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SHORT COMMUNICATION

Day/night variations of high-molecular-weight adiponectin and lipocalin-2 in healthy men studied under fed and fasted conditions

F. A. J. L. Scheer · J. L. Chan · J. Fargnoli · J. Chamberland · K. Arampatzi · S. A. Shea · G. L. Blackburn · C. S. Mantzoros

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Abstract
Aims/hypothesis Adiponectin and lipocalin-2 are adipocyte-derived plasma proteins that have been proposed to have opposite effects on insulin sensitivity. Given the epidemiological, physiological and molecular links between sleep, the circadian timing system and glucose metabolism, the aim of this study was to assess effects of the sleep/wake cycle and the fasting/feeding cycle on high-molecular-weight adiponectin (HMW-adiponectin; the biologically active form) and lipocalin-2. We also aimed to compare the 24 h rhythms in the levels of these proteins with those of cortisol, leptin, leptin-binding protein and total adiponectin.
Methods Lean men underwent a 3 day in-laboratory study, either in the fed state (n=8, age: 20.9±2.1 years, BMI: 22.8±2.3 kg/m^2) or fasting state (3 day fast, n=4, age: 25.3±3.9 years, BMI: 23.3±2.2 kg/m^2). The sleep episode was scheduled in darkness from 23:00 to 07:00 hours. Blood was sampled every 15 min for 24 h on the third day of each study.
Results While fed, HMW-adiponectin and lipocalin-2 had large daily rhythms with troughs at night (HMW-adiponectin: ~04:00 hours, peak-to-trough amplitude 36%, p<0.0001; lipocalin-2: ~04:00 hours, 40%, p<0.0001). On the third day of fasting, the timing and relative amplitudes were unchanged (HMW-adiponectin: ~04:00 hours, 38%, p=0.0014; lipocalin-2: ~05:00 hours, 38%, p=0.0043).
Conclusions/interpretation These data show that HMW-adiponectin and lipocalin-2 both have significant day/night rhythms, both with troughs at night, that these are not driven by the feeding/fasting cycle, and that it is important to report and/or standardise the time of day for such assays. Further studies are required to determine whether the daily rhythm of HMW-adiponectin levels influences the daily rhythm of insulin sensitivity.
Trial registration ClinicalTrials.gov NCT00140205
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Keywords Cortisol · Fasting · High-molecular-weight adiponectin · Leptin · Leptin-binding protein · Lipocalin-2 · Nutrition · Soluble leptin receptor · Sleep/wake cycle · Total adiponectin

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F. A. J. L. Scheer (✉) · S. A. Shea
Division of Sleep Medicine,
Brigham and Women's Hospital and Harvard Medical School,
221 Longwood Avenue,
Boston, MA 02115, USA
e-mail: fscheer@rics.bwh.harvard.edu

J. L. Chan · J. Fargnoli · J. Chamberland · K. Arampatzi · C. S. Mantzoros
Division of Endocrinology, Diabetes, and Metabolism,
Beth Israel Deaconess Medical Center, Harvard Medical School,
Boston, MA, USA

G. L. Blackburn
Department of Surgery,
Beth Israel Deaconess Medical Center, Harvard Medical School,
Boston, MA, USA

Abbreviations

HMW-adiponectin	high-molecular-weight adiponectin
sOB-R	soluble leptin receptor

Introduction

Adiponectin is an abundant adipocyte-derived hormone with insulin-sensitising and anti-atherogenic actions that circulates predominantly in multimeric complexes, of which high-molecular-weight (HMW) adiponectin is thought to represent the biologically active form [1]. Another adipocyte-derived hormone, lipocalin-2 (also called neutrophil gelatinase-associated lipocalin, growth factor-stimulated superinducible protein, 24p3 or oncogene neu-related lipocalin), has been suggested to have a role in decreasing insulin sensitivity [2]. There is extensive evidence that glucose tolerance and insulin sensitivity express clear day/night rhythms with troughs around the middle of the night [3, 4], which may be of clinical relevance for their diagnosis, management and treatment. However, the underlying mechanisms are not well understood. To begin to understand whether day/night changes in HMW-adiponectin and/or lipocalin-2 may be involved in the day/night rhythm of insulin sensitivity, we tested the primary hypotheses that: (1) HMW-adiponectin concentrations show a significant day/night variation; (2) HMW-adiponectin concentrations are low at night; (3) lipocalin-2 concentrations show a significant day/night variation; and (4) lipocalin-2 concentrations are high at night. We could not reliably determine the daily rhythm in insulin sensitivity in this study because of fluctuations related to meals during the fed state and possible gradual changes in metabolic state across the fasting state, and therefore based this on the well-known day/night profile of insulin sensitivity [3, 4]. Second, we evaluated whether any day/night rhythms in HMW-adiponectin and/or lipocalin-2 were dependent on fasting/feeding cycles. Finally, cortisol, leptin and total adiponectin express daily rhythms [5, 6] so to shed light on potential mechanistic links, we also studied correlations and relative timing of daily rhythms of HMW-adiponectin and lipocalin-2 with rhythms of cortisol, leptin, soluble leptin receptor (sOB-R) and total adiponectin.

Methods

Participants Ten healthy, lean, non-smoking men participated in a 3 day study in a general clinical research centre, either in a fed state (n=8; mean±SD age, 20.9±2.1 years; BMI, 22.8±2.3 kg/m^2, all <25 kg/m^2) or a fasting state (n=4; age, 25.3±3.9 years; BMI, 23.3±2.2 kg/m^2, all <25 kg/m^2), as previously described [7]. Two of the participants participated in both fed and fasted studies.

Study protocol The study was approved by the local Institutional Review Board and participants gave informed consent. Before the laboratory studies, participants were advised to maintain their activity level and a stable sleep routine, and to remain on an isoenergetic diet. For both studies, participants were admitted to the laboratory the evening before study day 1. For both studies, starting at 08:30 hours on day 3, blood samples were collected every 15 min through a peripheral intravenous catheter for 24 h. No strenuous activity was allowed and a 23:00 to 07:00 hours sleep opportunity was scheduled in darkness. During the fed study, participants were kept on an isoenergetic diet to maintain body weight. Following an evening snack before study day 1, fasted study participants received nothing to eat or drink except energy-free liquids and a standard multivitamin with minerals daily (to prevent vitamin and mineral deficiency) for 3 days. The fast ended with breakfast on study day 4 at 10:00 hours.

Hormone measurements Serum or plasma HMW-adiponectin levels were measured using ELISA (Linco Research, St Louis, MO; now Millipore, Billerica, MA, USA) with a sensitivity of 0.5 ng/ml and intra-assay CV of 1.0–3.4%. Lipocalin-2 levels were measured using ELISA (Biovendor, Modrice, Czech Republic) with a sensitivity of 0.02 ng/ml and intra-assay CV of 9.7–9.8%. Serum leptin, sOB-R, cortisol and total adiponectin concentrations were measured as previously reported [5].

Analysis Quantities of serum/plasma available were limited for some samples so data were grouped into 30 min bins. Daily variations of hormones were analysed by mixed model analysis of variance. Amplitude and timing of hormonal rhythms were assessed by two-harmonic non-orthogonal spectral analysis of group data, using an exact maximum likelihood fitting procedure. Analyses were carried out using SAS or JMP (both SAS Institute, Cary, NC, USA), and $\alpha<0.05$ was considered significant. Partial analysis of the data for cortisol, total adiponectin, sOB-R and leptin (in six of the eight participants) under fed conditions has been previously published [5].

Results

The daily patterns of the variables during the fed and fasting state (third day in the laboratory for both studies) are displayed in Fig. 1. The 24 h mean concentration, the

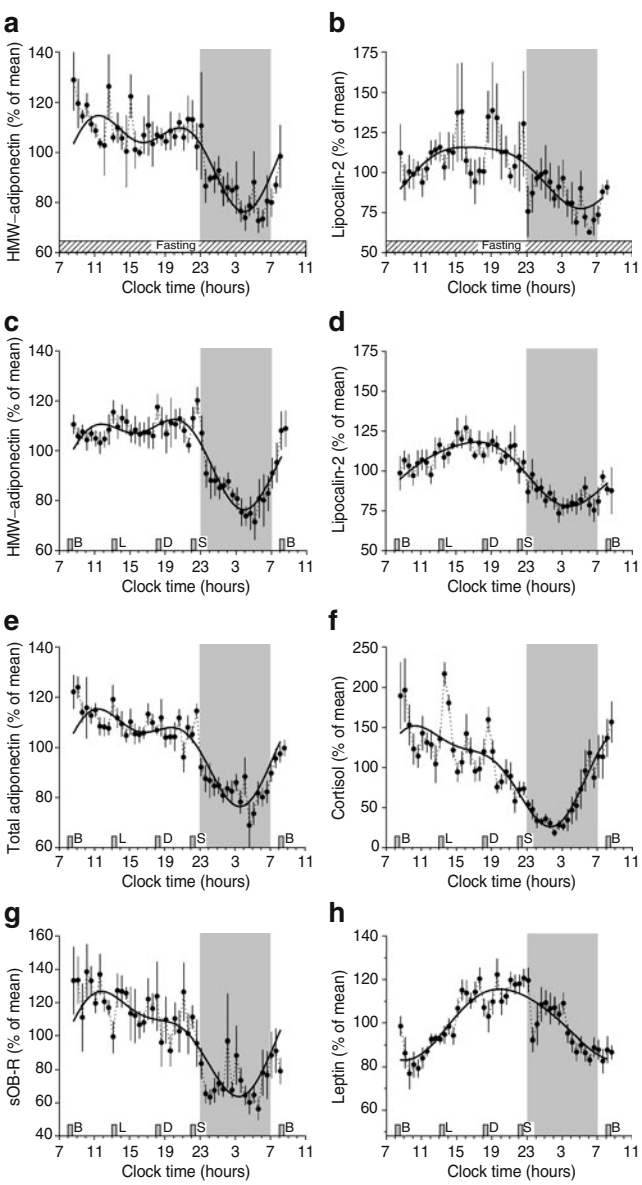

Fig. 1 Day/night variation in HMW-adiponectin, lipocalin-2, total adiponectin, cortisol, sOB-R and leptin, in the fed and fasting state. HMW-adiponectin had a significant daily rhythm that was nearly identical during fed (trough 03:53 hours, peak-to-trough amplitude 36%, n=8; p<0.0001) (**c**) and fasted (04:00 hours, 39%, n=4; p<0.0014) (**a**) conditions, with trough at night and rise before scheduled awakening. Lipocalin-2 had a significant daily rhythm that was similar during fed (03:45 hours, 40%, n=8; p<0.0001) (**d**) and fasted (05:04 hours, 38%, n=4; p=0.0043) (**b**) conditions, with a trough at night. During fed conditions, total adiponectin (03:30 hours, 39%, n=6; p<0.0001) (**e**) and sOB-R (03:23 hours, 63%, n=6; p<0.0001) (**g**) showed significant daily rhythms with timings similar to those of HMW-adiponectin, whereas the rhythm in cortisol preceded HMW-adiponectin by several hours (01:38 hours, 126%, n=6; p<0.0001) (**f**). In addition to the day/night rhythm, cortisol also had a superimposed ultradian rhythm, with a peak every 2–2.5 h, correlated in part with meal times. Leptin showed a significant rhythm, with a rhythm that lagged that of HMW-adiponectin and lipocalin-2 by about 5 h (09:00 hours, 33%, n=8; p<0.0001) (**h**). There was a rapid and transient fall in leptin in all eight participants within the first hour after bedtime (possibly related to sleep onset or a postural effect). Similar, but less consistent, falls were observed for the other variables. Circles, 30-min averages; error bars, SEM; smooth lines, two-harmonic cosine fits; large vertical grey area, sleep episode; small grey bars, meal times: B, breakfast; L, lunch; D, dinner; S, snack; horizontal hatched bar in **a** and **b**, third day of fasting; n, number of participants contributing to data; p values, statistical significance for day/night variation. Because of large inter-individual differences in hormone concentrations, the data on the graph are expressed as percentages of each individual's mean. For the absolute concentrations of 100% of mean for each variable under each condition, see the '24 h mean concentration' in Table 1

relative peak-to-trough difference, and the timing of the trough for all variables measured during the fed and fasting states are described in Table 1. In brief, HMW-adiponectin had significant and similar day/night variations during the fed and fasting states (trough ~04:00 hours; ~40% peak-to-trough difference; both p<0.005). Lipocalin-2 also had similar day/night variations during the fed and fasting states (~04:00 to 05:00 hours; ~40%; both p<0.005), which were similar to those of HMW-adiponectin. During fed conditions, total adiponectin (03:30 hours; 39%; p<0.0001) and sOB-R (03:23 hours; 63%; p<0.0001) showed significant day/night variability with timings similar to those of HMW-adiponectin, whereas the rhythm in cortisol preceded that of HMW-adiponectin and lipocalin-2 by several hours (01:38 hours; 126%; p<0.0001). The day/night rhythm of leptin lagged that of HMW-adiponectin and lipocalin-2 by about 5 h (09:00 hours; 33%; p<0.0001). The timing of peaks for all variables is shown in Table 1. The results of the cross-correlation analyses are shown in Electronic supplementary material Tables 1, 2 and 3. Significant highlights, which suggest potential causal mechanisms, include the observations that the daily rhythm in HMW-adiponectin lagged that of cortisol (2 h), led that of leptin (3.5 h) and was almost synchronous with those of lipocalin-2, sOB-R and total adiponectin.

Discussion

Our study shows that both HMW-adiponectin and lipocalin-2 have large diurnal variations, with troughs in the middle of the sleep episode, and timing and amplitude independent of the fasting/feeding cycle.

The strengths of the study include: (1) high-frequency assessment of the day/night variation of HMW-adiponectin and lipocalin-2, as well as of related hormones; (2) standardised in-laboratory conditions, including standardised sleep/wake schedule and meal timing; and (3) assessment of the relative role of feeding/fasting cycle in day/night variations by fasting participant for 3 consecutive days. Limitations include the small number of participants and that there was no assessment of insulin sensitivity.

Table 1 Day/night variation characteristics of serum lipocalin-2, HMW-adiponectin, total adiponectin, leptin, sOB-R and cortisol levels under fed and fasted conditions (where available)

	Lipocalin-2 (ng/ml)	HMW-adiponectin (μg/ml)	Total adiponectin (μg/ml)	Leptin (ng/ml)	sOB-R (ng/ml)	Cortisol (nmol/l)
Fed state						
24 h mean concentration	29.7±10.1	2.1±0.3	4.7±0.3	3.2±1.0	13.7±1.6	210±14
Peak-to-trough difference (%)	40.4	36.4	39.0	32.6	63.4	126.3
Timing of trough (hours)	03:45	03:53	03:30	09:00	03:23	01:38
Timing of peak (hours)	18:00	12:00 and 20:00	12:00 and 20:00	20:00	12:00	10:00
Fasted state						
24 h mean concentration	63.1±15.4	1.8±0.4				
Peak-to-trough difference (%)	38.2	38.6				
Timing of trough (hours)	05:04	04:00				
Timing of peak (hours)	16:00	12:00 and 21:00				

As a result of the broad and often plateau-like shape of the peaks, the cosine model gives a less precise description of the peaks (biphasic for some) than of the troughs. We therefore estimate the timing of the peaks to the closed whole hour. Values are means ± SE

The magnitude of the day/night variation in HMW-adiponectin would be likely to contribute to clinically relevant daily changes in insulin sensitivity. For example, the magnitude of the diurnal variation in HMW-adiponectin (~40%) was larger than the ~25% increase observed with 10% weight loss after weight-loss surgery [8] and similar to the ~50% difference between insulin-resistant and insulin-sensitive individuals [9]. Furthermore, with morning fasting concentrations as much as 25% lower than those around lunch time, this highlights the importance of considering the time of day when HMW-adiponectin measurements were taken in all clinical and research assessments. This is also important for lipocalin-2, total adiponectin, leptin, and especially sOB-R and cortisol, which showed even larger amplitude rhythms.

The night-time trough in HMW-adiponectin was consistent with our hypothesis related to the insulin-sensitising effect of HMW-adiponectin and so may be causally related to the robust night-time trough in insulin sensitivity [1, 3, 4, 10]. In contrast, the night-time trough in lipocalin-2 is inconsistent with our hypothesis related to the reported insulin-desensitising effect of lipocalin-2, indicating that lipocalin-2 does not drive the daily insulin sensitivity rhythm. This suggests that the daily rhythms in other regulatory factors—including possibly those in HMW-adiponectin—have a stronger influence in shaping the daily variation in insulin sensitivity than lipocalin-2 [2–4, 10]. Future studies are required to investigate whether the daily rhythm in lipocalin-2 may counterbalance and thereby 'fine-tune' the daily rhythm in the influence of HMW-adiponectin on insulin sensitivity.

The similar day/night profiles for HMW-adiponectin, lipocalin-2, total adiponectin and sOB-R suggest that they may be influenced by common regulatory factors. All these rhythms lag the robust daily rhythm in cortisol by approximately 2 h (Table 1). Future studies are required to determine whether or not the daily cortisol rhythm is causal in these day/night rhythms. The rhythm in leptin lagged the rhythms in HMW-adiponectin, lipocalin-2, total adiponectin and sOB-R by 4–6 h.

Recently, strong mutual links between the circadian timing system, sleep and metabolism have been identified [6, 10, 11]. For example, sleep curtailment decreases glucose tolerance and insulin sensitivity, and increases ghrelin, appetite and food intake [10], whereas circadian misalignment decreases leptin and increases postprandial glucose and insulin [6]. Human studies, such as presented here, may not only help to elucidate adipokine physiology, but also reveal potential underlying physiological mechanisms that explain the increased health risk in shift workers. For example, if the rhythm of HMW-adiponectin is driven by the endogenous circadian system, the resultant low HMW-adiponectin levels during the (night-time) waking hours in night workers may contribute to impaired glucose tolerance when these workers would be likely to consume most of their meals [6].

In summary, we demonstrate that HMW-adiponectin and lipocalin-2 show large (~40%) daily variation, with troughs at night, and with relative amplitude and timing that are unaffected by a 72 h fast. Future studies are required to determine whether HMW-adiponectin and lipocalin-2 show similar rhythms in obese and diabetic populations, whether endogenous circadian control and/or behavioural factors such as sleep drive their daily rhythms, and whether the daily rhythm in HMW-adiponectin is causal in the daily rhythm in glucose tolerance and insulin sensitivity.

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