Sleep enhances nocturnal plasma ghrelin levels in healthy subjects

Andrea Dzaja, Mira A. Dalal, Hubertus Himmerich, Manfred Uhr, Thomas Pollmächer,
Andreas Schuld

Max Planck Institute of Psychiatry, Munich, Germany

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Address correspondence and reprint requests to:
Andreas Schuld, M.D. E-mail: schuld@mpipsykl.mpg.de
Max Planck Institute of Psychiatry Phone: (+49) 89 30622 1
Kraepelinstrasse 10 FAX: (+49) 89 30622 562
80804 Munich, Germany
ABSTRACT
Ghrelin, an endogenous ligand of the growth hormone secretagogue receptor, has been shown to promote slow-wave sleep (nonREM-sleep stages 3 and 4). Plasma levels of ghrelin are dependent on food intake and increase in sleeping subjects during the early part of the night. It is unknown whether sleep itself affects ghrelin levels or whether circadian networks are involved. Therefore, we studied the effect of sleep deprivation on nocturnal ghrelin secretion. In healthy male volunteers plasma levels of ghrelin, cortisol and human growth hormone (hGH) were measured during two experimental sessions of 24 hours each. Once the subjects were allowed to sleep between 2300 and 0700 hours, and once they were kept awake throughout the night. During sleep, ghrelin levels increased during the early part of the night and decreased in the morning. This nocturnal increase was blunted during sleep deprivation and ghrelin levels increased only slightly until the early morning. Ghrelin secretion during the first hours of sleep correlated positively with peak hGH concentrations. We conclude that the nocturnal increase in ghrelin levels is more likely to be caused by sleep-associated processes than by circadian influences. During the first hours of sleep ghrelin might promote sleep-associated hGH secretion and contribute to the promotion of SWS.
INTRODUCTION
Ghrelin is a 28 amino acid polypeptide which has been discovered as an endogenous ligand for the growth hormone secretagogue receptor (1). Ghrelin is mainly synthesized in stomach endocrine cells but has also been identified in different other tissues including the hypothalamus (2-4). Ghrelin stimulates growth hormone secretion, increases food intake and appetite, and promotes body weight gain (5-7). Recent findings suggest that ghrelin might be involved in the regulation of sleep in humans by promoting slow-wave sleep (SWS) (8).
Plasma ghrelin levels increase during fasting and decline within one hour after food intake (9). Furthermore, ghrelin levels have been observed to increase during the night in sleeping subjects and to decrease in the morning some hours prior to awakening and, thus, prior to breakfast (9).
This time course in sleeping subjects suggests the existence of a mechanism regulating ghrelin production which is not dependent on food intake. Possible candidates are the circadian rhythms regulating numerous endocrine networks such as for example the secretion of cortisol or leptin (10;11), which, in turn, are related to insulin and blood glucose levels (12). Alternatively, sleep might directly influence ghrelin production as it has been demonstrated, for example, for the secretion of growth hormone by the pituitary (13).
To dissect diurnal variations from sleep-related influences on ghrelin production we performed a study on the 24-hour pattern of ghrelin secretion in healthy males. During two experimental sessions from 0900 hours to 1000 hours the following morning the subjects were allowed to sleep or were sleep-deprived in balanced order. Nocturnal ghrelin secretion was compared between the two conditions using a within-subjects design.
MATERIALS AND METHODS

Experimental Subjects

We investigated 10 healthy, non-obese male volunteers (age 28 ± 3.1 years, BMI 24.0 ± 2.9 kg/m²; range from 20.5 to 29.5 kg/m²) both under sleep and sleep deprivation conditions. Written informed consent was obtained from all participants prior to inclusion and the study was approved by an independent ethics committee. The subjects underwent detailed medical history, evaluation of sleeping habits, physical examination, laboratory investigations, EEG, and ECG to exclude acute or chronic diseases. Subjects with a personal or family history of psychiatric disorders, a history or symptoms of any sleep disorder, use of any medication, alcohol or substance abuse or dependence were not included. Furthermore, we excluded subjects with a history of recent irregular sleep-wake schedules, e. g. shift work or intercontinental flights within the last 4 weeks. To control for regular sleep-wake behavior, subjects kept a sleep log the week prior to both experimental sessions.

Experimental Procedure

The study was performed using a balanced, randomized within-subject design. Every subject participated in two 24-hour experiments. Once the subjects were totally sleep-deprived and the other time allowed to sleep from 2300-0700 hours. The time interval between the two sessions was at least two weeks. In order to adjust to the experimental settings, the subjects spent the night prior to each session (2300 to 0700 hours) in the sleep laboratory. At 0800 hours, electrodes for EEG, EMG, and EOG recordings were placed according to standardized criteria (14). The subjects remained in a semi-recumbent position and received standardized meals at four different time points. The subjects received a standardized diet from our hospitals kitchen with 1800 kcal per 24 hours, containing about 30% fat, 60% carbohydrates, and 10% protein. The subjects were not allowed to eat additional food. Measurements were
started at 0900 hours. Until 1000 hours the next morning subjects stayed in bed for 25 hours. Artificial light intensity was always below 200 Lux. Lights were turned off and the subjects were allowed to sleep between 2300 and 0700 hours in the sleep condition only. During sleep deprivation, another person stayed continuously in the same room with the subject to prevent lapses into sleep. Standardized meals were served at 0800, 1200, 1900 and again at 0800 hours the next morning. Water was freely available during the whole experimental session.

**Blood sampling**

Blood samples were taken hourly via an i. v. catheter placed into an antecubital vein which was kept patent by continuous 0.9% saline infusion containing 400 IU of Na-Heparin/l at a rate of 30 ml/h. To minimize disturbances of sleep continuity, blood sampling was done through the wall via a long line from a room adjacent to the sleep laboratory. Blood was collected in tubes, which were stored on ice and contained Na-EDTA (1 mg/ml of blood) and aprotinin (300 KIU/ml of blood). Immediately after withdrawal blood was centrifuged at 2600 x g for 7 min at 4°C and plasma was aliquoted and frozen to -20 °C until the various assays were performed.

**Sleep recordings**

To ensure that sleep deprivation was successful, polygraphic recordings were obtained throughout 24 hours. All records were visually scored in 30s epochs using standardized criteria (14) by the same experienced person blind to the experimental condition. During sleep deprivation, none of the subjects displayed REM-sleep or nonREM-sleep stages 2-4. The sleep parameters from the sleep phase during the sleep condition are shown in table 1. Sleep quality and distribution of the different sleep stages were comparable to data from other studies on healthy male volunteers who underwent sleep recordings with intermittent blood sampling (15-17) (see table 1).
**Determination of ghrelin, hGH and cortisol plasma levels**

Total plasma ghrelin levels were determined by a radioimmunoassay (Phoenix Pharmaceuticals, CA). The limit of detection was 80 pg/ml. The intra- and interassay coefficients of variation were below 13%. Human growth hormone plasma levels were determined using radioimmunoassays purchased from Nichols Institute Diagnostics, San Juan Capistrano, CA. The limit of detection was 1.5 ng/ml, the intra- and interassay coefficients of variation were below 7%. Plasma cortisol levels were determined by means of a radioimmunoassay from ICN Biomedicals, Carson, CA. The limit of detection was 0.2 ng/ml, the intra- and interassay coefficients of variation were below 7%.

**Statistics**

To compare nocturnal hormone plasma levels between the sleep condition and the sleep deprivation condition, ANOVA for repeated measures was performed with time and condition (sleep vs. sleep deprivation) as within-subject factors. To avoid too many posteriori tests, which would imply a strong reduction of the level of significance, only five time points between 2300 and 0700 hours were included (2300, 0100, 0300, 0500 and 0700 hours) in the ANOVA. In this analysis it was also tested, whether baseline ghrelin levels at 2300 hours (sleep onset) significantly differ between the two conditions. This was not the case. Additionally, ghrelin levels were compared statistically between conditions by a recently developed methodology for the comparison of the stability of hormone concentration curves (18). Finally, the peak level of hGH between 2300 and 0300 hours was determined and correlated with the ghrelin concentrations between 2300 and 0300 hours and their area under the curve (AUC) in this interval as well by using Pearson’s correlation coefficient. The level of statistical significance was set to 0.05. One subject had to be excluded from statistical
analysis, because no blood samples were available between 2300 and 0500 hours during the sleep condition due to technical reasons. In the figures, data were depicted as the mean ± standard error of mean (SEM), in the table data are given as mean ± standard deviation.

RESULTS

In the sleep condition, ghrelin levels rose around midnight and then slowly declined until the next morning. In contrast, when subjects were sleep-deprived, ghrelin levels increased slowly and steadily up to a plateau in the early morning hours and declined just after breakfast (see figure 1). ANOVA for repeated measures revealed a significant difference between the conditions with respect to nocturnal (2300 and 0700 hours) ghrelin secretion (F[4;32]=6.65, p<0.01). This was confirmed by a Z-score of –2.31 (p<0.05) in the comparison of the hormone curves between sleep and sleep deprivation using the above-mentioned stability testing (18).

ANOVA also revealed a significant difference in nocturnal hGH secretion between the conditions (F[4;32]=8.1, p<0.01), confirming the well established hGH peak around sleep onset and its strong attenuation by sleep deprivation (see figure 1). The peak hGH level during night sleep was positively correlated to ghrelin secretion during the first four hours of sleep (r = 0.705, p < 0.05; see figure 2). A similar, but non-significant relationship (r = 0.433, n.s.) was observed when hGH and ghrelin levels were suppressed during sleep deprivation. During the day, both ghrelin and hGH plasma levels increased prior to meals and declined thereafter (see figure 1).

Cortisol levels across the 24-hour experimental period showed the well known circadian variability, with a maximum in the morning and a nadir during the night. ANOVA for repeated measures revealed no significant differences between the two conditions (F[4;32]=2.3, n.s.).
DISCUSSION

In the present study we investigated the influence of nocturnal sleep compared to sleep deprivation on ghrelin plasma levels in healthy volunteers. We confirmed the finding of Cummings and colleagues (9) that in sleeping subjects ghrelin levels rise during the early part of the night and decline in the morning prior to breakfast. We discovered, however, that this increase is blunted by sleep deprivation. When subjects stayed awake ghrelin levels rose slowly and steadily to reach a plateau in the early morning and did not decrease until breakfast. This relative increase of about 25% during nocturnal wakefulness was similar to the effects of a 12-hour fast observed during the day (19). The suppressive effect of sleep deprivation on ghrelin levels suggests that the bell-shaped time course observed during sleep might not be due to an influence of circadian rhythms but rather related to sleep itself. Hence, nocturnal ghrelin secretion shows similarities to hGH production which is promoted by sleep as well, and particularly by sleep onset (20). In addition, peak hGH levels correlated positively to ghrelin secretion during the first hours of sleep (figure 2) further supporting the idea of a tight interaction.

Sleep-related growth hormone secretion is probably caused by an increased production of hypothalamic GH releasing hormone acting on the pituitary (13). Ghrelin, in contrast, is mainly produced by the stomach and other splanchnic organs (19;21). Small quantities might also be produced in the hypothalamus (for review see 22), but these are very unlikely to contribute to systemic levels (23). Hence, it is almost sure that the increased ghrelin levels which we found in sleeping subjects are due to peripheral production. Yet it is unclear at present how the brain might act on ghrelin-producing cells. Despite the positive correlation between ghrelin and hGH secretion a causative role of hGH is unlikely, because a number of studies demonstrated that hGH administration does not affect ghrelin levels (24-27). Studies investigating the interaction between ghrelin production and feeding suggest an influence of vagal cholinergic efferents on ghrelin production and transmission of the ghrelin signal to the
brain through vagal afferents (27;28). These studies indicate a negative influence of the vagus on ghrelin secretion. Therefore, the increased parasympathetic tone occurring during sleep (28) is unlikely to explain increased ghrelin secretion, because rather the opposite would be expected. Hence, although the present results clearly demonstrate increased ghrelin levels during sleep, the question remains to be solved how the sleeping brain stimulates ghrelin secretion.

At the first glance, it is not easy to understand why the production of an appetite-stimulating hormone should be increased during sleep. However, the interaction between metabolic, endocrine and sleep-regulating networks is complex and ghrelin has numerous functions. Ghrelin has recently been shown to increase slow-wave sleep (SWS) and this effect is probably dependent on the GHRH receptor (8;29). Therefore, ghrelin might represent a peripheral feed forward signal to support SWS initiation and/or maintenance during the first hours of night sleep in concert with central SWS-promoting actions of GHRH. Similarly, ghrelin being a potent GH secretagogue (1) might reinforce the central GHRH signal for enhanced hGH production. This idea is supported by the correlation between hGH and ghrelin production shown in figure 2.

Studies on the interactions between sleep and neuroendocrine factors date back to the late 1960s (e.g. 31) but it was only very recently that a particularly intriguing link between sleep and the regulation of appetite became evident. Orexins, hypothalamic peptides which were initially thought to mainly regulate food intake, but also have potent arousing properties (32), play a crucial role in the pathophysiology of narcolepsy. Narcolepsy is characterized by excessive daytime sleepiness, cataplexy (a sudden, short-lasting loss of muscle tone triggered by emotions) and an acquired complete loss of hypothalamic orexin production in the hypothalamus in most patients (32). In addition to sleep-related symptoms patients display various endocrine and metabolic abnormalities such as obesity and hypoleptinemia (33-35). Like ghrelin plasma levels, orexin CSF levels are increased at night (36) and ghrelin-induced
food intake is mediated by orexinsnergic pathways (37). Therefore, it might be of great interest to investigate ghrelin under baseline conditions, in association to sleep and sleep deprivation, and in association to meals in narcoleptic patients. To conclude, ghrelin production is enhanced during night sleep in healthy humans and correlates to the increased release of hGH occurring in parallel. Although the underlying mechanisms remain to be elucidated, the present study further supports the idea that ghrelin is a novel important player within the sleep-neuroendocrine interplay.

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   Central ghrelin production does not substantially contribute to systemic


Figure legends

Figure 1:
Plasma levels of ghrelin, hGH and cortisol in nine healthy subjects during sleep (closed squares) and sleep deprivation (open squares). Arrows indicate food intake, the black bar indicates the time subjects were allowed to sleep or kept awake, respectively.

Figure 2:
Scatter plot of hGH peak versus AUC of ghrelin secretion between 2300 and 0300 hours during sleep in nine healthy subjects. Pearson’s correlation coefficient revealed a significant positive correlation ($r=0.71$, $p<0.01$).
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<table>
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<tbody>
<tr>
<td><strong>Table 1:</strong> Sleep parameters during sleep condition</td>
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<tr>
<td>Time in bed (min)</td>
<td>478.2 ± 13.0</td>
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<tr>
<td>Sleep onset latency (min)</td>
<td>24.7 ± 11.5</td>
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<tr>
<td>Sleep period time (min)</td>
<td>452.5 ± 22.4</td>
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<tr>
<td>Total sleep time (min)</td>
<td>424.1 ± 24.2</td>
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<tr>
<td>Sleep efficiency index (%)</td>
<td>93.7 ± 0.05</td>
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<tr>
<td>REM sleep latency (min)</td>
<td>59.2 ± 30.2</td>
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<tr>
<td>Stage 1 (min)</td>
<td>30.0 ± 13.7</td>
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<tr>
<td>Stage 2 (min)</td>
<td>217.5 ± 36.8</td>
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<tr>
<td>Stage 3 (min)</td>
<td>38.9 ± 10.1</td>
</tr>
<tr>
<td>Stage 4 (min)</td>
<td>32.2 ± 28.7</td>
</tr>
<tr>
<td>REM sleep (min)</td>
<td>100.5 ± 20.3</td>
</tr>
<tr>
<td>Wake (min)</td>
<td>51.8 ± 21.9</td>
</tr>
<tr>
<td>Movement time (min)</td>
<td>5.0 ± 2.4</td>
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</tbody>
</table>

Sleep variables according to Rechtschaffen and Kales (13) during the sleep condition between 2300 and 0700 hours in nine healthy male volunteers. All values are expressed as means ± SD; time in bed = total time of scoring; sleep period time = time between sleep onset and last epoch of sleep in the morning; total sleep time = total time spent asleep; sleep efficiency index = percentage of time asleep of sleep period time; sleep onset latency = time between start of recording and 1st epoch scored as sleep stage 2; REM latency = time between sleep onset and first epoch scored as REM sleep.
Fig. 1:

[Graph showing changes in Ghrelin, hGH, and Cortisol levels over time (hours) with markers indicating specific time points.]
Fig. 2: