Published in final edited form as:
Blood Cells Mol Dis. 2008 ; 41(3): 265–269. doi:10.1016/j.bcmd.2008.06.009.

Serum prohepcidin is associated with soluble transferrin receptor-1 but not ferritin in healthy post-menopausal women

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Abstract

Hepcidin is a 25-amino-acid iron peptide hormone originated from its two precursors of prohepcidin (60-amino-acid) and preprohepcidin (84-amino-acid). Serum prohepcidin levels have been widely used to evaluate iron overload in clinical and preclinical studies. However, its usefulness is often questioned and its stepwise conversion mechanism remains largely unknown. Using New York University Women's Health Study subjects, we measured serum levels of prohepcidin with ELISA and hepcidin with mass spectrometry as well as ferritin and soluble transferrin receptor 1 (sTfR1) in 45 normal healthy postmenopausal women over a 1-year period with 2 samples per subject. We found that serum prohepcidin levels are correlated with the serum sTfR1 levels ($r=0.45$, $p<0.01$) but not to ferritin levels ($r=0.08$, $p=0.60$), suggesting that serum prohepcidin is not a biomarker of iron overload that was originally thought and designed for. Interestingly, serum hepcidin levels are associated with serum ferritin levels ($r=0.64$, $p<0.0001$) but not with sTfR1 levels ($r=0.04$, $p=0.69$), indicating that hepcidin is a measure of iron overload. Although hepcidin is a downstream product of prohepcidin, the amounts of hepcidin and prohepcidin are not related to each other ($r=-0.007$, $p=0.90$) under normal physiological conditions. The interrelationships between sTfR1 and prohepcidin or between ferritin and hepcidin suggest that ferritin- and sTfR1-sensed hepcidin conversion system exists in human body and maybe regulated at the post-translational level.

Keywords

Iron; homeostasis; transferrin receptor; ferritin; hepcidin

Introduction

Hepcidin is a peptide hormone synthesized in the liver and is the principal regulator of systemic iron homeostasis. Hepcidin controls plasma iron concentration and tissue iron distribution by inhibiting intestinal iron absorption, iron recycling by macrophages, and iron mobilization from hepatic stores [1,2]. Hepcidin influences iron absorption through direct binding to ferroportin at the basolateral membrane, leading to decreased export of iron to the circulation system [3]. The functional hepcidin molecule is derived from the 2-step conversion of an 84-

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amino-acid long peptide, the preprohepcidin, by N-terminus cleavage of a 24-amino-acid signal peptide to give first rise to prohepcidin, followed by a second cleavage of a 35-amino-acid peptide to yield the active 25-amino-acid hepcidin [4,5]. Prohepcidin is expressed at the basolateral membrane of hepatocytes and is found in the blood [6]. Serum levels of prohepcidin have been widely used to diagnose iron overload. However, clinical and preclinical studies have failed to show positive correlations between serum prohepcidin concentrations and serum iron overload markers such as serum ferritin levels [6–9]. Until now, it has been unclear whether serum prohepcidin is a measure of active hepcidin or simply a non-functional precursor [10], and mechanisms controlling its stepwise conversion in the circulation remain unknown.

Currently, prohepcidin can be easily measured in serum using the commercially available enzyme-linked immunosorbent assay (ELISA) [6]. The active form of hepcidin can be measured in serum by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) [11]. Our aim was to examine the relationship between active hepcidin and prohepcidin in sera of normal healthy subjects and to assess whether serum ferritin and soluble transferrin receptor 1 (sTfR1) play a part in hepcidin regulation at the post-translational level.

Materials and Methods

The New York University (NYU) Women's Health Study

Between March 1985 and June 1991, the NYU Women's Health Study enrolled a cohort of 14 274 healthy women, aged 34–65 years, at a breast cancer screening center in New York City and details about blood collection and completion of a self-administered questionnaire were previously described [12,13]. Forty-five women were selected at random from the pool of eligible women within the NYU Women's Health Study as previously described [14]. This pool consisted of the NYU Women's Health Study participants who were postmenopausal at entry, had given blood on 3 or more occasions at yearly intervals, had a yield of 11 or more aliquots at each visit, had not been diagnosed with cancer or cardiovascular disease, and had not been selected as a control in any case-control study nested within the cohort. The Institutional Review Board at the NYU School of Medicine approved this study. Two yearly samples were retrieved for each woman.

Laboratory analyses for serum prohepcidin, ferritin, and sTfR1

Serum samples were identified solely by a sample number so that laboratory personnel were not aware of the identity of the contributing participants. All samples from a subject were always assayed in the same batch. Levels of prohepcidin were determined by an ELISA assay based on a competitive principle (DRG International, Inc., Mountainside, NJ). sTfR1 in sera was determined by an ELISA technique using two different monoclonal antibodies specific for sTfR1 (R & D System, Minneapolis, MN). Ferritin in sera was determined according to a previously published protocol [15].

Analyses of serum hepcidin by mass spectrometry

Serum hepcidin measurements by SELDI-TOF MS were performed as previously described [11]. In brief, the hepcidin SELDI test involves the chromatographic retention of hepcidin and hepcidin variants using an immobilized metal affinity chromatographic ProteinChip® Array pre-loaded with copper ions. After binding the sample under optimized conditions of pH and ionic strength, non-specifically associated proteins are removed by washing with the binding buffer. The retained proteins are then detected by SELDI-TOF MS and the specific hepcidin variants are identified within the mass spectrometry by their unique mass/charge ratio. For the quantitation of hepcidin, the relative peak intensity of the hepcidin variant is compared against a standard curve generated by spiking in synthetic hepcidin peptide (American Peptide

Company, Sunnyvale, CA) into a reference serum. Data were expressed as μg/ml concentration.

Statistical Methods

Correlation among prohepcidin, hepcidin, ferritin, and sTfR1 were analyzed using the Spearman rank correlation.

Results

Women had a median age (range) at first blood donation of 62.3 years (49.5 – 68.1 years), with a median time since menopause of 12.6 years (2.2 – 25.3 years). The median weight and body mass index were 66.0 kg (43.0 – 86.0 kg) and 24.6 kg/m^2 (19.2 – 35.9 kg/m^2), respectively. Serum samples were in storage at −80°C for a median of 16.6 years (15.8 – 18.1 years) at the time when we performed all the measurements.

Figure 1 presents a linear standard curve obtained from peak intensity at 2792 m/z with the synthetic peptide ranging from 0 to 2 μg/mL (A) and representative protein mass spectra of a standard and a serum sample from one of the study subjects (B). We found interassay precisions of 24.8%, 11.2%, 8.7%, 7.0%, and 1.4% at 0.125, 0.25, 0.5, 1, and 2 μg/mL, respectively.

Table 1 shows the Spearman rank correlation coefficients among serum levels of prohepcidin, sTfR1, ferritin, and hepcidin over the two visits. Combining the data of both visits, we found that prohepcidin is positively correlated to sTfR1 ($r=0.45$, $p<0.01$) but not to ferritin ($r=0.08$, $p=0.60$). Active hepcidin is positively associated with ferritin ($r=0.64$, $p<0.0001$) but not with sTfR1 ($r=0.06$, $p=0.70$). To our surprise, serum prohepcidin levels are not correlated at all with serum active hepcidin levels ($r=-0.007$, $p=0.90$). Serum ferritin levels are not correlated with serum sTfR1 levels ($r=0.06$, $p=0.67$). In addition, we found that the reliability coefficient for active hepcidin was fairly low ($r=0.49$, 95% confidence interval 0.22–0.68). This is in contrast to the high temporal reliability coefficients that we previously observed for ferritin, sTfR1, and prohepcidin, which were 0.78 (95% CI: 0.67–0.86), 0.79 (95% CI: 0.69–0.87), and 0.89 (95% CI: 0.84–0.94), respectively [14].

Discussion

Hepcidin is a key regulator of body iron metabolism and, thus, there is an enormous interest in quantifying circulating hepcidin in clinical samples. Because SELDI-TOF MS is not easily accessible for hepcidin measurements, ELISA-based technique to measure prohepcidin has been widely used as an amenable tool with the assumptions that the concentrations of prohepcidin correlate with those of hepcidin and both of them have similar clinical implications in iron overload. However, previous studies failed to show prohepcidin changes as a function of iron status changes, which raised the question of the usefulness of prohepcidin in evaluating iron overload [6,9,16]. For example, it has been shown that serum prohepcidin concentrations in blood samples from hereditary hemochromatosis patients were not significantly different from those of normal healthy subjects and were not related to differences in iron stores as measured by ferritin [9]. Even in hemochromatosis patients undergoing phlebotomy, the range of serum prohepcidin was small, despite large differences in serum ferritin concentrations [9]. Serum prohepcidin concentrations in patients with sickle cell anemia with abnormally high serum ferritin concentrations were no different than in controls [16]. Similarly, no association was found between serum prohepcidin and iron status measures such as serum iron, transferrin saturation, or serum ferritin [6].

In view of the discordance between prohepcidin and iron overload, we measured serum levels of prohepcidin with ELISA and hepcidin with SELDI-TOS MS, as well as ferritin and sTfR1

in 45 health post-menopausal subjects. Surprisingly, our study shows that prohepcidin levels are correlated to sTfR1, suggesting that prohepcidin may be associated with hypoferremia or erythropoiesis. In contrast, active hepcidin is positively associated with ferritin but not with sTfR1, indicating that active hepcidin is a biomarker of iron overload. This positive association is in agreement with the data showing a significant and positive correlation between active hepcidin and ferritin in hemodialysis patients as well as in healthy controls [17,18]. Although our sample size is relatively small, the associations between active hepcidin and ferritin but not sTfR1, and between prohepcidin and sTfR1 but not ferritin are reproducible with repeated visits by the same subject. Moreover, subjects in our study were normal healthy post-menopausal women, so that levels of prohepcidin and hepcidin in these individuals should not be affected by disease conditions such as inflammation, anemia, iron overload, hemodialysis, or cancer [17,19–22]. No impact of estrogen or menstruation should be at play either because all women were post-menopausal and not taking hormone replacement therapy [23]. Therefore, levels of these four iron proteins should reflect only the physiological iron status in the healthy body.

Iron homeostasis is strictly controlled at the transcriptional level in the body and at posttranscriptional level in the cells [2,3]. Intracellular iron balance is achieved through posttranscriptional regulation of TfR1 and ferritin mRNA levels by iron regulator proteins [1,24]. Cellular iron deficiency increases iron uptake by over-expressing membrane TfR1 and down-regulating ferritin in the cells [25]. Hepcidin is a negative regulator of body iron uptake [2]. At the systemic level, iron overload up-regulates preprohepcidin mRNA levels and iron deficiency down-regulates preprohepcidin in the liver [26–28]. By incubating HepG2 cells with sera from iron deficiency anemia and thalassemia major patients, which contain elevated sTfR1, levels of preprohepcidin mRNA expression were significantly decreased [29]. Bone morphogenetic proteins have been found to use hemojuvelin as a coreceptor to regulate preprohepcidin expression and to be more potent than IL-6 in stimulating preprohepcidin transcription [30,31]. Recently, it has been identified that transmembrane serine protease 6 (TMPRSS6) is an essential component detecting iron deficiency and blocking preprohepcidin transcription [32]; germline mutations in the TMPRSS6 gene can cause iron-refractory iron deficiency anemia [33].

In addition to its regulation at the transcriptional level, post-translational cleavage of prohepcidin to hepcidin in human hepatocytes is mediated by the prohormone convertase furin [34]. Our results suggest that stepwise conversions of preprohepcidin after its secretion from liver may exist in the circulation (Figure 2). Serum sTfR1 is usually considered to be markers of hypoferremia and/or erythropoiesis. Ferritin is related to iron overload and/or inflammation. Under hypoferremic condition or high erythropoietic activity, increased sTfR1 may not only inhibit mRNA and protein synthesis of preprohepcidin in the liver [26–28], but also could mediate conversion of preprohepcidin to prohepcidin in sera. That is probably why in our study sTfR1 was positively associated with prohepcidin. This regulation could be a synchronized event in order to decrease the overall mRNA and protein levels of preprohepcidin.

Our data also showed that the reliability coefficient for prohepcidin is higher (r=0.89) than that of active hepcidin (r=0.48) over a 1-year period. The high reliability of prohepcidin indicates that this protein is fairly stable over time within an individual, relative to others. On the contrary, the lower reliability of hepcidin indicates variations over time for serum hepcidin levels. In view of the facts that 1) synthetic hepcidin causes rapid dose-dependent hypoferremia and immediate excretion of hepcidin metabolites in the urine; 2) furin quickly cleaves prohepcidin to hepcidin in hepatocytes; and, 3) both hepcidin and ferritin are acute phase proteins in response to inflammation [19,34–36], it is reasonable to assume that the step 2 conversion of prohepcidin to hepcidin is fast. Thus, the step 1 conversion of preprohepcidin to prohepcidin may be the rate-limiting step (Figure 2). This step 1 conversion is probably the first line of

defense to prepare the body to get ready when iron overload arises. When iron loading does occur, the step 2 conversion can quickly take place leading to the inhibition of iron absorption.

Overall, our results indicate that prohepcidin is a biomarker reflecting hypoferremic conditions and possibly erythropoietic activity, which was previously unexpected in its clinical and preclinical use. On the other hand, active hepcidin is a biomarker for iron overload such as hemochromatosis. Our results also suggest that a ferritin- and sTfR1-sensed hepcidin conversion mechanism may exist to regulate systemic iron homeostasis at the post-translational levels. Further studies should elucidate the mechanisms of how human body senses the needs for hepcidin conversions and what factors play a role in these processes.

Abbreviations

sTfR1, soluble transferrin receptor 1; TMPRSS6, transmembrane serine protease 6..

Acknowledgement

This work was funded by the US National Institute of Health (NIH CA132684) and in part by NIH grants ES00260, CA34588, and CA16087.

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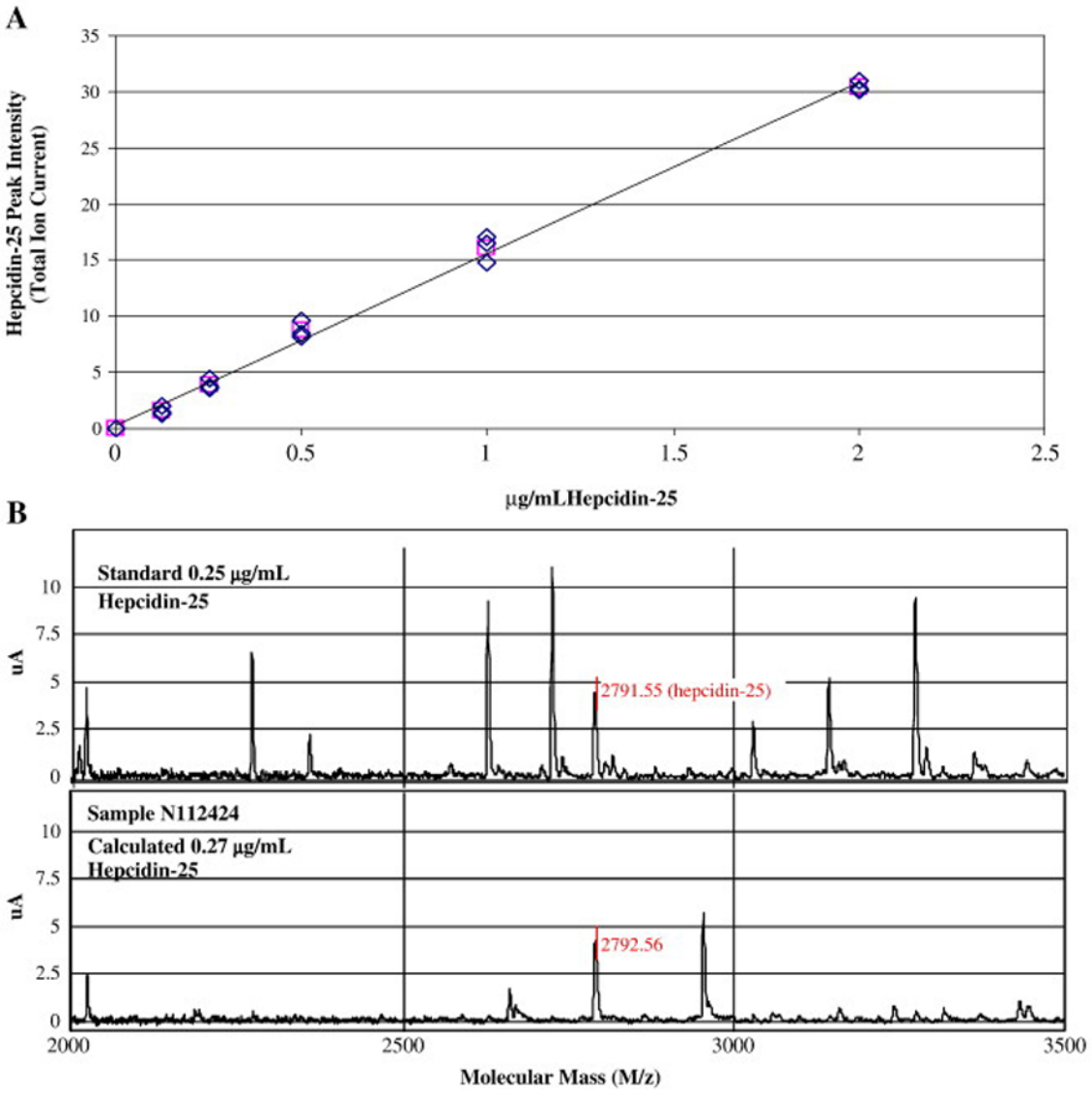

Figure 1. Standard curve of synthetic hepcidin and representative protein mass spectra of synthetic hepcidin and a serum sample

(A) Mean slope from a set of 4 standard curves with hepcidin-25; (B) Top: synthetic hepcidin; Bottom: Serum sample from one of the study subjects.

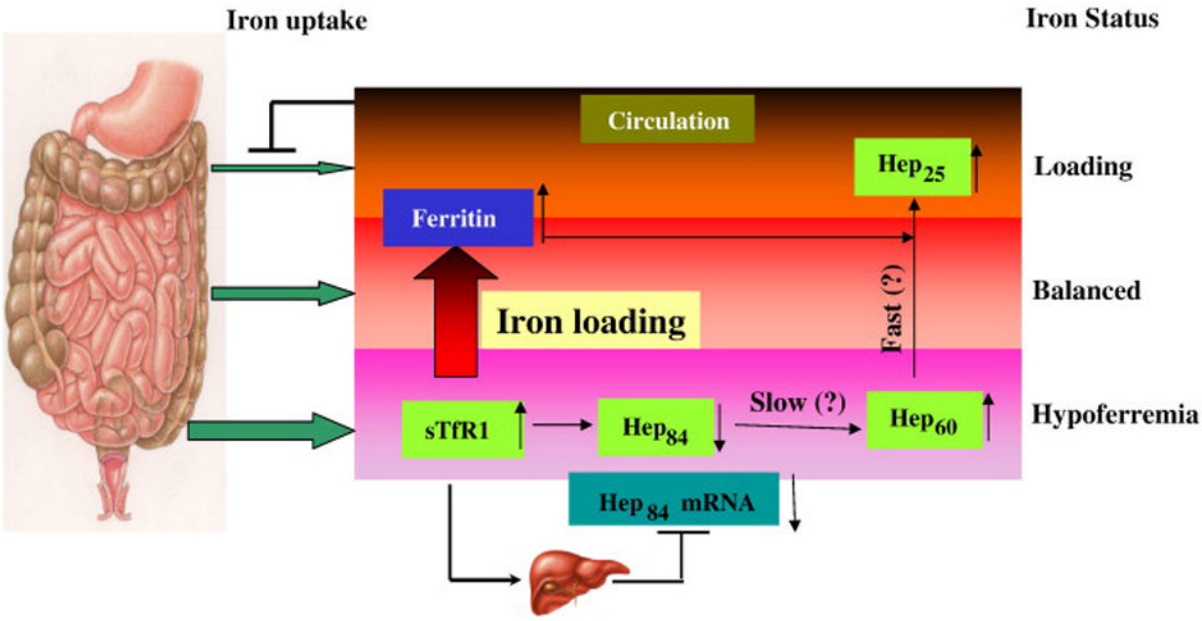

Figure 2. Proposed ferritin- and sTfR1-sensed hepcidin conversion mechanism

Iron uptake may be controlled by ferritin- and sTfR1-sensed preprohepcidin conversion mechanism in the circulation at the post-translational level. Regulation of preprohepcidin in the liver at the transcription level is also shown. Hep84: preprohepcidin; Hep60: prohepcidin; Hep25: hepcidin.

Table 1

Results of Spearman rank correlation among prohepcidin, hepcidin, ferritin, and sTfR1 ($N = 45$)

	Prohepcidin				**Ferritin**				**sTfR1**				**Hepcidin**			
	Visit 1		**Visit 2**		**Visit 1**		**Visit 2**		**Visit 1**		**Visit 2**		**Visit 1**		**Visit 2**	
	r	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Prohepcidin	1		1		0.079	0.60	0.08	0.60	0.42*	0.004	0.49*	0.007	−0.025	0.87	0.01	0.94
Ferritin	0.079	0.60	0.08	0.60	1		1		0.09	0.56	0.04	0.79	0.57*	< 0.0001	0.71*	< 0.0001
sTfR1	0.42*	0.004	0.49*	0.007	0.09	0.56	0.04	0.79	1		1		0.04	0.77	0.08	0.62
Hepcidin	−0.025	0.87	0.01	0.94	0.57*	< 0.0001	0.71*	< 0.0001	0.04	0.77	0.08	0.62	1		1	