Scheduled feeding results in adipogenesis and increased acylated ghrelin

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Ghrelin, the only orexigenic peptide secreted by the stomach and small intestine, plays an essential role in short-term energy balance by regulating appetite and meal-anticipatory behaviors (7, 17, 20). Ghrelin is also involved in long-term energy homeostasis, affecting a diverse array of processes involved in fuel utilization, all of which promote a positive energy balance (12, 22). Ghrelin directly promotes adipogenesis in most (3, 9, 40, 41, 45), but not all (52, 53), studies. In the plasma, ghrelin circulates under various forms: an unacylated peptide whose physiological significance through an unknown receptor is not clearly characterized and several acylated peptides (octanoyl, decanoyl, and des-Q14-ghrelin), that bind to the GHS-R1a and stimulate appetite (23, 24). In humans, total and acylated ghrelin circulating levels rise before meals and are suppressed following food consumption (13, 29). In ad libitum-fed rats, total and acylated ghrelin are secreted with an ultradian pulsatility in close correlation with feeding episodes (42). Initially thought to be a signal to initiate meals, preprandial ghrelin rise may occur as a conditioned response and is likely to be involved in complex processes associated with the preparation of meals (21). Under restricted feeding, total ghrelin secretion increases in anticipation of a scheduled meal (16, 37), and the amplitude of these preprandial secretory peaks depends on the feeding frequency (38). Recent studies have shown that the acylated form is likely to be modulated in these different feeding conditions (7, 29). Therefore, we hypothesized that changes in acylated ghrelin pulsatile secretion could be the missing link between the fixed-feeding schedule and the observed shift toward adipogenesis.

In the present study, we investigated the metabolic changes in rats fed a fixed-feeding schedule of three meals a day. The whole body composition was evaluated by DEXA analysis and compared with ad libitum-fed rats and to animals daily injected with ghrelin. Feeding patterns were analyzed over a 24-h period and compared with circadian activity and pulsatile acylated ghrelin variations within the meal time period.
MATERIALS AND METHODS

Animals

Four-week-old (75–90 g body wt) male Wistar rats (Janvier, Le Genest Saint Isle, France) were housed individually in cages under standardized conditions (room temperature of 21 ± 0.5°C, 40–60% relative humidity, inverse 12:12-h light-dark cycle with lights on at 2300), following European guidelines on animal care. The rats had free access to water. Complete rodent food (Sniff; Bioservices, Schaijk, The Netherlands) was provided as described in Experimental design. Animals were allowed to adapt to laboratory conditions, handling, and intraperitoneal (ip) injections for 3 wk prior to the experimental sessions. All research protocols were approved by the Ethics Commission for Experimental Use of Animals of the K. U. Leuven.

Drugs, Chemical Reagents and Other Materials

Octanoylated ghrelin was purchased from Genscript (Piscataway, NJ), dissolved in saline with 0.05% bovine albumin and stored at −20°C. Xylazine (XYL-M 2%, xylazine hydrochloride; Arendonk, Belgium) and ketamine (Eurivot Animal Health, Bladel, The Netherlands) were used as anaesthetics during DEXA analysis. Isoflurane anesthesia (Baxter, Maurepas, France) was used to catheterize the rats.

Experiment 1: DEXA Study

Long-term procedure. The rats were divided into three groups of 10, based on their body weight and total daily food intake. One group received a daily ip bolus injection of ghrelin (100 μg/kg body wt in 0.5 ml of saline) 15 min before dark onset for 14 consecutive days. The control group simultaneously received an ip injection of 0.5 ml saline. Both groups had free access to food, except for 1 h before dark onset, during which the mangers were withdrawn to synchronize the onset of food intake, measured 3, 6, and 23 h after dark onset. A third group was scheduled fed; the rats were not fed ad libitum but received their daily caloric intake in three meals at fixed time points: at dark onset and at 3 and 6 h after dark onset (Supplementary Table S1; see this article online at the Journal website). The schedule-fed rats consumed exactly the same amount of food as that consumed on the previous day by the control-treated rats but with a different mealtime distribution; consequently, the schedule-fed group was started a day later. The first meal, given at dark onset, was significantly reduced in size, on average 16%, compared with control intake in the same time period. The second meal was not reduced in size, and the last meal was significantly increased in size an average 8% (Suppl. Table S1). Total daily caloric intake of the schedule-fed rats was, as such, not reduced compared with control daily intake; only the mealtime distribution was different. Schedule-fed animals were ip injected with 0.5 ml of saline so as to create the same injection-induced stress. Body weights were measured daily 0.5 h before drug injection, and the amount of ghrelin injected was adapted to body weight changes.

Blood sampling. After drug injection on day 15, rats were anesthetized, and blood samples were collected at dark onset (1100) and stored in appropriate collection tubes (Vacutainer). Serum samples were centrifuged, aliquoted, and frozen at −80°C until analysis.

Blood analysis. Serum glucose and triacylglycerol concentrations were determined using the VetTest 8008 analyzer (Idexx Laborato- ries). The apparatus is based on dry-chemical technology and colorimetric reaction. Sample analysis is carried out on selective testing pads were excised and weighed. Several procedures were used to assess the reliability and validity of the DEXA measurements. First, the body weights of the rats were measured using a balance with dynamic animal weighing function (Sartorius, accuracy to 0.1 g) and compared with those calculated by the Hologic Discovery A. The Pearson correlation coefficient for the baseline and final body weights were, respectively, r = 0.89 (n = 38) and r = 0.92 (n = 38). Furthermore, the overall means (± SE) were almost identical (baseline body wt 232.0 ± 1.9 g for the balance vs. 232.5 ± 2.1 g for the DEXA analysis; final body weights 324.2 ± 2.8 g for the balance vs. 325.7 ± 2.6 g for the DEXA analysis). Second, randomly chosen subsets were analyzed in triplicate with the Hologic Discovery A. The precision or within-subject repeatability of the DEXA measurements was expressed as a coefficient of variation (standard deviation/average × 100); CVbaseline = 0.1%, CVfinal = 0.2% for the global mass estimations, CVbaseline = 6.2%, CVfinal = 3.0% for the global fat mass. Third, the perirenal body fat of each rat was excised and weighed during dissections on the day after the final DEXA analysis. The correlation between the perirenal fat mass determined by dissection and by DEXA (% global fat gain) was r = 0.69 (n = 38).

To define the fat distribution in different regions, whole body scans were divided into fixed regions, and a DEXA analysis was performed on every separate region by the software included in the machine (Suppl. Fig. S1 and Suppl. Table S2).

Experiment 2: Analyses of Meal Pattern and Home Cage Activity in Schedule-Fed Animals

Nine rats, not previously in another experiment, were schedule-fed for 14 consecutive days as described in experiment 1, and their feeding and activity patterns were compared with ad libitum-fed saline-injected controls (n = 9). Each rat was placed twice in the special food and water monitoring TSE cages (TSE Labmaster Sys- tem, Bad Homburg, Germany) to adapt to this new environment. In all cages, water was available ad libitum. On the 13th treatment day, each rat was placed in an individual Labmaster TSE cage for two consecu- tive days. On the first day, rats received the treatment, but no data collection was performed. On the second, i.e., the 14th day of treatment, the drinking and feeding behaviors were recorded from 1100 till 1000 the next day by means of high-precision sensors attached to the top of the cage lids. Water and food consumptions were monitored continuously by amount and time. Meal patterns were analyzed by processing the data, using the following definition of a meal: a meal is the consumption of >0.3 g of food, separated from the next feeding episode by at least 10 min (8, 26). Recorded meals...
accounted for ~96% of daily food intake. For each rat, latency to eat (min), meal size (g), meal duration (min), total duration of eating (min), total food intake (g), eating rate (g/min), and meal frequency were determined. The eating rate (g/min) was calculated for each separate meal by dividing meal size by meal duration. The intermeal intervals (min), which is the time between the end of one meal and the onset of the next meal, were also determined. Food efficiency was calculated with the following formula: 100 × weight gain (g)/food intake (g).

Besides food and water intakes, circadian activity was also monitored in the TSE cages. The ambulatory movement in the X dimension (Xa) was used as a parameter for spontaneous activity. The detection of movement is based on sensitive sensors integrated into a measuring platform that supports the cage. Load changes caused by the animal’s movements are converted into time-integrated pulses and accumulated in activity counters.

**Experiment 3: Effect of Meal Feeding Patterns on Circulating Acylated Ghrelin Levels**

Rats with identical specifications as mentioned above were divided into two treatment groups (n = 12): a control group with free access to food and another group that was fed on a fixed-feeding schedule similar to the one of the schedule-fed in previous experiments.

After 18 days, a Silastic cannula was inserted, under isoflurane anesthesia (1.8%, air flow: 500 ml/min), through the right external jugular vein to approach or enter the right atrium and was exteriorized to the back of the neck, as previously described (6). After surgery, the rats were returned to their individual home cages for recovery of 1 day during which they received no further treatment. On that day, the controls had free access to water and food, whereas the meal-fed animals had free access to water only and were maintained on the fixed-feeding regimen. Following the recovery day, blood was sampled (0.2 ml) in conscious, freely moving rats every 20 min from 0900 until 1700. Control rats had free access to food, and fixed-meal fed rats followed their fixed-feeding schedule. Two hours before the sampling period, the distal extremity of the cannulas was connected to a polyethylene catheter filled with 25 IU/ml heparinized saline. Blood samples were collected on EDTA (1 mg/ml) and p-hydroxymercuribenzoic acid (0.36 mg/ml PHMB; Sigma, Saint Quentin Fallavier, France) to avoid ghrelin degradation and immediately centrifuged, and plasma was stored at −20°C until hormone assays. Red cells were reinserted every hour to attenuate hemodynamic modifications. Acylated ghrelin was measured by an in-house immunoenzymatic assay (54), using polyclonal rabbit antibodies made against NH2-terminal ghrelin (kindly provided by Dr. Hosoda, Osaka, Japan) and human ghrelin coupled to acetylcholinesterase (Spibio, Saclay, France) as a tracer. This antiserum does not cross-react with unacylated ghrelin. The sensitivity was 20 pg/ml, and the intra-assay coefficient of variation was 7%.

**Data Analysis and Statistical Procedures**

The results are shown as means ± SE. One-way analysis of variance (ANOVA) with Tukey’s post hoc testing was used to analyse the data of experiments 1 and 2. Significant differences in hormone levels between control and schedule-fed animals were analyzed using an unpaired t-test or z-test against a fixed value. Pulse analysis was performed using Cluster® software with the t-value set to 2 to maintain false-positive rates under 1%. Number of points for a peak and number of points for a nadir were set at 1 and 2, respectively (47).

Approximate entropy (ApEn) was calculated using the MC-ApEn software using R value set to 0.2 and number of MC cycle set to 1,000. ApEn is a model-independent regularity measure that monitors sample-by-sample pattern irregularity. The area under the curve (AUC) was also determined. A P value of <0.05 was considered statistically significant. To analyze the body weight change of the growing rats and the cumulative food intake, the cumulative parameter (Y) was plotted vs. time (X) and was fitted by linear regression. All statistical procedures were carried out using GraphPad Prism (version 4; San Diego, C).

**RESULTS**

**Experiment 1: DEXA Study**

**Food intake.** As described in MATERIALS AND METHODS, schedule-fed rats received their daily food in three meals, respectively at dark onset and at 3 and 6 h after dark onset. The first meal given was significantly reduced compared with control intake (on average 4.6 ± 0.6 vs. 5.5 ± 0.5 g in controls, P = 0.0004), the second meal was equal to control intake (on average 6.2 ± 0.3 vs. 6.0 ± 0.7 g controls, P = 0.4425), and the third meal was significantly increased compared with control intake (on average 18.0 ± 2.1 vs. 16.7 ± 1.2 g, P = 0.0014). Despite the different distribution of the food intake over the three meals, the total daily food intake of the schedule-fed rats was not significantly different from that of the control rats (Fig. 1).

Compared with controls, ghrelin administration significantly increased food intake in the 3, 6, and overall food intake of, respectively, 28.9% (P < 0.001), 19.5% (P < 0.001), and 7.5% (P < 0.001) (Fig. 1A). The cumulative food intake (Fig. 1B) displayed a linear relationship for all treatment groups (control: slope = 28.00 ± 0.22, P < 0.0001; schedule fed: slope = 28.30 ± 0.29, P < 0.0001; ghrelin: slope = 30.27 ± 0.26, P < 0.0001). The slope of the cumulative food intake of the ghrelin-treated rats was significantly different from those of other two groups in which no differences were found.

Food efficiency was significantly decreased in the schedule-fed rats compared with control and ghrelin-treated rats (P < 0.0001; control: 23.5 ± 0.6, schedule-fed: 19.3 ± 0.6, ghrelin: 22.4 ± 0.5).

**Body weight evolution.** Linear regression analysis of cumulative body growth (Fig. 2) revealed a linear relationship between growth and time for the three treatments (control: slope = 6.36 ± 0.13, P < 0.0001; schedule-fed: slope = 5.55 ± 0.14, P < 0.0001; ghrelin: slope = 6.65 ± 0.15, P < 0.0001). The slope for the schedule-fed animals was significantly lower than the control and ghrelin-treated group slopes (P < 0.0001). No significant difference in slope was observed between the two latter groups.

**Serum blood variables.** Serum glucose concentrations were not affected by scheduled feeding or ghrelin injections (Table 1). Triacylglycerol concentration was significantly decreased in the schedule-fed group compared with the ghrelin-treated but not with the control group. NEFA and corticosterone levels were significantly and equally increased in both the ghrelin-treated and the schedule-fed rats.

**Whole body composition using DEXA analysis.** Baseline DEXA data indicated that the body compositions of all groups were equal before the start of the experiment (data not shown). Schedule-fed rats gained less global mass than ghrelin-treated and control rats (Fig. 3A). Compared with controls, the change in fat mass (g) over the 14-day treatment period was significantly higher in both schedule-fed and ghrelin-treated rats (P = 0.005; Fig. 3B). Moreover, the weight of the dissected perirenal fat pad of the schedule-feds (4.78 ± 0.2 g) and that of the ghrelin-treated rats (4.64 ± 0.2 g) was significantly increased compared with controls (3.52 ± 0.2 g). The schedule-fed rats
gained significantly less lean mass than the control and ghrelin-treated rats, which displayed an equal gain in lean mass (P < 0.009; Fig. 3C). Gain in BMC was similar in the three groups (Fig. 3D). Schedule-fed rats, but not ghrelin-injected rats, had a higher fat gain/lean gain ratio (P = 0.0010; Fig. 3E).

After 14 days of treatment, the fat-to-body weight ratio was significantly increased in schedule-fed rats compared with control and ghrelin-treated animals (P < 0.001; control: 2.9 ± 0.2; schedule-fed: 4.1 ± 0.2; ghrelin-treated 3.3 ± 0.2).

Division of the DEXA scans in subregions (Suppl. data, Suppl. Fig. S1) showed that, compared with control level, the fat mass gain of the schedule-fed rats was significantly higher in the trunk (R1), abdominal (R2), and gonadal (R3) areas (Suppl. Table S2). Ghrelin-treated rats gained significantly more fat in R1 than controls. Nevertheless, the distribution of the total amount of fat over the subregions was not different between the treatment groups. Compared with controls, the schedule-fed rats gained less lean mass in R3. The ghrelin-treated rats, on the other hand, gained significantly more lean mass in R4 and R5 compared with control lean mass gain. Nevertheless, the distribution of the total amount of lean mass was also not different after treatment.

Experiment 2: Analyses of Meal Pattern and Home Cage Activity in Schedule-Fed Animals

During the dark phase, the schedule-fed rats consumed significantly fewer meals than the ad libitum-fed control rats (Table 2 and Fig. 4). In the former case, however, meals were significantly larger in size and duration; therefore, total food intake (g) and duration of eating (min) during the dark phase were equal for both groups. Moreover, the mean eating rate (g/min) did not differ with treatment. The schedule-fed rats had a lower latency to eat their first meal, although this was not significant.

During the light phase, the schedule-fed rats could not eat, since they had eaten the complete amount of food provided in each meal in only a few hours during the dark period. As a result, their meal pattern was markedly different from that of the control rats, which did still eat during the light phase.

Schedule-fed rats increased their ambulatory activity in anticipation to each scheduled meal, resulting in increased global activity over the 24-h light-dark period (Fig. 5). The overall changes resulted from an increase in activity during the dark period (11,739 ± 626 and 15,462 ± 1,007 counts, P = 0.008 in control vs. schedule-fed rats), whereas activity during the lights-on period was unchanged (2,572 ± 402 and 2,851 ± 353 counts in control vs. schedule-fed rats).

Experiment 3: Effect of Meal Feeding on Circulating Ghrelin Level

As displayed in Fig. 6 and Table 3, circulating ghrelin levels in schedule-fed rats were increased during the whole sampling period. Pulsatility analysis of individual secretory patterns showed that mean and nadir ghrelin levels were significantly higher in schedule-fed rats compared with controls, whereas
peak amplitude, frequency, and interval were not different (Table 3). Similar ApEn values indicated that both patterns displayed an equal degree of orderliness.

**DISCUSSION**

The present study shows that scheduled feeding, associated with intermittent fasting periods, results in a slower growth rate, increased adiposity compared with ad libitum-fed animals despite an identical daily caloric intake. We demonstrated that this repartitioning effect, also observed in the ghrelin-treated rats, is associated with an increase in endogenous acylated ghrelin secretion.

DEXA analysis and dissections revealed that the slower growth rate observed in schedule-fed rats was due to an increased fat mass and a decreased lean mass. In addition, compared with control levels, schedule-fed animals displayed increased plasma leptin levels (data not shown) and elevated circulating FFA together with decreased triacylglycerol levels. These observations are congruous with the observed shift toward adipogenesis and indicate an enhanced lipogenesis instead of fat oxidation.

**Table 1. Effects of scheduled feeding or daily injection of ghrelin (100 µg/kg ip) or saline for 14 consecutive days on several serum parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 10)</th>
<th>Schedule Fed (n = 8)</th>
<th>Ghrelin Treatment (n = 10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/l</td>
<td>9.0 ± 0.2</td>
<td>9.6 ± 0.3</td>
<td>9.00 ± 0.8</td>
<td>0.6861</td>
</tr>
<tr>
<td>Triacylglycerol, mmol/l</td>
<td>1.53 ± 0.1&lt;sup&gt;a&lt;/sup,b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.90 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.71 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0205</td>
</tr>
<tr>
<td>Nonesterified fatty acid, mmol/l</td>
<td>0.51 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.69 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0031</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>224 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>378 ± 20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>337 ± 23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are expressed as means ± S. Different superscript letters indicate statistically significant differences within rows. P < 0.05; ANOVA, followed by Tukey’s multiple comparison test.

Fig. 3. Effects of scheduled feeding (n = 8) or daily ip injection of ghrelin (n = 10) for 14 consecutive days on global mass gain (g; A); global fat mass gain (g; B); global lean mass gain (g; C); global bone mineral content (BMC; g; D) and fat gain (g)/lean gain (g; E). Values are expressed as means ± SE. Statistical differences are indicated by different letters and determined by 1-way ANOVA followed by Tukey’s multiple comparison tests. P < 0.05 is considered statistically different.
Table 2. Mean meal parameters after scheduled feeding in juvenile rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Schedule Fed</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean meal size, g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night</td>
<td>2.5 ± 0.2b</td>
<td>5.4 ± 0.3a</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Day</td>
<td>1.0 ± 0.2</td>
<td>0.0 ± 0.0b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mean meal duration, min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night</td>
<td>17.8 ± 1.5b</td>
<td>34.8 ± 3.5a</td>
<td>0.0004</td>
</tr>
<tr>
<td>Day</td>
<td>6.2 ± 1.3b</td>
<td>0.0 ± 0.0b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mean food intake, g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night</td>
<td>25.3 ± 0.9</td>
<td>25.5 ± 0.2</td>
<td>0.8424</td>
</tr>
<tr>
<td>Day</td>
<td>2.0 ± 0.5b</td>
<td>0.0 ± 0.0b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mean total duration of eating, min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night</td>
<td>182.7 ± 13.8</td>
<td>169.3 ± 19.2</td>
<td>0.7909</td>
</tr>
<tr>
<td>Day</td>
<td>13.1 ± 4.1</td>
<td>0.0 ± 0.0b</td>
<td>0.0008</td>
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<tr>
<td>Mean eating rate, (g/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night</td>
<td>0.17 ± 0.0</td>
<td>0.17 ± 0.0</td>
<td>0.7920</td>
</tr>
<tr>
<td>Day</td>
<td>0.17 ± 0.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mean meal frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night</td>
<td>10.7 ± 0.9a</td>
<td>4.9 ± 0.3b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Day</td>
<td>2.0 ± 0.5a</td>
<td>0.0 ± 0.0b</td>
<td>&lt;0.0001x</td>
</tr>
<tr>
<td>Mean latency to first meal, min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Night</td>
<td>3.7 ± 0.8</td>
<td>1.8 ± 0.8</td>
<td>0.1005</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. P values were obtained with unpaired two-tailed t-test or with a z-test against fixed value 0 whenever the mean and SE of a group were equal to 0. P < 0.05 is considered statistically different and is displayed in boldface. Different superscript letters (a, b) indicate statistically significant differences within rows.

In accord with several other reports (40, 41, 45, 46), ghrelin treatment resulted in increased food intake, leading to a significant fat mass gain together with an equal lean mass compared with controls. Serum FFA levels in the ghrelin-treated animals were significantly increased compared with controls, and triacylglycerol levels also tended to increase, confirming the observed increased fat mass. The observations in the ghrelin-treated group and those in the schedule-fed animals displayed some remarkable similarities. Both groups showed an identical increase in FFA, a similar increase in perirenal fat pad weight, and an identical fat gain in the truncal region. The similarity in fat mass gain between the ghrelin-treated group and the schedule-fed group suggests that chronic elevated ghrelin levels in growing rats may potentially be responsible for the observed repartitioning effect. Although both groups invested in fat deposition, their growth rates, lean mass, and food efficiency were very different. This resulted in a significant increase in the fat/lean ratio in schedule-fed but not in ghrelin-treated rats. Although this difference in body composition suggests that the observed changes in schedule-fed animals are not exclusively due to ghrelin, these discrepant effects may also be explained by the fact that ghrelin-treated rats had free access to food, consequently leading to a higher food consumption, whereas the caloric intake of the schedule-fed rats was not different from that of the control group. In addition, comparison between these two groups is complicated by the fact that exogenous ghrelin injections once a day cannot mimic the pulsatile increase in endogenous ghrelin secretion and results in higher plasma concentrations than what can be seen in schedule-fed animals.

Previous observations in rats on scheduled feeding without caloric restriction displayed similar metabolic changes as those observed in the schedule-fed rats in the present experiments (2, 4, 10, 11). These reports, together with the observations in the ghrelin-treated animals, led us to evaluate whether the metabolic observations in the schedule-fed animals are a consequence of the scheduled feeding. A meal pattern analysis revealed that the schedule-fed rats anticipated the arrival of each scheduled meal and displayed a nonstop eating behavior each time they were given a portion of food, resulting in an intermittent period of nonfeeding or short fasting before receiving the next scheduled meal. This was accompanied by an increase in locomotor activity corresponding with the timing of scheduled meals.

The presence of intermittent food deprivation periods in the feeding pattern of schedule-fed animals might as such have resulted in increased ghrelin secretion. Ghrelin is upregulated upon fasting (1, 27, 30, 35, 44, 45). Like total ghrelin, acylated ghrelin, the form that specifically binds to GHS-R1a, is also secreted with an ultradian pulsatility in rats (42, 54) but the ghrelin, the form that specifically binds to GHS-R1a, is also secreted with an ultradian pulsatility in rats (42, 54) but the presence of intermittent food deprivation periods in the feeding pattern of schedule-fed animals might as such have resulted in increased ghrelin secretion. Ghrelin is upregulated upon fasting (1, 27, 30, 35, 44, 45). Like total ghrelin, acylated ghrelin, the form that specifically binds to GHS-R1a, is also secreted with an ultradian pulsatility in rats (42, 54) but the impact of fasting and meal pattern on the acylated hormone in rats is not well characterized yet.

Acylation, which depends on GOAT (ghrelin O-acyltransferase) activity and nutrient availability (25), is essential for
many of the physiological actions of the peptide like the stimulatory effects on food intake, GH secretion, and adipogenesis (43, 45). In addition, acylated ghrelin has a short half-life in plasma (23, 54) and is rapidly degraded by acyl-protein thioesterase (APT1)/lysophospholipase-1 (34) into desacyl ghrelin. Like acylated ghrelin, desacyl ghrelin was also shown to drive adipogenesis in bone marrow (41), but to our knowledge its role in white adipose tissue per se has not been demonstrated. In the present study, we did not measure the desacyl form to test a possible involvement of endogenous desacyl ghrelin or acyl/desacyl ghrelin ratio on adipogenesis.

To evaluate the impact of scheduled feeding on acylated ghrelin pulsatile secretion, the circulating ghrelin levels were monitored during the time period when animals were fed. Ghrelin secretory episodes were located at the time points of the customary meals of the schedule-fed animals, confirming that the imposed feeding schedule induced these changes in acylated ghrelin secretion. Moreover, these observations demonstrate that a component of ghrelin secretion results from anticipatory effects of habitual meals, as previously described with total ghrelin by Drazen et al. (16). The simultaneous occurrence of ghrelin peaks and scheduled meals also confirms ghrelin’s direct role in the preparation of the gastrointestinal tract for digestion and nutrient uptake (33). Moreover, rats on this scheduled feeding display an increased basal ghrelin secretion, resulting in increased AUC compared with ad libitum-fed animals. Interestingly, a transient ghrelin surge occurs before each meal in sheep on a restricted feeding regimen, and the amplitude of this ghrelin surge before scheduled feeding is higher in sheep fed twice daily than in sheep fed four times daily or fed ad libitum, resulting in elevated AUC (38).

Ghrelin contributes to the communication between the periphery and the central regulatory networks to maintain energy homeostasis (12, 15), as it signals the brain about the body’s nutritional status, sensing caloric deficit or nutrient availability (25). Moreover ghrelin acts as an anabolic signal molecule by provoking a central response to store the available energy as fat and reduce energy expenditure, as such increasing energy efficiency (12, 15, 45). Recently, acylated ghrelin was proposed to have a role in meal-anticipatory processes rather than simply being a signal of meal initiation, as the anticipatory locomotor activity preceding a scheduled meal was attenuated in GHS-R-deficient mice (7). Consistent with these observations.

**Fig. 5.** Spontaneous ambulatory activity patterns of ad libitum-fed controls (A) and schedule-fed animals (B) and mean spontaneous ambulatory activity over the 24-h period (C). Gray area corresponds to lights-off phase. Dotted lines in B and C indicate time points of food disposal into mangers of schedule-fed animals. Values are expressed as means ± SE.

**Fig. 6.** Effects of scheduled feeding regimen of 3 meals a day on acylated ghrelin secretion in freely moving rats. Dotted lines indicate fixed time points on which meals were daily offered to schedule-fed animals. Gray area corresponds to lights-off phase. Values are depicted as means ± SE.

**Table 3. Analysis of acylated ghrelin pulsatility parameters in schedule-fed rats**

<table>
<thead>
<tr>
<th></th>
<th>Control (Free Fed)</th>
<th>Schedule Fed</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Mean levels, pg/ml</td>
<td>3.794 ± 258.4</td>
<td>4.549 ± 180.1</td>
<td>0.027</td>
</tr>
<tr>
<td>AUC</td>
<td>2.1·10⁶ ± 0.40·10⁶</td>
<td>2.5·10⁶ ± 0.30·10⁶</td>
<td>0.029</td>
</tr>
<tr>
<td>Nadir, pg/ml</td>
<td>2.177 ± 266</td>
<td>3.100 ± 157</td>
<td>0.005</td>
</tr>
<tr>
<td>Peak amplitude, pg/ml</td>
<td>4.754 ± 428</td>
<td>5.542 ± 210</td>
<td>0.081</td>
</tr>
<tr>
<td>Peak frequency, no.</td>
<td>3.7 ± 0.3</td>
<td>4.3 ± 1.0</td>
<td>0.226</td>
</tr>
<tr>
<td>Peak interval, min</td>
<td>104.8 ± 7.8</td>
<td>112.2 ± 12.7</td>
<td>0.680</td>
</tr>
<tr>
<td>ApEn</td>
<td>0.95 ± 0.07</td>
<td>0.96 ± 0.02</td>
<td>0.784</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. ApEn, approximate entropy. P values were obtained by t-test. P < 0.05 is considered statistically different and is displayed in boldface.
sions, the rise in plasma acylated ghrelin levels before the scheduled meals as observed in the present study was associated with increased spontaneous activity in schedule-fed animals. As such, increased adiposity in this model is not the consequence of reduced locomotor activity but may likely result from decreased basal metabolic activity. On the basis of this literature and the observations in the schedule-fed animals, it appears that the observed shift toward adipogenesis is a result of the particular imposed feeding schedule of three meals a day, which resulted in a feeding pattern with intermittent periods of food deprivation, anticipatory effects on the habitual meals, and increased foraging. The presence of these periods of food deprivation, together with the anticipatory responses on the habitual meals, is linked to the increased circulating acylated ghrelin levels. The increased acylated ghrelin levels in the schedule-fed animals can as such be interpreted as a biological feedback mechanism to cope with the forced feeding schedule, since it prepared the body for even scarcer food periods by increasing energy efficiency and investing in fat deposition instead of lean mass.

Although schedule-fed rats display increased ghrelin secretion, we cannot rule out that this increase may not be the primary factor responsible for the fat deposition. Pathologically increased circulating cortisol concentration in humans is associated with central obesity (32). In genetic models of obesity, excessive glucocorticoid tone has also been observed but is not considered to be the primary mechanism for obesity (36), and reductions of circulating corticosterone by adrenalectomy does not correct diet-induced obesity (31). In the current study, the increase in corticosterone levels is unlikely to be the primary mechanism for the observed metabolic changes in schedule-fed animals. Indeed, we did not observe defects associated with hypercorticoteronomia, like short stature or central obesity. The fat gain was equal for all subareas and was not limited to the abdominal area. Chronic ghrelin treatment has been shown to induce abdominal obesity (resulting either from retroperitoneal or epididymal depots rather than visceral) (14). In the current study, we observed increased adipose depots in the abdominal area as well but also in the trunk or leg areas in both ghrelin-treated and schedule-fed rats, suggesting a global rather than an abdominal fat distribution. Comparison of both studies is difficult due to different methodological approaches, including the method to measure adipose depot, the dose, and mode and/or duration of administration. In addition, the impact of ghrelin on adiposity may be different in growing and adult animals.

In conclusion, this scheduled feeding model points out the delicate role of meal frequency in adiposity. It shows that it is not only the amount of caloric intake that matters, but also that the mealtime contribution by itself severely affects metabolism. As forced schedule feeding may affect metabolism, the use of pair-fed groups in metabolic studies should be interpreted with caution.

Interestingly, this schedule-fed group provides an efficient animal model to further study the impact of elevated ghrelin on energy metabolism or on other parameters without exogenous ghrelin administration. Although our observations could not be directly extrapolated to human obesity syndromes, an animal model displaying a continuous rise in endogenous ghrelin levels might provide an effective investigative tool for the study of dysregulated energy balance associated with increased ghrelin secretion.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


SCHEDULED FEEDING INDUCES ADIPOGENESIS


