, no correlation between intestinal iron absorption and serum prohepcidin was apparent, despite a considerable range of both iron absorption and serum prohepcidin concentrations. Possible explanations, further considered below, include the following: 1) serum prohepcidin may not reflect the active form of hepcidin that influences intestinal iron absorption; 2) the range of iron status and related prohepcidin concentrations of healthy, premenopausal women is limited compared with clinical conditions that affect iron metabolism, such as anemia or hemochromatosis; and 3) hepcidin may not be a primary factor that influences intestinal iron absorption as related to iron stores in healthy people.

The prohepcidin molecule is a precursor to the hepcidin peptides composed of 20, 22, or 25 AAs. The 60-AA prohepcidin found in serum (11, 13) is apparently cleaved from the 84-AA pre-propeptide gene product (1) and expressed substantially, but not exclusively, in the liver (1, 13, 14). The metabolic influence of prohepcidin is likely modified further by factors that affect cleavage to produce the active form of hepcidin (7). The possible significance of specific extrahepatic cleavage is suggested by the observation that both the 20- and 25-AA forms predominate in urine (11), but only the 25-AA hepcidin peptide appears to bind to and induce internalization of the iron transporter, ferroportin, on the apical surface of cultured enterocytes (7).

An antibody useful for detecting the smaller hepcidin peptides in serum has been technically difficult to produce, probably because of hepcidin’s small size, unique folding, and extensive conservation among animal species. However, much of the current knowledge of hepcidin related to iron absorption was based on rodent (2−5, 23, 24) or human (25) studies of hepcidin gene expression, for which the gene product prohepcidin, in serum, may serve as an indicator.

The value of serum hepcidin has been questioned, with the suggestion that urinary hepcidin excretion may more accurately represent hepcidin production over several hours (26). However, the consistency observed with serum prohepcidin concentrations under fasting conditions in the present investigation, in contrast with that of urinary prohepcidin, suggests that serum prohepcidin concentrations are more stable than are urinary concentrations. Iron absorption is relatively stable in healthy subjects over periods of several weeks (27, 28). Because hepcidin is expressed in the kidney (14), urinary hepcidin excretion may reflect localized renal production rather than blood concentrations that are more likely to influence intestinal absorption. The present results confirm those of Kulaksiz et al (14), who found no correlation between circulating prohepcidin and urinary hepcidin. No such comparison has been made between serum and urinary hepcidin, and none of these hepcidin or prohepcidin indexes has previously been evaluated with iron absorption measurements in humans. Differences were observed when an inflammatory stimulus (intravenous injection of bacterial lipopolysaccharide) administered to 10 healthy subjects decreased serum iron and increased urinary hepcidin without affecting serum prohepcidin (29). In patients who had liver surgery, urinary hepcidin correlated positively with hepatic hepcidin mRNA and hepatic iron concentrations (30). At this time, limitations of the serum prohepcidin assay cannot be ruled out as an explanation for finding no correlation between serum prohepcidin and iron absorption in healthy women.

The more narrow range of ferritin and associated body iron stores of healthy premenopausal women than of subject groups that also include men and postmenopausal women and conditions such as anemia or hemochromatosis is a possible limitation for detecting an association between serum prohepcidin and iron absorption. However, the range of iron status in the present study sample was sufficient for serum ferritin values to explain 33% of the variation in iron absorption (Figure 2). Serum ferritin is a sensitive biochemical indicator of body iron stores in healthy subjects (31−34), and the well-established inverse relation between iron absorption and serum ferritin accounts for up to 65% of the variation when more subjects or both male and female subjects are observed (35−38). Thus, this sample of premenopausal women with a limited range of iron status was sufficient.
transferrin saturation may influence hepcidin expression and iron absorption. In that study, the increase in iron absorption was observed in a mouse model with inherited mild anemia (hemoglobin deficiency) characterized by reduced iron uptake into immature erythroid cells (40). A nearly 3-fold increase in iron absorption was observed in rats 6 d after switching from an iron-adequate to iron-deficient diet (5). Hepcidin expression was greater in patients with higher than with lower liver iron staining (basal expression was greater in the control subjects than in those who were homozygous for the HFE C282Y hemochromatosis mutation) (39). Although these associations show that hepcidin expression is related to body iron stores, it is not known whether iron stores per se control basal hepcidin expression.

Hepcidin expression may be more responsive to changes in erythropoiesis or iron intake than to changes in body iron stores. Consistent with this, hepcidin expression was down-regulated by phenylhydrazine-induced hemolytic anemia, despite increased liver iron (5, 24). This hemolytic anemia significantly increased iron absorption (whole-body retention of oral $^{59}$Fe) in rats, coinciding with reduced liver hepcidin expression and increased duodenal expression of the iron transporters DMT1, cytochrome b, and Ireg1 (also called ferroportin) (24). Increased hepcidin expression and increased transferrin saturation were observed, without differences in liver iron concentration or iron absorption, in a mouse model with inherited mild anemia (hemoglobin deficient or hbd) characterized by reduced iron uptake into immature erythroid cells (40). A nearly 3-fold increase in iron absorption was observed in rats 6 d after switching from an iron-deficient diet to an iron-deficient diet was accompanied by similar changes in iron absorption before iron stores or erythropoiesis are affected (9).

The effect of hepcidin on iron absorption apparently differs from the effect of dietary iron. Synthetic hepcidin injections substantially reduced iron absorption, as measured in tied duodenal segments, in mice fed either iron-deficient or iron-replete diets for 3 wk (6). Hcpidin reduced both the mucosal uptake and transfer of iron to similar degrees with each dietary treatment. However, the dietary treatment, but not the hepcidin injection, significantly altered the proportional mucosal transfer of iron, suggesting that an additional factor other than hepcidin was involved in the control of mucosal iron transfer as influenced by dietary iron (6).

The present results suggest that neither serum nor urinary prohepcidin is positively correlated with the variations in human iron absorption that are associated with a normal range of body iron stores. Consistent with the positive correlation observed between serum ferritin and serum prohepcidin in the present study, serum hepcidin correlated with serum ferritin (ranging from 10 to $>13$ 000 μg/L) but not transferrin saturation, transferrin receptor, or hemoglobin in patients evaluated for ferritin or for anemia (26). In 36 patients undergoing liver surgery, with iron status that spanned from anemia to excessive iron stores, hepatic hepcidin expression correlated with liver iron but not with transferrin saturation and, after elimination of 16 subjects with liver fibrosis, correlated with serum ferritin (30). Urinary hepcidin in the same patients correlated with liver hepcidin expression, liver iron, and serum ferritin but not with transferrin saturation (30). Serum prohepcidin concentrations of patients with hemochromatosis tended to be lower than those of control subjects (nonsignificantly) but did not correlate with serum ferritin or transferrin saturation (13). Together, these data suggest only an inconsistent correlation of hepcidin indexes (hepcidin expression, serum prohepcidin, or urinary hepcidin) with serum ferritin and no correlation with transferrin saturation in humans.

We note the lack of a demonstrated relation with transferrin saturation in the human studies, because diferric transferrin, which interacts with the transferrin receptor complex, together with immune-related factors such as the hemochromatosis gene product, HFE, appears to play a central role in hepatic hepcidin gene expression (9, 41–44). However, compared with ferritin, transferrin saturation is less closely associated with iron absorption in humans (35).

Before the present study, neither serum nor urinary concentrations of either hepcidin or prohepcidin had been tested with sensitive iron absorption measurements in humans. Such absorption measurements will be useful as knowledge accrues on the role of the hepcidin system in the control of iron absorption. The present findings showed that neither serum nor urinary prohepcidin was significantly related to the association between iron absorption and serum ferritin in healthy humans. This association needs to be explained as mechanisms that control iron absorption are further elucidated.

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JRH conceived the study. Both KBH and JRH participated in the experimental design, data collection and analysis, and writing of the manuscript. LKJ participated in the experimental design and performed the statistical analyses of the data. None of the authors had a conflict of interest.

REFERENCES