Overeating of palatable food is associated with blunted leptin and ghrelin responses

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Abstract

Palatable food is rich in fat and/or sucrose. In this study we examined the long-term effects of such diets on food intake, body weight, adiposity and circulating levels of the satiety peptide leptin and the hunger peptide ghrelin. The experiments involved rats and mice and lasted 5 weeks. In rats, we examined the effect of diets rich in fat and/or sucrose and in mice the effect of a high fat diet with or without sucrose in the drinking water. Animals fed with the palatable diets had a larger intake of calories, gained more weight and became more adipose than animals fed standard rat chow. Fasted animals are known to have low serum leptin and high serum ghrelin and to display elevated serum leptin and lowered serum ghrelin postprandially. With time, a sucrose-rich diet was found to raise the fasting level of leptin and to lower the fasting level of ghrelin in rats. A fat-rich diet suppressed serum ghrelin without affecting serum leptin; high sucrose and high fat in combination greatly reduced serum ghrelin and raised serum leptin in the fasted state. The mRNA expression of leptin in the rat stomach was up-regulated by sucrose-rich (but not by fat-rich) diets, whereas the expression of ghrelin seemed not to be affected by the palatable diets. Mice responded to sucrose in the drinking water with elevated serum leptin (fasted state) and to all palatable diets with low serum ghrelin. The expression of both leptin and ghrelin mRNA in the stomach was suppressed in fasted mice that had received a high fat diet for 5 weeks. We conclude that the expression of leptin mRNA in stomach and the concentration of leptin in serum were elevated in response to sucrose-rich rather than fat-rich diets, linking leptin with sucrose metabolism. In contrast, the expression of ghrelin and the serum ghrelin concentration were suppressed by all palatable diets, sucrose and fat alike. In view of the increased body weight and adiposity neither elevated leptin nor suppressed ghrelin were able to control/restrain the overeating that is associated with palatable diets.

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1. Introduction

The incidence of obesity in the world is on the increase. Obesity is thought to be responsible for a number of disorders, such as type-2 diabetes and atherosclerosis. Much of the current increase in obesity worldwide can be ascribed to the easy access to palatable food, i.e., diets rich in fat and/or sugar.

Fat is claimed to cause overeating, and sucrose (liquid sucrose in particular) has recently been reported to do the same \cite{1}. The purpose of the present study was to investigate the effects of dietary fat and sucrose, both in solid and liquid form, on body composition and on the circulating concentrations of two important appetite-controlling hormones, namely ghrelin and leptin.

Ghrelin is a gastric hormone, known to stimulate the release of growth hormone \cite{2}. It has been claimed to initiate food intake; a prolonged treatment with ghrelin causes increased body weight \cite{3–8}. These effects are thought to be mediated by neuropeptide Y (NPY) and Agouti-related protein, mobilized from neurons in the
Arcuate nucleus [3,4,7,9]. Serum ghrelin concentrations increase upon fasting and decrease in response to food intake; the signalling pathways that control the secretion of ghrelin from the ghrelin cells in the oxyntic mucosa have not yet been identified [5,10–13]. Dietary glucose and fat have been shown to lower circulating ghrelin levels, whereas a low-protein diet has been reported to raise serum ghrelin [14–18].

Leptin is produced and synthesized by white adipose tissue [19] and by the chief cells of the stomach [20]. Hyperleptinemia (induced by infusion of adenovirus carrying the rat leptin cDNA) causes visible body fat to disappear [21], probably because of increased energy expenditure [22–25]. Centrally [23] and peripherally [26] administered leptin suppresses food intake; sustained treatment with leptin causes weight loss. Serum leptin increases after feeding and is thought to induce satiety by interfering with the release of neuropeptides, such as NPY [27,28] and proopiomelanocortin-derived peptides [29,30], from neurons in the hypothalamus. The role of leptin during high fat/high carbohydrate feeding has been investigated in several studies [31–33]. Also the regulation of leptin mRNA expression in white adipose tissue has been examined previously [34,35]. In this study, we measured serum concentrations (fasted and postprandial levels) and the gastric mRNA expression of leptin and ghrelin in rats maintained on palatable diets for 5 weeks. We also examined the effect of fat and of sucrose in the drinking water on the serum concentrations and gastric mRNA expression of leptin and ghrelin in mice.

2. Materials and methods

2.1. Animals and diets

Female Sprague–Dawley rats and NMRI mice (B&K, Sollentuna, Sweden) were used. Upon arrival to the laboratory they were given free access to standard rat chow (R36, Lactamin, Kimsta, Sweden). A week later, they were divided into four groups and put on an 8 h-feeding schedule (between 8 a.m. and 4 p.m.). At this point, the rats weighed 200–225 g and the mice 25 g. Each group then received a special diet for 5 weeks. As a consequence of the introduction of the strict 8 h-feeding schedule, all animals (all diets including the standard rat chow) had a slow weight gain for 2 weeks (rats) or actually lost weight (mice). The composition of the diets is described in Table 1. The diets were custom-made in our own laboratory. The animals had free access to tap water at all times and were kept in plastic Macrolon cages (one rat per cage and three mice per cage).

2.1.1. Rats

One group of rats continued on the standard chow (low fat, LF), another group was fed a low fat, high sucrose diet (LFS), the third group received a high fat, diet (HF) and the fourth group was fed a high fat, high sucrose diet (HFS). One day, 15 days and 35 days after the start of the experiment, blood was drawn from the tail. Each group consisted of 5 animals.

2.1.2. Mice

One group continued receiving the LF diet and water (LF), another group received the LF diet and a 23% sucrose solution instead of water (LF+S), a third group received the HF diet and water (HF) and the fourth group was offered the HF diet and sucrose in the drinking water (HF+S). Each group consisted of 6 animals.

The experiments described were approved by the Local Animal Welfare Committee, Lund, Sweden.

2.2. Measurement of food intake and drink consumption

2.2.1. Daily food intake

The daily food intake was monitored throughout the study. The food was weighed before it was given to the animals and again at the end of the day when it was removed from the cage. The daily food intake was calculated as the difference between the amount of food given and the amount of food removed.

2.2.2. Daily drink consumption

Bottles containing water or sucrose solution were filled on a daily basis with a known volume. The following morning the volume of the remaining liquid was measured. Daily drink consumption was calculated as the difference between the volume given and the volume that remained.
2.3. Rat study

At the start of the study, the rats were fasted overnight and placed in Bollman cages for blood sampling. Blood was drawn from the tail and serum was stored at −20 °C until analysis. The rats were returned to their cages, refed for 3 h (between 9 and 12 in the morning) and then subjected to renewed blood sampling. After 2 and 5 weeks on the different diets, blood was again collected in the same way. At the end of the study (5 weeks) the refed rats were killed by exsanguination from the abdominal aorta under anesthesia (ketamin, 100 mg/kg and xylazin, 20 mg/kg). The acid-producing part of the stomach (fundus) was dissected out, frozen in liquid nitrogen and stored at −80 °C until analysis. Serum was kept at −20 °C.

2.4. Mouse study

At the end of the study (5 weeks), the mice were fasted overnight, anesthetized (ketamin and xylazin) and decapitated. Blood was collected from the neck and serum was stored at −20 °C until analysis. White adipose tissue (mesenteric, retroperitoneal, parametrial, perirenal and inguinal fat pads) was dissected out and weighed. The fundus was also dissected out and immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Serum was collected and serum was stored at −20 °C.

2.5. Techniques of molecular biology

2.5.1. Isolation of RNA and Northern blot

Total RNA was extracted from the fundus using acid guanidine thiocyanate—phenol—chloroform extraction as described previously [36]. The quality and concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm; \( A_{260}/A_{280} > 1.7 \) was thought to indicate sufficient purity. 20 μg of total RNA was separated on 1.0% agarose–formaldehyde gel and transferred to a nylon membrane (Zeta-Probe, Bio-Rad). The ghrelin and leptin probes were labeled using \( [\alpha^{-32}P] \) deoxycytidine triphosphate (Amersham Pharmacia Biotech UK, Buckinghamshire, UK) according to a Nick Translation kit (Roche Applied Science, Basel, Switzerland). Hybridization was carried out overnight at 65 °C and the membranes were then placed on an imaging plate (BAS-II, Fuji, Tokyo, Japan). The plate was analyzed using an FLA 3000 phosphoimagier (Fuji) and the software Image Reader (Fuji). The signals were quantified using the software Image Gauge (Fuji). As reference for the amount of RNA in each lane, the membranes were rehybridized with an 18S probe at 37 °C overnight (the 18S probe was end-labelled using a polynucleotide kinase and \( [\gamma^{-32}P]ATP (10 \mu Ci/\mu l) \)). All rehybridisation was done after the membranes were stripped in boiling 0.1 x SSC and 0.5 x SDS for 2 x 30 min. The ghrelin and leptin mRNA levels were compared with that of the 18S control and expressed as arbitrary units.

2.5.2. Probe construction

1–2 μg of total RNA from the fundus was used to construct the probes. After an initial reverse transcription reaction at 48 °C for 45 min, the following step-cycle programme was used: denaturation for 2 min at 94 °C followed by 35 cycles at 94 °C for 10 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min, followed by a final elongation at 72 °C for 7 min. All primers were designed on the basis of the sequence available at the NCBI gene bank. For leptin (accession no. NM_013076), the primer sequences were: 5' aggtccaagaagaagacccca 3' (position 28–51) and 5' tgtaagtcagcaggagagtgctgg 3' (position 698–765). For ghrelin (accession no. AB029433) the primer sequences were: 5' ttccagacatctgcctaccacc 3' (position 1–24) and 5' tgtaaagtcagcaggagagtgctgg 3' (position 483–459). All primers were used in a concentration of 50 pmol. The RT-PCR products were run on a 2% agarose gel and then extracted using a gel extraction kit (QIAEX II Gel Extraction kit, Qiagen). All RT-PCR reactions were performed using an Access RT-PCR System from Promega and RT-PCR-and PCR-amplifications were performed on an Eppendorf Master Cycler Personal.

2.6. Methods of immunochemical analysis and X-ray

2.6.1. Radioimmunoassay of serum ghrelin

Ghrelin concentration in the serum was determined using a commercially available kit from Phoenix Pharmaceuticals (Belmont, CA, USA). The tracer was radioiodinated (\( ^{125}I \)) rat ghrelin and ghrelin concentrations were expressed as pmol equivalents of rat ghrelin-28 per liter. The antisera recognizes both octanoylated and des-octanoylated ghrelin-28 but does not recognize des-Gln-ghrelin [37].

2.6.2. Enzyme-linked immunosorbent assay for leptin

Serum leptin concentrations were determined using a commercially available kit from Crystalchem (Downers Grove, IL, USA). Serum leptin concentrations were expressed as pmol equivalents of mouse leptin per liter.

2.6.3. Dual-energy X-ray analysis (DEXA) of body composition in mice

Lean tissue and body fat were assessed (whole mouse) by dual energy X-ray absorptiometry (DEXA; PIXImus, Lunar Corporation, Madison, MI, USA).

2.7. Statistical analysis

All data are presented as mean±SEM. Data were analysed by Student t-test (paired comparisons) or by two-way analysis of variance (ANOVA) with the Fischer post hoc test for individual comparison. The difference was considered significant if \( p<0.05 \).
3. Results

3.1. Rat study

3.1.1. Food intake

Fig. 1A shows the cumulative food intake in grams and Fig. 1B shows the cumulative caloric intake in kcal during the 5 weeks of study. Rats fed LFS consumed larger quantities of food than rats fed LF, whereas those fed HF and HFS consumed less food than LF fed rats (Fig. 1A). Rats fed the palatable diets (HF, HFS and LFS) consumed more calories than those fed LF (Fig. 1B; \( p < 0.001 \) for all dietary groups vs. LF).

3.1.2. Body weight gain and adiposity

After 5 weeks, rats on HF, HFS and LFS had gained more weight than LF rats (Fig. 1C; \( p < 0.05 \) for LF vs. HF and \( p < 0.01 \) for LF vs. HFS). Fat pads (mesenteric, retroperitoneal, parametrial, perirenal and inguinal) were dissected out and weighed. The pooled weights of the different fat depots are shown in Fig. 1D, which also illustrates the finding that rats given diets rich in fat and/or sucrose (fat in particular) had more adipose tissue than LF rats (Fig. 1D; \( p < 0.05 \) for all dietary groups vs. LF), although there was no linear relationship between caloric intake and adiposity (compare Fig. 1B and D).

3.1.3. Serum leptin

Fig. 2 shows the effect of the different diets on the serum leptin concentration during both fasting and postprandial conditions. At the start of the study (Day 1), the postprandial serum leptin concentration was 3 times higher than in the fasted state (Fig. 2A and B). After 5 weeks on HF or LFS, the fasting serum concentration of leptin had increased; at this point in time it did not differ much from the postprandial level (Fig. 2C). With HFS there was a further elevation in both fasted and postprandial serum leptin levels (Fig. 2C). LF induced no change in the fasting serum leptin levels after 5 weeks of feeding (Fig. 2A), and the LF rats continued to respond to food by elevated postprandial levels throughout the study (Fig. 2C).

![Graphs showing food intake, caloric intake, body weight gain, and fat pads weights](image-url)
3.1.4. Serum ghrelin
As expected, the postprandial serum ghrelin concentration was lower than the concentration in the fasted state in all dietary groups at all times (Fig. 3), except in rats given LFS for 2–5 weeks. In these rats, the serum ghrelin concentration did not differ significantly between the fasted and postprandial state. Another notable effect was that 2–5 weeks of HFS greatly suppressed both fasted and postprandial serum ghrelin concentrations. With all the palatable diets (HF, HFS and LFS) there was a prompt and clear-cut decline of the serum ghrelin concentration in the fasted state (Fig. 3A), whereas postprandial serum ghrelin levels seemed unaffected (Fig. 3B). Two diets, LFS and HFS, notably suppressed the fasting serum ghrelin levels. In the case of the LFS rats this resulted in an abolished difference between postprandial and fasted serum ghrelin concentrations. HF and HFS rats displayed a suppression (versus LF rats) of postprandial ghrelin levels (Fig. 3C). From these observations it seems that with time sucrose as well as fat lowers the serum ghrelin concentration in both the fasted and the postprandial state.

3.1.5. Expression of leptin and ghrelin mRNA in the stomach
Leptin mRNA and ghrelin mRNA was measured using total RNA from the fundus of rats in the postprandial state. Leptin mRNA was clearly higher in HFS and LFS rats than in LF and HF rats (Fig. 4A) and hence, seems to be up-regulated by sucrose-rich diets, whereas dietary fat had no effect on the gastric leptin mRNA expression. The ghrelin mRNA expression did not differ significantly between the different dietary groups (Fig. 4B).

3.2. Mouse study
3.2.1. Food intake
LF mice consumed more food (in grams) than the other groups tested (Fig. 5A). Sucrose in the drinking
Fig. 3. Circulating ghrelin concentrations were high in the fasted (F) LF rats and low postprandially (P). (A) Fasting serum ghrelin concentration at different times after start of diet: After 2–5 weeks, HFS and LFS rats had lower serum ghrelin levels than LF rats (## \( p < 0.01 \) for LF vs. LFS and * \( p < 0.05 \) for LF vs. HFS, respectively). (B) Postprandial serum ghrelin: After 5 weeks, HFS rats had lower serum ghrelin than LF rats (*** \( p < 0.005 \)). After 2–5 weeks, the ghrelin levels in the LFS rats did not differ between the fasted and the postprandial state (C). Stars above columns indicate comparison between the fasted and the postprandial state: * \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.005 \).

Fig. 4. Gastric leptin mRNA and ghrelin mRNA expression (relative to 18S) in rats maintained on different diets for 5 weeks. The leptin mRNA expression was higher in HFS and LFS rats than in LF and HF rats (postprandial state) (A). The ghrelin mRNA expression did not differ significantly between the different groups (B). Unless otherwise stated, stars above columns indicate comparison with LF: * \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.005 \).
water greatly lowered the intake of food in terms of grams (Fig. 5A; \( p < 0.005 \) for both LF+S vs. LF and for HF+S vs. HF) but raised it in terms of calories (Fig. 5B; \( p < 0.005 \) for LF+S vs. LF and for HF+S vs. HF). Mice fed HF+S consumed more calories than HF mice (B) and LF+S and HF+S mice consumed much larger volumes of drinking water than LF and HF mice (C). Mice on the palatable diets did not lose as much weight as the LF mice (D) (\( p < 0.005 \) for all groups compared to LF). Data are presented as mean ± SEM \((n=6)\). Unless otherwise stated, stars above columns indicate comparison with LF: *\( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.005 \).

Fig. 5C shows that mice given sucrose in the drinking water drank much more than those given plain water (\( p < 0.005 \) for LF+S vs. LF and for HF+S vs. HF). Mice fed the different palatable diets gained weight unlike the LF mice (Fig. 5D; \( p < 0.001 \) for all groups vs. LF).

Fig. 6. DEXA analysis of body fat (A) and lean tissue (B) in mice maintained on different diets for 5 weeks. Data are presented as % of total body compartment. Mice on palatable diets had more body fat than LF mice and less lean tissue. Unless otherwise stated, stars above columns indicate comparison with LF: **\( p < 0.01 \); ***\( p < 0.005 \).
3.2.2. Body composition

Fig. 6 shows body fat (A) and lean tissue mass (B) after 5 weeks on the different diets. Mice fed the palatable diets (particularly HF and HF+S) had more body fat and less lean tissue than mice on LF.

3.2.3. Serum leptin and ghrelin

Mice given sucrose in the drinking water displayed a robust increase in the fasting level of serum leptin compared to mice given water, regardless of the fat content of the diet (Fig. 7A; \( p < 0.005 \) for LF+S vs. LF, \( p < 0.05 \) for HF+S vs. HF and \( p < 0.05 \) for HF+S vs. LF). The circulating leptin concentration in the HF mice, in fact, did not differ from that in the LF mice (Fig. 7A). The fasting serum ghrelin concentration was much lower in all groups fed palatable diets than in LF mice (Fig. 7B; \( p < 0.05 \), \( p < 0.01 \) and \( p < 0.005 \), respectively).

3.2.4. Expression of leptin mRNA and ghrelin mRNA in the fundus

Mice on HF and HF+S showed a greatly reduced gastric leptin mRNA expression compared to LF and LF+S mice (Fig. 8A; \( p < 0.005 \) for both diets). The same pattern was observed for gastric ghrelin mRNA (Fig. 8B; \( p < 0.005 \) for both HF vs. LF and LF+S and HF+S vs. LF).

Fig. 7. Serum concentrations of leptin (A) and ghrelin (B) in mice maintained on different diets for 5 weeks. Measurements were made in the fasted state. Mice given sucrose in the drinking water (LF+S and HF+S) had much higher serum leptin levels than mice given plain water (LF and HF)(A). Mice on LF+S, HF and HF+S had much lower serum ghrelin concentrations than mice on LF. High fat (HF and HF+S) seemed to be slightly more effective than high sucrose (LF+S) in lowering serum ghrelin. Unless otherwise stated, stars above columns indicate comparison with LF: *\( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.005 \).

Fig. 8. Leptin and ghrelin mRNA expression (relative to 18S) in the fundus of mice maintained on different diets for 5 weeks. Measurements were made in the fasted state. Gastric leptin mRNA expression (A) was much lower in HF and HF+S mice than in LF and LF+S mice. Sucrose in the drinking water did not seem to affect the gastric leptin mRNA expression. The gastric ghrelin mRNA expression (B) was much lower in HF and HF+S mice than in LF and LF+S mice. Interestingly, high sucrose (LF+S) did not seem to affect the ghrelin mRNA expression. Unless otherwise stated, stars above columns indicate comparison with LF: ***\( p < 0.005 \).
4. Discussion

In this study we demonstrate that diets rich in fat and/or sucrose cause overeating in rats and mice (Figs. 1 and 5). A surprising finding was that sucrose, in particular in liquid form, was a powerful inducer of overeating and that dietary fat was less powerful. An attempt to identify the causes and consequences of overeating revealed that the three palatable diets (LFS, HF and HFS) were associated with elevated serum leptin (fasted state) and with suppressed serum ghrelin compared to the non-palatable diet (LF). This is an anticipated and highly appropriate response [6,8,38]. Despite the increase in the satiety peptide leptin and the decrease in the hunger peptide ghrelin overeating occurred, suggesting that neither leptin nor ghrelin are able to control food intake and energy expenditure in rats exposed to palatable food. The decreased serum ghrelin concentration in response to food is in line with the idea that food intake lowers ghrelin and that satiety is associated with low ghrelin (Fig. 3). The ghrelin response to food was abolished in the LFS rats and blunted in the HF and HFS groups, suggesting that these diets do not affect ghrelin formation/mobilization as much as LFS (Fig. 3C). The blunted postprandial ghrelin response can be ascribed to the decreased fasting level of the hormone, which was manifested already after 2 weeks on the palatable diets (Fig. 3). In humans, both obesity [39] and anorexia nervosa [40] are states that show loss of meal-induced decrease in serum ghrelin levels. Perreau et al. show that prolonged long-term feeding of high fat diet to mice results in the disappearance of the difference between fasting and postprandial serum ghrelin levels [41]. The expression of ghrelin mRNA in the fundus of postprandial rats was not affected (Fig. 4), suggesting that ghrelin secretion is affected by the palatable diets more than the expression.

With standard food (LF), the serum leptin concentration increased postprandially, which is in line with the idea that leptin acts as a satiety hormone. The sucrose-rich diets also raised the fasting serum leptin levels (Figs. 2 and 7), suggesting that leptin responds to sucrose intake more than to fat intake. This has been suggested earlier [33,42] and leptin is thought to regulate glucose metabolism [43].

Mice received low fat or high fat diets with or without sucrose added to the drinking water: LF, LF+S, HF and HF+S. All mice displayed a dip in body weight between Day 1 and Day 7 (Fig. 5D), probably reflecting their slow adjustment to the new feeding schedule. The dip was most prominent in the LF mice. The fact that the LF diet contained the lowest amount of calories (kcal/g) probably explains why this effect was particularly noticeable in these animals. The sucrose solution supplied along with the food in the LF+S and HF+S groups raised the serum leptin concentration (Fig. 7A). Nonetheless, these mice gained more weight (Fig. 5D) and developed more body fat (Fig. 6A) than LF mice, indicating that the increased serum leptin concentration did not induce a reduced food intake. The ineffectiveness of leptin to suppress food intake suggests that these animals develop resistance to leptin [44]. We also found the ghrelin response to food intake to be blunted by the palatable diets. Fasted mice on HF and HF+S had lower mRNA expression of leptin (Fig. 8A) and ghrelin (Fig. 8B) than LF and LF+S mice, suggesting that the gastric leptin and gastric ghrelin mRNA expression depends on the type of nutrient ingested.

In conclusion, we have shown that 5 weeks on diets rich in sucrose and/or fat raised the serum concentration of the satiety peptide leptin and lowered that of the hunger peptide ghrelin, yet overeating and increased body weight and adiposity still occurred. We conclude that ghrelin and leptin signalling in response to palatable diets is insufficient to control/restrain appetite, body weight gain and adiposity.

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References
