Am J Physiol Endocrinol Metab 287: E297–E304, 2004.
First published March 23, 2004; 10.1152/ajpendo.00582.2003.

Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues

D. E. Cummings,[1] R. Scott Frayo,[1] Corinne Marmonier,[2] Roberte Aubert,[3] and Didier Chapelot[2]

[1]*Division of Metabolism, Endocrinology, and Nutrition, Department of Medicine, University of Washington, Veterans Affairs Puget Sound Health Care System, Seattle, Washington 98108;* [2]*Laboratoire de Physiologie du Comportement Alimentaire, Unité de Formation et de Recherche Santé Médecine and Biologie Humaine, Université Paris 13, Bobigny 93014; and* [3]*Laboratoire de Nutrition Humaine, Faculté Xavier Bichat, Université Paris VII, Paris, 75018 France*

Submitted 22 December 2003; accepted in final form 9 March 2004

Cummings, D. E., R. Scott Frayo, Corinne Marmonier, Roberte Aubert, and Didier Chapelot. Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. *Am J Physiol Endocrinol Metab* 287: E297–E304, 2004. First published March 23, 2004; 10.1152/ajpendo.00582.2003.—Ghrelin is an orexigenic hormone that is implicated in meal initiation, in part because circulating levels rise before meals. Because previous human studies have examined subjects fed on known schedules, the observed preprandial ghrelin increases could have been a secondary consequence of meal anticipation. A causal role for ghrelin in meal initiation would be better supported if preprandial increases occurred before spontaneously initiated meals not prompted by external cues. We measured plasma ghrelin levels among human subjects initiating meals voluntarily without cues related to time or food. Samples were drawn every 5 min between a scheduled lunch and a freely requested dinner, and hunger scores were obtained using visual analog scales. Insulin, glucose, fatty acids, leptin, and triglycerides were also measured. Ghrelin levels decreased shortly after the first meal in all subjects. A subsequent preprandial increase occurred over a wide range of intermeal intervals (IMI; 320–425 min) in all but one subject. Hunger scores and ghrelin levels showed similar temporal profiles and similar relative differences in magnitude between lunch and dinner. One subject displayed no preprandial ghrelin increase and was also the only individual whose insulin levels did not return to baseline between meals. This finding, along with a correlation between area-under-the-curve values of ghrelin and insulin, suggests a role for insulin in ghrelin regulation. The preprandial increase of ghrelin levels that we observed among humans initiating meals voluntarily, without time- or food-related cues, and the overlap between these levels and hunger scores are consistent with a role for ghrelin in meal initiation.

appetite; insulin

ALTHOUGH MEALTIME HUNGER IS a common, daily experience, the nature of the molecular determinants underlying this sensation is debated. Ghrelin is a recently discovered enteric peptide hormone that is the only known circulating orexigen and one of very few substances shown to stimulate appetite and food intake when administered to humans (22, 36, 46, 51). Orexigenic actions of ghrelin have been demonstrated after modest-dose peripheral injections that generate approximately physiological blood levels in humans and rodents, suggesting that normal fluctuations in endogenous circulating ghrelin can affect appetite (51, 52). Considerable evidence in multiple species implicates ghrelin in the control of preprandial hunger and meal initiation. Additional evidence suggests that ghrelin may also participate in long-term body-weight regulation, and blockade of ghrelin signaling is actively being explored as a potential anti-obesity modality (13, 14).

The following observations are consistent with the hypothesis that ghrelin contributes to preprandial hunger and meal initiation (12, 14). *1*) The greatest amount of ghrelin is produced by the stomach and duodenum, organs that are well positioned to sense the presence or absence of recently ingested food (3, 16, 19, 22, 24). *2*) As predicted for a meal initiator, ghrelin levels increase with fasting and are suppressed within minutes by refeeding or enteral infusions of nutrients but not water (3, 46). *3*) Exogenous ghrelin stimulates eating when administered at times of minimal spontaneous food intake (4, 36, 46, 53). *4*) Ghrelin's orexigenic actions are extremely rapid and short-lived, as required for a signal influencing individual meal-related behavior (4, 36, 46, 53). *5*) A detailed analysis of meal patterns after ghrelin injections reveals that the primary orexigenic effect of ghrelin is to decrease the latency to feed, leading to one extra episode of eating that occurs directly after ghrelin administration (17). *6*) Ghrelin stimulates gastric motility and acid secretion, both of which increase in anticipation of meals (4, 29). *7*) Hypothalamic neurons that cosecrete the orexigenic substances neuropeptide Y and agouti-related protein are the most clearly documented targets of ghrelin action in the brain and are implicated in the central control of meal initiation (13, 14). Levels of these neuropeptides increase at times of maximal feeding in rodents, whereas the expression of other neuropeptides involved in energy balance is relatively constant throughout the day and night (25).

Among the most compelling evidence suggesting a role for ghrelin in meal initiation in humans is the observation that circulating levels increase before, and decrease after, every meal (12, 15, 40, 48, 49). Likewise, sheep habituated to various feeding schedules demonstrate preprandial ghrelin surges occurring as many times per day as meals are provided (43, 44). In the studies reporting these findings, however, subjects knew when food was to be served; therefore, it is possible that the preprandial increases in ghrelin levels were part of a cognitive, anticipatory response to upcoming meals. A causal role for ghrelin in meal initiation would be supported more strongly if such increases were observed among individuals initiating meals spontaneously when isolated from external cues related to either time or

Address for reprint requests and other correspondence: D. E. Cummings, Univ. of Washington, VA Puget Sound Health Care System, 1660 South Columbian Way, S-111-Endo, Seattle, WA 98108 (E-mail: davidec@u.washington.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

food, and also if there were a temporal relationship between ghrelin levels and hunger.

In the current study, we sought to determine, with very frequent blood sampling, whether preprandial increases in circulating ghrelin levels occur among human subjects initiating meals voluntarily, in the absence of any time- or food-related cues. We also assessed whether the timing of fluctuations in ghrelin levels in this setting overlaps with that of the subjective sensation of hunger. Because insulin and leptin are strongly involved in the biological cascade of eating behavior and may modulate ghrelin action, these hormones were also evaluated, as were the important fuel substrates glucose, triglycerides, and nonesterified fatty acids (NEFA).

RESEARCH DESIGN AND METHODS

Subjects. After approval of the procedure by the Ethics Committee in Human Research at Bocage Hospital, subjects were recruited through advertisements posted at the Faculties of Science and Medicine of Dijon. To be included, subjects had to be male, healthy, aged 19–25 yr, and have a body mass index (BMI) between 19 and 25 kg/m^2. We excluded smokers, trained athletes, and individuals who drank alcohol more than occasionally, who had food allergies, and who used medication. Subjects were also excluded if they reported a personal or family history of obesity, eating disorders, diabetes, other metabolic diseases, or a change in body weight of >1 kg over the 3 yr preceding the study. All subjects had to have low dietary restraint scores (≤6 out of a possible 18) on the Three Factor Eating Questionnaire (42). Candidates were excluded if they reported fear of blood withdrawal or aversion to any of the foods provided in the study. Moreover, subjects had to be accustomed to eating three meals per day, not including any habitual food intake between lunch and dinner. This criterion was not specified to candidates before they recorded all of their food intake on 5-day diary reports, upon which this criterion was verified. The selected subjects all gave written informed consent before the experiment and were financially compensated for completing the study. Characteristics of these individuals are shown in Table 1.

The experimental session was designed to determine, in subjects deprived of cues related to time and food, the temporal pattern of plasma ghrelin concentrations and hunger sensations from the beginning of lunch (provided at a fixed time) to the beginning of dinner (provided when subjects voluntarily requested it).

Body composition. Body composition was determined by whole body dual-energy X-ray absorptiometry using the Hologic QDR 4500 (Hologic, Waltham, MA). Scans were analyzed with Hologic software version 8.07. A Step Phantom calibration was performed daily, as recommended by the manufacturer. After having urinated, subjects were scanned for 5 min in a supine position, wearing only undergarments and no metal objects. The same investigator performed all scans.

Table 1. *Subject characteristics*

Subject No.	Age, yr	Weight, kg	BMI, kg/m^2	Total Fat Mass, kg	Body Fat, %	Restraint Score*
1	21	74.6	22.5	12.7	17.0	6
2	20	58.6	19.0	6.1	10.4	2
3	22	76.1	23.2	10.1	13.3	6
4	20	75.4	21.3	8.6	11.4	3
5	23	67.1	19.4	7.6	11.4	4
6	21	73.7	22.5	7.1	9.6	3
Mean±SD	21.2±1.2	70.9±6.8	21.3±1.8	8.7±2.4	12.2±2.7	4.0±1.7

BMI, body mass index. *On the Dietary Restraint subscale of the Three-Factor Eating Questionnaire (42), in which the maximum possible score is 18.

Meals. On the test day, lunch was a two-course meal, consisting of spaghetti bolognese (17, 34, and 49% of energy derived from protein, fat, and carbohydrate, respectively) and fruit yogurt (14, 17, and 69% of energy derived from protein, fat, and carbohydrate, respectively). Dinner consisted of chicken with rice, vegetables, corn and mushrooms, bread, apple stew (Andros), and biscuits (Palets bretons). All items were served in large portions, and water was provided ad libitum.

Subjective appetite ratings. Hunger was assessed using 100-mm visual analog scales preceded by the question "Do you feel hungry?" These scales were anchored with "not at all" and "extremely" at the left and right ends, respectively. The distance from the extreme left to the subject's vertical dash represented the rating score, expressed in millimeters. Hunger ratings were determined every 30 min throughout the study, and subjects were unaware of the duration of this interval. Additional measurements were also randomly intercalated to avoid providing any time cues.

Study protocol. Subjects arrived at the hospital at 1900 on the evening before the study. After consuming an ad libitum dinner, they slept in private bedrooms at the facility. They were awakened at 0800 the following day and were served their usual breakfast, as determined from their home food intake reports. They were allowed to eat as much of this meal as they desired.

After breakfast, subjects were seated in comfortable armchairs and deprived of time cues by exposing them to artificial light only and by removing all sources of visual and auditory time indicators (e.g., clocks, watches, radios, television, telephones, etc.). They were isolated in individual rooms, which were quiet and comfortable enough to allow them to pursue their studies. At 1100, an anesthetic cream (Emla; AstraZeneca) was spread over the area where the catheter was to be inserted to eliminate any pain-induced stress resulting from the puncture. At 1200, the sampling catheter (see below) was inserted in an antecubital vein; blood withdrawal started immediately thereafter and continued uninterrupted throughout the experiment. Lunch was served 30 min after the first blood sample; meal duration was not constrained, to encourage ad libitum intake. Subjects were served sufficient portions to ensure that they were not limited in their spontaneous desire to eat. Before and after consumption, foods were accurately and covertly weighed to determine actual intake. Subjects were instructed to request their next meal whenever they felt hungry enough to do so. Regardless of the time of this request, they were informed that they would not be allowed to leave the center until 2200 to avoid any premature meal requests. Subjects were served dinner when they asked to eat and were again instructed to consume as much as they desired. After they finished this meal, their catheter was withdrawn, and they were asked to guess the time of day. At 2200, they were allowed to leave the center.

Blood sample collection. Blood was harvested using a specially designed, double-lumen catheter (MTB Medezintechnik, Amstetten, Germany) fitted into a 21-gauge, indwelling cannula in the antecubital vein. This system allowed heparinized blood to be withdrawn continuously throughout study sessions without any heparin being infused in the vein (9). The catheter was heparinized by pumping sterile heparin-saline solution (200 U/ml) via a peristaltic pump at 24 μl/min through the distal lumen of the catheter to the tip of the cannula. The heparinized blood was continuously withdrawn through the proximal lumen of the cannula at 400 μl/min. Blood samples were collected every 5 min. The transit time of this continuous sampling line was ~10 min, a lag time that was precisely measured for each test in every subject and taken into account for data analysis. Blood samples were immediately centrifuged for 15 min at 2,000 *g* at 4°C, after which plasma was separated into aliquots and stored at −80°C until being assayed.

Plasma measurements. Total immunoreactive ghrelin concentration was determined with our modification of a commercial RIA (Phoenix Pharmaceuticals, Belmont, CA). This assay uses a ^{125}I-labeled ghrelin tracer and a rabbit polyclonal antibody against full-

length, octanoylated human ghrelin that recognizes the acylated and des-acyl forms. Although only acylated ghrelin is bioactive (22), total ghrelin appears to be a reasonable surrogate for the acylated form because the ratio of the two levels remains constant under a wide variety of conditions that affect ghrelin (2, 34, 35). The lower and upper detection limits were 80 and 2,500 pg/ml, respectively, and the inter- and intra-assay coefficients of variation were 5.9 and 10.3%, respectively.

Insulin concentration was determined by RIA using the SB-INSI-5 kit (7% accuracy, sensitivity of 2 IU/l; CEA, Gif-sur-Yvette, France). Leptin concentration was determined by RIA using the commercial Sensitive Human Leptin kit (Linco Research, St. Charles, MO). Mean intra- and interassay coefficients of variation were 3.5 and 5.3%, respectively.

Glucose was measured by the glucose oxidase enzymatic method (kit Hycel, Pouilly en Auxois, France). Triglycerides and NEFA were quantified using a colorimetric enzymatic method (kit C, Wako, both 5% accuracy; Oxoïd, Dardilly, France). All substrate measurements were performed with an automat device (Lisa 200).

Statistics. Statistical analyses were conducted on SYSTAT software (version 7.01; SPSS, Chicago, IL). All results are expressed as means ± SE, except where standard deviations are specified. Areas under the curve (AUC) of substrate and hormone profiles were determined using the trapezoidal method after subtracting the basal AUC from the calculated AUC. Because AUC does not take into account the differences among subjects in duration of the intermeal interval (IMI), a mean value of each blood factor during the IMI was calculated by dividing the sum of the values by the number of tubes. To obtain a common representation of the IMI for all subjects, a time scale based on percentages of IMI was constructed (see Ref. 10 for details). The IMI of each subject was divided into 20 equal intervals, and, for each of these periods, a mean value of the measurements was calculated for every variable. The changes of concentrations were expressed as percent change from the baseline, defined as the mean of three samples taken at 5-min intervals immediately before the beginning of lunch.

The significance of the changes during the IMI (either in minutes or as %IMI) was evaluated by ANOVA for repeated measures. When an interaction with time was found, post hoc analyses were conducted using Scheffé's test.

Pearson's correlations were used to assess relationships between ghrelin levels and anthropometric measures (BMI, fat mass, fat-free mass, and %body fat), behavioral measures (duration of the IMI and energy intake at dinner), and levels of blood factors (insulin, leptin, glucose, NEFA, and triglycerides). Predictive equations were fitted by multivariate linear regression using a backward stepping procedure. Significance was fixed at $P < 0.05$.

RESULTS

The mean time taken to eat lunch was 20 min (range 17–23), and the mean energy intake at that meal was 3,356 kJ (range 2,558–3,989; Table 2). The mean IMI between the fixed lunch and freely requested dinner was 359 min (range 320–425). The mean guessed time at the point when dinner was requested was 34 ± 5 min (range 15–55 min) later than the actual time ($P = 0.005$).

Table 2. *Energy intake and intermeal interval*

Subject No.	Lunch, kJ	Dinner, kJ	IMI, min
1	2,558	3,875	320
2	3,989	4,286	390
3	3,733	5,039	340
4	2,949	4,241	350
5	3,657	6,568	425
6	3,252	3,961	330
Mean±SD	3,356±538	4,662±1,020	359±40

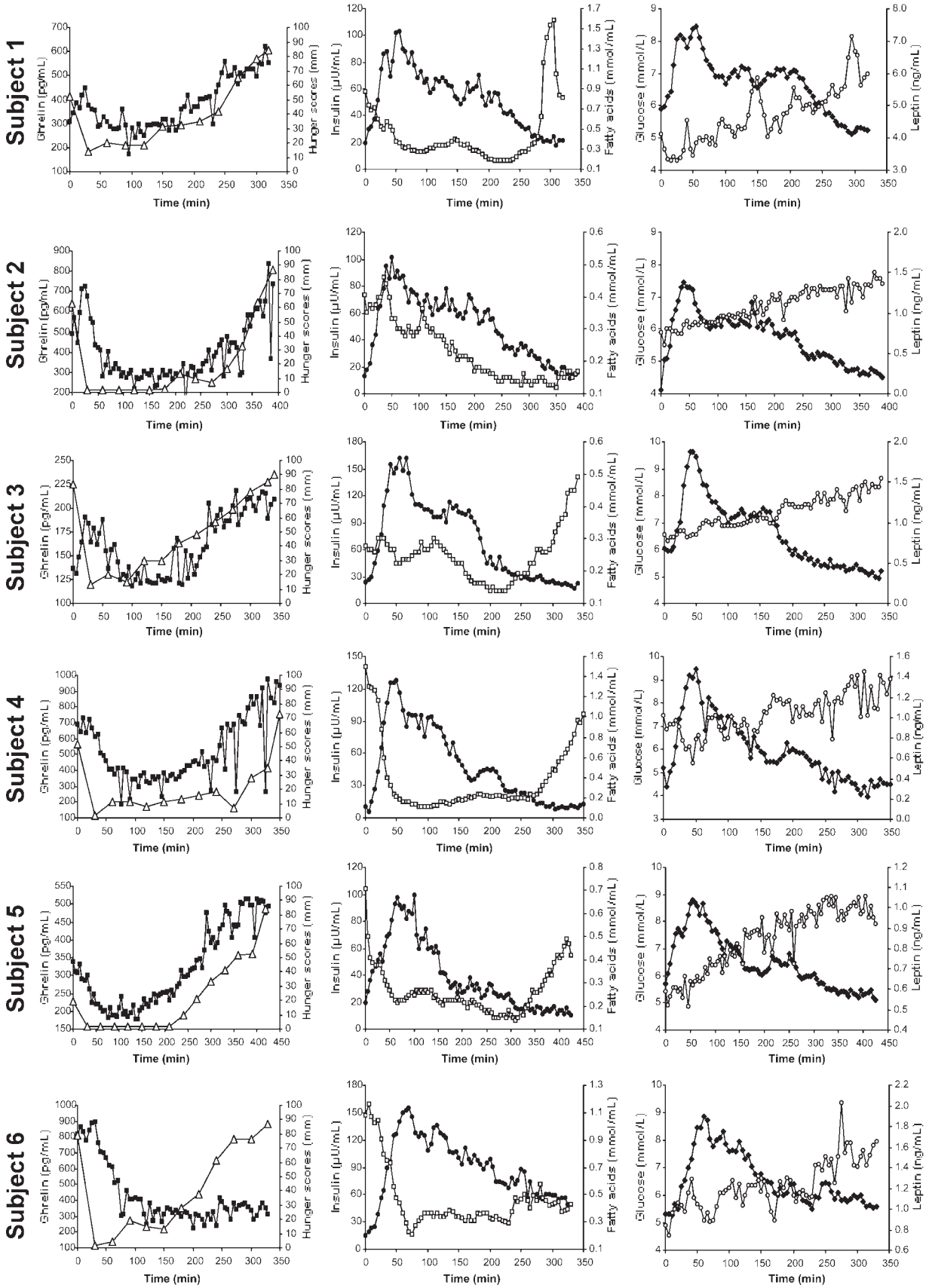
IMI, intermeal interval.

Individual subject profiles for ghrelin and other analytes are shown in Fig. 1. Presumably as a continuation of the preprandial rise before lunch, mean ghrelin levels generally increased briefly after the start of this meal, for 21 ± 2 min (an average of 7.5% of lunch-dinner IMI; Fig. 2). Ghrelin levels then began to fall shortly after lunch was consumed in all subjects, and this decline reached statistical significance for the group by 60 min after the start of the meal ($P = 0.035$). Calculated as percentage of individual IMI, the change from baseline was significant by 22.5% of the IMI ($P = 0.021$; Fig 2). The trough was reached ~70 min after the start of lunch, and the nadir was 34.6 ± 8.4% lower than the baseline value. The duration of the trough varied widely across subjects, e.g., from ~150 min in *subject 5* to 250 min in *subject 2*. A progressive increase was then observed until the voluntary dinner request in all subjects except *subject 6*. Mean ghrelin levels were no longer different from baseline by 57.5% of the IMI, and they reached an average of 116 ± 15% of trough values at the time when dinner was freely requested (Fig. 2). At this time, mean ghrelin levels were 31.2 ± 19.5% above baseline values, but this difference did not reach significance because of *subject 6*.

The temporal profiles of mean ghrelin levels and subjective hunger scores overlapped closely with each other throughout the IMI (Fig. 2). This relationship was observed, regardless of the duration of the IMI, in all subjects except *subject 6* (Fig. 1). In *subjects 1*, *2*, *4*, and *5*, the increase in ghrelin levels slightly preceded the increase in hunger scores, whereas the two variables were synchronized in *subject 3*. It is noteworthy that, in all but *subject 6*, the differences in ghrelin levels between dinner and lunch mimicked those in hunger scores, i.e., a higher ghrelin level corresponded to a higher hunger score and vice versa. This relationship was particularly evident in *subject 5*, who displayed considerably lower levels of both ghrelin and hunger before lunch than were observed for both measurements before dinner. The only time throughout the IMI when the mean curves for ghrelin and hunger diverged substantially was shortly after lunch was consumed, during which time appetite scores fell more rapidly from preprandial values than did ghrelin levels (Fig. 2).

To assess a possible role for insulin, glucose, NEFA, leptin, or triglycerides in the variability of ghrelin levels among subjects, concentrations of these hormones and substrates were analyzed in the same manner as was ghrelin (Figs. 1 and 2). *Subject 6* displayed two profiles that differed substantially from those of the other subjects, in addition to a markedly different ghrelin profile lacking the usual preprandial increase. This individual's profiles were unusual in that, before dinner, NEFA levels did not increase and insulin levels never reached baseline values after their postlunch peak (Fig. 1). Fatty acid levels also did not increase before dinner in *subject 2*, who nevertheless showed a robust preprandial ghrelin increase. Thus the failure of insulin levels to reach baseline values during the IMI in *subject 6* seems the most parsimonious explanation for the lack of a preprandial rise in this individual's ghrelin levels.

The only association between AUC values for ghrelin and the other measured blood parameters was a negative correlation between ghrelin and insulin AUC ($r = -0.814$, $P = 0.049$). We found no correlations between ghrelin AUC and



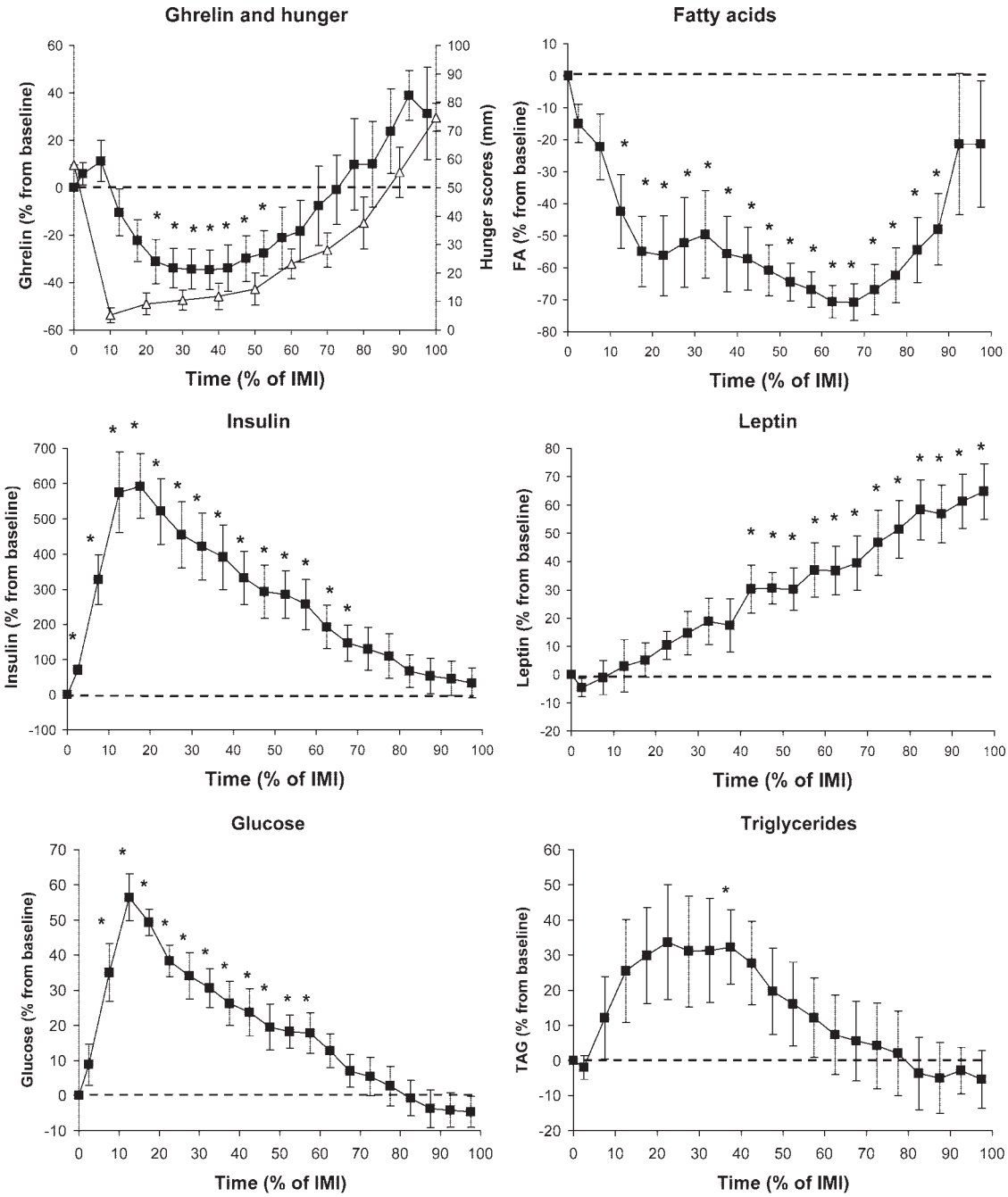


Fig. 2. Mean±SE values for hunger scores (△) and plasma levels of ghrelin, insulin, glucose, NEFA, leptin, and triglycerides (TAG) among 6 human subjects isolated from time- and food-related cues. For clarity, data are displayed as %change from the initial baseline, and time is expressed as a percentage of each individual's intermeal interval (IMI) between the fixed lunch (begun at *time 0*) and the freely requested dinner (begun at time 100%). Each subject's IMI was divided into 20 equal intervals, and, for each of these periods, the mean value of every variable is displayed. FA, fatty acid. *$P < 0.05$ compared with baseline values at *time 0*.

energy intake at lunch, intake at dinner, duration of the IMI, or anthropometric measures. To assess the accuracy of our anthropometric measurements of adiposity, we evaluated the correlation between percent fat mass and leptin levels, two factors known to be tightly associated. As expected, there was a significant correlation between percent fat mass and both mean leptin levels ($r = 0.872$, $P = 0.023$) and leptin AUC ($r = 0.870$, $P = 0.024$).

Fig. 1. Analysis of individual subjects initiating meals voluntarily while isolated from time- and food-related cues. Data are shown from the time when lunch was provided (*time 0*) until dinner was freely requested, at a time of their choosing. Displayed are subjective hunger scores (△) and plasma levels of ghrelin (■), insulin (●), nonesterified fatty acids (NEFA; □), glucose (◆), and leptin (○). Hunger was quantified using visual analog scales every 30 min. For plasma measurements, samples were taken automatically every 5 min from continuously withdrawn blood.

The multiple-regression analyses showed that a model could be constructed for ghrelin AUC. The only significant predictive factors were insulin AUC and NEFA AUC, with negative and positive coefficients, respectively ($r^2 = 0.950$, $P = 0.011$; Table 3). The correlation between estimated and actual ghrelin AUC was $r = 0.975$, $P = 0.001$.

DISCUSSION

In this study, the very frequent blood measurement (every 5 min from continuously withdrawn blood) and the avoidance of all environmental conditional cues associated with eating allowed a detailed analysis of variations in plasma ghrelin levels relative to spontaneous eating. The postprandial decrease and preprandial increase of plasma ghrelin levels that have been reported among individuals fed on fixed schedules was also observed among all but one of our subjects initiating meals voluntarily, in the absence of cues related to time or food. Moreover, except in this one subject, the temporal profiles of ghrelin levels overlapped considerably with those of subjective hunger scores, with ghrelin increases generally preceding hunger increases by a short interval.

It is important to note that, although our protocol reduces the effect of external cues on the parameters we measured, it does not exclude circadian influences on these parameters, since subjects were deprived of time cues for only a short period. Durable circadian rhythms could clearly affect our results, and elimination of these potentially confounding effects would require long-term studies of people deprived of time cues for many weeks or months. Furthermore, we studied a small number of subjects, each examined quite intensively. The large amount of data resulting from frequent blood sampling allows us to draw conclusions regarding the interrelationships among our measured end points within individuals, but this small sample size limits our ability to extrapolate our findings to broad populations.

Not withstanding these caveats, however, the close association that we observed between a subjective measure of appetite and a blood parameter is, to our knowledge, the first to be described in the area of ingestive behavior. Moreover, it is striking that the relative difference in hunger scores between lunch and dinner in each individual mimicked those of ghrelin levels. Although NEFA and ghrelin levels showed similar IMI profiles, the relationship of hunger scores to NEFA levels was, on a group or individual basis, much less tightly linked than were hunger scores and ghrelin levels. Because our subjects were isolated from any time indicators and also from the sight, smell, sound, or mention of food, their decision regarding when to eat should have been driven more by putative endogenous appetite signals than by external influences. The observed preprandial increase in ghrelin levels and their close association with hunger scores under these conditions is, therefore, consistent with the hypothesis that ghrelin may function as part of the mechanism leading to an appetite signal. Although our observations are correlative and do not prove a causal role for ghrelin in meal initiation, they are consistent with that model and represent more compelling evidence in its favor than has been provided from our prior work on subjects fed according to a fixed schedule (12, 15).

Table 3. *Regression model for ghrelin AUC*

Dependent Variable	β	SE	*P*
Constant	136,841.81	24,538.17	0.011
Insulin AUC, $\mu U \cdot min^{-1} \cdot ml^{-1}$	−9.37	1.43	0.007
NEFA AUC, $mmol \cdot min^{-1} \cdot ml^{-1}$	207.31	49.76	0.025

The following variables were tested: energy intake at lunch; the interval between lunch and the next spontaneous meal request, i.e., IMI; BMI; lean and fat mass (in absolute weight and % total body weight); and incremental area under the curves (AUC) for insulin, leptin, nonesterified fatty acid (NEFA), and triglycerides. β, Standard regression coefficient; *P*, significance fixed at $P < 0.05$. The r^2 and significance of the equation are indicated in text.

Because one subject did not show a preprandial increase in ghrelin levels, however, a mandatory relationship between ghrelin and meal initiation is questioned. One explanation might be that this subject's decision regarding when to eat was not driven by physiological factors. Although this possibility cannot be excluded, some facts argue against it. First, the subject's hunger scores manifested the usual profile observed in a spontaneous IMI and reached high values before the request for dinner. Second, the duration of the IMI and the energy intake at dinner were not reduced compared with those in other subjects. Third, a small increase in ghrelin levels was actually apparent ~250 min after lunch, but, contrary to those in other subjects, this level plateaued until the dinner request. Other blood profiles were explored to provide hypotheses to explain this unusual ghrelin profile, and additional atypical features were observed. By the end of this subject's IMI, NEFA levels had not increased from trough values, and insulin levels had not declined to their baseline. The unusual NEFA profile is unlikely to explain the unusual ghrelin profile, however, since *subject 2* showed a similar NEFA profile but had a clear preprandial increase in ghrelin levels. Moreover, it is important to note that a preprandial increase in NEFA levels is not consistently observed and requires a long IMI and low insulin levels (10, 27, 28). This subject's unusual insulin profile was, therefore, the only parameter we measured that, together with the ghrelin profile, contrasted with that of all other subjects. Although insulin levels reached baseline values 250–350 min after lunch in all other subjects, they remained >250% higher than baseline in *subject 6*. In summary, the most likely explanation for the lack of a preprandial increase in ghrelin levels in *subject 6* appears to be the failure of this individual's insulin levels to return to baseline during the IMI.

A relationship between ghrelin and insulin is further supported in our study by the inverse correlation of AUC values of the two hormones during the IMI. This observation is consistent with recent reports supporting a role for insulin as a negative regulator of ghrelin (1, 18, 26, 30, 33, 39). Modeling of ghrelin AUC by multivariate linear regression, however, suggests that NEFA could be a potential cofactor linking insulin and ghrelin. The relationship between ghrelin and NEFA being positive, this means that, for a similar insulin level, ghrelin would be higher if NEFA were elevated. It is noteworthy that NEFA were also found to be tightly related to leptin in a previous study conducted with a similar procedure (10). In sparing glucose from oxidation via the glucose-fatty acid cycle (37) in the late part of the IMI (after the insulin-induced inhibition of lipolysis is weakening), mobilized NEFA can help maintain glucose levels and prolong satiety (21). However, NEFA contribute to metabolism mainly in the late part of the IMI (28). Beginning its increase sooner, ghrelin may

represent a precocious enteric signal enhancing the disposition of a person toward food seeking, i.e., the interest in food.

Leptin showed its usual daytime increase in all subjects, but the onset and the magnitude of this increase varied among individuals. The highest values during the lunch-dinner interval were reached between 275 and 380 min across subjects. The predinner decline in leptin levels that we previously reported in five out of six subjects (10) was observed in only four out of six subjects here. In summary, it appears that spontaneous meal initiation can occur without a prior decrease in leptin levels, an increase in NEFA or ghrelin levels, or a return of insulin levels to baseline. The most consistent preprandial profile across subjects in this and several other studies in which very frequent blood sampling was performed is the preprandial decline in glucose (7, 8, 11, 23, 31, 32).

Our results, however, still support a role for ghrelin in the dynamic situation leading to meal initiation, and additional lines of recent evidence are also compatible with such a role. Spontaneous increases in circulating ghrelin levels correlated with episodes of feeding (but not growth hormone release) among ad libitum-fed rats evaluated with formal pulsatility analysis (45). The assertion that similar preprandial increases might affect human meal initiation is strengthened by observations that three different single-nucleotide polymorphisms in the human genes encoding either ghrelin or its receptor are associated with abnormal meal patterns characterized by excessive nibbling (or "grazing"), findings consistent with a role for ghrelin in dictating the timing of meals (20). A positive correlation was reported between preprandial ghrelin levels and gastric emptying time, a determinant of the duration of satiety (47). Finally, we have shown in humans that the depth and duration of postprandial ghrelin suppression is related dose dependently to caloric load, when all other features of meals are held constant (6). In other words, large meals suppress ghrelin more thoroughly than do small meals, similar to the manner in which caloric load affects appetite. Although these observations and our current findings present a picture of ghrelin as participating in meal initiation, the data are largely correlative and thus are not yet definitive. Critical loss-of-function experiments with ghrelin-blocking agents or genetic ablations are required to prove or disprove the hypothesis that ghrelin is an important participant in meal initiation.

The temporal profiles of ghrelin levels and hunger scores in our study overlapped more closely during the preprandial increase than during the postprandial decline. This finding is more consistent with a physiological role for the preprandial rise of ghrelin in meal initiation than with a key role for the postprandial fall of ghrelin in mediating meal termination. The latter event results from sensory cues (5, 38), gastric distention, and several short-acting, meal-stimulated intestinal satiation factors (e.g., CCK; see Refs. 41 and 50). These forces presumably cause the sensation of gastrointestinal fullness via ghrelin-independent mechanisms and are likely to be responsible for the postprandial fall of hunger scores that we observed preceding the fall of ghrelin levels.

In summary, we observed postprandial suppression and preprandial increases of plasma ghrelin levels among human subjects initiating meals voluntarily, in the absence of time- and food-related cues. Our data favor a role for insulin in both this short-term prandial regulation of ghrelin and in overall ghrelin AUC. We also found a close overlap between the temporal profiles of ghrelin levels and hunger scores. These correlative data are consistent with, but do not prove, the hypothesis that ghrelin functions as an endogenous meal-initiation signal. Further studies in which ghrelin signaling is abolished are required to establish definitively if ghrelin is, indeed, an important physiological meal initiator.

ACKNOWLEDGMENTS

We thank Dr. Jeanine Louis-Sylvestre for extensive and insightful input into the work and this manuscript.

GRANTS

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1 DK-61516 (D. E. Cummings), the Veterans Affairs Puget Sound Health Care System, and by a grant from Danone Vitapole Nutrivaleur, Danone, Co. (Palaiseau France).

REFERENCES

1. **Anderwald C, Brabant G, Bernroider E, Horn R, Brehm A, Waldhausl W, and Roden M.** Insulin-dependent modulation of plasma ghrelin and leptin concentrations is less pronounced in type 2 diabetic patients. *Diabetes* 52: 1792–1798, 2003.
2. **Ariyasu H, Takaya K, Hosoda H, Iwakura H, Ebihara K, Mori K, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, and Nakao K.** Delayed short-term secretory regulation of ghrelin in obese animals: evidenced by a specific RIA for the active form of ghrelin. *Endocrinology* 143: 3341–3350, 2002.
3. **Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, and Nakao K.** Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab* 86: 4753–4758, 2001.
4. **Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, and Kasuga M.** Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 120: 337–345, 2001.
5. **Booth D, Mather P, and Fuller J.** Starch content of ordinary foods associatively conditions human appetite and satiation, indexed by intake and eating pleasantness of starch-paired flavors. *Appetite* 3: 163–184, 1982.
6. **Callahan HS, Cummings DE, Pepe MS, Breen PA, Matthys CC, and Weigle DS.** Postprandial suppression of plasma ghrelin level is proportional to ingested caloric load in humans. *J Clin Endocrinol Metab* 89: 1319–1324, 2004.
7. **Campfield LA and Smith FJ.** The glucostatic hypothesis-1995 update-transient declines in blood glucose as signals for meal initiation (Abstract). *Obes Res* 3: 311s, 1995.
8. **Campfield LA, Smith FJ, Rosenbaum M, and Hirsch J.** Human eating: evidence for a physiological basis using a modified paradigm. *Neurosci Biobehav Rev* 20: 133–137, 1996.
9. **Chabert M, Verger P, and Louis-Sylvestre J.** A method for long-term and accurate measurement and recording of blood glucose level in man. *Physiol Behav* 260: R756–R763, 1991.
10. **Chapelot D, Aubert R, Marmonnier C, Chabert M, and Louis-Sylvestre J.** An endocrine and metabolic definition of the intermeal interval in humans: evidence for a role of leptin on the prandial pattern through fatty acid disposal. *Am J Clin Nutr* 72: 421–431, 2000.
11. **Chapelot D, Marmonier C, Aubert R, Gausseres N, and Louis-Sylvestre J.** A role for glucose and insulin preprandial profiles to differentiate meals and snacks. *Physiol Behav* 80: 721–731, 2004.
12. **Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, and Weigle DS.** A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 50: 1714–1719, 2001.
13. **Cummings DE and Schwartz MW.** Genetics and pathophysiology of human obesity. *Annu Rev Med* 54: 453–471, 2003.
14. **Cummings DE and Shannon MH.** Roles for ghrelin in the regulation of appetite and body weight. *Arch Surg* 138: 389–396, 2003.
15. **Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, and Purnell JQ.** Human plasma ghrelin levels after diet-induced

weight loss and gastric bypass surgery. *N Engl J Med* 346: 1623–1630, 2002.
16. **Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, and Nakazato M.** Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 141: 4255–4261, 2000.
17. **Faulconbridge LF, Cummings DE, Kaplan JM, and Grill HJ.** Hyperphagic effects of brainstem ghrelin administration. *Diabetes* 52: 2260–2265, 2003.
18. **Flanagan DE, Evans ML, Monsod TP, Rife F, Heptulla RA, Tamborlane WV, and Sherwin RS.** The influence of insulin on circulating ghrelin. *Am J Physiol Endocrinol Metab* 284: E313–E316, 2003.
19. **Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, and Korbonits M.** The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* 87: 2988–2991, 2002.
20. **Gueorguiev M, Lecoeur C, Meyre D, Weil J, Grossman AB, Froguel P, and Korbonits M.** Ghrelin and growth hormone secretagogue receptor: a role in eating behaviour? *Proc Dig Horm Appetite Energy Bal* OC2, 2003.
21. **Himaya A, Fantino M, Antoine JM, Brondel L, and Louis-Sylvestre J.** The satiety power of dietary fat: a new appraisal. *Am J Clin Nutr* 65: 1410–1418, 1997.
22. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, and Kangawa K.** Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402: 656–660, 1999.
23. **Kovacs EM, Westerterp-Plantenga MS, Saris WHM, Melanson KJ, Goosens I, Geurten P, and Brouns F.** Associations between spontaneous meal initiations and blood glucose dynamics in overweight men in negative energy balance. *Br J Nutr* 87: 39–45, 2002.
24. **Krsek M, Rosicka M, Haluzik M, Svobodova J, Kotrlikova E, Justova V, Lacinova Z, and Jarkovska Z.** Plasma ghrelin levels in patients with short-bowel syndrome. *Endocr Res* 28: 27–33, 2002.
25. **Lu XY, Shieh KR, Kabbaj M, Barsh GS, Akil H, and Watson SJ.** Diurnal rhythm of agouti-related protein and its relation to corticosterone and food intake. *Endocrinology* 143: 3905–3915, 2002.
26. **Lucidi R, Murdolo G, DiLoreto C, DeCicco A, Parlanti N, Fanelli C, Santeusanio F, Bolli GB, and DeFeo P.** Ghrelin is not necessary for adequate hormonal counterregulation of insulin-induced hypoglycemia. *Diabetes* 51: 2911–2914, 2002.
27. **Marmonier C, Chapelot D, and Louis-Sylvestre J.** Metabolic and behavioral consequences of a snack consumed in a satiety state. *Am J Clin Nutr* 70: 854–866, 1999.
28. **Marmonier C, Chapelot D, and Louis-Sylvestre J.** Snacks consumed in a no-hunger state have poor satiating efficiency: influence of snack composition on substrate utilization and hunger. *Am J Clin Nutr* 76: 518–528, 2002.
29. **Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, and Kangawa K.** Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun* 276: 905–908, 2000.
30. **McCowen KC, Maykel JA, Bistrian BR, and Ling PR.** Circulating ghrelin concentrations are lowered by intravenous glucose or hyperinsulinemic euglycemic conditions in rodents. *J Endocrinol* 175: R7–R11, 2002.
31. **Melanson KJ, Westerterp-Plantenga MS, Campfield LA, and Saris WHM.** Appetite and blood glucose profiles in humans after glycogen-depleting exercise. *J Appl Physiol* 87: 947–954, 1999.
32. **Melanson KJ, Westerterp-Plantenga MS, Saris WHM, Smith FJ, and Campfield LA.** Blood glucose patterns and appetite in time-blinded humans: carbohydrate versus fat. *Am J Physiol Regul Integr Comp Physiol* 277: R337–R345, 1999.
33. **Mohlig M, Spranger J, Otto B, Ristow M, Tschop M, and Pfeiffer ARH.** Euglycemic hyperinsulinemia, but not lipid infusion, decreases circulating ghrelin levels in humans. *J Endocrinol Invest* 25: RC36–RC38, 2002.
34. **Murakami N, Hayashida T, Kuroiwa T, Nakahara K, Ida T, Mondal MS, Nakazato M, Kojima M, and Kangawa K.** Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats. *J Endocrinol* 174: 283–288, 2002.
35. **Nakai Y, Hosada H, Nin K, Ooya C, Hayashi H, Akamizu T, and Kangawa K.** Plasma levels of active form of ghrelin during oral glucose tolerance test in patients with anorexia nervosa. *Eur J Endocrinol* 149: R001–R003, 2003.
36. **Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, and Matsukura S.** A role for ghrelin in the central regulation of feeding. *Nature* 409: 194–198, 2001.
37. **Randle PJ.** Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab Rev* 14: 263–283, 1998.
38. **Rolls BJ, Rolls ET, Rowe EA, and Sweeney K.** Sensory-specific satiety in man. *Physiol Behav* 26: 215–221, 1981.
39. **Saad MF, Bernaba B, Hwu CM, Jinagouda S, Fahmi S, Kogosov E, and Boyadjian R.** Insulin regulates plasma ghrelin concentration. *J Clin Endocrinol Metab* 87: 3997–4000, 2002.
40. **Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, and Matsukura S.** Plasma ghrelin levels in lean and obese humans and effect of glucose on ghrelin secretion. *J Clin Endocrinol Metab* 87: 240–244, 2002.
41. **Smith GP.** Introduction to the reviews on peptides and the control of food intake and body weight. *Neuropeptides* 33: 323–434, 1999.
42. **Stunkard AJ and Messick S.** The three-factor eating questionniare to measure dietary restraint, disinhibition, and hunger. *J Psychosom Res* 29: 71–83, 1985.
43. **Sugino T, Hasegawa Y, Kikkawa Y, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K, and Terashima Y.** A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep. *Biochem Biophys Res Commun* 295: 255–260, 2002.
44. **Sugino T, Yamaura J, Yamagishi M, Ogura A, Hayashi R, Kurose Y, Kojima M, Kangawa K, Hasegawa Y, and Terashima Y.** A transient surge of ghrelin secretion before feeding is modified by different feeding regimens in sheep. *Biochem Biophys Res Commun* 298: 785–788, 2002.
45. **Tolle V, Bassant M-H, Zizzari P, Poindessous-Jazat F, Tomasetto C, Epelbaum J, and Bluet-Pajot M-T.** Ultradian rhythmicity of ghrelin secretion in relation with GH, feeding behavior, and sleep-wake patterns in rats. *Endocrinology* 143: 1353–1361, 2002.
46. **Tschop M, Smiley DL, and Heiman ML.** Ghrelin induces adiposity in rodents. *Nature* 407: 908–913, 2000.
47. **Tschop M, Statnick MA, Suter TM, and Heiman ML.** GH-releasing peptide-2 increases fat mass in mice lacking NPY: indication for a crucial mediating role of hypothalamic agouti-related protein. *Endocrinology* 143: 558–568, 2002.
48. **Tschop M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, and Folwaczny C.** Post-prandial decrease of circulating human ghrelin levels. *J Endocrinol Invest* 24: RC19–RC21, 2001.
49. **Weigle DS, Cummings DE, Newby PD, Breen PA, Frayo RS, Matthys CC, Callahan HS, and Purnell JQ.** Roles of leptin and ghrelin in the loss of body weight caused by low-fat, high-carbohydrate diet. *J Clin Endocrinol Metab* 88: 1577–1586, 2003.
50. **Woods S, Seeley R, Porte D, and Schwartz M.** Signals that regulate food intake and energy homeostasis. *Science* 280: 1378–1383, 1998.
51. **Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, and Bloom SR.** Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* 86: 5992–5995, 2001.
52. **Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, and Bloom SR.** Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 50: 2540–2547, 2001.
53. **Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, and Bloom SR.** The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 141: 4325–4328, 2000.