

## A Preprandial Rise in Plasma Ghrelin Levels Suggests a Role in Meal Initiation in Humans

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The recently discovered orexigenic peptide ghrelin is produced primarily by the stomach and circulates in blood at levels that increase during prolonged fasting in rats. When administered to rodents at supraphysiological doses, ghrelin activates hypothalamic neuropeptide Y/agouti gene-related protein neurons and increases food intake and body weight. These findings suggest that ghrelin may participate in meal initiation. As a first step to investigate this hypothesis, we sought to determine whether circulating ghrelin levels are elevated before the consumption of individual meals in humans. Ghrelin, insulin, and leptin were measured by radioimmunoassay in plasma samples drawn 38 times throughout a 24-h period in 10 healthy subjects provided meals on a fixed schedule. Plasma ghrelin levels increased nearly twofold immediately before each meal and fell to trough levels within 1 h after eating, a pattern reciprocal to that of insulin. Intermeal ghrelin levels displayed a diurnal rhythm that was exactly in phase with that of leptin, with both hormones rising throughout the day to a zenith at 0100, then falling overnight to a nadir at 0900. Ghrelin levels sampled during the troughs before and after breakfast correlated strongly with 24-h integrated area under the curve values ( $r = 0.873$  and  $0.954$ , respectively), suggesting that these convenient, single measurements might serve as surrogates for 24-h profiles to estimate overall ghrelin levels. Circulating ghrelin also correlated positively with age ( $r = 0.701$ ). The clear preprandial rise and postprandial fall in plasma ghrelin levels support the hypothesis that ghrelin plays a physiological role in meal initiation in humans. *Diabetes* 50:1714–1719, 2001

Ghrelin, the endogenous ligand for the growth hormone secretagogue (GHS) receptor (1), has recently been implicated in the control of food intake and energy balance. This highly conserved acylated peptide is produced primarily by cells in the oxyntic glands of the stomach as well as in the intestine (2), and it is secreted into the bloodstream. When administered either peripherally or centrally to rodents, ghrelin rapidly increases food intake and body weight (3–6) in addition to stimulating gastric motility and acid secretion (6,7). Ghrelin is a more potent stimulant of short-term feeding than any known peptide except neuropeptide Y (NPY), with which it has approximately equal potency (5,6). Its orexigenic effects are independent of growth hormone (GH) stimulation (3,4) and appear to be mediated at least in part through activation of NPY/agouti gene-related protein (AGRP) neurons in the hypothalamic arcuate nucleus, 94% of which express ghrelin receptors (8). Activation of the ghrelin receptor stimulates *c-fos* in these cells (4,9,10) and also increases hypothalamic NPY and AGRP expression (4,6,11,12). A role for NPY and AGRP as mediators of ghrelin's feeding effects is suggested by studies in which antagonism of either NPY or AGRP signaling in the brain was shown to attenuate the orexigenic potency of injected ghrelin (4,6,12). In rodents, ghrelin expression increases with prolonged fasting (3,6), and fasting blood levels are suppressed by refeeding or by infusion of nutrients (but not water) into the stomach (3). Based on these findings, it has been proposed that ghrelin is a hormone that contributes to the initiation of individual meals.

Because the above studies used pharmacological doses of exogenous ghrelin or measured serum levels after prolonged fasting, they did not address whether ghrelin plays a physiological role in initiating meals. If circulating ghrelin does perform such a function, its levels would be expected to rise before each meal and fall shortly after food is consumed. Human plasma ghrelin levels were reported in one recent study (13), but only fasting values were examined. We sought to determine the daily pattern of ghrelin secretion in normal humans as well as to assess any potential diurnal variation. Plasma ghrelin levels were measured 38 times during a 24-h period in healthy subjects given three meals per day at specified times. These values were compared with 24-h profiles of insulin and leptin. Our results demonstrate a dramatic preprandial rise and post-

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AGRP, agouti gene-related protein; AUC, area under the curve; CV, coefficient of variation; GH, GH; GHS, GH secretagogue; NPY, neuropeptide Y; RIA, radioimmunoassay.

prandial fall in circulating ghrelin levels, a pattern that is consistent with the hypothesis that ghrelin is a physiological meal initiator.

## RESEARCH DESIGN AND METHODS

A total of 10 apparently healthy subjects (9 women and 1 man) were recruited through local newspaper advertising. Subjects were >18 years old, weight-stable for at least 3 months preceding the study, and at their lifetime maximal weight. Exclusion criteria were as follows: BMI >30 kg/m<sup>2</sup>, diabetes, chronic medical illness, pregnancy, use of tobacco products, regular intense exercise (>30 min of aerobics 3 times per week), and alcohol consumption of >2 drinks per day. None of the subjects had undergone gastrointestinal surgery. The age range was 29.1–63.7 years, and the BMI range was 22.0–30.0 kg/m<sup>2</sup>. After giving informed consent, eligible subjects were enrolled into the study. All procedures and protocols took place at the General Clinical Research Center (GCRC) and were approved by the Human Subjects Review Committee at the University of Washington.

**Diet and GCRC protocols.** Before blood sampling, subjects were placed for 2 weeks on an outpatient diet prepared by the metabolic kitchen of the GCRC at the University of Washington. The diet consisted of 35% fat, 45% carbohydrate, and 20% protein, a macronutrient content that approximates the average American diet (14). During this time, subjects were seen and weighed by GCRC dietitians twice weekly, and total ingested calories were adjusted to maintain weight stability. At the end of the 2-week feeding period, subjects were admitted to the GCRC, where they were given the same diet administered as breakfast, lunch, and dinner at 0800, 1200, and 1730, respectively. An intravenous catheter was placed in the subject, and blood was drawn into EDTA tubes at 30-min intervals from 0800–2100, then hourly until 0800 the next morning (24 h total). Samples were stored at 4°C during the collection period and then centrifuged. The plasma was separated into four aliquots and stored at –70°C.

**Hormone assays.** Plasma immunoreactive ghrelin levels were measured in duplicate using a commercial radioimmunoassay (RIA) that uses <sup>125</sup>I-labeled bioactive ghrelin as a tracer and a rabbit polyclonal antibody raised against full-length octanoylated human ghrelin (Phoenix Pharmaceuticals, Belmont, CA). In our study, the lower and upper limits of detection for this assay were 80 and 2,500 pg/ml, respectively. All assays included 12 plasma control samples from common stocks that were transferred to aliquots and frozen at the beginning of the study, used to normalize each test for interassay variability. Based on these controls, the intra-assay coefficient of variation (CV) was 8.7% and the interassay CV was 14.6% ( $n = 10$ ). No cross-reactivity was seen with human leptin, which was assessed at doubling dilutions from 100 to 1 ng/ml.

Plasma insulin was measured in duplicate using a modification of a double-antibody RIA (15). The lower and upper limits of detection were 13 and 1,680 pmol/l, and the intra-assay CV was <10%. Leptin was measured with a commercial RIA kit that uses the double-antibody/polyethylene glycol technique (Linco Research, St. Charles, MO). The lower and upper limits of detection were 0.5 and 100 ng/ml. The intra- and interassay CVs were 5.0 and 5.5%, respectively. For all three hormones, all samples from a single individual were run in duplicate in the same assay.

**Statistical analysis.** Plasma hormone concentrations are expressed as the means  $\pm$  SE. Total 24-h integrated area under the curve (AUC) values for plasma ghrelin were calculated using the trapezoidal rule, and integrated 24-h averages were determined by dividing the AUC by 24 h. Linear regression was used to determine the correlation between 24-h integrated AUC ghrelin values and either the 0600 or 0930 levels as well as the correlation between age and ghrelin levels. Multivariate regression analysis was used to determine the correlation between ghrelin values (dependent variable) and age, BMI, and total calories consumed in 24 h (independent variables). As a test for diurnal variation, the 24-h profile of average plasma leptin concentrations was fit to a cosine function using the NLMREG program. The cosine equation used was  $[y = 20.27 + 3.23\cos(0.27x)]$ , where  $y$  is plasma leptin concentration,  $x$  is clock time, 20.27 is the offset, 3.23 is the amplitude, and 0.27 is the frequency.

## RESULTS

Plasma ghrelin levels rose by an average of 78% 1–2 h before the onset of each meal and fell to trough levels within 1 h after food was first consumed (Fig. 1A). Although there was considerable variation in the range of ghrelin values among individuals, all 10 subjects displayed preprandial ghrelin surges, the only exception being the occasional absence of a surge before breakfast. The 24-h

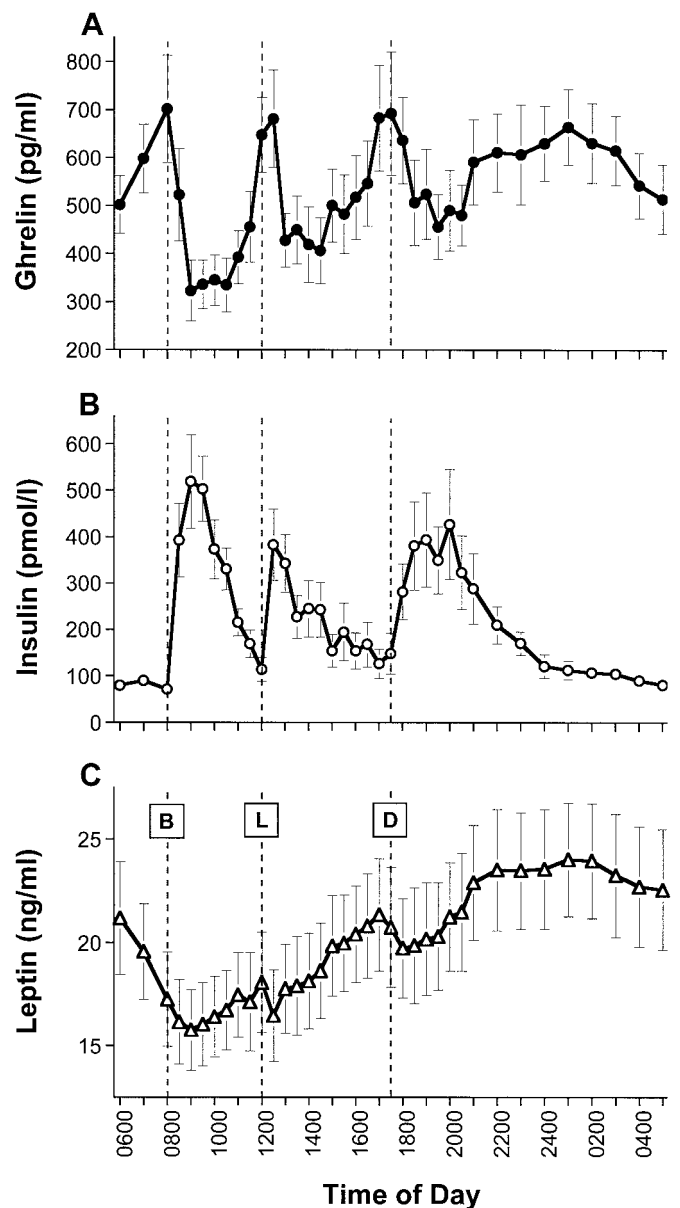


FIG. 1. Average plasma ghrelin (A), insulin (B), and leptin (C) concentrations during a 24-h period in 10 human subjects consuming breakfast (B), lunch (L), and dinner (D) at the times indicated (0800, 1200, and 1730, respectively).

plasma ghrelin profiles for two subjects are shown in Fig. 2, illustrating the interindividual variability in the range of ghrelin values and the heterogeneity in prebreakfast surges.

With regard to its temporal relationship to meals, the 24-h time course of plasma ghrelin was reciprocal to that of insulin (Fig. 1B). While ghrelin levels rose sharply before each designated meal time and declined precipitously to trough values within 60 min after meal ingestion, insulin levels increased from premeal trough values by 3.0- to 7.3-fold within 30–60 min after meal consumption. Similarly, during the intermeal interval, ghrelin levels gradually rose toward their next premeal peak, whereas insulin levels decreased toward their basal value.

Plasma leptin levels did not change acutely before meals, but they displayed clear diurnal variation, with a daily nadir at 0900 and a zenith at 0100 (Fig. 1C). Levels at

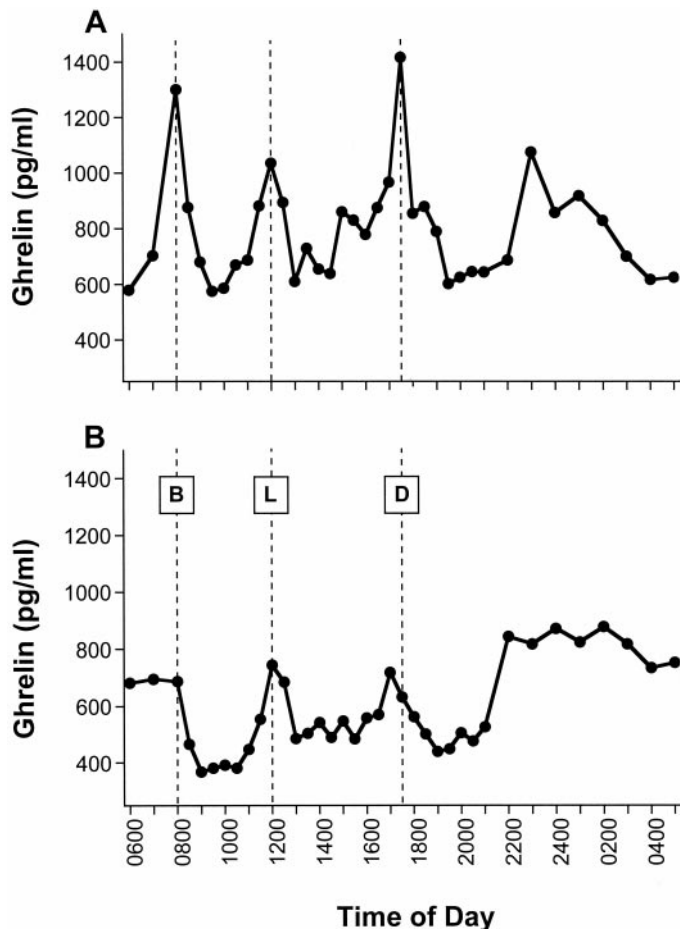


FIG. 2. Representative 24-h profiles of plasma ghrelin levels from two subjects (A and B) consuming breakfast (B), lunch (L), and dinner (D) at the times indicated. Note the wide range of circulating ghrelin values between individuals and the heterogeneity of the surge before breakfast.

the nadir were 23% lower than the integrated 24-h average value. Consistent with a diurnal variation in leptin levels, the 24-h profile correlated significantly with a cosine function ( $r = 0.906$ ,  $P < 0.00001$ ). Superimposed on this diurnal pattern, small (8–9%) drops in circulating leptin occurred within 1 h after the beginning of each meal.

Ghrelin levels between meals rose progressively through-

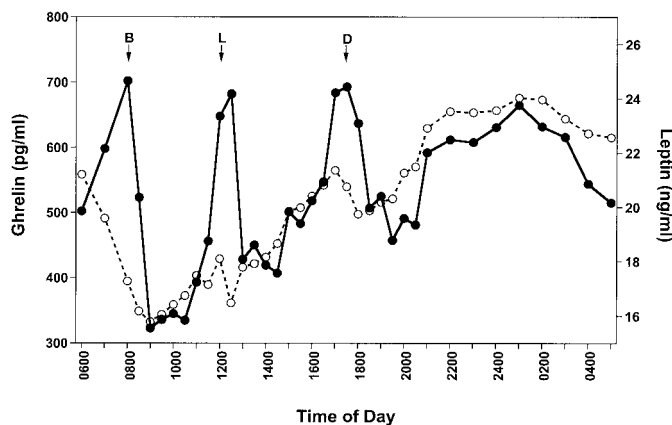


FIG. 3. Overlaid average plasma ghrelin (●) and leptin (○) concentrations during a 24-h period in 10 human subjects consuming breakfast (B), lunch (L), and dinner (D) at the times indicated.

out the day, reaching a zenith at 0100, then gradually fell overnight to a trough at 0600, before the prebreakfast surge (Fig. 1A). This diurnal pattern resembled that of plasma leptin, for which daily nadir and zenith values both occurred at the same hours as did those for intermeal ghrelin values (Fig. 3). Levels of both hormones also fell after each meal, although the amplitude of this oscillation was far greater for ghrelin than for leptin. No cross-reactivity was seen in the ghrelin RIA, with human leptin measured at concentrations as high as 100 ng/ml, which is 71 times greater than the highest measured plasma ghrelin level. Because ghrelin intermeal trough values steadily increased while the height of preprandial surges remained constant, the amplitude of the postprandial fall in ghrelin levels diminished with each successive meal. The magnitude of the average decrease in plasma ghrelin from peak to trough was 54% after breakfast, 40% after lunch, and 34% after dinner.

We sought to determine whether a single, conveniently obtainable plasma ghrelin value could serve as a surrogate for the integrated 24-h AUC ghrelin value in human subjects. Accordingly, linear regression analysis was performed between each subject's 24-h integrated AUC ghrelin value and either the 0930 level (from the trough after breakfast) or the 0600 level (from the trough occurring just before the prebreakfast surge after an overnight fast) (Fig. 1A). As shown in Fig. 4A, postprandial ghrelin levels measured at 0930 correlated very strongly with 24-h AUC values ( $r = 0.954$ ,  $P < 0.0001$ ). This correlation was seen even though the size of breakfast preceding the 0930 sampling was not standardized, and it varied among subjects from 520 to 768 cal. Overnight fasting ghrelin levels at 0600 also correlated significantly with 24-h AUC values ( $r = 0.873$ ,  $P = 0.0004$ ) (Fig. 4B).

Both of these associations remained statistically significant regardless of whether a single low-ghrelin outlier was omitted from the analyses. The 24-h ghrelin profiles were measured twice on this subject at times spaced 2 weeks apart. On both occasions, her 24-h AUC values were substantially below the group mean (3,073 and 4,121 pg-day/ml for the low-ghrelin subject vs. an average of 14,222 pg-day/ml for all other subjects). The low-ghrelin subject's serum was found to be positive for anti-parietal cell antibodies at a 1:20 dilution and showed a mildly low  $B_{12}$  level of 205 pg/ml (normal  $>224$  pg/ml).

The 24-h AUC values of plasma ghrelin showed a significant positive correlation with age ( $r = 0.701$ , univariate  $P = 0.022$ ). In a multivariate regression analysis with 24-h AUC ghrelin as the dependent variable and with age, BMI, and total calories ingested over 24 h as the independent variables, age was the only variable that correlated significantly with ghrelin levels ( $P = 0.045$ ). Similar results were derived using 0600 or 0930 ghrelin levels as the dependent variables compared with the same three independent variables (multivariate  $P = 0.038$  and 0.046, respectively, for ghrelin vs. age).

## DISCUSSION

In this study, human plasma ghrelin levels were shown to rise nearly twofold shortly before each meal and fall to trough levels within 1 h after eating, a profile that is consistent with a physiological role for ghrelin in initiating

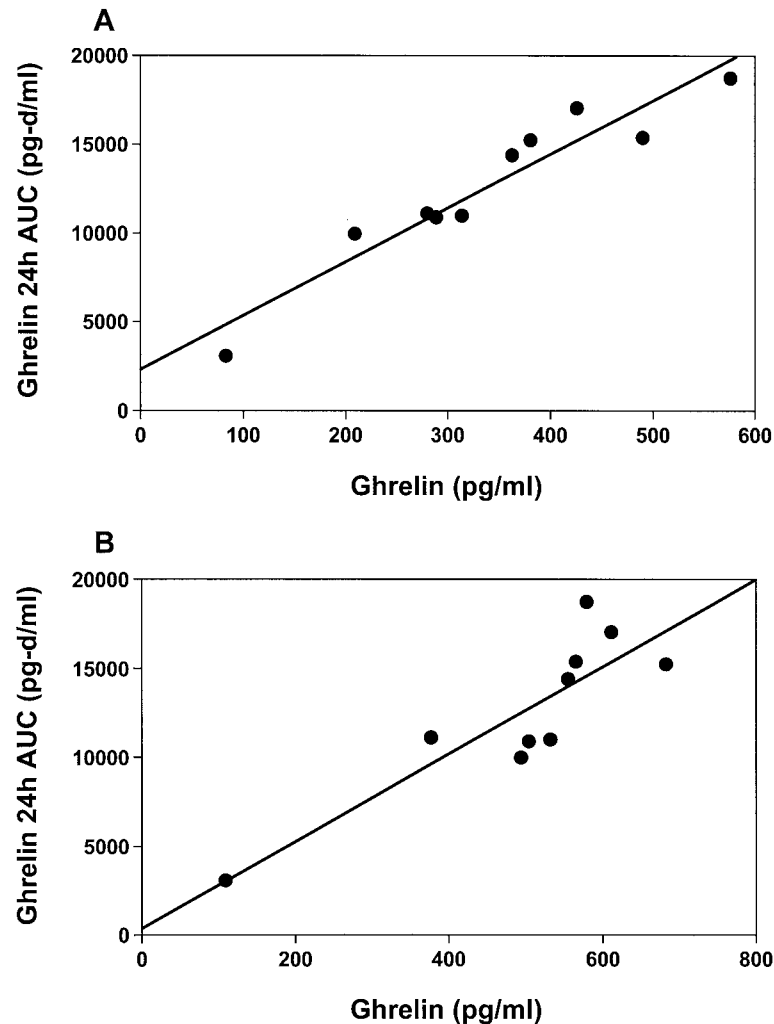


FIG. 4. Correlation between integrated 24-h AUC ghrelin values and plasma ghrelin levels measured at either 0930 ( $r = 0.954$ ,  $P < 0.0001$ ), during the trough after breakfast (A), or 0600 ( $r = 0.873$ ,  $P < 0.0004$ ), during the trough before breakfast after an overnight fast (B).

individual meals. The temporal patterns of ghrelin and insulin surges were reciprocal, occurring just before and after the designated meal times, respectively. Because it is well established that insulin surges are postprandial, these data confirm that meals were consumed at the times planned in the study protocol and that increases of circulating ghrelin occurred before each meal. Intermeal ghrelin levels rose progressively throughout the day, peaking at 0100, then decreased steadily until shortly before breakfast. Plasma leptin displayed a very similar diurnal variation, as has been shown by others (16), with daily nadir and zenith levels occurring at the same hours as those for intermeal ghrelin levels. Both hormones also decreased after meals, although the amplitude of this decline was greater for ghrelin than for leptin. Ghrelin levels measured at the 0600 and 0930 troughs before and after breakfast correlated significantly with individual 24-h integrated AUC ghrelin values. Circulating ghrelin levels correlated positively with age.

Several observations from rodent studies support the hypothesis that ghrelin is a physiological meal initiator. First, ghrelin is synthesized primarily by the stomach (1), an organ that is well positioned to sense short-term fluxes in energy balance. Second, despite being produced peripherally, ghrelin acts centrally to stimulate food intake (3–6).

Third, ghrelin affects feeding rapidly, increasing both food intake (6) and gastric acid secretion (7) within 20 min of intraperitoneal injection, a time course that is consistent with a role in meal initiation. Fourth, exogenous ghrelin triggers eating in rodents during the day (4–6), a time when food intake is usually nominal. Finally, ghrelin activates hypothalamic NPY/AGRP neurons and increases AGRP gene expression (vide supra). AGRP has been implicated as a central mediator of meal initiation because mRNA levels in the hypothalamus rise shortly before the onset of maximal daily food intake in ad libitum-fed rats, whereas levels of other neuropeptides involved in energy balance are stable throughout the day (17).

Together with our findings of a large preprandial rise and postprandial fall in plasma ghrelin levels in humans, these observations support a model in which ghrelin acts as a physiological meal initiator. Because subjects in our study were provided food at specified times, however, we cannot conclude that the observed preprandial surges in circulating ghrelin levels actually contributed to meal initiation. It is possible that the surges occurred as an anticipatory response to meals because the subjects knew when food was to be provided. Additional studies to distinguish between anticipatory responses and true meal initiation are now warranted.

Ingested nutrients are the most likely mediator of the rapid postprandial fall in circulating ghrelin levels. This contention is supported by the finding that fasting serum ghrelin levels are decreased in rats by filling the stomach with a 50% dextrose solution but not with an equal volume of water (3). Neither this experiment nor our data distinguish whether ingested nutrients suppress ghrelin production directly or indirectly (e.g., through insulin), a possibility that is consistent with the reciprocal 24-h profiles of these hormones (Fig. 1).

The factors that dictate the relatively wide range of ghrelin levels among individuals remain to be determined. It has recently been reported that fasting plasma ghrelin concentrations are negatively correlated with percent body fat and are decreased in obesity (13). If ghrelin is involved in regulating overall energy balance, then an ineffective compensatory modulation of ghrelin levels in obesity, rather than a causative role of ghrelin levels, is suggested. Among our subjects, 24-h AUC ghrelin values tended to be lower with increasing BMI ( $r = -0.519$ ,  $P = 0.128$ ), although the narrow range of BMI in our study cohort (22.0–30.0 kg/m<sup>2</sup>) limited our ability to detect a statistically significant relationship between these parameters.

One subject's plasma ghrelin levels were significantly lower than those of all other subjects on two separate 24-h measurements, although on both occasions she did display meal-related ghrelin oscillation. Because she has hypothyroidism, presumed to arise from an autoimmune etiology, we evaluated the possibility that her ghrelin levels might be low because of autoimmune gastrointestinal disease. Her serum proved to have anti-parietal cell antibodies and a low B<sub>12</sub> level. Although these findings were not dramatic in magnitude, they are unusual for a young woman (29 years old) and suggest an evolving gastric autoimmune process. This may be the cause of her low ghrelin levels, a possibility that seems plausible in view of the close juxtaposition of parietal and ghrelin cells within gastric oxyntic glands (2).

The observation that leptin and intermeal ghrelin levels display diurnal rhythms that are in phase with one another suggests that the two may be coordinately regulated. The diurnal pattern of leptin has been shown to be entrained to meal timing (18), and the nocturnal rise in circulating levels is thought to reflect the overall accumulation of ingested calories throughout the day (19,20). Thus, it is possible that in addition to being acutely negatively regulated by the ingestion of individual meals, circulating ghrelin is also positively regulated by fluxes in overall energy balance. This would explain both the gradual rise of basal levels throughout the day and evening and the fall at night, during which times humans experience states of positive energy balance followed by negative balance. If ghrelin is a physiologically important circulating orexigen, its effects may be counterbalanced during the late night by high leptin levels.

In view of these in-phase diurnal rhythms, it is conceivable that leptin directly stimulates ghrelin secretion. There are conflicting data from rodent studies that show both positive (21) and negative (6) regulation of ghrelin by leptin. Alternatively, ghrelin might induce leptin, as ghrelin receptors are expressed in adipose tissue (1) and the stomach (22), the principal sites of leptin synthesis (23,24), and

this possibility warrants further investigation. The subtle postprandial drop in leptin levels that we detected has been observed, though not commented upon, by others (18,25). It is conceivable that this reflects meal-related regulation of gastric leptin.

The 24-h AUC ghrelin values correlated significantly with levels at 0600 after an overnight fast as well as with 0930 levels measured 90 min after consumption of a non-standardized breakfast. If our findings can be replicated in larger studies, they indicate that these convenient single measurements may serve as indexes of 24-h ghrelin concentrations in situations where 24-h blood sampling is precluded. This has important implications for scientific studies and for possible future uses of plasma ghrelin levels in a clinical context.

Circulating ghrelin, assessed as 24-h AUC, 0600, or 0930 values, correlated positively with age. Although the validity of this observation is limited because of our small sample size, the possibility may now be entertained that rising ghrelin levels could play a role in the gradual increase of body fat content that occurs throughout the breadth of adult life in humans (26).

If ghrelin is proven to be a physiologically important meal initiator, the medical implications would be considerable. Bioactive ghrelin and orally bioavailable ghrelin agonists have been synthesized (27) and can be tested as remedies for the pathological anorexia that can accompany cancer, AIDS, tuberculosis, and aging. In this regard, it is noteworthy that although ghrelin is implicated in triggering individual meals, chronic administration causes significant weight gain in rodents (3,4). We have recently shown that central administration of ghrelin increases food intake and body weight in anorexic rats bearing prostate adenocarcinoma (28). Ghrelin has been delivered to humans in two small trials designed to determine its effects on GH secretion (29,30). Although neither of these studies examined food intake, one of them noted that three of four subjects reported feeling hungry after receiving intravenous ghrelin (29). Whether ghrelin antagonism could reduce food intake and be developed as a treatment for obesity is an important question for future studies.

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