CLINICAL STUDY

Longitudinal changes of circadian leptin, insulin and cortisol plasma levels and their correlation during refeeding in patients with anorexia nervosa

Stephan Herpertz¹, Norbert Albers², Richard Wagner³, Barbara Pelz¹, Werner Köpp⁴, Klaus Mann³, Werner F Blum^{5,6}, Wolfgang Senf¹ and Johannes Hebebrand⁷

¹Clinic of Psychotherapy and Psychosomatics, University of Essen, Postfach 103043, D-45030 Essen, Germany, ²Children's Hospital, University of Bonn, Germany, ³Division of Endocrinology, Department of Internal Medicine, University of Essen, Germany,

⁴Department of Psychosomatics and Psychotherapy, Benjamin Franklin University Berlin, Germany, ⁵Children's Hospital, University of Gießen, Germany, ⁶Lilly Deutschland GmbH, Bad Homburg, Germany, ⁷Department of Child and Adolescent Psychiatry of the University of Marburg, Germany

(Correspondence should be addressed to S Herpertz, Email stephan.herpertz@uni-essen.de)

Abstract

Objective: To study the longitudinal changes in plasma levels of leptin, insulin and cortisol during the transition from the state of starvation to the state of refeeding focussing on diurnal secretion characteristics and their temporal relationships.

Design: Leptin, insulin and cortisol were measured every 2 h for 24 h during acute starvation (T1). Sampling was repeated after reaching half the target-body mass index (BMI) (T2) and again at target-BMI (17.5 kg/m²; T3). The temporal relationships between the diurnal secretion patterns were assessed by cross-correlation analysis.

Results: Although BMIs at T1 were uniformly low, leptin levels varied widely within a range clearly below normal levels (0.03–1.7 µg/l). With increasing body fat during the course of refeeding, mean leptin levels increased from 0.64 µg/l (range: 0.27–1.73 µg/l) (T1) to 1.61 µg/l (range: 0.36–4.2 µg/l) (T2) and to 3.67 µg/l (range: 0.7–9.8 µg/l) (T3). Circadian leptin secretion patterns showed maximal values uniformly around 0200 h and minimal values around 0800 h at all stages of the study. At all three weight levels, plasma leptin levels were highest between midnight and the early morning hours and lowest around the late morning hours. Refeeding neither profoundly changed secretion patterns of leptin nor did it change the positive, time-delayed relationship between leptin and insulin with increments in insulin secretion preceding those of leptin by 6 h. A temporal relationship between leptin and cortisol could not be demonstrated in the state of semistarvation but emerged after a substantial weight gain; at that time, leptin increases preceded cortisol increases by 8 h.

Conclusions: Absolute leptin, insulin and cortisol levels are profoundly changed during starvation in anorectic patients, while refeeding, paralleled by a BMI gain, reverses these changes. During refeeding the relationship between leptin and cortisol changed profoundly, showing no significant correlation in the state of starvation, whereas at T3 after refeeding a strong inverse relationship could be observed. Leptin and insulin did not correlate significantly at any of the three stages studied.

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Introduction

The *ob* gene encodes leptin, which is a protein secreted specifically by adipocytes and involved in body weight regulation (1, 2). In the mouse, as well as in humans, defects in the leptin coding sequence (*ob/ob*) (1–5) or the leptin receptor/signalling system (*db/db*) (6, 7) cause severe obesity. Leptin is not only of interest in the pathogenesis of obesity, but also at the other extreme of body weight, in anorexia nervosa (AN). Fasting leads to a rapid decrease in circulating leptin levels

which sets in prior to weight loss (8–10). Serum leptin levels are correlated with weight and percentage body fat in normal- and overweight probands (11). Studies on the exact relationship of leptin to body weight in AN produced contradictory findings (12–16). In humans, serum leptin levels show a circadian rhythm (17). Whereas in the study of Sinha *et al.* (17) the circadian pattern did not appear to be influenced by meal ingestion and meal-related increases in circulating insulin and glucose levels, we could demonstrate a clear temporal relationship

not only of insulin but also of cortisol with leptin in humans of different weight categories (18). These findings were in line with the results of former studies in rats (19).

Secretion of leptin occurs in a pulsatile manner and serum leptin levels show an inverse relationship to those of adrenocorticotrophic hormone (ACTH) and cortisol (20). Furthermore the ultradian fluctuations in leptin levels show pattern synchronicity with those of both luteinizing hormone (LH) and estradiol (21). Recently, we compared diurnal secretion characteristics of leptin, insulin and cortisol of anorectic patients prior to refeeding, and in the state of refeeding, and in under- and normal-weight controls (18). Beside exceedingly low plasma leptin levels, semistarvation in the non-refed anorectic patients was associated with a qualitative alteration in the circadian rhythm of leptin and cortisol levels and an alteration in the temporal coupling between cortisol and leptin. Increments in insulin secretion preceded those of leptin by 4–6 h in both anorectic patients and in controls. However, 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. In the present study we investigated the intra-individual dynamics of the diurnal secretion of the three hormones in five anorectic patients. All of them reached a BMI of 17.5 kg/m^2 as the minimal weight criterion for AN according to the Diagnostic Manual of Mental Disorders, DSM-IV (22) during their inpatient treatment.

Subjects and methods

Clinical research protocol

Eleven anorectic patients and 11 under- and normal-weight probands were studied after giving informed consent for a clinical research protocol approved by the Ethics Committee of the University of Essen (18). AN was diagnosed on the basis of the structured interview for anorexia nervosa and bulimia nervosa (SIAB) (23) which is a standardized interview for the assessment of eating disorders according to the DSM-IV (22).

From this cohort, hormone levels of five patients (1 male, 4 females) with AN (restricting type) in the state of semistarvation were studied twice: once after admission (T1) and once when the target body mass index (BMI) of 17.5 kg/m^2 was reached (T3). Four patients were also studied at time T2 (BMI (T2) = $(17.5 + \text{BMI (T1)}) \times 0.5$). Age ranged from 22 to 31 years and the BMI at T1 from 13.15 to 15.10 kg/m^2 . All four female patients with AN were amenorrheic and none had received estrogens within the 3 months prior to the study. Apart from the eating disorder, clinical histories of concurrent illnesses could be excluded. Thyrotropin (TSH) levels of the patients were in the normal range and none of them received medications during the study period. According to the

therapeutic and feeding regimen within the eating disorder unit, patients with AN were offered a balanced diet consisting of 2500 kcal/day that was distributed as a breakfast buffet in the morning between 0730 h and 0830 h and as ready-to-eat meals for lunch and dinner between 1200 h and 1230 h and 1730 h and 1800 h, respectively. Patients were requested to gain 0.5 kg/week.

Sampling and hormone determinations

Blood sampling was initiated at 2000 h via an indwelling venous catheter that had been inserted at 1900 h; samples were collected every 2 h for 24 h totalling 12 samples per subject. Plasma samples were frozen at -80°C prior to determination of leptin levels. Leptin levels were measured using a highly sensitive non-equilibrium version of a radioimmunoassay (sensitivity of $0.003 \mu\text{l/l}$) described previously in detail (24). The intra- and interassay coefficients of variation were 3.6 and 9.2% respectively. Insulin levels were determined by RIA (Insulin-CT, CIS Bio International, France) with an intra-assay coefficient of variation of 4.2% and a sensitivity of $2.0 \mu\text{g/l}$. Cortisol levels were measured using the Ciba Corning ACS Cortisol immunoassay with an intra-assay coefficient of variation of 5.7% and a sensitivity of $0.20 \mu\text{g/l}$.

Statistics

Mean leptin, insulin and cortisol concentrations within the 24 h period were computed for each patient. Data are expressed as means $\pm$ S.D. or range. Correlation analyses were performed using Pearson product moment correlation coefficient. For all hormone values, areas under the curve (AUC) were computed using the parallelogram method. Group comparisons were performed with analysis of variance (ANOVA) followed by an unpaired Student's *t*-test as appropriate. Comparisons of values during the time course of refeeding (T1 to T3) were performed using ANOVA for repeated measures in those four subjects with complete data for T1 and T3. Significant differences were assumed at the 5% level. In addition, comparison of values for T1 and T3 was performed in those 4 patients with available data using Student's paired *t*-test. As data were not normally distributed, analysis was done after logarithmic transformation which yielded better normalization (skewness ranged from a maximum of 1.2 to <0.7).

Hormone profiles were subjected to cross-correlation analysis (leptin values against insulin and cortisol values) as described previously with minor modifications (18). In short, one set of data was fixed on the time axis, the second profile was shifted towards increasing time points (positive phase shift) or towards decreasing time points (negative phase shift). Shifting interval length equaled blood sampling interval length (2 h).

Figure 1 Leptin levels of five anorectic patients over a period of 24 h at three different weight levels. T1: at admission in the state of semistarvation, five patients (A); T3: at target-BMI of 17.5 kg/m²; five patients (C); T2: after reaching half the target-BMI; four patients (B). Please note the logarithmic scale of the y-axis and the expanded range at T3.

For each phase shift, the correlation coefficient r was calculated. Once the cross-correlation had been calculated for each subject, a mean correlation coefficient R for every phase shift was computed for both groups of subjects. These mean R values with their respective standard deviations were plotted against the phase shift. A significant departure of the mean correlation from 0 was assumed if correlation coefficients were outside the 95% confidence interval defined by $-1/N \pm 2/(N^{-2})$ (25). Calculations were performed using Excel (Microsoft, USA) and StatView 5.0 (SAS Institute, USA) on an Apple Power Macintosh computer.

Results

Within the whole sample of 22 anorectic patients and under- and normal-weight healthy probands the relationship of leptin to BMI showed a significant correlation ($r=0.585$; $P=0.004$). Although the BMI of the five anorectic patients who took part in the longitudinal study ranged from only 13.15 to 15.0 kg/m² at T1, mean serum leptin levels showed large differences. Three patients had exceedingly low leptin levels with a mean between 0.03 and 0.06 µg/l, while two other patients had mean values of 1.3 and 1.7 µg/l, close to the range of underweight, non-anorectic patients (18). Despite this heterogeneity, values increased drastically in all patients. Conversely, mean cortisol levels decreased from 425 µg/l (range: 334–601 µg/l) (T1) to 336 µg/l (range: 307–404 µg/l) (T2) and to 274 µg/l (range: 196–336 µg/l) (T3).

Figure 1 shows the 24 h leptin secretion patterns of the five anorectic patients during refeeding (T1 to T3, data of one patient at T2 are missing).

At T1 the five anorectic patients in the state of semistarvation showed the lowest mean leptin levels at 0800 h (mean: 0.34 µg/l, range: 0.02–0.85 µg/l) and the highest levels at 0200 h (mean: 0.77 µg/l, range: 0.03–2.1 µg/l) (Fig. 1A). At T2 the BMI of the four anorectic patients in the state of refeeding ranged from 15.40 to 16.70 kg/m² with the lowest levels at 0800 h (mean: 0.82 µg/l, range: 0.21–1.59 µg/l) and the highest levels at 0200 h (mean: 2.02 µg/l, range: 0.41–5.39 µg/l) (Fig. 1B). At T3, the lowest levels could be detected at 0800 h (mean: 2.52 µg/l, range: 0.54–6.25 µg/l) and the highest levels at 0200 h (mean: 4.70 µg/l, range: 0.94–12.92 µg/l) for all five anorectic patients (Fig. 1C). Because values at T2 were not available in one patient, ANOVA analysis was confined to four patients. Table 1 shows the strong increase of leptin values throughout the refeeding period.

Leptin levels and leptin AUCs increased significantly ($P=0.012$ and $P=0.011$, respectively, Student's paired t -test on log-transformed data). Cortisol levels and AUCs decreased, barely missing statistical significance ($P=0.082$ and $P=0.091$ respectively), while insulin levels did not change.

Table 1 Mean increases of leptin, cortisol and insulin levels in five anorectic patients during acute starvation (T1) and during the period of refeeding with increasing BMI (T2 and T3, details in text). Individual values were transformed to percentages of the T1 levels. Analysis by ANOVA for repeated measures was followed by Fisher's PLSD to describe the probability of a true departure (increase or decrease) from the initial value of T1.

Hormone	T1 (% T1)	Mean T2 (% T1)	Mean T3 (% T1)	Mean ANOVA P value
Leptin	100	1430 ± 910	3340 ± 2520	0.033
Cortisol	100	88 ± 4	76 ± 8	0.004
Insulin	100	98 ± 40	130 ± 83	n.s.

n.s., not significant with $P>0.05$.

Cross-correlation analysis of leptin and insulin revealed no significant correlation at T1, T2 and T3 (Fig. 2), but leptin increases tended to follow insulin increases by 6 h at all three points in time. Statistical significance was narrowly missed for T2 at a lag of 6 h. The cross-correlation profiles were similar to profiles published earlier (18). For the leptin/cortisol cross-correlation no significant coefficients were obtained at T1, while at refeeding, the profile changed from T2 to T3. At T3, a strong inverse correlation between cortisol and leptin was observed at a lag of zero and 2 h (correlation coefficients -0.672 and -0.783 , respectively, the 95% confidence interval limit being -0.661). A tendency to a positive correlation between leptin and cortisol was seen at a lag of -8 h, i.e. leptin increases preceded cortisol increases by 8 h with a relatively large interindividual variation (Fig. 2).

Discussion

Multiple hormonal disturbances in AN have been well described (26–28). Very low leptin levels during starvation were documented by several authors (12–16). Within our sample of 22 anorectic patients and probands of under- and normal-weight, leptin levels correlated with the BMI.

In this study, we examined for the first time the longitudinal changes of leptin, insulin and cortisol levels in anorectic patients during the transition from the state of starvation to the state of refeeding, defined by the attainment of a BMI of 17.5 kg/m². All patients were studied at an intermediate point of this transition, arbitrarily defined by the acquisition of 50% of the difference between the individual baseline and the target BMI. This study design enabled us to evaluate changes in hormone levels in a longitudinal fashion. Only with this design was ANOVA analysis of repeated measures possible, increasing the statistical impact of the data collected. Leptin levels increased in a BMI-dependent fashion as T2 levels were intermediate between T1 and T3, supporting the notion of leptin being a signal generated by the increasing

Figure 2 Cross-correlograms for leptin/insulin (A–C) and leptin/cortisol (D–F) in patients with anorexia nervosa. The correlation coefficient r between the two hormone series is depicted on the y-axis at 0 on the x-axis. If the hormone series are shifted against each other by one sampling interval, the correlation coefficient changes. This situation is shown at phase shift '1' on the x-axis (the second hormone has been shifted by one sampling interval, the correlation has been recalculated). Further shifting of the hormone series by single sampling intervals gives rise to different r values as shown in the graph. For better readability, the data points have been connected with a line. Correlation coefficients are shown as $r \pm 1$ s.d. If the second hormone (insulin or cortisol) is shifted to the opposite side, the phase shift is negative. As the sampling interval equaled 2 h, each phase shift unit is 2 h. Thus, a phase shift of 3 units equals a phase shift of 6 h. Shown are cross-correlograms of leptin/insulin (left hand graphs) and of leptin/cortisol (right hand graphs) from the state of semistarvation (T1) through refeeding at T2 and T3 (see text for exact definition of T2 and T3). Data at time points T1 and T3 stem from one cohort of five patients studied sequentially. From one patient, data at T2 are missing, thus T2 represents data from only four patients. Note that for leptin/insulin and for leptin/cortisol the pattern of the correlogram does not change qualitatively, although a quantitative change emerges at T3. Significant levels were reached at leptin/cortisol T3 ($P < 0.05$ with r below -0.661 , statistics see text).

fat mass. Although leptin levels oscillate in a circadian fashion (18, 20), the substantial increases in leptin during refeeding in AN were only achieved over a period of about 8 weeks for the first period (T1-T2) and another 10 weeks for the second period (T2-T3). Although these time lags were similar to those of Casanueva *et al.* (29) we could not confirm their assumption of a BMI level below which leptin levels do not drop further but also do not increase despite weight gain.

The intraindividual leptin secretion dynamics during the course of refeeding up to a BMI of 17.5 kg/m² yielded an almost uniform secretion pattern at all three weight levels with maximal values around 0200 h and minimal values around 0800 h. These circadian variations are in line with 24 h studies on leptin secretion in normal weight individuals published previously (16, 20). However, contrary to the results of Balligand *et al.* (16) who postulated a complete abolition of circadian variation in anorectic patients, our results support a preservation of diurnal secretion patterns even in the severe state of emaciation. One explanation of these controversial findings may be the insufficient sensitivity of the leptin RIA (0.5 µg/l) used by Balligand *et al.* (16) and their 8 h intervals of leptin measurement.

In our study, cortisol levels decreased during refeeding in a BMI-dependent fashion with intermediate T2 levels. The hypercortisolism with elevated corticotrophin-releasing hormone (CRH) levels in CSF and blunted ACTH responses to CRH is not specific for AN. The up-regulation in the hypothalamo-pituitary-adrenal axis is probably a consequence of nutritional factors and starvation. It most likely reflects a suprapituitary effect at or above the hypothalamus (28, 30, 31) and already normalizes after only small weight gains (26). It may be hypothesized that a negative feedback between leptin and cortisol secretion also exists in humans, similar to rodents, and is effective at both the hypothalamo-pituitary level and the adrenals. Alternatively, cortisol levels are regulated independently of leptin and decrease due to the lessening of the stress situation of starvation. We favor the first hypothesis, as we have shown in a previous study (18) and also in this paper that cortisol and leptin are negatively correlated on a short-term, ultradian basis. Insulin levels increased during refeeding, but due to a wide interindividual variation and a lack of an increase from T1 to T2, statistical significance was not reached.

Another aim of our study was to determine the temporal relationship between leptin and cortisol as well as insulin. We have previously demonstrated a positive, time-delayed relationship of leptin and insulin with insulin peaks preceding (and stimulating?) leptin increases (18). In the present study this relationship could be observed qualitatively at all three states of nutrition (T1-T3); however, no significant correlation could be obtained. Conversely, a temporal relationship between leptin and cortisol could not be demonstrated at starvation, but it emerged during

refeeding, reaching significance only at T3, i.e. a BMI of 17.5 kg/m². Our present results differ only slightly at T1 from our previous findings (18). The negative relationship around a phase shift of -6 h was preserved as was the positive relationship at a phase shift of +4 to +6 h. Only around 0 phase shift was a tendency towards negative correlations observed in this study, while values were positive in the previous sample. As none of the correlation coefficients were significantly different from zero, this difference is probably due to interindividual variations, as patients studied in these two experiments were not totally identical. This finding points to a pronounced but reversible disturbance of the relationship between cortisol and leptin in AN. It remains unclear if this disturbance contributes to the disease or if it follows on from the multiple pathophysiological changes in anorexia.

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