Protein appetite is increased after central leptin-induced fat depletion

Michael F. Wiater,* Bryan D. Hudson,* Yvette Virgin, and Sue Ritter

Programs in Neuroscience, Washington State University, Pullman, Washington

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Leptin, the product of the ob gene, is an adipose-derived hormone with diverse effects on physiology and behavior (37). Two well-described consequences of exogenous leptin administration are the suppression of food intake and the potent stimulation of lipolysis, which result in rapid depletion of fat from adipose stores (11, 12). Leptin treatment in rats and mice reduces white adipose tissue; dual X-ray absorptiometry

LEPTIN, THE PRODUCT OF THE ob GENE, IS AN ADIPOSE-DERIVED HORMONE WITH DIVERSE EFFECTS ON PHYSIOLOGY AND BEHAVIOR (37). TWO WELL-DESCRIBED CONSEQUENCES OF EXOGENOUS LEPTIN ADMINISTRATION ARE THE SUPPRESSION OF FOOD INTAKE AND THE POTENT STIMULATION OF LIPOLYSIS, WHICH RESULT IN RAPID DEPLETION OF FAT FROM ADIPOSE STORES (11, 12). LEPTIN TREATMENT IN RATS AND MICE REDUCES WHITE ADIPOSE TISSUE; DUAL X-RAY ABSORPTIOMETRY

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Cannula implantation. Cannulas for lateral ventricular administration of leptin and saline control solutions were fabricated from stainless steel tubing. For surgery, rats were anesthetized by intramuscular injection (0.1 ml/100 g body wt) of a cocktail of ketamine HCl (Fort Dodge Animal Health, Fort Dodge, IA), xylazine (Vedco, St. Joseph, MO), acepromazine (Vedco), and sterile saline in a ratio of 10:5:2:3. Guide cannulas (26-gauge tubing), occluded with removable obturators, were stereotaxically implanted into the lateral ventricle (1.0 mm caudal to bregma, ±1.5 mm lateral to midline, and 3.9 mm ventral to the dura mater) and screened for proper placement using an angiotensin II (All)-induced drinking test (10). For this test, All (100 ng/ml; Chemicon, Phoenix Pharmaceuticals, Burlingame, CA) was injected using an injection cannula (33-gauge stainless steel tubing) that extended 0.5 mm beyond the tip of the intraventricular guide cannula and was connected by polyethylene tubing to a Gilmont (St. Louis, MO) syringe. Animals were then placed back into their home cages, and latency to drink was recorded. All animals began to drink within 5 min and drank at least 5 ml of H2O, indicating that all cannulas were patent and correctly placed. Therefore, all animals were used in the experiments.

Central leptin injections. Intracerebroventricular (ICV) injections of either mouse recombinant leptin (Calbiochem, San Diego, CA; 2.5 μg/3 μl) (n = 5) or control aCSF (1 μg/3 μl) (n = 6) were given for 38 consecutive days. Central injections were consistently started at 1 PM, using the injection procedure described above for the All test. To avoid complications of infections and cross contamination, a separate injector, autoclaved on a daily basis, was used for each rat. The drug delivery system was filled with the leptin (2.5 μg/3 μl) or control aCSF (3 μl) solution. Movement of a tiny indicator bubble in the calibrated infusion line was used to verify drug delivery. Leptin was delivered over a 3-min period. The injector was left in the lumen of the guide cannula for 1 min after the injection, after which it was replaced with a sterile obturator. The rat was then returned immediately to its home cage.

In vivo and in vitro analysis of body fat during chronic leptin treatment. Nineteen male rats, with an initial mean body weight of 359 ± 9 g, were used to examine leptin’s effects on adipose stores using one in vivo and one postmortem measure of body fat. For this experiment, rats were given daily lateral ventricular injections of aCSF (3 μl) or leptin (2.5 μg/3 μl) on days 6 and 11 of leptin or aCSF treatment, rats were lightly anesthetized and subjected to dual-energy X-ray absorptiometry (DEXA or DXA) as an in vivo means of measuring body fat loss during chronic leptin treatment (n = 17 and 19, respectively). DEXA scans for these experiments were made with the Hologic QDR 4500A machine (Bedford, MA) using 11.1:3 software. Rats were lightly anesthetized with the ketamine–xylazine–acepromazine cocktail (described above) shortly before the scans were taken. DEXA has been extensively discussed elsewhere and validated for the direct determination of fat levels in the rat (21, 28). DEXA detects fatty tissues indiscriminately and thus cannot distinguish between fat sources. The detected fat includes metabolically active white adipose tissue, thermogenic brown adipose tissue, and lipolysis-resistant mechanical fat pads, such as the orbital fat pad that supports the eyeball (25). For this reason, DEXA measures are most reliable for the determination of relative values, either between or within individual subjects, as we use them here.

On the same days (days 6 and 11) on which DEXA scans were done, subsets of rats were killed, and the visceral retroperitoneal and epididymal fat pads were dissected and weighed. Of all dissectible fat pads, the retroperitoneal fat pad has been validated as the strongest correlate of total body adiposity in the rat (23).

Effects of chronic leptin treatment on food intake, body weight, and macronutrient selection. A separate group of 11 rats, with a mean initial body weight of 346 ± 4 g, were used for macronutrient studies. Before presentation of macronutrients, rats were given ad libitum access to standard pelleted rodent chow and injected daily with leptin or aCSF, as described above, for 29 days, at which time chow intake had returned to control levels, but body weight was still at its nadir. Body weight and food intake measurements were taken at 10 AM every day, beginning 6 days before the onset of leptin/aCSF injections. Spillage was collected and weighed, and daily intake values were adjusted accordingly.

On day 30, the standard chow diet was discontinued and replaced with separate macronutrient sources for 8 days. Leptin and aCSF injections were continued during this 8-day macronutrient test (days 30–38 of leptin/aCSF injections). Rats had no previous experience with commercial diets other than pelleted chow before day 30. We reasoned that, by using macronutrient-naïve rats, we could reduce any bias in their diet selections introduced by previously learned associations or preferences. By extending the test over an 8-day period, we provided the rats with an opportunity to experience feedback from the diet and to develop novel preferences based on and appropriate for their ongoing metabolic state. Carbohydrate (corn meal), protein (casein), and fat (corn oil) were supplemented ad libitum from separate food cups. Corn meal and casein were supplemented with vitamin mix (Rodent F8135, Bio-Serv, 2 g/100 g), mineral mix (F8575, Bio-Serv, 5 g/100 g), and cellulose (ICN Biochemicals, 16.9 g/100 g). DL-Methionine (ICN Biochemicals, 0.6 g/100 g) was also added to the casein diet, because casein is deficient in this amino acid. Fat was not supplemented with vitamin and mineral mix due to the difficulty of maintaining a homogeneous mix of the supplements in the oil. Based on previous experience with macronutrient diets, we expected each of these diets to be acceptable to and consumed by the rats (26, 27). The position of the food cups in the rats’ cages containing each macronutrient was varied each day when food intake was measured and fresh food was provided. Although spillage was minimal, it was collected and accounted for by adjustment of the total amount consumed on that day.

Statistics. Two-way repeated-measures analysis (leptin or aCSF by time) with Tukey-Kramer post hoc tests was used throughout, except on DEXA and visceral fat data, where t-tests were used. Alpha level was set at P < 0.05.

RESULTS

In vivo and in vitro analysis of body fat during chronic leptin treatment. The effect of our leptin injection protocol on body weight and body fat, assessed in a study parallel to the main behavioral study, is shown in Fig. 1. Figure 1A shows leptin’s effect on body weight. Body weight reached a nadir on about day 9. Figure 1B shows corresponding changes in total body fat content, as estimated by in vivo DEXA scans, displayed with the combined weight of dissected visceral fat pads at these same time points in a subset of rats killed on the indicated days. The coefficient of variation for these DEXA scans was 5.9% for fat in grams and 0.2% for total mass in grams, as determined with four scans of one control rat. This coefficient of variation percent number is typical for adult control rat DEXA fat in grams (cf., 5.2–5.8% (21)) and for DEXA body weight in grams and 0.2% for total mass in grams, as determined with four scans of one control rat. This coefficient of variation percent number is typical for adult control rat DEXA fat in grams calculations (cf., 5.2–5.8% (21)) and for DEXA body weight in grams calculations (cf., 0.55–0.67% (21) and 0.04% (28)). There was a significant repeated-measures interaction [F(1,14) = 168.82, P < 0.05], and leptin significantly reduced body weight every day. Measures of fat differed significantly between leptin and aCSF-treated rats for each comparison (P < 0.05). For dissected fat, the mean weight (±SE) on day 6 was 5.2 ± 0.4 g for aCSF-treated rats and 0.1 ± 0.0 g for leptin-treated rats. On day 11, dissected fat weights were 5.8 ± 0.4 and 0.1 ± 0.0 g, respectively, for the two groups. As predicted from many other studies, these results show that loss of body weight due to our leptin treatment protocol was associated with profound loss of body fat, apparent at both day 6 and day 11 of leptin treatment with both in vivo and in vitro measures. There was a significant repeated-measures interaction for food intake [F(1,15) = 11.39, Fig. 2] until day 11, when food intake began to normalize after visceral body fat was lost.

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Macronutrient selection. Body weights and food intake of rats used in the macronutrient study (Fig. 3, A and B, respectively) were not significantly different at the beginning of leptin treatment. Chronic injection of ICV leptin on days 1–30 significantly reduced body weight for all days [repeated-measures interaction $F(1,29) = 91.02, P < 0.05$; Fig. 3]. In addition, intake of the standard pelleted chow diet was significantly decreased from day 1 to day 23 [repeated-measures interaction $F(1,28) = 4.44, P < 0.05$, Fig. 3]. However, intake of the chow diet in leptin-injected rats returned, thereafter, to control levels. Mean intakes of the chow diet for leptin and aCSF treatment groups on days 27–29 were statistically indistinguishable and were used as the caloric baselines (“base”) for the macronutrient selection data. Intake of macronutrient diets was measured for 8 days, beginning on day 30 of leptin treatment. Days 1–8 of macronutrient testing are equivalent to days 30–38 of ICV leptin treatment.

Results shown in Fig. 4 depict major differences in macronutrient selection by leptin and aCSF-treated rats expressed as a percentage of total calories. However, these differences emerged after day 1. Remarkably, rats of both groups exhibited identical selection profiles on day 1. Compared with the macronutrient composition of the chow diet (calculated from manufacturer’s information), the diets self-selected on day 1 were higher in fat, lower in protein, and similar in carbohydrate content. The self-selected diets diverged after day 1 for the two groups, revealing between-group differences in protein and carbohydrate intake, but no differences in fat intake. Protein intake of leptin-treated animals was significantly different from that of aCSF controls [$F(1,8) = 20.71, P < 0.05$]. Protein consumption was abruptly and significantly increased in leptin-treated rats after day 3, compared with aCSF controls (Fig. 4, top). Leptin and aCSF groups also differed in their intake of the carbohydrate diet [$F(1,8) = 4.00, P < 0.05$]. Carbohydrate consumption was significantly elevated in the aCSF group, compared with the leptin group, on days 2–8 (Fig. 4, middle). There was no significant difference between groups in fat consumption (Fig. 4, bottom), but there was a significant main effect of day [$F(1,8) = 15.54, P < 0.05$] due to increased fat intake by both groups on day 1 of macronutrient choice.

Total caloric intake and body weights during the 8 days of macronutrient testing are shown in Fig. 5. Both leptin and aCSF groups were able to maintain their initial body weight during the transition and adaptation to macronutrient diets. For body weight percent change from baseline (Fig. 5A) during this period, there was a significant repeated-measures main effect for day [$F(1,7) = 4.16, P < 0.05$] and for interaction [$F(1,7) = 5.16, P < 0.05$] between leptin treatment and day, but there was not a significant main effect for leptin treatment [$F(1,7) = 0.77, P = 0.4$], nor was there a significant difference between groups by day. Total daily caloric intake (Fig. 5B) did not change significantly within the leptin-treated group during the macronutrient test. However, there was a significant repeated-measures interaction [$F(1,8) = 2.91, P = 0.007$] for total caloric intake due to a significant increase of intake by aCSF-treated rats on day 2 of macronutrient choice. It is interesting that the total calories consumed on day 1 of macronutrient diets did not differ in either group from the caloric intake on the 3 previous days of pelleted chow feeding (i.e., “the base”), despite the major differences in the nature of the diets. Moreover, it is notable that the total daily caloric intake of all macronutrients combined for the two groups did not differ significantly from each other after day 2, despite the between-group differences in macronutrient selection shown in Fig. 4.

Macronutrient selection. Body weights and food intake of rats maintained on a standard chow diet and injected daily into the lateral ventricle with leptin (2.5 μg/3.0 μl, n = 17) or artificial cerebrospinal fluid (aCSF, n = 19). Mean initial BWs for the two groups were 356 ± 11 and 359 ± 9 g, respectively. A: two distinct stages of BW change during leptin treatment are apparent: a stage of dynamic weight loss, lasting for 10 days in this particular experiment, followed by stable maintenance stage near the BW nadir that lasts for the remainder of the treatment. Dual-energy X-ray absorptiometry (DEXA) scans were performed on days 6 and 11 for analysis of body fat. B: body fat content as measured by DEXA and visceral fat dissection from subsets of the same animals as in A. Rats used for dissection were killed after aCSF or leptin injection for 6 days (aCSF, n = 4 and leptin, n = 5) or 11 days (aCSF, n = 4 and leptin, n = 3). Both measures indicate a profound reduction of body fat at both time points in the leptin-treated rats.

Fig. 1. A: mean percent change in body weights (BW) of rats maintained on a standard chow diet and injected daily into the lateral ventricle with leptin (2.5 μg/3.0 μl, n = 17) or artificial cerebrospinal fluid (aCSF, n = 19). Mean initial BWs for the two groups were 356 ± 11 and 359 ± 9 g, respectively. A: two distinct stages of BW change during leptin treatment are apparent: a stage of dynamic weight loss, lasting for 10 days in this particular experiment, followed by stable maintenance stage near the BW nadir that lasts for the remainder of the treatment. Dual-energy X-ray absorptiometry (DEXA) scans were performed on days 6 and 11 for analysis of body fat. B: body fat content as measured by DEXA and visceral fat dissection from subsets of the same animals as in A. Rats used for dissection were killed after aCSF or leptin injection for 6 days (aCSF, n = 4 and leptin, n = 5) or 11 days (aCSF, n = 4 and leptin, n = 3). Both measures indicate a profound reduction of body fat at both time points in the leptin-treated rats.

Fig. 2. Mean intake of standard chow diet by the rats used for fat analysis by DEXA scan and visceral fat pad dissection (Fig. 1). Rats were injected daily into the lateral ventricle with leptin (2.5 μg/5.0 μl, n = 17) or aCSF (n = 19). DEXA scans were performed on days 6 and 11. Note that food intake is suppressed by leptin until about day 7 or 8 in this example, after which it gradually increases to control levels. The point at which food intake begins to increase coincides with the depletion of body fat by leptin, shown in Fig. 1. A and B. There is no significant difference between leptin-treated rats and aCSF-treated rats for food intake from day 13 to 15.
DISCUSSION

DEXA scan analysis and visceral fat dissection confirmed that leptin reduced body weight until white adipose tissue was depleted. Food intake was reduced during the period of leptin-induced lipolysis, but began to increase at approximately the point of adipose depletion. Body fat measurements also confirmed that body fat depletion was not reversed by the increase of food intake to near normal levels. Body weight and food intake changes in response to leptin followed a pattern in the macronutrient study that was similar to the pattern observed in the DEXA study. Therefore, we assume the leptin-treated rats to be in an adipose-depleted state during the macronutrient study.

In this study, rats inexperienced with food choice adopted a pattern of macronutrient selection over a period of days that reflected their distinct metabolic states and appeared to be guided by feedback from their dietary choices. On the first day of macronutrient exposure, both groups exhibited similar macronutrient choices. Thereafter, leptin-treated rats exhibited macronutrient choices that differed strikingly from control rats. Leptin-treated rats rapidly developed a strong preference for protein, with a reciprocal decrease in carbohydrate selection. In contrast, controls rapidly developed a preference for carbohydrate that was sustained throughout the test period, with protein intake increasing only modestly with continuing exposure to the diets. Intake of fat did not differ between groups at any time. Moreover, after a brief adjustment period, rats of both groups modulated their intake of the macronutrients without increasing total caloric intake above base levels.

Fig. 3. A: BW (percent change) of rats during central leptin (n = 5) or aCSF (n = 6) treatment before macronutrient selection testing. Initial BWs for the two groups were 361 ± 6 and 350 ± 6 g, respectively. BW reached its nadir after ∼10 days of leptin treatment and remained relatively stable thereafter. B: intake of the standard pelleted chow maintenance diet (shown as percentage of baseline) by the same rats as in A. Baseline intakes for the two groups for the 3 days before leptin injection were 23.0 ± 0.3 and 22.1 ± 0.05 g, respectively. Food intake began to return to control levels after day 11 in the leptin-treated group. On the 3 days before the macronutrient selection experiment (days 27–29 of leptin treatment), the intakes of the two groups did not differ significantly. The mean intake and BW for each group during this 3-day period (bar) were used as the baseline (base) for the macronutrient selection test.

Fig. 4. Intake of macronutrients in an 8-day test by rats given daily injections of leptin or aCSF into the lateral cerebroventricle. Protein (top), carbohydrate (middle), and fat intake (bottom), expressed as percentage of total caloric intake, are shown. Before presentation of macronutrient diets, rats were maintained on standard chow and given leptin or aCSF injections for 30 days. The daily leptin and aCSF treatments were maintained for the additional 8 days of macronutrient testing. At the start of the macronutrient test, the leptin-treated rats were fat deplete, but their chow intake had returned to control levels. Rats had no experience with diets other than pelleted chow before the test. The baseline (base) is the calculated macronutrient content of the chow diet consumed during the 3 days before the macronutrient selection test. The two groups differed dramatically in their protein and carbohydrate intake during the 8-day test. Initially, both groups reduced their intake of protein below levels present in chow. Subsequently, leptin-treated rats increased their intake of protein and decreased their intake of carbohydrate, while aCSF-treated rats increased their carbohydrate, but not their protein intake. The two groups did not differ in their fat intake, which was initially elevated above the level consumed in chow diet, but returned to baseline levels by day 3. See Fig. 5B for total daily caloric intake for each group.
In the latter cases, both fat and lean body mass are reduced. However, the hypothesis that protein appetite is stimulated directly by leptin is discredited by previous studies reporting that protein intake is suppressed during short-term leptin treatment and in response to a single leptin injection. Moreover, the high levels of central leptin present in our fat-depleted model would not occur normally in the presence of low levels of body fat, arguing against leptin as a physiological mechanism for control of protein appetite.

Increased protein appetite in our study was more likely to be an indirect effect of leptin treatment. Previous studies have shown that moderately low dietary protein is associated with an increase in food intake (13, 34). Intake of isocaloric diets was maximal for those containing protein levels between 8 and 12.5% by weight (13), which is about the minimum protein requirement for rats. Our rats were maintained on standard chow during the 29 days of leptin treatment before presentation of macronutrient diets. Chow consists of a relatively low fixed proportion of protein (~25%). Since chow intake was profoundly suppressed during the initial stages of leptin treatment, reducing protein intake to roughly 11% of daily intake, rats may have developed a protein debt that contributed to the stimulation of protein appetite in the later phases of chronic treatment (16). Another factor that may increase protein intake is the additional demand on protein as a metabolic fuel when body fat is unavailable. In fat-depleted rats, increased protein intake may be required to avoid or repair deficiency of indispensable amino acids, a condition with profound and adverse physiological consequences (15).

Another potential stimulus for the protein appetite observed in the present experiment is fat depletion per se and the associated reduction in the availability of fat for ongoing oxidation. In support of this hypothesis, we found previously that acute blockade of fatty acid oxidation by administration of mercaptoacetate (3, 26, 27) produces a robust and selective stimulation of protein intake without altering 24-h caloric intake. In these studies, the effect of mercaptoacetate was studied using diets similar to those used here, as well as with diets composed of alternative fat, protein, and carbohydrate sources. Rats were tested under conditions in which a single macronutrient was present during the test and under conditions in which all three macronutrients were present. Blockade of fatty acid oxidation stimulated a robust increase in protein consumption under all testing conditions that were not related to prior preference. The present experimental findings suggest that leptin-induced fat depletion produces a metabolic signal that is similar to that produced by pharmacological blockade of fatty acid oxidation. Perhaps the function of this signal is not to protect fat stores, but to protect body protein, which is threatened when total body energy stores are severely diminished. This interpretation is compatible with the observation that protein consumption is decreased, not increased, in the initial stages of leptin treatment (32, 33) when the animal’s fat deposits are still within the normal range and fat utilization is enhanced, as indicated by a shift in respiratory quotient (30).

Perhaps the most intriguing aspect of the present study is that the return of food intake to normal levels during sustained adipose depletion, the expression of macronutrient preferences appropriate to metabolic state, and the relatively precise adjustment of caloric intake during the macronutrient selection tests all occurred in the presence of (despite) chronic ICV leptin administration. Intake of calories and nutrients appears to be precisely controlled to maintain ongoing activities of the animal. Thus controls based on...
ongoing metabolic and nutritional requirements supersed the negative feedback controls that inhibit feeding in the interest of maintaining constancy of stored fat.

The existence of and need for controls capable of superseding leptin’s negative influence on food intake are intuitively clear, since deficits in particular nutrients and minerals are life threatening, even in the presence of excess body fat. For example, glucose is required for brain energy metabolism and cannot be replaced by fat. The glucoprivic control, which stimulates feeding in response to glucose deficit, is not impaired by either acute or chronic peripheral leptin administration (36). Also, the need for protein certainly cannot be offset by availability of fat. The model of leptin treatment used in the present study, as well as the similar approach using central leptin gene therapy (20), provides a useful approach to identify and examine controls of feeding that are independent of leptin’s actions. Moreover, this model provides a means of unconfounding the direct effects of leptin on food intake from effects arising from leptin-induced lipolysis, since the contribution of lipolysis to energy metabolism is minimal after fat pad depletion. Finally, this model provides a window for examining the chronic leptin model will increase our understanding of the pathways involved in control of food intake in both the presence and the absence of body fat.

GRANT
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REFERENCES