Circulating Human Hepcidin-25 Concentrations Display a Diurnal Rhythm, Increase with Prolonged Fasting, and Are Reduced by Growth Hormone Administration

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BACKGROUND: Hepcidin-25 reduces iron absorption by binding to the intestinal iron transporter ferroportin and causing its degradation. Currently, little is known about the basal regulation of circulating hepcidin-25. In addition, although erythropoietin administration has been reported to decrease the circulating hepcidin concentration, information is limited regarding how other stimulators of erythropoiesis, such as growth hormone (GH), might alter hepcidin-25 concentrations.

METHODS: We used a sensitive and specific hepcidin-25 dual–monoclonal antibody sandwich immunoassay to measure hepcidin-25 in healthy human volunteers at various time points throughout the day and during 3 days of fasting and subsequent refeeding. We also measured hepcidin-25 concentrations in healthy volunteers after GH administration.

RESULTS: In healthy individuals, hepcidin-25 concentrations displayed a diurnal variation, with concentrations being lowest in the early morning and steadily increasing throughout the day before declining during the evening hours, a pattern that was not influenced by food intake. Prolonged fasting produced statistically significant increases in hepcidin-25 concentrations. Refeeding reversed this process, and GH administration markedly decreased hepcidin-25 concentrations.

CONCLUSIONS: Our results indicate that in humans, hepcidin-25 exhibits diurnal changes that can be altered by prolonged fasting, which increases hepcidin-25 concentrations approximately 3-fold after 3 days of fasting, possibly owing to a suppression of erythropoiesis that may occur during the fasting state to preserve tissue iron concentrations. In contrast, GH administration decreased hepcidin-25 concentrations by approximately 65%, presumably by stimulating erythropoiesis. These results indicate that circulating hepcidin-25 concentrations display much more dynamic and rapid variation than might have been anticipated previously.

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has been the difficulty in developing straightforward immunoassays for measuring the active form of the hormone, hepcidin-25, in human serum (16). This difficulty stems in part from the small size of the active form of the molecule (25 amino acid residues) and the fact that it contains 8 cysteine residues and 4 disulfide bonds (17). Furthermore, hepcidin-25 is derived from preprohepcidin, which furin cleaves at the N terminus to yield the inactive prohepcidin peptide (64 amino acid residues) (18). Prohepcidin is further processed via removal of its N-terminal 39 amino acid residues to yield hepcidin-25 (the active form of the hormone), which can then undergo further N-terminal processing to yield the inactive forms hepcidin-20, hepcidin-22, and hepcidin-24 (18). Owing to this complex processing, it has proved difficult to develop specific, robust immunoassays for hepcidin-25.

To address the need for a highly specific and robust immunoassay, we recently developed a sandwich ELISA with 2 monoclonal antibodies for measuring hepcidin-25 in human serum (19) and demonstrated that hepcidin-25 concentrations were significantly increased in patients with cancer and rheumatoid arthritis. In the present study, we further applied our assay to better understand some of the basic questions about the normal physiology of hepcidin-25, including investigations of its diurnal variation and the effects of prolonged fasting and sleep deprivation on hepcidin-25 concentrations. In addition, because erythropoietin has been reported to decrease circulating hepcidin concentrations (20–22), we also investigated the effect of growth hormone (GH)6 administration, which has been reported to stimulate erythropoiesis (23–25), on hepcidin-25 concentrations.

Materials and Methods

HUMAN SAMPLES
Serum samples collected and analyzed in the course of prior studies (26–29) were stored at −70 °C before analysis of their hepcidin-25 concentrations. Diurnal changes in hepcidin-25 were monitored during standard feeding in 5 healthy individuals, who were sampled as previously described [every 90 min, from 0900 on the first day to 1030 on the following day (26, 27)]. The importance of food intake was studied in the same individuals on another occasion, in which the overnight fast was extended another 7 h [from 0900 to 1600, with samples drawn every hour (27)].

The possible relationship of the hepcidin-25 concentration to the variation in the enterohepatic circulation (EHC) of bile acids was studied in 2 groups of participants. In the first group, samples were obtained from 10 individuals (5 men, 5 women) before and during chronic cholestyramine treatment (mentioned in Results). In the second group, 10 healthy individuals (5 men, 5 women) received 4 g cholestyramine with each meal for 1 day (16 g total), and blood samples were taken every 90 min over 25.5 h, as previously described (26, 27).

The dynamic effects of 66 h of fasting and 50 h of sleep deprivation were also evaluated. We used samples obtained from a survival physiology study with 12 healthy participants (5 women, 7 men), who were divided into 2 groups, as previously described (28). Up to 66 h of fasting was compared with up to 50 h of sleep deprivation in a crossover design, with baseline and washout measurements taken on days 1 and 8, respectively. During the fasting period, samples were taken at 18, 42, and 66 h of fasting. Afterward, the participants consumed a 500-kcal meal (divided into 4 parts) over 24 h, followed by unrestricted caloric intake. During the other days, participants were sleep deprived. The effect of GH treatment was evaluated with samples obtained from 8 healthy young individuals [mean (SE) age, 28 (1) years; range, 23–33 years] and 7 elderly individuals [mean age, 66 (1) years; range, 62–70 years]. These individuals were treated daily with GH for 3 weeks, with the dosage increasing each week (0.1 IU·kg−1·day−1 administered during the final week), as previously described (29). All participants gave informed consent, and all studies were approved by the Ethics Committee of Karolinska Institute.

HEPCIDIN-25 ELISA
Hepcidin-25 concentrations were measured with a high-sensitivity sandwich ELISA specific for hepcidin-25, as previously described (19). In brief, a human hepcidin-25 Meso Scale Discovery (MSD6) ELISA was performed by using streptavidin-coated and blocked wells that had been incubated for 1 h with biotinylated antihepcidin capture antibody (2 mg/L). After aspiration, the wells were washed 3 times with TBST [Tris-buffered saline (10 mmol/L Tris, pH 7.40, 150 mmol/L NaCl) containing 1 mL/L Tween 20]. Then, 100 μL hepcidin-25 calibrators (Peptides International) (different hepcidin-25 concentrations in assay buffer, consisting of 50 mmol/L HEPES, pH 7.40, 150 mmol/L NaCl, 10 mL/L Triton X-100, 5 mmol/L EDTA, and 5 mmol/L EGTA) were added to the wells to generate a standard calibration curve. Serum samples were diluted with 20 parts assay buffer and added to their respective wells, and the ELISA plate was incubated for 1 h at room temperature. After aspiration, wells were

6 Nonstandard abbreviations: GH, growth hormone; EHC, enterohepatic circulation; MSD, Meso Scale Discovery (ELISA); TBST, Tris-buffered saline (10 mmol/L Tris, pH 7.40, 150 mmol/L NaCl) containing 1 mL/L Tween 20.
washed 3 times with TBST, and 100 μL of a solution of conjugate antibody (ruthenium-labeled anti-hepcidin-25 detection antibody, 1 mg/L) diluted with 1000 parts TBST was added to each well for a 1-h incubation at room temperature. After aspiration, wells were washed 3 times with TBST, and the plate was developed with an MSD reader, which recorded ruthenium electrochemiluminescence.

DATA ANALYSIS
MSD software and SigmaPlot (version 8.0; Systat Software) were used for fitting ELISA calibration curves. For each group of participants or time points studied, results were expressed as the mean (SE). Data were plotted and graphed with the Fig.P program (version 2.98; Biosoft). Comparisons of groups or time points with respect to the hepcidin-25 concentration were performed with the same program. Data were analyzed by one-way ANOVA; means were then compared with the least significant difference test. In each case, a P value <0.05 was considered statistically significant. Diurnal variation was analyzed by fitting individual curves to a sine function by nonlinear regression. Estimates of period length close to 24 h were considered evidence of a diurnal rhythm, provided that all model parameters were estimated with high statistical significance (P < 0.001).

Results
We first examined whether hepcidin-25 shows diurnal variation in healthy individuals. For this purpose, we measured serum hepcidin-25 concentrations in 5 individuals who had been sampled every 90 min over a 25.5-h period, from 0900 on day 1 to 1030 on day 2. Fig. 1 shows the results of this experiment. Hepcidin-25 concentrations displayed a diurnal rhythm, with concentrations being lowest during the morning hours and then steadily increasing throughout the day before declining during the later evening hours. These results suggested that hepcidin-25 concentrations display a diurnal variation and prompted us to study the effect of food intake on circulating hepcidin-25 concentrations.

The 5 participants whose results are shown in Fig. 1 were reinvestigated on a second occasion, in which their overnight fast was extended from 0900 to 1600 (Fig. 2). The mean hepcidin-25 concentration gradually increased approximately 3.5-fold during this fasting period, from 3.4 (1.3) μg/L at 0900 to 11.7 (4.7) μg/L at 1500, but it then again declined slightly to 8.0 (3.0) μg/L at 1600. This pattern is very similar to that for the same time period shown in Fig. 1, suggesting that food intake is not crucial for the diurnal variation in hepcidin-25 concentrations.

Bile acids undergo EHC, which may influence hepatic metabolism; however, interruption of the EHC by chronic treatment with cholestyramine for 21 days (8-g dose twice per day) did not influence the fasting hepcidin-25 concentrations in 10 healthy individuals [mean (SE), 4.2 (1.2) μg/L for untreated individuals vs
4.8 (1.1) µg/L for treated individuals. The distinct pattern of diurnal variation in the hepcidin-25 concentration shown in Fig. 1 was also evident in a study of a second group of 10 healthy individuals who were treated acutely with cholestyramine and sampled every 90 min over a 25.5-h period, from 0830 on day 1 to 1000 on day 2. The results of this experiment (Fig. 3) show that hepcidin-25 concentrations display a clear diurnal rhythm, with hepcidin-25 concentrations being lowest during the morning hours, steadily and significantly increasing throughout the day, and then declining again during the later evening hours.

The nonlinear fit to a sine curve indicated significant diurnal variation (i.e., an approximately 24-h periodicity) in 11 of the 15 individuals in the 2 groups investigated. Thus, the hepcidin-25 concentration displayed a marked diurnal variation that was both independent of food intake and unaffected by the interruption in the EHC of bile acids. This result prompted us to study the effect of longer periods of fasting on circulating hepcidin-25 concentrations.

To address the question of how prolonged fasting would influence circulating hepcidin-25 concentrations, we studied 12 additional participants over an 8-day period, which included a 66-h fast and refeeding, and then a 50-h period of sleep deprivation in a crossover design. In this study, hepcidin-25 measurements were made at baseline and washout (on days 1 and 8, respectively), with additional samples collected on a daily basis (Fig. 4). During the 66-h fasting period, blood samples were obtained after 18, 42, and 66 h of fasting. Extended fasting produced significant increases in the mean hepcidin-25 concentration, which increased from 11.5 (2.0) µg/L at baseline to 24.5 (2.2) µg/L at 18 h of fasting ($P < 0.01$, vs baseline), to 31.4 (5.0) µg/L at 42 h of fasting ($P < 0.01$, vs baseline), and to 38.5 (4.3) µg/L at 66 h of fasting ($P < 0.01$, vs baseline).

Fig. 4 also shows that refeeding with 500 kcal after the 66-h fast led to a significant decrease in the mean hepcidin-25 concentration, from 38.5 (4.3) µg/L to 27.0 (3.6) µg/L ($P < 0.01$, vs the mean concentration after 66 h of fasting). Prolonged sleep deprivation, in contrast, had no significant effect on the hepcidin-25 concentration, compared with the baseline concentration. Interestingly, after the marked increases in hepcidin-25 caused by the prolonged fasting period, the mean washout hepcidin-25 concentration appeared to overcorrect to 4.5 (0.8) µg/L, which was actually significantly lower than the mean baseline hepcidin-25 concentration ($P < 0.01$, vs baseline).

We next explored whether GH secretion could explain the fasting-induced hepcidin-25 concentrations. We treated 15 healthy participants—8 young individuals (mean age, 28 (1) years; range, 23–33 years) and 7 elderly individuals (mean age, 66 (1) years; range, 62–70 years)—with GH for 3 weeks, with the daily GH dose administered increasing each week (to 0.1 IU·kg$^{-1}$·day$^{-1}$ during the final week), as previously
Our observations regarding the variation in hepcidin-25 concentrations build on the work of several researchers. Recently, Galesloot and coworkers assessed serum hepcidin concentrations in a large, well-phenotyped sample of almost 3000 healthy control individuals and reported that serum hepcidin concentrations were lower for premenopausal women than for postmenopausal women, whereas hepcidin concentrations in men remained constant with age (33). Kemna and colleagues measured hepcidin and prohepcidin in patients with iron metabolism disorders and in control individuals and demonstrated that inflammation was strongly related to increased hepcidin concentrations, regardless of the iron-store and erythropoietic-activity status (34). The same group has also presented evidence for a circadian rhythm for hepcidin in 3 healthy individuals (35).

Ganz and colleagues reported in 2008 that healthy volunteers demonstrated increases in serum hepcidin
concentrations at the times of 2400 and 2000, compared with 0800 (36). In 2009, Kroot and coworkers studied serum hepcidin concentrations in 24 healthy controls by TOF mass spectrometry at 4 different times during the day and showed that serum concentrations increased significantly through the morning and afternoon (37). In our current study, we confirmed previous reports regarding diurnal changes in hepcidin concentrations and extended these observations by using frequent sampling (every 90 min) of large numbers of healthy volunteers. The results demonstrate that hepcidin-25 concentrations in healthy individuals follow a sinusoidal diurnal rhythm, as evidenced by a significant fit to a sine curve with a 24-h periodicity. In addition, our current study showed that hepcidin-25 concentrations increased significantly with prolonged fasting and that this trend was reversed with refeeding. Furthermore, hepcidin-25 concentrations were not altered significantly by prolonged sleep deprivation, suggesting that interruption of the sleep–wake cycle has a modest effect on circulating hepcidin-25.

The dramatic effect of a longer fasting period in increasing circulating hepcidin-25 concentrations was striking and somewhat unexpected. We originally thought that prolonged fasting might decrease the intestinal iron available for uptake and that this decrease would cause circulating hepcidin-25 concentrations to decrease as a response to promote more iron to be absorbed from the intestine. Instead, we observed the opposite, with hepcidin-25 values increasing dramatically as the duration of fasting increased. These results prompted us to investigate what has been reported for the effect of prolonged fasting on serum iron concentrations. Unfortunately, this aspect of iron metabolism does not appear to have been extensively studied, although Furugouri previously reported that tissue iron stores are maintained or even somewhat increased during prolonged fasting (38).

If that is indeed the case, it might explain why hepcidin-25 concentrations increase with extensive fasting. During prolonged fasting periods, it would make sense that erythropoiesis would be suppressed to preserve the tissue iron concentrations required for crucial cellular processes, such as oxidative respiration. Because the life span of red blood cells is approximately 120 days, erythropoiesis could be safely suppressed to maintain adequate tissue iron concentrations required for the iron-dependent mitochondrial cytochrome system. In this case, the decreased erythropoiesis caused by prolonged fasting would be sensed by the liver (via an unknown mechanism), leading to an increase in hepatic hepcidin-25 synthesis and secretion.

With regard to the effect of erythropoietin administration on hepcidin concentrations, erythropoietin has previously been shown to decrease hepcidin (20–22). The mechanism proposed for this effect is direct stimulation of erythropoiesis, which requires increased iron to be incorporated into hemoglobin in the maturing red blood cell precursors. To meet this need for iron, the liver senses the increased erythropoiesis (again via an unknown mechanism) and secretes less hepcidin to allow the absorption of more iron from the intestine as well as the release of iron from the reticuloendothelial system. This mechanism may be a key means by which erythropoietin is effective in treating anemia.

In light of these reports, we also wanted to examine the effect of GH on circulating hepcidin-25 concentrations. GH has been reported to stimulate erythropoiesis (23–25), and we therefore hypothesized that GH administration might also decrease hepcidin-25 concentrations. In this case, our prediction was correct, because GH treatment produced a clear, dose-dependent decrease in hepcidin-25, a result consistent with our hypothesis that GH may decrease hepatic iron concentrations via the stimulation of erythropoiesis. This concept seems plausible, especially in light of our observations suggesting that prolonged fasting may increase hepcidin-25 concentrations owing to the suppression of erythropoiesis to preserve tissue iron concentration. Also of note is that in addition to the effect of GH on erythropoiesis, one should also consider the possible direct effect of growth factors in the liver that could affect hepcidin synthesis and secretion. Goodnough and colleagues recently demonstrated that hepatocyte growth factor and epidermal growth factor suppressed hepatic hepcidin synthesis (39). In light of these interesting data, a direct effect of GH on the liver to suppress hepcidin synthesis may be another potential mechanism by which GH suppressed hepcidin concentrations in the current study.

Our findings in the present study shed interesting light on the physiology and regulation of hepcidin-25, which have turned out to be much more complex than originally anticipated (40). The dramatic increase in hepcidin-25 after prolonged fasting that we observed may be best explained by the fact that during an extended fasting state the body suppresses erythropoiesis to maintain tissue iron concentrations. Because red blood cells have a relatively long life span, erythropoiesis can safely be suppressed for several days. Decreased erythropoiesis is somehow sensed by the liver, and hepcidin-25 synthesis and secretion are increased accordingly. The opposite occurs during refeeding, with the compensatory increased erythropoiesis signaling the liver to decrease hepcidin-25 synthesis and secretion to meet the increased demand for iron required for hemoglobin synthesis. This mechanism most likely ex-
plans why after refeeding for several days, hepcidin-25 concentrations were actually significantly lower than those at baseline (Fig. 4). The fasting and refeeding data are consistent with the observations for GH administration, which decreased hepcidin-25 concentrations. Because GH stimulates erythropoiesis (23–25), the increased erythropoiesis is somehow sensed by the liver, which responds by decreasing hepcidin-25 synthesis and secretion. Taken together, these observations indicate that circulating hepcidin-25 concentrations have greater dynamic variation than may have previously been anticipated. In light of these data, further studies are needed to understand the mechanism through which erythropoiesis regulates hepatic hepcidin-25 synthesis and secretion.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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