Protein appetite is increased after central leptin-induced fat depletion

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Submitted 8 May 2007; accepted in final form 21 June 2007

Wiater MF, Hudson BD, Virgin Y, Ritter S. Protein appetite is increased after central leptin-induced fat depletion. Am J Physiol Regul Integr Comp Physiol 293: R1468–R1473, 2007. First published June 27, 2007; doi:10.1152/ajpregu.00322.2007.—Leptin reduces body fat selectively, sparing body protein. Accordingly, during chronic leptin administration, food intake is suppressed, and body weight is reduced until body fat is depleted. Body weight then stabilizes at this fat-depleted nadir, while food intake returns to normal caloric levels, presumably in defense of energy and nutritional homeostasis. This model of leptin treatment offers the opportunity to examine controls of food intake that are independent of leptin’s actions, and provides a window for examining the nature of feeding controls in a “fatless” animal. Here we evaluate macronutrient selection during this fat-depleted phase of leptin treatment. Adult, male Sprague-Dawley rats were maintained on standard pelleted rodent chow and given daily lateral ventricular injections of leptin or vehicle solution until body weight reached the nadir point and food intake returned to normal levels. Injections were then continued for 8 days, during which rats self-selected their daily diet from separate sources of carbohydrate, protein, and fat. Macronutrient choice differed profoundly in leptin and control rats. Leptin rats exhibited a dramatic increase in protein intake, whereas controls exhibited a strong carbohydrate preference. Fat intake did not differ between groups at any time during the 8-day test. Despite these dramatic differences in macronutrient selection, total daily caloric intake did not differ between groups except on day 2. Thus controls of food intake related to ongoing metabolic and nutritional requirements may supersede the negative feedback signals related to body fat stores.

macronutrient choice; homeostasis; lipoprivation; 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 so dramatically that the fat pads are no longer visible to the naked eye (6, 9, 31). Body protein, however, appears to be conserved during leptin treatment (1, 9). Rats that have been treated with central leptin gene therapy remain fat free throughout their lifespan, with no apparent metabolic pathology (20). In contrast to the persisting suppression of body weight by chronic leptin administration or gene therapy, leptin’s effects on food intake are more transient. During chronic leptin administration, food intake is initially reduced by exogenous leptin, but then gradually normalizes. Normalization of chow feeding during chronic leptin treatment has been demonstrated in a number of published papers (2, 5, 6, 9, 11, 12, 14, 17, 18) and does not appear to be due to leptin resistance, since suppression of body weight by leptin is maintained. The fact that chow feeding is able to return to preleptin levels and that lean body mass is preserved, despite continued leptin administration, indicate that controls of food intake not suppressed by leptin are able to maintain energy homeostasis.

This model of leptin treatment, therefore, provides a potentially powerful approach with which to examine controls of feeding that are independent of or supersede leptin’s direct actions and to study these controls under metabolic conditions that are not confounded by leptin-induced lipolysis. In the present paper, we describe the phases of food intake during long-term chronic leptin treatment with respect to the changes in body fat stores to validate this model. Second, we begin to characterize the controls of food intake operative after adipose tissue has been depleted by leptin, but when food intake has returned to levels similar to controls. We hypothesized that food intake during this fat-deplete phase of leptin treatment may be controlled in part by the need for particular macronutrients. Surprisingly few studies have examined the effects of leptin administration on macronutrient selection, and the studies that have been done have examined macronutrient selection during acute or short-term chronic leptin treatment (e.g. Refs. 1, 32, 33). None have examined macronutrient selection subsequent to leptin-induced fat depletion. It seems likely that the profound metabolic alterations induced by leptin treatment, including transition from a fat-replete to a fat-deplete energy economy, would impact macronutrient preference. We found that leptin-treated rats developed a strong and sustained preference for protein, whereas artificial cerebrospinal fluid (aCSF) controls revealed a strong preference for carbohydrate. However, despite these differences in macronutrient selection, total caloric intakes of the leptin-treated and control groups were indistinguishable except on day 2 of the macronutrient selection test. These results recapitulate the powerful effects of leptin in reducing body fat, but clearly demonstrate the operation of controls of food intake that are independent of these effects.

MATERIALS AND METHODS

Subjects and housing conditions. Adult male Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA) and individually housed in suspended wire-mesh cages, under standard Association for Assessment and Accreditation of Laboratory Animal Care-approved conditions, in a temperature-controlled room (21 ± 1°C), illuminated between 0700 and 1900. Throughout the experiment, the rats had ad libitum access to pelleted rat chow (Harlan Teklad F6 Rodent Diet W, Madison, WI) and tap water, except during macronutrient studies. Before surgery, the rats were handled daily for at least 1 wk and habituated to the laboratory environment and to the testing procedures. The Washington State University Institutional Animal Care and Use Committee, which conforms to National Institutes of Health rules and regulations, approved all experimental animal protocols.

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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 (AII)-induced drinking test (10). For this test, AII (100 ng/3 μl) or saline (3 μl) was injected into a guide 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 AII 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 11.13 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-naive 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 provided 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 test 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 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.
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.
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

![Graphs showing BW, Food Intake, Protein, Carbohydrate, and Fat intake changes over days for leptin and aCSF treatments.](Figures)
LEPTIN-INDUCED FAT DEPLETION AND PROTEIN APPETITE

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

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**Fig. 5.** A: BW, expressed as percent change from baseline for leptin and aCSF-treated rats during the 8-day macronutrient selection test conducted on days 30–38 of leptin or aCSF treatment. Baseline BWs were 313 ± 10 and 400 ± 6 g, respectively. BW changes did not differ significantly between groups during the macronutrient test. B: total daily caloric intake of leptin and aCSF-treated rats during the 8-day macronutrient selection test. Between-group differences after day 2 were not significant. For both A and B, the baseline (base) is the average BW and caloric intake over the 3 days of the standard chow diet just before macronutrient choice.

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the preceding basal level of the chow diet immediately preceding the macronutrient study. Thus, despite differences in texture and possibly palatability among the three diets, the initial selections by the two naive groups were similar, making our key finding the dramatic divergence of their respective responses during the 8-day test.

The dramatic stimulation of protein appetite in leptin-treated rats during the fat-depleted phase of leptin treatment was probably not due to a starvation-induced ketotic state (22, 29). This state has also been shown to be associated with increased protein intake, decreased carbohydrate intake, and unaltered fat intake (19). However, since our rats were neither starving nor ketotic (unpublished data). Furthermore, our results differed from those produced by brief caloric restriction (such as overnight food deprivation), which has been reported to increase intake of carbohydrate and fat (4, 7, 24).

It is possible that the dramatic increase in protein intake we observed in leptin-treated rats reflects a direct action of leptin. If so, this action might be a mechanism for preserving lean body mass. Preservation of lean body mass is a striking feature of leptin so, this action might be a mechanism for preserving lean body mass. Preservation of lean body mass is a striking feature of leptin so, this action might be a mechanism for preserving lean body mass.
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
This study was supported by Public Health Service Grant DK40498 and Juvenile Diabetes Research Foundation Grant 1-2006-308 to S. Ritter.

REFERENCES