Increased levels but preserved diurnal variation of serum leptin in GH-deficient patients: lack of impact of different modes of GH administration

Helene Nørrelund, Claus Højbjerg Gravholt, Piera Englaro², Werner F Blum^{1,2}, Wolfgang Rascher², Jens S Christiansen and Jens O L Jørgensen

Medical Department M (Endocrinology and Diabetes), Aarhus University Hospital, Aarhus, Denmark, ¹Lilly, Bad Homburg, Germany and ²Department of Pediatrics, University of Giessen, Germany

(Correspondence should be addressed to H Nørrelund, Medical Department M, Aarhus Kommunehospital, DK-8000 C, Aarhus, Denmark)

Abstract

The regulation of leptin production in humans is poorly understood but appears to depend on total body fat, changes in energy intake and insulin levels. Since growth hormone (GH) is an important regulator of both lipid metabolism and insulin secretion and action, we tested whether GH status directly or indirectly regulates leptin secretion.

Circadian serum leptin concentrations were measured in GH-deficient patients in two different protocols involving different modes of acute and prolonged GH exposure. In study I, eight GH-deficient patients all underwent three 4 week study periods in random order: (1) evening (2000 h) s.c. GH injections (2 IU); (2) morning (0800 h) s.c. GH injections (2 IU); (3) no GH administration. At the end of each period the patients were admitted to hospital for 24-h measurements of hormones and metabolites. For comparison, 10 age- and sex-matched healthy untreated subjects were hospitalised under identical conditions. In study II, six GH-deficient patients were hospitalised for 44 h on three occasions, separated by at least 4 weeks without GH treatment. On each occasion they received 2 IU GH, administered i.v. as (1) two boluses (at 2000 and 0200 h), (2) eight boluses (at 3 h intervals starting at 2000 h) or (3) a continuous (2000–2000 h) infusion. In both studies, serum leptin levels peaked between midnight and early morning followed by low day-time levels ($P < 0.01$). The mode of GH treatment or previous discontinuation did not affect the leptin level ($P > 0.05$), but the patients had significantly higher leptin levels than the controls ($P < 0.01$). The diurnal variation in leptin was compared with changes in GH, insulin, non-esterified fatty acids, 3-hydroxybutyrate, insulin-like growth factor I and glucose, but no robust cross-correlations could be demonstrated.

The following conclusions were made. (1) The circadian pattern of serum leptin is not influenced by either experimental or spontaneous changes in serum GH concentrations. (2) GH deficiency is associated with elevated leptin levels which most likely reflects increased fat mass in these patients.

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Introduction

The discovery of leptin, the product of the obese (*ob*) gene expressed in adipose tissue of several species including humans, has expanded our knowledge of the regulation of fat mass and energy balance (1, 2). Leptin is suggested to serve as both an index of fat mass and a sensor of energy balance (3). Administration of recombinant murine leptin to *ob/ob* mice, which do not produce leptin, decreases food intake and increases thermogenesis both of which result in a reduction in body weight and adipose tissue mass.

The regulation and action of endogenous leptin in humans are, however, less well understood. Serum leptin correlates with total body fat in both normal weight and obese subjects, which suggests insensitivity

to leptin in obese patients (4). More rapid changes in serum leptin have furthermore been reported after short-term changes in energy intake (4) and administration of insulin (5). Moreover, a modest circadian variation in serum leptin has been described, with highest levels between midnight and the early morning hours and lowest levels from noon to mid-afternoon (6).

Growth hormone (GH) exerts pronounced acute and long-term effects on lipid metabolism and resting energy expenditure (7). Experimental studies in GH-deficient patients have furthermore shown that the timing of GH administration is an important determinant of the circadian patterns of circulating lipid intermediates (8), and the relative obesity of untreated GH deficiency is reversed and normalised after long-term substitution (9). GH in the physiological range also

acutely antagonises the actions of insulin on glucose metabolism, which may be secondary to its lipolytic effects, and GH excess leads to hyperinsulinaemia and insulin resistance (10). It is therefore tempting to speculate that GH status directly or indirectly influences leptin secretion.

To test this hypothesis, we measured circadian serum leptin concentrations in GH-deficient patients in two different protocols involving different modes of acute and prolonged GH exposure (8, 11). The protocols also allowed a comparison with age-matched untreated normal subjects, and an investigation of possible correlations between circadian patterns of serum leptin and pertinent hormones and metabolites.

Subjects and methods

Participants

Protocol I Eight GH-deficient patients (two females and six males; mean $\pm$ S.E.M. age, 15.3 ± 1.0 years; Table 1) participated after informed consent had been obtained from patients and parents. Diagnosis was based on auxiological data (height and height velocity more than 2 S.D. below the mean for sex and age and retardation of bone age) and ultimately on a peak serum GH ($<5 \mu\text{g/l}$

after two conventional stimulation tests). Before the study, all patients were treated with GH (Norditropin; Novo Nordisk, Copenhagen, Denmark) at a daily dose of 2 IU, administered s.c. in the evening. The medications listed in Table 1 were continued unchanged throughout the study period.

Ten healthy subjects (three girls and seven boys; mean $\pm$ S.E.M. age, 14.9 ± 1.6 years), without any significant medical history and without any medication, volunteered as control subjects.

Protocol II Six GH-deficient patients (two females and four males; mean $\pm$ S.E.M. age 20.5 ± 1.1 years; Table 1) participated. The diagnosis was based on a peak serum GH of less than $5 \mu\text{g/l}$ after at least two provocation tests. Apart from GH, the medications listed in Table 1 were continued unchanged during the study period.

Study design

Protocol I All patients underwent three consecutive 4 week study periods in random order receiving (1) daily s.c. injections of 2 IU GH (Norditropin; 3 IU/mg) given in the evening (at 2000 h), (2) daily s.c. injections of 2 IU GH given in the morning (at 0800 h), or (3) no GH. At the end of each period the patients were hospitalised in

Table 1 Details of patients in the two studies.

Study I

Patient no.	Sex (M/F)	Chronological age (years)	Height (cm)	Weight (kg)	Tanner stage	Diagnosis	Medication other than GH
1	F	15	150	45	2/2	IGHD	None
2	F	13	142	43	2/1	Craniopharyngeoma	T ₄ , cortisone, desmopressin
3	M	13	138	33	1/1	IGHD	None
4	M	11	134	31	1/1	IGHD	None
5	M	17	159	56	2/1	IGHD	None
6	M	17	165	53	3/2	Pineal tumour	T ₄ , cortisone, desmopressin, testosterone
7	M	20	170	57	4/4	Craniopharyngeoma	T ₄ , cortisone, desmopressin, testosterone
8	M	16	170	60	4/4	Chromophobe adenoma	
Mean $\pm$ S.E.M.		15.3 ± 1.0	153 ± 5.1	47.3 ± 3.9			

Study II

Patient no.	Sex (M/F)	Age (years)	Height (cm)	Weight (kg)	Tanner stage	Diagnosis	Medications
1	F	17	165	56.2	4/4	IGHD	Estrogen, GH
2	F	23	153	50.0	5/5	IGHD	Nil
3	M	23	158.5	45.3	5/5	IGHD	Levothyroxine
4	M	22	165	57.0	5/5	Epidermoid cyst	Cortisone, levothyroxine, desmopressin, testosterone
5	M	14	171	85.0	4/4	Pinealoma	Cortisone, levothyroxine, testosterone
6	M	21	173	55.5	5/5	Craniopharyngeoma	Cortisone, levothyroxine, testosterone, GH
Mean $\pm$ S.E.M.		20.5 ± 1.1	164.3 ± 3.1	58.2 ± 5.7			

IGHD, isolated growth hormone deficiency; T₄, thyroxine.

the evening for 24 h blood sampling at 1 h intervals starting on the following day at 0800 h by means of a cannula inserted in an antecubital vein. Meals were served at 0800, 1200 and 1700 h. The meals were identical in terms of composition and caloric content for each patient on each occasion, and no calories were given in-between. Normal daily activities other than sport were allowed until 2000 h, after which the patients stayed in bed.

The control subjects were hospitalised once under similar conditions. They did not receive any treatment.

Protocol II Each patient underwent three i.v. GH administration schedules during hospitalisation in random order. Each study was separated by 4 weeks, when the patients did not receive GH. The two patients who were receiving GH replacement therapy (nos 1 and 6) had this withdrawn 4 weeks before and during the entire study period. Each study started at 1600 h on the first day and lasted 44 h, including periods before and after GH administration, as outlined below. Meals, which were standardised for each patient with respect to composition and total caloric content, were served at 1700, 0730, 1200, 1700 and 0730 during each study. No calories were allowed between meals. The patients were confined to bed from 2000 to 0800 h during both nights, whereas in the remaining interval ambulant activities other than sports were allowed. In each of the three studies the same total amount of 2 IU biosynthetic human GH (3 IU/mg; Norditropin) was administered. The three administration schedules were: (1) two i.v. boluses at 2000 and 0200 h on the first evening and night, (2) eight i.v. boluses at 3 h intervals from 2000 to 1700 h, starting on the first evening, or (3) a continuous i.v. infusion (0.6 IU/ml) from 2000 to 2000 h, starting on the first evening. One i.v. catheter was placed in an antecubital vein in each arm for infusion and blood sampling. Samples were drawn at 1-h intervals from 1800 to 2400 h on the first evening, then every second hour from 2400 to 1200 h and every fourth hour from 1200 to 2400 h. Finally one sample was drawn at 0800 and 1200 h on the last day of the study. Not all parameters were assayed in every sample.

The studies were performed according to the Helsinki Declaration, which included oral and written consent from patients or parents. Data from the two studies have been published previously (8, 11).

Assays

Serum leptin was measured by a newly developed RIA as described previously (12). Serum GH and insulin and plasma glucagon were measured by RIA (13) as was insulin-like growth factor-I (IGF-I), using acid/methanol-extracted serum (14). Blood glucose was measured by a glucose oxidase method, and serum non-esterified fatty acids by a radiochemical technique (15). The whole blood content of 3-hydroxybutyrate was

measured by an automated enzyme fluorimetric method (16). Blood for determination of serum concentrations was allowed to clot at room temperature for 1–2 h, and samples for measurement of metabolites were centrifuged immediately and stored at -30°C .

Statistical analysis

The variation in serum leptin levels over time was evaluated by single-factor ANOVA after the data had been expressed as percentage change from fasting morning values. To characterise further the circadian leptin pattern, autocorrelation analysis was performed to obtain nadir and peak time points. Two-way ANOVA with repeated measures was used to compare leptin levels between patients and controls, and between the different GH protocols. One-way ANOVA was used to test for differences in total area under the curve (AUC) of serum leptin between patients and controls, and between the different GH protocols. To compare leptin levels between patients and healthy subjects further, analysis of covariance was employed using body mass index (BMI) as the covariate. This approach was used because of the well-known dependence of leptin on body composition. Cross-correlation analysis was used to detect significant correlations between time series of leptin and pertinent hormonal and metabolic variables, and to identify possible synchronicities between such time series at the same or lagged time points. The significance of these correlations was further examined in a regression analysis, and the predicted data were generated from the regression equation and plotted. Since leptin data were normally distributed, as tested by the Kolmogorov-Smirnov test, the data are expressed as mean $\pm$ S.E.M. All statistical computations were performed with SPSS for Windows version 7.0 (SPSS Inc., Chicago, IL, USA).

Results

Study I

Figure 1 shows mean circulating levels of leptin during the different GH treatment schedules and in the control group. A significantly higher leptin level (adjusted by using baseline BMI as a covariate) was found in the GH-deficient patients than in the control group ($P < 0.01$), while mode of GH treatment or previous discontinuation of GH for a period of 4 weeks did not affect serum leptin level ($P > 0.05$). No differences in BMI between GH-deficient patients and controls were found ($19.75 \pm 0.62 \text{ kg/m}^2$ and $18.97 \pm 0.6 \text{ kg/m}^2$ respectively ($P > 0.05$)).

We observed time-dependent changes in leptin levels when they were represented as percentage change from fasting levels ($P < 0.01$), while no effect of time was found when absolute values were analysed because of

Figure 1 Mean $\pm$ S.E.M. circulating levels of leptin in subjects on different GH treatment schedules (no GH (●), morning (■) and evening (▲) injections) and the reference group (▼).

considerable individual differences in leptin level ($P > 0.05$). In both groups, i.e. GH-deficient patients in all three studies and controls, we observed increased leptin levels between midnight and early morning compared with lowest leptin levels in the morning (Fig. 1). The average circadian amplitude between acrophase (0200 h) and nadir (1200 h) was 75.6% in GH-deficient patients without treatment, 153.5% after morning injections, 88.7% after evening injections, and 184.8% in the control group. The peak night-time leptin levels were 28, 20, 30 and 48% higher than nadir leptin levels when represented as percentage change from 0800 h fasting levels in GH-deficient patients without treatment (102.68 ± 9.07 vs 131.47 ± 7.13), after morning injections (131.4 ± 31.3 vs 157.46 ± 14.7), after evening injections (93.67 ± 7.84 vs 121.4 ± 5.51), and in control groups (109.45 ± 23.9 vs 161.6 ± 11.7).

The AUC of serum leptin in the patient group was significantly higher than in the control group ($P < 0.05$), while no differences were found between the different treatment schedules.

Diurnal changes in leptin were compared with changes in GH, insulin, non-esterified fatty acids, 3-hydroxybutyrate and glucose. Figure 2 shows profiles of circulating leptin, insulin and 3-hydroxybutyrate among GH-deficient patients after different GH administration schedules, and in the reference group. Circadian variation in leptin did not correlate unambiguously with either insulin or 3-hydroxybutyrate, although a significant cross-correlation was identified between diurnal change in leptin and insulin among GH-deficient patients without treatment ($P < 0.01$), while P values in the remaining correlations between leptin and insulin were between 0.05 and 0.08. One series did

not uniformly lead to another, and the time lag between the series was from 4 to 10 h. 3-Hydroxybutyrate correlated significantly with serum leptin level in patients receiving morning and evening injections and in the control group ($P < 0.05$). Changes in serum leptin and 3-hydroxybutyrate occurred at the same time points. No significant correlations were observed between circulating levels of leptin and GH, IGF-I, non-esterified fatty acids or glucose levels.

Study II

No significant differences in serum leptin were found when the three regimens were compared ($P > 0.05$) (Fig. 3). Increased leptin levels were observed between midnight and the early morning hours compared with lowest values in the morning (acrophase 2400 h; nadir 0800 h). The significant nightly increases in leptin were more pronounced on the second night (1800–0800 h: 8.11 ± 0.31 vs $11.48 \pm 0.88 \mu\text{g/l}$ ($P < 0.01$)). The average circadian amplitude between acrophase and nadir was 66.8% after infusion, 95.3% after two bolus injections and 92.3% after eight bolus injections. The peak night-time leptin levels were 142, 165 and 122% higher than nadir leptin levels when represented as percentage change from 0600 h fasting levels in GH-deficient patients treated by infusion (189.97 ± 44.55 vs 78.56 ± 17.63), two boluses (211.46 ± 65 vs 79.73 ± 3.02) and eight boluses (174.05 ± 23.05 vs 78.51 ± 5.23) respectively.

Profiles of circulating leptin, insulin and 3-hydroxybutyrate are shown in Fig. 4. Significant cross-correlations were identified between diurnal change in leptin and insulin after infusion and two bolus injections

Figure 2 Profiles of circulating leptin (▲), insulin (●) and 3-hydroxybutyrate (▼) in GH-deficient patients on different GH administration schedules (no GH, morning and evening injections) and in the reference group.

Figure 3 Mean $\pm$ S.E.M. circulating levels of leptin between different GH regimens (GH infusion (▼), bolus $\times$ 2 (●), bolus $\times$ 8 (▲)).

($P < 0.01$). Circadian variation in 3-hydroxybutyrate correlated significantly with leptin in patients receiving bolus injections ($P < 0.05$). Again there was no consistency as to whether changes in leptin were influenced by changes in insulin or 3-hydroxybutyrate or vice versa. Circulating levels of leptin did not correlate with GH, non-esterified fatty acids, IGF-I or glucose.

Discussion

The present studies provided an evaluation of the impact of GH status on the circadian pattern of serum leptin concentrations. The first protocol involved measurements of 24-h serum profiles after 4 weeks of no GH, evening administration of GH or morning administration of GH. This study also included an age- and sex-matched group of healthy untreated children. From that study we have previously reported that GH and the timing of its administration significantly influence the circadian patterns of circulating insulin and lipids (8). The second protocol (11) compared acute pulsatile versus continuous i.v. GH administration in GH-deficient patients, who had been off GH therapy for at least 4 weeks before each study. We have consequently been able to address the following questions. (1) Does the 24-h pattern of leptin concentration depend on circadian changes in GH levels? (2) Is there a difference in 24-h leptin levels between GH-deficient patients and normal subjects? (3) Does acute GH exposure influence leptin secretion? (4) Is there a robust cross-correlation between 24-h levels of circulating leptin and pertinent hormones and metabolites?

A similar and subtle but significant diurnal variation in serum leptin concentration was detected in all studies. This variation was characterised by peak

levels between midnight and early morning, and nadir levels around noon. This pattern is compatible with previous studies in both lean and obese subjects (6, 17). In protocol I, both the absolute level and pattern of serum leptin were almost identical in the three patient studies. Moreover, the three different modes of i.v. GH exposure in protocol II yielded very similar changes in leptin levels. It is therefore unlikely that the 24-h serum leptin levels depend on the corresponding GH pattern. This observation is substantiated by the lack of a consistent cross-correlation between the time series of leptin and GH.

The circadian pattern of leptin concentration was similar in patients and controls, but the absolute levels were significantly higher in the former. This difference was present in spite of an equal sex distribution and comparable BMI levels. At first glance, this could suggest a direct effect of GH status on leptin secretion, but we consider such an interpretation invalid for two reasons. First, it is well documented that BMI provides a poor estimate of adiposity in GH-deficient patients, in whom a normal BMI in the presence of distinctly increased total body fat is usually encountered (18). In the present study we have no additional measures of adiposity, but we expect the total body fat of the patients to be increased compared with the controls. Secondly, the leptin levels in the patients did not change after 4 weeks without GH. The latter probably reflects the fact that 4 weeks of discontinuation of GH treatment is insufficient time to induce significant alterations in fat mass. In accordance with this, it has recently been reported that more prolonged GH therapy in GH-deficient adults prompted a decrease in both total body fat and leptin without affecting BMI (19).

Figure 4 Profiles of circulating leptin (▲), insulin (●) and 3-hydroxybutyrate (▼) in GH-deficient patients subjected to different modes of acute GH exposure (GH infusion, bolus×2, bolus×8).

Acute GH exposure from 2000 h to 2000 h of patients with untreated GH deficiency induced a significant increase in serum leptin levels during the subsequent day (from 2000 h to 1200 h). The underlying mechanism is unclear, and it is important to realise that a protocol involving acute GH exposure to GH-deprived

GH-deficient patients represents a rather unphysiological and non-steady-state condition. From the same study (11), we have previously reported a gradual increase in serum IGF-I levels from hypopituitary levels, but only when the patients received constant infusion or eight boluses. Since an increase in leptin was observed in all

three studies, it seems less likely that IGF-I causes a stimulation in leptin. Both insulin and lipid intermediates increased to the same extent after the three modes of GH administration. At present it can be concluded that acute GH exposure in GH-deficient patients, irrespective of its pattern of administration, may cause a modest increase in leptin levels under certain conditions.

Acute changes in leptin levels in situations of negative and positive energy balance suggest short-term regulation. Blum *et al.* (20) showed a decline in leptin in fasting subjects which could be inversely correlated with IGF-binding protein-1. Since IGF-binding protein-1 is thought to reflect insulin suppression integrated over hours, this finding suggests that the decrease in leptin is secondary to hypoinsulinaemia. This is compatible with clinical studies demonstrating a clear correlation between serum leptin and insulin concentration (4), and *in vitro* studies of fat cell cultures in which insulin directly stimulates leptin (12). Studies using the euglycaemic-hyperinsulinaemic clamp have, however, failed to show an increase in serum leptin, at least during short-term hyperinsulinaemia (21, 22).

In the present study I, diurnal serum insulin levels exhibited substantial differences among the three studies, whereas leptin levels were similar. Significant cross-correlations between change in leptin and insulin were only detected among GH-deficient patients taken off GH treatment. In study II no significant differences in serum insulin were found when the three regimens were compared in terms of changes with time or AUC. In all three studies, insulin was lowest during the first night and tended to increase during the second night. No significant change in leptin level was observed between the different treatment schedules. Circadian variations in insulin correlated significantly with leptin only in patients receiving infusion and two bolus injections. Thus cross-correlation and time series analysis between diurnal variation in serum leptin and insulin did not show homogeneity and did not allow any conclusions about whether one series leads to another.

Kolaczynski *et al.* (23) studied the effect of fasting and refeeding on leptin level and observed a reverse relationship between leptin and 3-hydroxybutyrate. The fasting-induced fall in leptin could be prevented by infusion of glucose adjusted to prevent ketogenesis, whereas infusion of 3-hydroxybutyrate to produce hyperketonaemia of the same magnitude as observed after a 36-h fast had no effect on leptin. Our study confirmed a distinct circadian pattern in lipid intermediates and ketone bodies characterised by low postprandial levels and high levels before each meal and during the night (Fig. 2). The most consistent association between leptin and 3-hydroxybutyrate appeared to be parallel nocturnal elevations, which does not support the hypothesis that ketone bodies suppress leptin production.

In summary, the present studies enabled us to investigate the association between circadian serum leptin levels, GH status, insulin and pertinent metabolites. Serum leptin levels exhibited a distinct diurnal rhythm, with high levels between midnight and the early morning hours, and low levels around noon. We did not detect any differences in leptin level between different GH treatment schedules, whereas significant differences between the control group and patients were found. The diurnal changes in leptin do not appear to depend strongly on simple variations in circulating levels of GH, insulin, glucose or lipid metabolites.

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