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Circadian Plasma Leptin Levels in Patients with Anorexia nervosa: Relation to Insulin and Cortisol

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Abstract

In anorexia nervosa, underweight results from a loss of body mass due to a restricted energy intake. Circulating leptin levels have been shown to be low in the acute stage of the disorder. We studied diurnal secretion characteristics of leptin, insulin and cortisol in a study group of anorectic patients prior to refeeding, a second study group of anorectic patients after initiation of refeeding and study groups of healthy underweight and normal-weight controls. Spontaneous secretion of leptin, insulin and cortisol was measured by drawing blood samples every 2 h for 24 h. The temporal relationships between the diurnal secretion patterns of the three hormones were assessed by cross-correlation analysis in every study group. Plasma levels of leptin and cortisol were secreted with a specific circadian rhythmicity and displayed an intricate temporal relationship in anorectic patients. Semistarvation in the non-refed patients was associated with (1) exceedingly low plasma leptin levels, (2) a qualitative alteration in the circadian rhythm of leptin and cortisol levels and (3) an alteration in the temporal coupling between cortisol and leptin. In contrast, in the patients who had gained weight, leptin levels were higher; furthermore, the diurnal pattern of leptin and the temporal relationship between leptin and cortisol were similar to controls. Increments in insulin secretion preceded those of leptin by 4–6 h in both anorectic patients and in controls. Leptin levels increased 4 h prior to those of cortisol in controls and in refed patients, whereas in the non-refed patients cortisol increased prior to leptin. Thus, anorexia nervosa leads to pronounced, albeit reversible changes in the secretion dynamics of leptin and cortisol.

Key Words
Leptin
Anorexia nervosa
Phase shift
Circadian rhythmicity

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Introduction

Leptin is a protein encoded by the *ob* gene that is expressed in adipocytes [1–3]. It has an influence on both energy intake and expenditure via central neuroendocrine mechanisms [4, 5]. Serum leptin levels are correlated with weight and percentage of body fat [3]. Fasting leads to a rapid decrease in circulating leptin levels which sets in prior to weight loss [6–8]. However, levels do not decrease if glucose concentration is maintained at a constant level [6]. Moreover, insulin has been shown to be a delayed modulator of leptin secretion using a clamp technique [8].

In humans, serum leptin levels show a circadian and ultradian rhythm [9]. In their study of 24-hour profiles of circulating leptin levels in 26 lean and obese subjects, Sinha et al. [9] demonstrated that leptin levels are highest between midnight and early morning hours and lowest around noon to mid-afternoon. Interestingly, in this study the circadian pattern did not appear to be influenced by meal ingestion and meal-related increases in circulating insulin and glucose levels. In contrast, Saladin et al. [10] demonstrated that in rats the diurnal variations of ob gene expression are linked to food intake probably through a direct action of insulin on adipocytes. Secretion of leptin occurs in a pulsatile manner [11]. Serum leptin levels show an inverse relation to those of adrenocorticotropic hormone (ACTH) and cortisol [11]. Laughlin and Yen [12] compared 24-hour leptin levels of highly trained women athletes with and without menstrual cyclicity with regularly cycling female controls. Leptin levels were correlated with insulin and inversely with cortisol levels. Contrary to the normally cycling controls and the cycling athletes, no diurnal pattern of leptin secretion was observed in the amenorrheic athletes suggesting [12] that the absence of a diurnal rhythm in athletes with amenorrhea might be explained by the observed reduction of meal-induced insulin excursions so that the stimulatory effect of insulin on leptin levels is below a certain threshold. Moreover, hypocortisolemia in women athletes might be indicative of increased central CRH levels inhibiting leptin gene expression and release via activation of central adrenergic activity and adipocyte-specific β_3-adrenergic receptors [12].

In view of leptin's role in semistarvation [13] and in reproductive function [14, 15] this hormone warrants further studies related to its pathophysiological significance especially in anorexia nervosa (AN). Reduced serum leptin levels have been observed in females with acute AN [15–18]. Low serum leptin levels in underweight females with and without eating disorders have been shown to predict past or present episodes of amenorrhea [15].

This study was conducted in order to investigate the relationships between serum leptin levels and both insulin and cortisol levels over a period of 24 h in patients with AN in comparison to underweight and normal-weight controls. To evaluate the influence of semistarvation on the secretion of these hormones, we compared the hormone levels of patients in the acute state of semistarvation (72 h after admission for inpatient treatment) with levels of patients who had regained 2–6 kg of weight.

Material and Methods

Subjects

Five patients (2 males, 3 females) with AN (4 of the restricting type, 1 of the binge eating/purging type) aged 19–32 years were assessed within 72 h after initiation of inpatient treatment at the Clinic of Psychotherapy and Psychosomatics of the University of Essen. The average weight gain during this 72-hour period was <400 g. Their mean (± SD) body mass index (BMI, kg/m^2) at referral was 13.4 ± 2.2 kg/m^2 (range 9.6–15.0). Three additional female patients with AN (1 with the restricting type and 2 with the binge eating/purging type) aged 19, 27 and 28 with BMIs at referral of 14.2, 14.9 and 14.2 kg/m^2, respectively, were investigated 4 weeks after referral. By this time they had gained 2.2, 4.7 and 5.8 kg of body weight, respectively. All patients with AN were amenorrheic: none had received estrogens within the 3 months prior to the study. None of the subjects had a clinical history of diabetes mellitus. All patients had TSH levels in the normal range. None of the patients received medications during the study period. AN was diagnosed on the basis of the Structured Interview for Anorexia nervosa and Bulimia nervosa (SIAB) [19, 20] which is a standardized interview for the assessment of eating disorders according to the Diagnostic Statistical Manual of Mental Diseases (DSM-IV) [21].

Controls

The healthy controls consisted of 5 underweight female individuals aged 24–30 years (BMIs 17.6–18.6 kg/m^2) and of 3 normal-weight males (BMIs 22.0–23.6 kg/m^2) aged 26–27 and 3 normal-weight females (BMIs 20.0–24.0 kg/m^2) aged 25–27. Pregnancy, eating disorders (excluded by SIAB) and somatic diseases were excluded. Written informed consent was obtained and the study was approved by the local ethics committee. With the exception of contraceptives (n = 2), none of the healthy controls took medications. According to the therapeutic and feeding regimen within the eating disorder unit, patients with AN were offered a balanced diet consisting of 2,500 kcal/day that was distributed as a breakfast buffet between 07:30 and 08:30 h and as ready-to-eat meals for lunch and dinner between 12:00 and 12:30 h and 17:30 and 18:00 h, respectively. Patients were requested to gain 0.5 kg/week. The healthy controls received the same diet.

Sampling and Hormone Determinations

Blood sampling was initiated at 20:00 h via an indwelling catheter that had been inserted at 19:00 h; samples were collected every 2 h for 24 h thus totalling 12 samples/subject. Plasma samples were

zen at −80 °C prior to determination of leptin levels. Leptin levels re measured using a radioimmunoassay described previously in ail [22]. Because of the extremely low leptin levels in anorectic ients, the assay was modified to further improve its sensitivity. In ticular, the assay volume was reduced by 50% to a total volume of) µl and the assay was performed as a nonequilibrium RIA. In el, 100 µl of serum sample or standard were incubated with 25 µl the first antibody (1:6,000) overnight. Then 25 µl leptin tracer .000 cpm per tube) were added and incubation was continued rnight. Separation of bound and free tracer was achieved by a ble antibody technique as described previously. This modifica- n improved the sensitivity of the assay by an order of magnitude wn to 0.003 µl/l. The intra- and interassay coefficients of variation re 3.6 and 9.2% respectively. Insulin levels were determined by A (Insulin-CT, CIS bio international, France) with an intra-assay fficient of variation of 4.2% and a sensitivity of 2.0 µg/ml. Corti- levels were measured using the Ciba Corning ACS™ Cortisol munoassay with an intra-assay coefficient of variation of 5.7% l a sensitivity of 0.20 µg/l.

Statistics

Mean leptin, insulin and cortisol concentrations within the 24- ır period were computed for each patient. The data were ex- ssed as mean ± SD. Statistical analyses were performed with nn-Whitney U tests for independent samples and Pearson corre- ons. In each individual, the relationship between leptin and the) other variables (insulin, cortisol) was determined by linear ression analysis. Profiles were subjected to pairwise cross-correla- ı analysis [23–25]. Cross-correlation analysis measures the degree linear correlation between two sets of data at varying time lags ase shifts). For this study, one set of data was fixed on the time s, while the second profile was shifted towards increasing time nts (positive phase shift) or towards decreasing time points (nega- phase shift). Shifting of the profiles was by multiples of the inter- length corresponding to the 2-hourly blood sampling interval. For h phase shift, the correlation coefficient r and Fisher's R to Z were culated. The latter method tests for a significant departure of r m 0. Once the cross-correlation had been calculated for each sub- .. a mean r for every phase shift was computed for both groups of jects. These mean correlation coefficients were plotted against the ıse shift. To test for a significant departure of mean r values from ı one-group t test was performed. Calculations were performed ıg Excel™ (Microsoft, Redmond, Va., USA) and StatView 4.5 acus Concepts, Berkeley, Calif., USA) on an Apple Power-Macin- ı™ computer.

Results

Circadian Pattern of Leptin Secretion

In normal-weight controls (fig. 1a) mean leptin concentions during the 24-hour study period were lowest (3.85 4.29 µg/l) at 22:00 h and highest (7.03 ± 7.02 µg/l) 16:00 h. Leptin levels increased between 24:00 and 00 h and 14:00 and 16:00 h.

In underweight subjects (fig. 1b) lowest leptin concentions (2.93 ± 2.18 µg/l) occurred at 12:00 h, highest levels (4.91 ± 2.63 µg/l) at 16:00 h. Although highest and lowest leptin levels occurred at different times in these two control groups, patterns of mean leptin levels were similar in that leptin levels increased between 24:00 and 04:00 h and 12:00 and 16:00 h. Mean leptin levels in the anorectic patients in the state of semistarvation were about 100-fold lower than in the underweight in normal-weight controls. These anorectic patients showed lowest levels at 04:00 h (0.02 ± 0.01 µg/l and highest levels at 12:00 h (0.04 ± 0.02 µg/l) (fig. 1c). Contrary to the control groups, leptin levels of these patients showed a clear decrease between 24:00 and 04:00 h. However, leptin secretion of the 3 anorectic patients who had gained between 2.2 and 5.8 kg was similar to that of the controls (fig. 1d). Among the anorectic patients in the state of semistarvation, mean leptin levels during the 24-hour period did not differ between males and females ($p = 0.2$). In normal-weight controls females had higher mean leptin levels than males ($p = 0.049$).

Circadian Pattern of Cortisol Secretion

Mean cortisol levels of the non-refed anorectic patients were significantly higher compared to the underweight ($p = 0.028$) and normal-weight controls ($p = 0.004$). In the group of anorectic patients in the state of semistarvation, episodic secretory spikes occurred at unusual times and the number of peaks was increased (fig. 1c). In contrast, patterns of cortisol secretion in the group of anorectic patients with moderate weight gains (fig. 1d) were similar to the underweight and normal-weight controls (fig. 1a, b) and revealed a pronounced diurnal cycle.

Temporal Coupling

Cross-correlation analysis was used to characterize temporal coupling of leptin levels with insulin and cortisol levels. In normal-weight and in underweight controls the maximal positive correlation was found for a phase shift of +6 h with insulin maxima preceding leptin maxima ($r = +0.470$, $p = 0.0047$ and $r = +0.380$, $p = 0.009$ respectively) (fig. 2a, b). In the state of semistarvation and without a phase shift, leptin and insulin were negatively correlated (mean $r = -0.185$, $p = 0.042$) (fig. 2c); the maximal positive correlation was found for a phase shift of +4 h ($r = +0.287$, $p = 0.1939$) with insulin maxima preceding leptin maxima. In the refed patients the temporal relationship between leptin and insulin was similar: without a phase shift mean r was −0.373 ($p = 0.058$), the maximal positive correlation was detected with a phase shift of +4 h ($r = +0.189$, $p = 0.452$) (fig. 2d). Thus, the pattern of cross-correlation was similar in all four groups (fig. 2a–d).

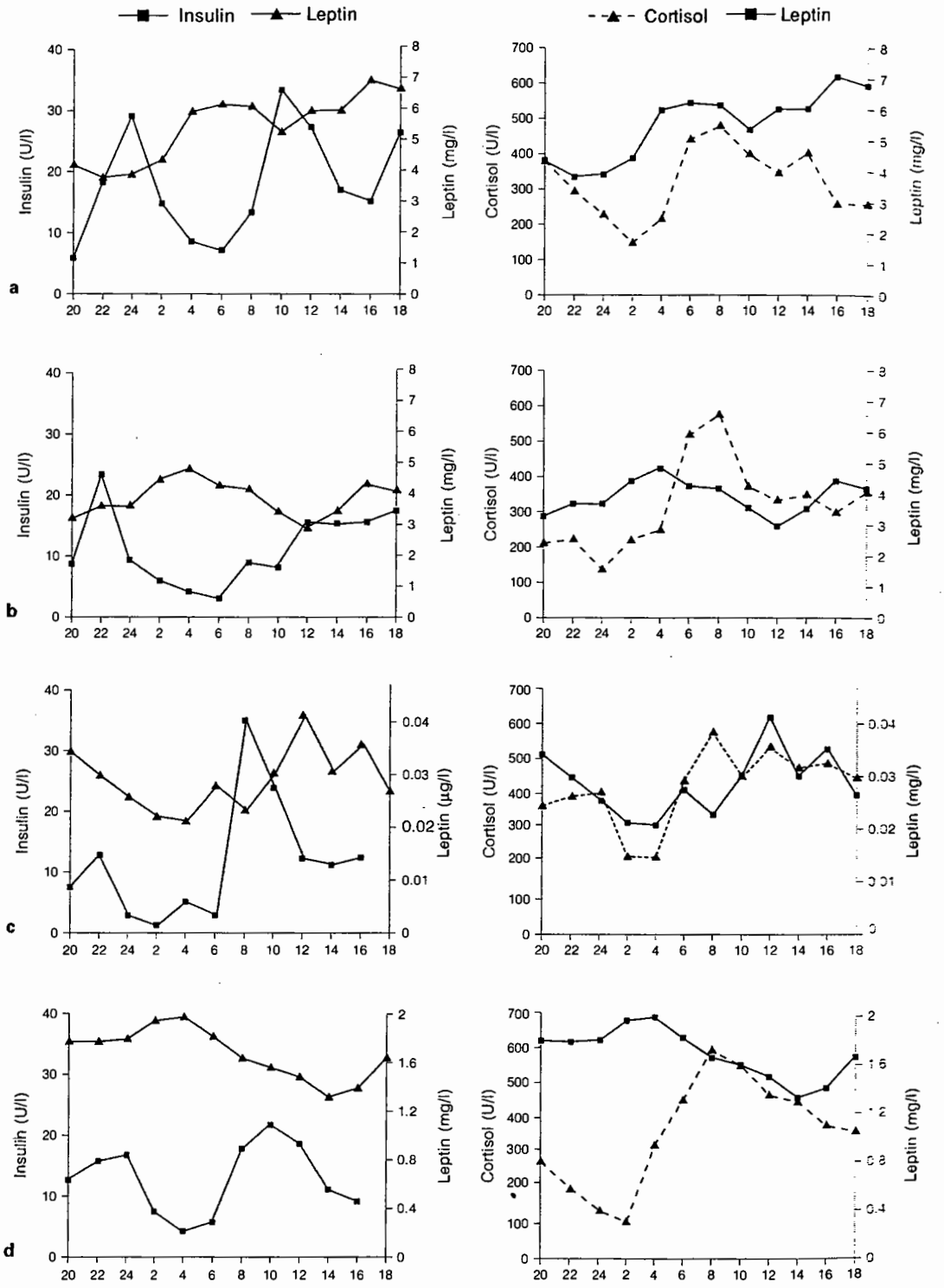

Fig. 1. Mean levels of leptin, insulin and cortisol. **a** Six healthy subjects with normal weight. **b** Five healthy underweight subjects. **c** Five anorectic patients in the state of semistarvation. **d** Three anorectic patients in the state of refeeding.

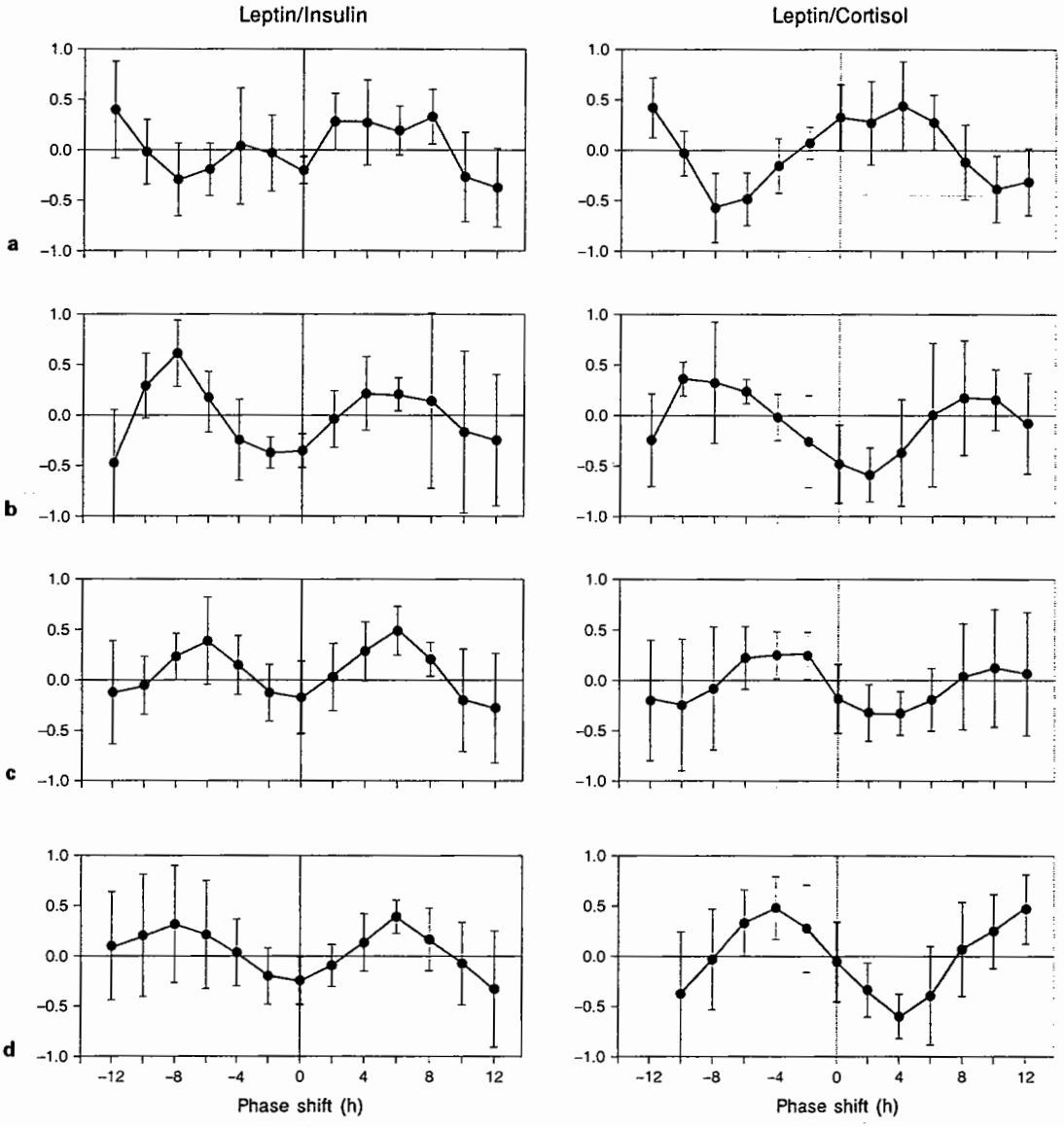

Fig. 2. Cross-correlation analysis of leptin with insulin and cortisol in patients with anorexia nervosa during semistarvation and during refeeding and in controls. X-axis: phase shift; Y-axis: correlation coefficient (mean ± SD). A positive phase shift indicates that insulin or cortisol increases precede leptin increases. a negative phase shift stands for leptin increases preceding insulin or cortisol. Cross-correlation pairs: left side leptin/insulin, right side leptin/cortisol. **a** Anorectic patients during semistarvation. **b** Anorectic patients in the state of refeeding. **c** Normal-weight controls. **d** Underweight controls.

Correlations between leptin and cortisol were mostly weaker than between leptin and insulin. Despite p values >0.05, the correlograms indicated nonrandom correlation coefficients (fig. 2). Leptin and cortisol were positively correlated in semistarvation (r = +0.350, p = 0.114), the maximal correlation at a phase shift of +4 h (r = +0.466, p = 0.112) showed that increases in cortisol levels preceded those of leptin by 4 h (fig. 2c). In the group of anorectic patients who had gained weight, a change in cross-correlation patterns was apparent: the correlation without phase shift was r = –0.479 (p = 0.169). The maximal correlation at a phase shift of +4 h was negative (r = –0.372,

p = 0.338) as shown in figure 2d. Cross-correlation patterns in controls were similar to this second group of patients whose refeeding had commenced, but distinctly different from the first group of patients who had been assessed upon referral and thus in the state of semistarvation. In normal-weight and in underweight controls a positive correlation was obtained with leptin maxima preceding cortisol maxima by 4 h (i.e. phase shift −4 h; r = +0.278, p = 0.038 and r = +0.518, p = 0.0014, respectively) (fig. 2a, b). At a phase shift of +4 h a negative correlation was detected in controls, similar to anorectic patients in the state of refeeding but different from patients during semistarvation (r = −0.284, p = 0.018 for normal-weight controls and r = −0.542, p = 0.005 for underweight controls) (fig. 2c, d).

Discussion

In the group of anorectic patients in the state of semistarvation, analysis of diurnal variation of leptin levels revealed a decrease in leptin levels from 20:00 until 04:00 h, while levels were highest at daytime. This finding is in contrast to the circadian leptin rhythm in controls and in patients in whom refeeding had been initiated. Contrary to Licinio's [11] and Sinha's [7] sample of healthy probands, normal-weight and underweight controls in our study showed peaks of leptin levels in the afternoon. Similar to these previous studies, however, they showed a definite increase in leptin levels between 24:00 and 04:00 h. Thus, the normal diurnal rhythmicity was abolished in anorectic patients in the state of semistarvation. Since anorectic patients in the state of refeeding, who were assessed 4 weeks after referral, had leptin patterns similar to the controls, we conclude that leptin levels return to a normal diurnal pattern soon after refeeding has been initiated. The absence of a normal diurnal pattern in the semistarved patients is similar to results observed in amenorrheic athletes [12].

Several groups have observed higher leptin levels in females than in males even after correction for BMI or percent body fat [22, 26–28]. However, neither the study of Licinio et al. [11] nor our study point to a sex-related difference in leptin secretion patterns and temporal coupling to insulin or cortisol. To assess temporal relationships between leptin, insulin and cortisol we chose cross-correlation analysis. This method allows to unveil synchronous relationships between fluctuations of hormone levels [23]. Licinio et al. [11] showed that leptin pulses occur with a frequency of 1 pulse every 44 min in healthy men. As our sampling intervals were long, true pulsatile bursts of leptin could not be analyzed appropriately.

Previous studies have shown a positive correlation of insulin with leptin [6]. Other groups have claimed that insulin has a long-term effect on leptin synthesis [8]. While we were able to confirm the correlation between leptin and insulin, our results, using cross-correlation analysis, demonstrate a previously undescribed time lag between leptin and insulin secretion maxima with a phase shift of 6 h in controls. A quantitatively similar pattern was observed in patients with AN without clear-cut differences between patients during acute semistarvation and refeeding. Thus, even at extremely low leptin levels, the temporal relationship to insulin secretion was preserved. The link underlying the synchronization of the two hormones may have been food intake, which stimulates insulin secretion. With a time lag, insulin then probably stimulates leptin release. Since the patients who were assessed upon referral also had a periodic, albeit characteristically very limited food intake, preservation of the temporal relationship would not contradict this hypothesis. Temporal variations in food intake might possibly underlie the observed temporal differences in peak leptin secretion between our normal-weight and underweight controls and the subjects in other studies [11, 12].

Cortisol was also synchronized with leptin secretion, and cross-correlation showed that increases in cortisol levels were preceded by increases in plasma leptin values in controls and in refed patients. This relationship, hpyothetically, may have been induced by a direct stimulation of cortisol secretion by leptin, although cross-correlation analysis does not permit to exclude indirect influences of unknown factors responsible for the synchronization of both cortisol and leptin levels. Our results are supported by recent findings of Licinio et al. [11] who reported a negative correlation between leptin and cortisol values. As no cross-correlation was performed in their study, only correlations without phase shift were calculated. This negative correlation contrasts with the stimulating effect of glucocorticoids on leptin expression [29] and leptin levels [30], which is most probably mediated through a glucocorticoid response element on the ob promoter. Without a phase shift, we were also able to demonstrate a negative relationship of leptin and cortisol in normal-weight controls as well as in anorectic patients after weight gain. This observation is in line with the results of Laughlin and Yen [12] in cyclic sedentary and athlete females with and without menstrual cyclicity and strengthens the hypothesis that the inverse relationship between cortisol and leptin levels and rhythms might be explained by an increase of

tral CRH underlying hypocortisolemia both in women letes [31, 32] and patients with AN [33, 34]. CRF ibits leptin gene expression and release via stimulation :entral adrenergic activity, peripheral norepinephrine :ase and adipocyte-specific β_3-adrenergic receptors. In trast, in the state of semistarvation the opposite pat- of cross-correlation was found. Here, cortisol levels reased prior to leptin maxima. It may be speculated t profound alterations in the relationship of leptin and tisol during semistarvation in anorectic patients are idly reversible after initiation of refeeding. On admis- , cortisol levels of the anorectic patients were elevated l episodic secretory spikes occurred frequently. As cribed previously [35, 36] these abnormal secretory terns normalize after weight gain.

n summary, serum levels of leptin, insulin and cortisol e secreted with a specific circadian rhythmicity and displayed an intricate temporal relationship in anorectic patients. Acute semistarvation is associated with exceedingly low plasma leptin levels, with a qualitative alteration in the circadian rhythm of leptin levels and finally with an alteration in the temporal coupling between cortisol and leptin. Leptin levels of anorectic patients in the state of refeeding seem to normalize rapidly even after a modest weight gain. In addition, temporal relationships between leptin and cortisol are restored. Thus, anorexia leads to dramatic, albeit reversible changes in the secretion dynamics of leptin, insulin and cortisol.

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