Nocturnal ghrelin pulsatility and response to growth hormone secretagogues in healthy men

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1Program in Nutritional Metabolism and Neuroendocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston 02114; 2General Clinical Research Center, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; and 3Pharmacology Department, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

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Koutkia, Polyxeni, Bridget Canavan, Jeff Breu, Michael L. Johnson, and Steven K. Grinspoon. Nocturnal ghrelin pulsatility and response to growth hormone secretagogues in healthy men. Am J Physiol Endocrinol Metab 287: E506–E512, 2004. First published May 11, 2004; 10.1152/ajpendo.00548.2003.—The physiological importance of endogenous ghrelin in the regulation of growth hormone (GH) secretion is still unknown. To investigate the regulation of ghrelin secretion and pulsatility, we performed overnight ghrelin and GH sampling every 20 min for 12 h in eight healthy male subjects [age 37 ± 5 (SD) years old, body mass index 27.2 ± 2.9 kg/m2]. Simultaneous GH and ghrelin levels were assessed to determine the relatedness and synchronicity between these two hormones in the fasted state during the overnight period of maximal endogenous GH secretion. Pulsatility analyses were performed to determine simultaneous hormonal dynamics and investigate the relationship between GH and ghrelin by use of cross-approximate entropy (X-ApEn) analyses. Subjects demonstrated 3.0 ± 2.1 ghrelin pulses/12 h and 3.3 ± 0.9 GH pulses/12 h. The mean normalized ghrelin entropy (ApEn) was 0.93 ± 0.09, indicating regularity in ghrelin hormone secretion. The mean normalized X-ApEn was significant between ghrelin and GH (0.89 ± 0.12), demonstrating regularity in cosecretion. In addition, we investigated the ghrelin response to standard GH secretagogues [GH-releasing hormone (GHRH) alone and combined GHRH-arginine] in separate testing sequences separated by 1 wk. Our data demonstrate that, in contrast to GH alone, which had little effect on ghrelin, combined GHRH and arginine significantly stimulated ghrelin with a maximal peak at 120 min, representing a change of 66 ± 14 pg/ml (P = 0.001 by repeated-measures ANOVA and P = 0.02 for GHRH vs. combined GHRH-arginine by MANOVA). We demonstrate relatedness between ghrelin and GH pulsatility, suggesting either that ghrelin participates in the pulsatile regulation of GH or that the two hormones are simultaneously coregulated, e.g., by somatostatin or other stimuli. Furthermore, the differential effects of GHRH alone vs. GHRH-arginine suggest that inhibition of somatostatin tone may increase ghrelin. These data provide further evidence of the physiological regulation of ghrelin in relationship to GH.

Ghrelin is a novel 28-amino acid peptide that is the endogenous ligand for the growth hormone secretagogue (GHS) receptor (30). The stomach is the primary source of circulating ghrelin, but it is also produced in small amounts by the intestines, kidneys, pituitary, hypothalamus, and placenta (5, 13, 21, 22, 33, 37). Intravenous ghrelin has been shown to increase appetite and food intake in humans (57). Ghrelin has dual actions to stimulate both food intake and GH secretion (4, 26, 30, 41, 46). Therefore, a role for ghrelin in regulating food intake and body weight has been proposed. The mechanisms of ghrelin regulation are not completely understood. Ghrelin levels are lower in obese than in lean subjects (15, 24, 52). In contrast, ghrelin levels are increased in malnutrition, preprandially (14), and in patients with anorexia nervosa (3, 40). Ghrelin levels are suppressed by food intake and may be elevated during late evening or early morning hours (14, 15). These relative changes are in agreement with the known effects of nutrition on GH regulation and existence of a nocturnal GH rise, implying that ghrelin may be an important regulator of GH secretion in adults or that GH and ghrelin are coregulated in response to nutritional status and/or other stimuli.

The physiological importance of endogenous ghrelin as a potential participant in the regulation of GH secretion is still unknown. In this study, we investigated ghrelin pulsatility compared with GH during overnight fasting. We studied eight healthy male subjects to determine ghrelin pulsatility, pattern of coupling, synchronicity to GH, and response to standard GH secretagogues, including GH-releasing hormone (GHRH) alone and in combination with arginine. Using simultaneous frequent sampling, we also investigated whether fluctuations in plasma ghrelin concentrations during overnight fasting exhibited pattern coupling and synchronicity to GH by use of cross-approximate entropy (X-ApEn). We analyzed ghrelin and GH concentration during overnight fasting when ghrelin and GH peak. These data extend our understanding of the physiology and regulation of ghrelin and the potential relationship of ghrelin to GH secretion and neuroendocrine function during fasting. Different results might be obtained if the subjects were to undergo frequent sampling during daytime; however, we wanted to eliminate the effects of feeding on ghrelin and the variability of GH secretion during the day.

METHODS

Research Protocol

Eight healthy, young (<45 yr of age), moderately overweight male volunteers [37 ± 5 yr of age and body mass index (BMI) of 27.2 ± 2.9 kg/m2] (Table 1) underwent a series of three visits (two outpatient and one inpatient). Written informed consent was obtained from each subject before testing, in accordance with the Committee on Human Studies at the Massachusetts General Hospital. Study subjects reported to the General Clinical Research Center for two outpatient visits separated by ≥1 wk. Subjects were randomized to receive

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Ghrelin pulsatility in healthy men

Table 1. Demographics and pulse characteristics in healthy men

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, ytt</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.2 ± 2.9</td>
</tr>
<tr>
<td>CT VAT/SAT</td>
<td>0.43 ± 0.10</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>94 ± 12</td>
</tr>
<tr>
<td>Insulin, μIU/ml</td>
<td>7.0 ± 2.2</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>6.2 ± 3.0</td>
</tr>
<tr>
<td>IGF-I, ng/ml</td>
<td>358 ± 110</td>
</tr>
<tr>
<td>Ghrelin pulse characteristics</td>
<td></td>
</tr>
<tr>
<td>No. of peaks/12 h</td>
<td>3.0 ± 2.1</td>
</tr>
<tr>
<td>Peak width, min</td>
<td>122 ± 68</td>
</tr>
<tr>
<td>Peak height, pg/ml</td>
<td>866 ± 375</td>
</tr>
<tr>
<td>Area, pg/ml-1·min</td>
<td>11,505 ± 7,671</td>
</tr>
<tr>
<td>No. of valleys</td>
<td>3.4 ± 2.2</td>
</tr>
<tr>
<td>Valley width, min</td>
<td>93 ± 101</td>
</tr>
<tr>
<td>Nadir, pg/ml</td>
<td>590 ± 117</td>
</tr>
<tr>
<td>Growth hormone pulse characterstics</td>
<td></td>
</tr>
<tr>
<td>No. of peaks/12 h</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>Peak width, min</td>
<td>150 ± 37</td>
</tr>
<tr>
<td>Peak height, ng/ml</td>
<td>2.23 ± 0.04</td>
</tr>
<tr>
<td>Area, ng/ml-1·min</td>
<td>123 ± 127</td>
</tr>
<tr>
<td>No. of valleys</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>Valley width, min</td>
<td>40 ± 16</td>
</tr>
<tr>
<td>Nadir, ng/ml</td>
<td>0.15 ± 0.14</td>
</tr>
</tbody>
</table>

Values are for 8 men. BMI, body mass index; CT VAT/SAT, computed tomography abdominal visceral-to-subcutaneous fat area ratio.

Ghrelin alone or combined GHRH+arginine testing at the first visit and vice versa at the second outpatient visit. Baseline GH and ghrelin levels were drawn at 0800 after an overnight fast, and immediately thereafter 1 μg/kg sermorelin acetate (Geref Diagnostic, Serono, Waltham, MA) was given as an intravenous bolus. l-Arginine [0.5 g/kg (maximum dose = 30 g)] was administered intravenously over 30 min. Subsequent GH and ghrelin levels were determined at +15, +30, +45, +60, +90, and +120 min. Body composition was determined on the morning of the first outpatient admission. At least 1 wk after the second outpatient visit, study subjects were admitted to the Clinical Research Center, had dinner at 1700, but were not permitted to eat or drink after 1800. Simultaneous GH and ghrelin levels were drawn at 20-min intervals for 12 h overnight (1900–0740 24-h clock time). Subjects were not allowed to perform strenuous activity and were exposed to dark overnight (2300–0800 24-h clock time). Sleep and wake periods were recorded by the General Clinical Research Center nursing staff during overnight sampling.

**Laboratory Methods**

**Hormonal assays.** GH was measured by two-site radioimmunometric assay (RIA) with an intra-assay coefficient of variation (CV) of 2.8–4.2% (Corning, Nichols Institute Diagnostics, San Juan Capistrano, CA). The sensitivity of the assay was determined to be 0.01 μg/l on the basis of multiple dilutions with a standard sample, and linearity of the assay was confirmed to a GH concentration of 0.05 μg/l. Insulin-like growth factor I (IGF-I) was measured by two-site RIA (intra-assay CV 4.93%, DSL, Webster, TX).

Serum ghrelin was measured by RIA (Phoenix Pharmaceuticals, Belmont, CA). The RIA uses 125I-labeled bioactive ghrelin as a tracer and a polyclonal antibody raised against full-length, octonoylated human ghrelin that recognizes both the octanoyl and des-octanoyl forms of the hormone. The intra-assay CV was 7.6%. All samples from each subject series were measured in a single assay for GH and ghrelin. Leptin was measured by RIA (Linco, St. Charles, MO). The intra-assay CV was <5%.

**ApEn calculation.** ApEn comprises a family of translation-, model-, and scale-independent regularity statistics designed to compare the relative orderliness of time series. ApEn comprises a two-parameter family of statistics, ApEn (m,r), with m a run length and r the tolerance for closeness. Higher ApEn values denote greater disorderliness of secretory patterns. In the present analysis, m was assigned a value of 1. An ApEn with m = 1 serves to evaluate the statistical consistency of contiguous pairs of data points, i.e., ApEn for a given value of m is the conditional probability that if a series of length m is similar, then the m + 1 points are also similar. Thus with m = 1, ApEn is looking at m + 1, or pairs, of data points. The parameter r was set to 20% as appropriate for shorter time series. The foregoing ApEn parameters, designated by ApEn (1, 20%), provide a replicable ApEn statistic with an approximate SD of 0.06–0.08 (42, 43). A normalized ratio of observed to random ApEn was calculated for each time series as the mean ratio of observed to random ApEn values calculated by shuffling the original data series times 1,000 times.

**X-ApEn computation.** X-ApEn is the bivariate analog of the ApEn statistic described above. This metric quantifies the joint synchrony of patterns in paired hormone series by using standardized (z-score transformed) data. X-ApEn quantifies the degree of lag-independent pattern synchrony between paired time series (43). We applied standard X-ApEn measurements on the following pairs of low-pass-filtered time series from each subject: ghrelin vs. GH.

**Cluster analysis.** To assess ghrelin pulsatility, we used Cluster, a largely model-free computerized pulse analysis algorithm to identify statistically significant pulsations in relation to dose-dependent measurement error in each hormone time series. In performing the analysis, we specified individual test cluster sizes for the nadir and peak width of (2 × 2), a minimum and maximum intraseries CV, a t-statistic to identify significant increase, and a t-statistic to define a significant decrease. For this purpose, a CV of 7.6%, the intra-assay CV for our assay, was used in the settings of the program. The data were also analyzed using a 1 × 2 analysis (peak size = 1, nadir size = 2), and the results were not significantly different (data not shown). The Cluster program does not provide information about the secretion of the hormone into the serum. The details of the protocol for analysis have been previously described (54–56). Pulses were characterized with the following parameters, reported as mean ± SD: peak frequency (number of peaks/12 h), mean peak duration (minutes), mean interval between peaks (minutes). A 20-min frequent sampling for this study was chosen, as this is a well-established sampling frequency for GH (GH half-life of 30 min) (1, 53). Prior studies have used less frequent sampling periods for ghrelin (e.g., every 1–2 h in overnight sampling) (8, 14, 15, 16, 47). We thus chose every 20 min as a sampling frequency that was reasonable for both ghrelin and GH, as we compare these by X-ApEn. Deconvolution analysis was not performed, because the half-life for ghrelin is not known in humans.

**Body composition.** Body composition was determined by cross-sectional abdominal computed tomography (CT) scanning. A lateral scout image was obtained to identify the level of the L4 pedicle, which served as a landmark for the single-slice image. Scan parameters for each image were standardized (144 cm table height, 80 kV, 70 mA, 2 s, 1 cm slice thickness). Fat attenuation coefficients were at ~50 Hounsfield units, as described by Borkan et al. (9). Abdominal visceral (VAT) and subcutaneous fat areas (SAT) were then determined. The ratio of visceral adipose tissue to subcutaneous adipose tissue area (VAT/SAT) was determined.

**Statistical Analysis**

Ghrelin and GH changes from baseline were determined in response to GHRH and combined GHRH-arginine testing. We compared the time-course response of ghrelin during GHRH and GHRH-arginine stimulation testing with repeated-measures analysis of variance (ANOVA) for within-group testing and MANOVA (multivariate
ANOVA) for group responses over time. Ghrelin data were analyzed using Cluster for 20-min sampling and also pooled for all subjects using 1-h data points to minimize variability. A cubic polynomial regression was fitted to the data to determine time of peak and nadir. We compared ApEn for ghrelin during wake and sleep by use of a paired t-test. All statistical analyses were made using SAS JMP Statistical Database Software (version 5; SAS Institute, Cary, NC). Statistical significance was defined as a two-tailed α-value of $P \leq 0.05$. Results are means ± SD for baseline descriptive data and means ± SE for response data.

RESULTS

The demographic characteristics of the subjects are shown in Table 1.

Pulsatility Analysis

Pulsatility of ghrelin was detected by independent methods including Cluster and ApEn (Fig. 1). There were $3.0 \pm 2.1$ ghrelin pulses/12 h and $3.3 \pm 0.9$ GH pulses/12 h (Table 1). GH pulsatility was similar to that seen in a prior study of healthy control subjects of similar age and BMI (45). Seven of the eight patients demonstrated ghrelin pulses during overnight fasting by Cluster analysis. Some of the visually apparent ghrelin excursions did not register as formal pulses in the Cluster analysis in either a $2 \times 2$ analysis (peak size = 2 and nadir size = 2) or a $1 \times 2$ analysis (peak size = 1 and nadir size = 2) (data not shown). These points may represent
variability in biological secretion. Entropy (ApEn) analysis confirmed that ghrelin dynamics in study subjects are both real and nonrandom. The mean normalized ghrelin entropy (ApEn) was 0.93 ± 0.09, indicating regularity in ghrelin hormone concentration. The mean normalized X-ApEn was significant between ghrelin and GH (0.89 ± 0.12), demonstrating a degree of regularity and pattern frequency.

In a pooled analysis restricted to 1-h sampling points, ghrelin demonstrated a nadir between 1900 and 2000 of 640 pg/ml and a peak between 0100 and 0200 of 705 pg/ml by use of a cubic polynomial regression equation ($r^2 = 0.58$; Fig. 2).

Sleep time is recorded in Fig. 1. The X-ApEn during the sleep period is 0.93 ± 0.18 ($P = 0.51$ vs. whole night, by paired $t$-test) and during the wake period is 0.90 ± 0.26 ($P = 0.92$ vs. whole night, by paired $t$-test; $P = 0.83$ vs. sleep phase, by paired $t$-test). The X-ApEn values are <1 for both sleep and wake cycles and not significantly different between cycles, indicating regularity between ghrelin and GH independent of sleep. There is no correlation between the percentage of time asleep and the synchronicity between hormones as assessed by X-ApEn values.

Ghrelin peak height ($r = -0.92, P = 0.004$) and ghrelin pulse area ($r = -0.95, P = 0.0009$) were inversely correlated with the VAT/SAT ratio. In contrast, ghrelin pulse characteristics did not correlate with fasting morning leptin, insulin, glucose, or response to standard oral glucose tolerance testing (data not shown).

Effects of GHRH vs. Combined GHRH-Arginine on Ghrelin and GH

The changes in ghrelin in response to GHRH alone and combined GHRH-arginine are shown in Fig. 3. With use of repeated-measures ANOVA, the ghrelin change from baseline during combined GHRH-arginine testing (+66 ± 14 pg/ml after 120 min) was significant ($P = 0.001$), whereas the change from baseline during GHRH alone was not significant ($P = 0.46$). The responses to GHRH alone vs. the response to combined GHRH-arginine were compared using MANOVA and were significantly different ($P = 0.02$; Fig. 3).

The time course of ghrelin and GH responses to GHRH-arginine are demonstrated in Fig. 4. In response to GHRH-arginine, the maximal decrease in ghrelin and the maximal increase in GH occurred at approximately the same time in mirror image responses (Fig. 4).

DISCUSSION

In this study, we investigated simultaneous ghrelin and GH pulsatility in healthy male subjects and determined synchronicity and relatedness in pulsatile hormone secretion. Our data demonstrate an increased rhythmic pattern of ghrelin discharge during overnight fasting in healthy, young, moderately overweight men. Ghrelin, a natural GHS (41), has a wide spectrum of endocrine action. There is evidence that ghrelin exerts its effects via the activation of the GHS receptor (GHS-R), likely enhancing the activity of GHRH-secreting neurons and also acting as a functional somatostatin antagonist (34).

To our knowledge, previous studies have not investigated ghrelin pulsatility in humans. Okimura et al. (39) investigated ghrelin pulsatility in relationship to feeding behavior in male rats. Endogenous plasma ghrelin levels exhibited pulsatile variations (39). No significant correlation between GH and ghrelin circulating levels was found, although mean interpeak intervals and pulse frequencies were close for the two hor-
mones (39). Tolle et al. (51) examined the relationships between ghrelin and GH secretion in rats. Ghrelin concentrations were not related to pulsatile GH patterns. Bagnasco et al. (6) demonstrated that ghrelin pulsatility was increased with fasting and decreased with feeding in rats (6).

Prior studies have examined overnight ghrelin patterns by using pooled data restricted to 1-h sampling intervals in humans. In these analyses, a nocturnal increase in ghrelin secretion during overnight fasting was seen (16, 47). In a similar analysis of pooled data, we show a nocturnal rise in ghrelin, with a nadir between 1900 and 2000 of 640 pg/ml and a peak between 0100 and 0200 of 705 pg/ml.

The results of our study suggest that ghrelin is secreted in a nonrandom model. Taken together, our findings suggest a nocturnal rise and a nonrandom, pulsatile pattern of ghrelin discharge in humans during overnight fasting.

We did not see an effect of sleep, and ApEn was not different during periods of sleep and wakefulness. Furthermore, there was no correlation between the percentage of time asleep and the synchronicity between ghrelin as assessed by X-ApEn values. In a prior study (16), Dzaja et al. demonstrated that ghrelin secretion during the first few hours of sleep correlated positively with peak GH concentrations. In this study, we show no relationship of ghrelin ApEn to sleep or wake periods.

The present investigation compares patterns of monohormonal ghrelin and bihormonal (coordinate) ghrelin-GH release in healthy male subjects with normal BMI. The normalized cross-ApEn value was 0.89 ± 0.12 for ghrelin and GH, suggestive of hormonal regularity and synchrony in the release of these hormones. However, it remains unclear whether the relatedness and synchronicity suggest a direct stimulatory effect of ghrelin on GH, whether ghrelin has a permissive role in GH secretion (49), or whether there is coordinated regulation of both GH and ghrelin by other hormones, such as somatostatin.

The GHS-R is predominantly expressed in the pituitary and brain (21, 23), but ghrelin expression is highest in the stomach (21). Ghrelin may signal hypothalamic regulatory centers that control energy balance in fasting and undernutrition (3, 7). In humans, increases in serum ghrelin during fasting are followed by similar quantitative changes in serum GH levels (enhanced GH secretion and amplified secretion rhythm), suggesting that ghrelin could drive GH secretion during fasting (35).

Smith et al. (50) proposed that GHS acts on the arcuate nucleus in the hypothalamus, upregulating the GHRH neurons as a functional somatostatin antagonist (50). GH secretion is regulated by the interplay of GHRH and somatostatin, and our data now suggest that ghrelin may be coordinately regulated with GH in a pulsatile fashion. Our data suggest that circulating ghrelin concentrations are influenced by body fat distribution but not by levels of IGF-I.

In this study, we compared the effects of GHRH alone to those of combined GHRH+arginine on ghrelin. We found that combined GHRH+arginine increased ghrelin compared with the effects of GHRH alone. Importantly, this increase in ghrelin is likely to be independent of any changes due to extended fasting over the hours of 0800–1000 after an overnight fast. Barkan et al. (8) studied plasma ghrelin and GH concentrations in young healthy men and demonstrated that there was no stimulation of ghrelin during normal saline infusion during the hours of 0800–1000 after an overnight fast.

Because arginine is thought to enhance the effects of GHRH by decreasing somatostatin tone (2, 20), our data suggest potential regulation of ghrelin by changes in endogenous somatostatin. There are only limited ways in which to block somatostatin in human physiology, and the testing paradigm we chose, i.e., to compare ghrelin responses to GHRH alone vs. GHRH plus arginine, is one such method (19). Previously, Freda et al. (18) and Barkan et al. (8) showed that somatostatin infusion lowers ghrelin. In contrast, we show for the first time that inhibition of somatostatin by addition of arginine to GHRH increases ghrelin. These data complement existing data in the literature suggesting an inhibitory effect of somatostatin on ghrelin secretion (8, 10, 25, 38, 48). Further studies are needed to determine more directly the effects of somatostatin on ghrelin secretion.

Our data provide initial evidence against significant effects of GHRH on ghrelin, because we saw no significant stimulatory response of ghrelin to the large doses of GHRH used in standard stimulation testing in healthy control subjects. Exam-
ination of the pattern of ghrelin response to GHRH and combined GHRH-arginine suggests that ghrelin may decrease as a result of GH feedback. Ghrelin decreases briefly at the point of maximal GH in response to the GHRH-arginine stimulation test. The changes in ghrelin and GH in response to GHRH-arginine appear to be mirror images. One interpretation for this pattern is that ghrelin transiently decreases coincident with peak stimulated GH levels from the GH stimulation testing. Evidence that GH may downregulate ghrelin was shown recently in acromegaly studies, in which low ghrelin levels normalize after treatment (18). Furthermore, Engstrom et al. (17) demonstrated that chronic GH treatment decreases ghrelin in GH-deficient patients.

In contrast to prior reports (8, 11, 12, 27–31, 32, 36), our data suggest more acute effects of changing GH concentrations on ghrelin in a tightly regulated feedback loop. Our data are also consistent with recent animal data demonstrating that GH administration decreases circulating ghrelin levels and mRNA expression in the stomach (44). Furthermore, Qi et al. (44) demonstrated that ghrelin is decreased in rats overexpressing GHRH as a result of feedback inhibition of GH on ghrelin. Further studies of the effects of GH on ghrelin in human physiology are needed.

In conclusion, our data suggest ghrelin pulsatility and relatedness to GH during fasting. Relatedness of GH and ghrelin may result from direct stimulation of GH by ghrelin and/or coregulation by other neuroendocrine factors. Stimulation of ghrelin by combined GHRH-arginine, more than GHRH alone, suggests potential regulation of ghrelin by somatostatin. Coordinated regulation of ghrelin and GH may be an important component in nutrient signaling to the brain, and ghrelin may be an important physiological regulator of GH. In addition, GH may feed back and inhibit ghrelin, resulting in a classical neuroendocrine feedback loop (Fig. 5). Further investigation is needed of the pathways by which ghrelin participates in the coordinated regulation of GH.

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REFERENCES


Ghrelin Pulsatility in Healthy Men


