Dietary modulation of ghrelin and leptin and gorging behavior after weight loss in the obese Zucker rat

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Abstract

In this study, we measured ghrelin and leptin in obese Zucker rats after weight loss induced by calorie restriction using either a low-fat (LF) or high-energy palatable (HEPa) diet. After weight loss, the animals were refed lab chow and offered one hour-palatable test meals on the second and fifteenth days of refeeding. Both LF and HEPa rats lost 10% of their initial body weight ($P < 0.0001$). Plasma ghrelin increased with calorie restriction in both groups ($P < 0.002$) with a tendency to a higher increase in the HEPa group while plasma leptin decreased only in the LF group ($P < 0.01$). Both groups ate the same quantity of chow

during refeeding and both groups gorged on palatable diet during test meals at a very high constant intensity in HEPa rats. After one week of refeeding, ghrelin levels remained elevated in HEPa rats ($+33.2\%$; $P < 0.001$) while returning to baseline in LF rats. Plasma leptin remained low in LF rats. We conclude that weight loss on a palatable diet is possible if total energy intake is controlled. After stopping restriction, when a palatable diet is available, observed gorging might be dependent on specific ghrelin and leptin changes.

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Introduction

Ghrelin is a powerful stimulator of food intake and has been detected in both the brain and the periphery (vanderLely *et al.* 2004). Ghrelin release into the blood is dependent on the nutritional state and diet composition (Tschop *et al.* 2000, Cummings *et al.* 2001, Beck *et al.* 2002, 2003, Gomez *et al.* 2004, Moesgaard *et al.* 2004, Sanchez *et al.* 2004). In association with proteins, fats play an important role in regulating ghrelin secretion (Lee *et al.* 2002, Beck & Richy 2008, Qi *et al.* 2008).

Various types of dietary manipulations have been proposed for inducing weight loss (LaraCastro & Garvey 2004, Dansinger *et al.* 2005). These dietary alterations are often associated with calorie restriction. Thus, these methods are often successful and induce variable but significant weight loss. However, the most important problem is the long-term maintenance of weight loss after the cessation of weight loss program. The reasons for weight rebound are not fully understood as calorie restriction modifies many regulatory systems, including that of ghrelin. Baseline levels, meal peaks, and nocturnal rises in ghrelin levels are increased by chronic calorie restriction in both normal humans and rats (Barazzoni *et al.* 2003, Leidy *et al.* 2007), likely in order to restore energy

balance. However, increased (Cummings *et al.* 2002, Hansen *et al.* 2002) or unchanged (Morpurgo *et al.* 2003, Reinehr *et al.* 2005) ghrelin levels have been reported in obese subjects after restriction.

The Zucker fa/fa rat is a widely used animal model of obesity with hyperinsulinemia and hypertriglyceridemia (Bray 1977). It is also characterized by the down-regulation of ghrelin in order to limit adiposity development (Beck *et al.* 2004) as in obese subjects and rodents (Tschop *et al.* 2001, Williams *et al.* 2006). Moreover, its ghrelin system remains operational and responds to food deprivation (Beck *et al.* 2003). To ascertain the role of ghrelin in weight regulation in obesity and its link with diet type, we studied the effects of calorie restriction and refeeding on body weight and changes in circulating ghrelin in obese Zucker rats. Energy restrictions were imposed by providing limited and fixed isoenergetic quantities of either a low-fat (LF) diet or a highly palatable (high-fat/sweet) diet. We also investigated leptin variations in our experiment because ghrelin and leptin are closely and reciprocally regulated (Barazzoni *et al.* 2003, Kalra *et al.* 2003) and despite the mutation in the leptin receptor gene (Chua *et al.* 1996, Iida *et al.* 1996), the obese Zucker rat is not totally leptin unresponsive. It shows a blunted response after leptin injection (AlBarazanji

et al. 1997, Wang *et al.* 1998) presenting therefore a leptin resistance that is also present in obese subjects (Considine *et al.* 1996).

Materials and Methods

The study was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Animals and protocol

For this experiment, we used 48 male six-month-old obese Zucker (fa/fa) rats bred in our laboratory. The rats were placed in individual wire cages in an air-conditioned vivarium with a 12 h light:12 h darkness cycle (lights on at 0900 h). They were given access to standard lab chow (A04, SAFE, Villemoisson sur Orge, France) and tap water *ad libitum*.

After 2 weeks of habituation to these conditions, 12 rats were killed by decapitation (T0) 3 h after the beginning of the light period. Food was withdrawn during this 3-h period in order to ensure that all animals were in the same nutritional state. This time was also chosen in order to minimize interference with circadian hormonal rhythms as both ghrelin and leptin present a nocturnal rise (Sinha *et al.* 1996, Cummings *et al.* 2002). These conditions were used for all blood samplings.

The remaining rats were randomly divided into two body weight-matched groups that were food-restricted for 3 weeks. The animals were provided 48 kcal/day of either a LF diet (12 g/day; *n*=18) or a high-energy palatable diet (HEPa; 9.8 g/day; *n*=18). Food was given in food cups during the first hour of the light period. The LF diet contained 20% protein, 64% carbohydrates, and 16% fats (% of total energy), whereas the HEPa diet contained 18% protein, 32% carbohydrates, and 50% fats (cf. Table 1). We have previously shown that the HEPa diet is particularly appetizing to normal rats (Beck *et al.* 2005).

After 3 weeks of energy restriction, 12 randomly chosen rats (six per group) were killed by decapitation (T21). All remaining rats had access to A04 chow *ad libitum* for 3 weeks. The A04 chow diet contained 5% of energy as fats and had an energy density of 2.9 kcal/g. At the end of the first day of refeeding on chow (T22), all rats were offered a test meal consisting of HEPa diet available *ad libitum* for 1 h. This procedure was repeated 2 weeks later (T35). The rats were killed by decapitation at the end of the 3-week period of A04 chow refeeding (T42) *ad libitum*.

Samplings and measurements

Blood samples were taken from the tail vein at the beginning (T0) and end of the calorie restriction period (T21) and 1 week after refeeding on A04 chow (T28). All samples (0.8 ml/rat) were collected in aprotinin-EDTA chilled plastic

Table 1 Composition of the high-energy palatable (HEPa) and low-fat (LF) diets

Ingredient	Quantity (g/kg)	
	HEPa	LF
Starch	113	427
Sucrose	225	213
Margarine and oil (50/50)	263	70
Casein	209	200
Sweet condensed milk	100	—
Salts ^a	40	40
Vitamins ^b	10	10
Cellulose	40	40
Caloric density (kcal/g)	4.9	4.0
Energy (%) supplied by		
Carbohydrates (C)	32	64
Fat (F)	50	16
Protein	18	20

^aMixture 205 b.
^bMixture 200 (UAR – Villemoisson sur Orge-France). Each diet was supplemented with 0.2% methionine.

tubes, and centrifuged at 4 °C for 20 min; plasma was aliquoted for subsequent assays.

When the rats were killed at T0, T21, and T42, different fat pads (perirenal, epididymal, and abdominal s.c. adipose tissue) were sampled and weighed to estimate adiposity.

Chow intake was measured every other day from T21 to T42 except on the first day of chow refeeding when it was measured after 1, 6, and 24 h of refeeding and during the 24 h that followed each test meal. Intake of HEPa diet during the 1-h test meal was also measured.

Assays

All samples were measured in the same assay for each hormone.

Total ghrelin was measured in duplicate in diluted plasma from the tail vein and trunk blood samples by a specific RIA kit (RK-031-31, Phoenix Europe GmbH, Karlsruhe, Germany) as previously described (Beck *et al.* 2003). For this experiment, maximal binding was 47.2%, non-specific binding was 2.0%, and IC50 was 31.9 pg/tube.

Leptin was measured in duplicate in diluted plasma of the same samples by a specific RIA kit (RL-83K, Linco, St Charles, MO, USA). For this experiment, maximal binding was 54.2%, non-specific binding was 2.3%, and IC50 was 2.9 ng/ml.

Statistical analysis

Results are reported as means ± S.E.M. Means were compared by two-way variance analysis (diet type × feeding condition) and variance analysis for repeated measures followed by PLSD Fisher's test when necessary using the Statview software (SAS Institute Inc, Cary, NC, USA). A probability of <0.05 was considered statistically significant.

Figure 1 Body weight and plasma ghrelin and leptin variations induced by the low-fat (LF) and high-energy palatable (HEPa) diets given in restricted but isocaloric quantities in obese Zucker rats for 3 weeks and after 1 week of refeeding. ^{###} $P < 0.001$ versus initial BW for both groups; ^{*} $P < 0.02$ versus same group at T0; ^{***} $P < 0.01$ versus same group at T0; ⁺⁺ $P > 0.01$ versus same group at T21; ^a $P > 0.03$ versus T21 for both groups.

Results

Effects of energy restriction on body weight, adiposity, and hormone levels

Body weight and adiposity Initial body weights of the LF and HEPa rats were similar. There was a significant effect of energy restriction ($P < 0.0001$) but not of diet type on body weight. Both groups lost about 10% of their initial body weight during the 3 weeks of restriction (Fig. 1 left). At the end of this period, there was no significant difference in body weight between LF and HEPa rats. Changes in adipose tissue weights are shown in Table 2. There was a significant effect of energy restriction on the weight of each fat depot (epididymal, $P < 0.001$; perirenal, $P < 0.02$; and s.c. abdominal, $P < 0.01$).

Plasma hormones There was a significant effect of energy restriction ($P < 0.01$) and a strong trend for diet type

($P = 0.06$) on plasma ghrelin levels. After weight loss, plasma ghrelin concentration rose by 19% in LF rats ($P < 0.02$) and by 30% in HEPa rats ($P < 0.001$; Fig. 1 top right). The difference between the two groups did not reach statistical significance.

Energy restriction led to a significant decrease in leptin levels in LF rats (-17% ; $P < 0.01$; Fig. 1 bottom right), but not in HEPa rats. At the end of the restriction period, leptin concentration in HEPa rats tended to be higher than in LF rats ($P = 0.07$).

Effects of refeeding ad libitum on feeding behavior, body weight, and hormone levels

Feeding and body weight Chow intake measured after 1, 6, and 24 h of feeding *ad libitum* is shown in Fig. 2 (left). There was no difference in food intake between the groups at any time. All of the rats ate a large meal (6–7 g of chow) during the first hour of refeeding.

Intakes of the HEPa diet during the two 1 h test meals on days 22 and 35 are shown in Fig. 2 (right). There was a significant effect of group ($P < 0.0001$). On day 22, HEPa rats consumed more than twice as much food as LF rats ($P < 0.0001$). During test meal two after 2 weeks of refeeding, both groups ate a very large meal but the difference in intake between HEPa and LF rats was no longer significant. Total energy intake during the second day of refeeding (test meal + chow) was significantly greater in HEPa than in LF rats (103.4 ± 3.5 vs 84.8 ± 2.8 kcal; $P < 0.001$). However, there was no significant difference in total energy intake during the entire first week of refeeding (539.2 ± 14.4 kcal (LF) vs 548.1 ± 19.3 kcal (HEPa)) or during the entire refeeding period (1470.4 ± 42.2 kcal (LF) vs 1466.1 ± 46.7 kcal (HEPa)).

After 3 weeks of refeeding, all rats had returned to their initial body weight and were even slightly heavier. Refed HEPa rats weighed significantly more at the end of the experiment than at the beginning (622.1 ± 13.7 vs 610.2 ± 12.3 g; $P = 0.02$); however, the difference between final and initial body weight was not significant for LF rats

Table 2 Changes in adipose tissue depots and body weights (mean $\pm$ S.E.M. in g) after energy restriction and refeeding in high-energy palatable (HEPa) and low-fat (LF) diet-restricted obese Zucker rats

	Epididymal AT	Perirenal AT	S.c. abdominal AT	BW
Depot				
Initial T0	14.4 $\pm$ 0.8	16.7 $\pm$ 1.8	45.5 $\pm$ 2.7	606.3 $\pm$ 8.7 ^a
End of energy restriction T21				
LF rats	10.0 $\pm$ 1.1 [*]	12.4 $\pm$ 1.0 [*]	35.6 $\pm$ 1.6 ^{†,¶}	539.2 $\pm$ 8.3 ^{*,§}
HEPa rats	11.9 $\pm$ 1.2 [*]	12.7 $\pm$ 1.6 [*]	37.6 $\pm$ 4.8	547.5 $\pm$ 9.4 ^{*,§}
End of refeeding T42				
LF rats	14.1 $\pm$ 0.4	16.9 $\pm$ 1.2	43.6 $\pm$ 1.6	611.1 $\pm$ 12.0
HEPa rats	15.0 $\pm$ 0.7	18.6 $\pm$ 1.5	46.2 $\pm$ 2.1	622.1 $\pm$ 13.7

AT, adipose tissue; s.c., subcutaneous. ^{*} $P < 0.001$ versus same group refed; [†] $P < 0.01$ versus same group refed; [‡] $P < 0.03$ versus same group refed; [§] $P < 0.001$ versus initial; ^{||} $P < 0.01$ versus initial; [¶] $P < 0.03$ versus initial.

^aMean for all rats.

Figure 2 Food intake during the first hour, 6 first hours, and first day of refeeding on chow diet (left), and during the two test meals given during refeeding (right) in the low-fat (LF) and high-energy palatable (HEPa)-restricted obese Zucker rats ($n=12$ per group). *** $P<0.001$ versus HEPa rats.

(611.1 ± 12.0 g (final) vs 602.8 ± 12.6 g; $P=0.10$). The rats regained 63% and about 25% of their weight during the first and second weeks respectively. In both groups, the final weights of the three fat depots were no longer significantly different from the initial weights (Table 2).

Plasma hormones After 1 week of refeeding (Fig. 1), plasma ghrelin levels decreased significantly in LF rats ($P<0.01$) and were no longer different from the initial levels. In HEPa rats, plasma ghrelin levels had not changed since the end of calorie restriction and remained significantly higher than the initial level ($P<0.02$). Plasma leptin levels did not vary and remained significantly lower than the initial levels in LF rats only ($P<0.001$).

At the end of the refeeding period, there was no difference in ghrelin and leptin levels between LF and HEPa rats.

Discussion

While behavioral approaches to managing obesity have been successful in achieving clinically significant weight loss, the long-term maintenance of weight loss remains a problem. Mechanisms for weight regain, particularly those involving hedonic processes are not fully understood. In this study, we had two objectives. First, we wanted to evaluate whether diet palatability influenced weight loss and regain; secondly, we wanted to evaluate whether the hormone changes induced by energy restriction and refeeding could explain the weight changes. We used 6-month-old obese Zucker rats that had developed a marked adiposity and focused on ghrelin and leptin, two hormone systems with opposing effects on BW regulation. Ghrelin stimulates food intake and BW gain, whereas leptin inhibits feeding and induces weight loss (Kalra *et al.* 2003). Dietary BW manipulations were done by providing restricted and fixed isoenergetic quantities of either a LF or HEPa diet. Both diets contained the same ingredients except for sweet condensed milk,

which contributed with margarine and oil to the enhanced palatability of the high-energy diet.

We showed that similar weight loss and adiposity changes could be achieved with intake of a restricted amount of a palatable diet as with intake of a LF diet. These results underscore the fundamental importance of calorie restriction as opposed to macronutrient composition for weight loss. These results agree with studies in humans (Golay *et al.* 1996). Thus, it is possible for obese people to lose weight by consuming limited amounts of pleasant food instead of less palatable meals. Incorporating this information into dietary regimens could facilitate greater weight loss by promoting better adherence to weight loss programs. It has been shown that adherence to a low carbohydrate diet is better than adherence to LF diet (Yancy *et al.* 2004).

Although differences in diet composition did not affect gross body parameters (BW and adiposity), diet composition appeared to have a more subtle influence on the hormone systems that participate in BW regulation. Plasma ghrelin and leptin concentrations were modified differently by a food restriction induced with a LF diet or a HEPa diet. At the end of the restriction period, plasma ghrelin was increased in both groups of rats with a trend towards a greater increase in HEPa rats. This confirms that the ghrelin system is upregulated by a sufficient level of weight loss. According to previous reports (Cummings *et al.* 2002, Hansen *et al.* 2002, Morpurgo *et al.* 2003, Reinehr *et al.* 2005) and our results, this threshold is around 10% of the initial BW. Plasma leptin levels were unaffected by 3 weeks of a calorie-restricted high-fat diet, whereas plasma leptin decreased in rats fed a LF diet for 3 weeks. These data suggest that the dietary fat content plays a role in regulating leptinemia, consistent with previous reports showing that ingestion of HF diets is associated with an increase in leptin levels (Ahren *et al.* 1997, Stricker-Krongrad *et al.* 1998, Qi *et al.* 2008). The changes in the plasma concentrations of both hormones reflect an activation of hunger and a blockade of satiety in order to encourage food intake.

These different hormone profiles influenced the qualitative aspects of feeding behavior differently during the post-restriction period. First, it should be noted that providing access to chow *ad libitum* to the two groups of rats did not allow them to maintain their weight loss even though the fat content in the chow (5%) was lower than that in the LF diet. BW rebound was complete after 3 weeks. In the first 24 h of refeeding, there was no difference in chow intake between HEPa and LF rats. Both groups ingested a large meal during the very first hour of refeeding. Therefore, drive to feed was activated because the quantity of food ingested during this time corresponded to what is generally taken in by overnight food-deprived rats (Beck *et al.* 2005). This drive did not decrease after 24 h, as shown by the fact that when an HEPa diet was provided during the 25th hour as a test meal, the HEPa rats ingested about the same quantity of energy as during the entire preceding day. The LF rats ate 22% more calories than during the first hour of their refeeding.

Thus, neophobia for food does not explain the difference between LF and HEPa rats; satiated LF rats still ingested a supranormal quantity of food in 1 h. The excess of palatable food intake by HEPa rats was rapidly compensated, as, at the end of the first week of refeeding, energy intake between both groups no longer differed.

Binge eating episodes are frequent in animals provided intermittent access to a very low carbohydrate, a high-fat, or a preferred diet (SegallSaacson *et al.* 2004, Davis *et al.* 2007, Cottone *et al.* 2008). Enthusiasm for the palatable diet but not for the regular chow was noted in both groups, and it persisted throughout the second week. The mechanisms behind this gorging behavior appear to be different for LF than HEPa rats as reflected by differences in the hormone changes. In LF rats, the gorging during the first test meal might be related to the decrease in plasma leptin and the increase in plasma ghrelin. In HEPa rats, gorging was related to the only increase in ghrelin; however, there could also have been a learning component involved as these rats had already been exposed to this diet. Thus, previous dietary experience appeared to have a much stronger influence on feeding behavior than down-regulation of leptin as demonstrated by the differences in HEPa diet intake between the groups during the first test meal. This interpretation was confirmed by the hormone levels measured after 1 week of refeeding. HEPa rats continued to have high ghrelin levels, which stimulated their food intake and induced their second gorging episode consistent with a role for ghrelin in the brain reward pathways (Naleid *et al.* 2005). LF rats continued to have lower leptin levels, whereas their ghrelin levels returned to normal. The second gorging episode in the LF rats should have been less substantial than the first one because their ghrelin levels had returned to baseline. However, they had an initial positive experience with the palatable diet during the first test meal, which reinforced their interest for the high-fat diet during the second test meal. This supports the importance of associative learning in feeding behavior. A recent report has also substantiated the influence of leptin on gorging behavior. As with the LF rats in our experiment, selected mice showing gorging episodes are characterized by a hormone profile including lower leptin levels associated with unmodified ghrelin levels (Hambly *et al.* 2007). Our data confirm that in obese Zucker rats, the brain reward system is sensitive to leptin despite the deficiency of fully operational leptin receptors (Fulton *et al.* 2004).

We cannot discount a possible influence on gorging behavior by other parameters sensitive to weight loss, such as hypothalamic neuropeptides that are dependent on the nutritional state and diet composition, that interact with leptin or are engaged in reward like neuropeptide Y (Brown *et al.* 2000, Beck 2006). Further studies will show whether qualitatively modulated restriction affects the brain profiles of these peptides.

Increasing the number of such gorging episodes would accelerate weight gain and restore initial fat stores. Thus, it appears that the period directly following the restriction

period is critical for BW regulation since gorging was only noted when highly palatable food was available and not with chow. The HEPa diet has some characteristics (energy density and high fat content) similar to those of craved foods (Gilhooly *et al.* 2007). Thus, greater vigilance in terms of dietary patterns and a combined therapy with drugs that could limit the ghrelin surge and enhance leptin secretion might help obese people to maintain their weight, leading to a healthier life. Drugs that modulate the ingestion of palatable food such as cannabinoid antagonists might also be useful.

Our study needs to be extended in the diet-induced obese rat, which better reflects common human obesity and in different rat strains, since genetic background is an important parameter controlling an animal's adaptation strategy in response to food restriction (Evans *et al.* 2005, Gelegen *et al.* 2006).

Declaration of interest

The authors have no conflict of interest to declare.

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