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Leptin inhibits prehibernation hyperphagia and reduces body weight in arctic ground squirrels

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Ormseth, Olav A., Margery Nicolson, Mary Ann Pelleymounter, and Bert B. Boyer. Leptin inhibits prehibernation hyperphagia and reduces body weight in arctic ground squirrels. *Am. J. Physiol.* 271 (*Regulatory Integrative Comp. Physiol.* 40): R1775–R1779, 1996.—The *ob* gene product leptin is thought to play a physiological role in the fine tuning of a homeostatic mechanism regulating satiety and adiposity. Mouse recombinant leptin was administered to seasonally hyperphagic arctic ground squirrels as a first step in demonstrating the evolutionary conservation of leptin function and the potential involvement of leptin in the seasonal regulation of adiposity in hibernators. Continuous infusion of leptin for 3 wk via miniosmotic pumps resulted in a reduction in food intake and body weight in a manner consistent with its proposed role as a satiety hormone. During the recovery period after leptin administration, squirrels that had received leptin became hyperphagic relative to controls. Percent body fat was estimated at weekly intervals by measuring total body electrical conductivity and decreased after 3 wk of leptin administration. Our observations support the role of leptin as a regulatory hormone involved in the control of satiety, adiposity, and possibly energy expenditure in hibernating mammals.

obesity; satiety; adiposity; hibernating mammals

ENERGY BALANCE is maintained by environmental, behavioral, and genetic factors. When energy intake exceeds energy expenditure, overall adiposity increases. A "lipostat" theory, in which a feedback loop connects white adipose tissue and the brain, has been proposed to explain how a set level of adiposity is maintained throughout adult life in many organisms, including humans (12). Parabiosis experiments with two genetically obese strains of mice (*ob/ob* and *db/db*) supported the lipostat theory and led to the suggestion that a fat-secreted satiety factor circulates in the blood and is involved in the regulation of food intake and body weight (5).

The recently cloned *ob* gene codes for a 16-kDa protein named leptin (22), which is synthesized in and secreted from adipose tissue and appears to fulfill many properties of a satiety factor. Injections of recombinant leptin into *ob/ob* mice normalizes food intake and reduces overall body weight and adiposity (3, 9, 16). Recombinant leptin has no effect on food intake and body weight in *db/db* mice (9), lending support to the

hypothesis that *db/db* mice have a leptin receptor defect. Injection of leptin directly into the lateral ventricles of *ob/ob* mice produces a more rapid reduction of food intake and body weight, indicating that the brain is likely to be involved in a feedback loop involving leptin secreted from adipose tissue (3).

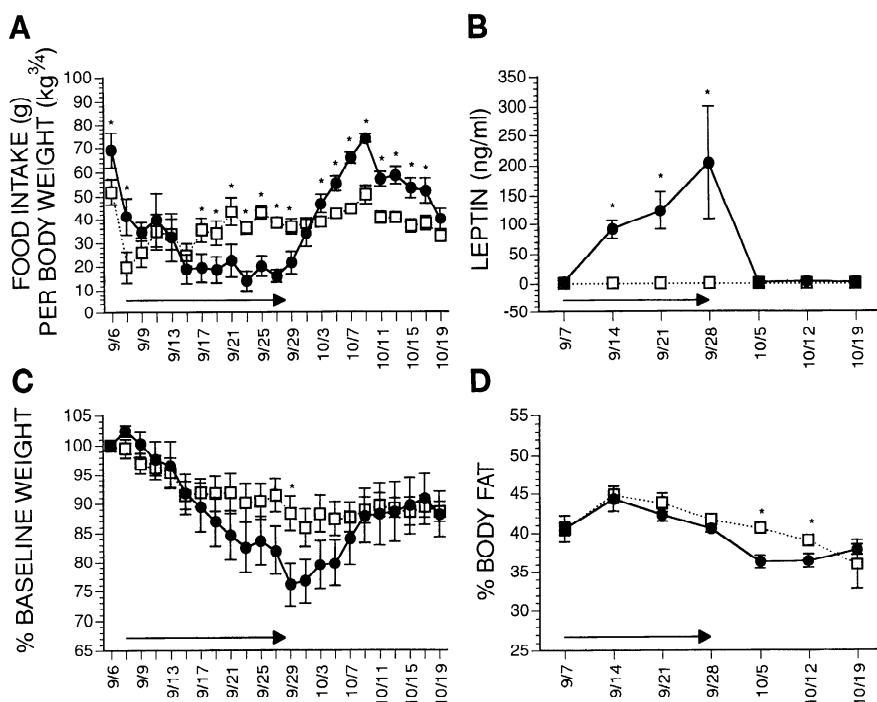
The sequence of the *ob* gene is highly conserved in mammals (22), suggesting that the function of leptin may also be conserved. Mammals undergoing seasonal changes in adiposity appear uniquely able to adjust the set point of their lipostat. Because arctic ground squirrels (*Spermophilus parryii*) hibernate up to nine months of the year and deposit considerable fat in preparation for hibernation (15), we predicted that they would be a useful model to investigate the control of whole body adiposity. As a first step, we administered mouse recombinant leptin to *S. parryii* during prehibernation hyperphagia. Our experimental results support the potential for involvement of leptin in the seasonal control of adiposity in hibernating mammals.

METHODS

Arctic ground squirrels were trapped on August 23–24, 1995, at Donnelly Dome in the Alaska Range, Alaska (64°N, elevation 3,000–4,000 ft). They were transported to the animal quarters facility at the University of Alaska at Fairbanks and housed at 20°C on a 8:16-h light-dark cycle. Mazuri rodent chow and water were provided ad libitum.

Twelve squirrels were divided into two groups of six, evenly matched for weight and sex. One group was chosen at random to receive mouse recombinant leptin, which was prepared as previously described (16), and the control group received saline. Leptin (1.28 mg/ml) was delivered via constant infusion using miniosmotic pumps (model 2ML1, Alza) with delivery rates of 10 μ l/h and a total volume of 2.2 ml. Therefore, average daily dose varied from 0.45 to 0.85 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, depending on individual weight (362–679 g). Thus the reservoir was sufficient to deliver leptin for 1 wk at a dose greater than the 0.3 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ constant infusion shown to be effective in mice (16). The control group was treated identically, except that saline was infused rather than leptin. Anesthesia was induced by exposure to methoxyflurane (Metofane) and maintained with a vaporizer using halothane. A 1.5-cm incision was made in the intrascapular region of each squirrel, the pump was inserted subcutaneously, and the wound was closed with surgical staples. The leptin delivery period lasted for 3 wk, requiring the implantation of three successive pumps in each animal. The delivery

Fig. 1. Food intake (A), circulating leptin concentrations (B), percent baseline body weight (C), and percent body fat (D) in squirrels receiving leptin (●) or saline (□) miniosmotic pumps. Arrows indicate period of leptin administration. Calendar date is displayed on x-axis. Food intake is expressed in g food intake/kg body weight^{0.75}, and body weight is shown as percent of individual baseline. Values are means \pm SE; $n = 6$ (except in leptin group; after 9/21, $n = 4$). *Significant difference between data points ($P < 0.05$, 2-sample t -test).



period was followed by a 3-wk recovery period in which no surgeries were performed on either group of animals.

Food intake and body weight were measured every other day throughout the 6 wk of the study. Food intake was determined by weighing the food left in the cage. Weight was expressed as a percentage of individual baseline levels established before leptin administration. Food intake was normalized to metabolic body size.¹ Weekly 2-ml blood samples were collected by cardiac puncture and kept on ice with EDTA and spun for 10 min, and the plasma was stored at -70°C until assayed for metabolites and leptin. Total body electrical conductivity (TOBEC) was measured weekly using an SA-3000 small animal body composition analysis system (EM-SCAN). Plasma glucose, triglycerides, and free fatty acids were assayed using a Hitachi 717 chemistry analyzer. Plasma insulin was measured by radioimmunoassay (Linco Research, St. Louis, MO). Plasma leptin levels were measured in a solid phase sandwich enzyme immunoassay utilizing an affinity purified polyvalent antibody immobilized in microtiter wells. Bound leptin was detected with affinity purified antibody conjugated to horseradish peroxidase and quantitated with a chromogenic substrate. Leptin concentrations were derived from standard curves generated in the same assay with recombinant leptin. Validity of the immunoassay was tested by neutralization with cold mouse antibody.

TOBEC data were used to compare levels of body adiposity between the control and leptin group (see Refs. 1 and 19 for reviews). To use TOBEC as a measure of total body fat, TOBEC numbers must be transformed using a calibration equation. At the end of the study the squirrels were euthanized by an overdose of halothane, and body composition was determined by fat extraction. Carcasses were frozen, sawed into 1-cm slices, and homogenized using a commercial food grinder (Hobart). Three samples (16 g each) were taken from the homogenate of each squirrel and dried to constant weight at 60°C . Aliquots of ~ 3 g from each sample were analyzed for

percent body fat using a Soxhlet extraction apparatus as directed by the manufacturer (Soxtec System HT6; Tecator, Höganäs, Sweden). The data from this analysis were combined with TOBEC values in a calibration equation as described in the SA-3000 operator's manual (7). This equation was used to convert the raw TOBEC numbers into grams of lean mass, from which percent body fat and percent lean mass were calculated. The reliability of this calibration was confirmed by regression analysis of the TOBEC and extraction values ($r^2 = 0.89$).

RESULTS

Plasma leptin concentration increased in each successive week of leptin administration² and fell to control levels after pump removal (Fig. 1B). Leptin levels in the control group (0.3–2.7 ng/ml) did not change throughout the study and were above the limits of detection (0.05 ng/ml), indicating that the antibody cross-reacted to squirrel leptin. The 3-wk constant infusion of leptin resulted in significantly decreased levels of food intake and body weight relative to a constant infusion of saline. Food intake dropped for both groups on the day of the first surgery (Sept. 7, Fig. 1A). From that point, food intake for the group receiving leptin continued to decline until it plateaued at 20 g/kg body wt^{0.75}, whereas food intake in the control group leveled out at ~ 40 g/kg body wt^{0.75}. On removal of the miniosmotic pumps, food intake in the leptin group increased rapidly and exceeded the level of the control group after 5 days (Oct. 3).

² The timing of this experiment was specifically chosen so as to test the effect of leptin during prehibernation hyperphagia, based on seasonal patterns previously described (15). Therefore, "dates" have been used to delineate the x-axis in Figs. 1 and 2.

¹ Food intake in grams was divided by body weight in kilograms raised to the 0.75 power, as described by Kleiber (12a).

Two squirrels were removed from the leptin group before completion of the study. One died on Sept. 28 while recovering from anesthesia. A second animal was removed from the study on Sept. 22 because it was approaching moribundity; it lost excessive amounts of body weight (169 g in 16 days), and its daily food intake was <1 g/day. The animals were not replaced and the experiment was not repeated because of the importance of seasonality in the experimental design.

Body weight also decreased as a result of leptin administration (Fig. 1C), although a significant reduction was not seen until well after reductions in food intake. Major reductions in body weight (71–147 g) were observed in all four of the animals weighing <600 g during weeks 2–3 of the leptin administration period. Weight for both groups decreased steadily throughout the first week of the study, from which point the leptin-infused squirrels continued to lose weight, while the control squirrels' weight stabilized at 80–90% of their baseline level. After 3 wk of steady decline during leptin administration, body weight in the leptin group had fallen to 76% of baseline, compared with 88% in the controls. Within 2 wk after pump removal, body weight in the leptin group increased to the level of the control group (89% of baseline), and both groups roughly maintained that level throughout the remainder of the study.

Although percent body fat for both groups significantly increased during the first week, values from both groups were indistinguishable from each other and from those of Sept. 7 in the second and third week of leptin administration (Fig. 1D). Percent body fat was significantly lower in the leptin group in the first and second week after pump removal. Circulating levels of triglycerides in the leptin group declined during leptin

administration and increased during the recovery period; triglyceride levels in the control group were variable throughout the study (Fig. 2A). Although free fatty acids seemed to increase in several of the leptin squirrels during the leptin administration period (data not shown), the lack of this response in other squirrels in the same group resulted in noticeable variability in plasma free fatty acids. Free fatty acids thus appear to remain steady for both groups throughout the study, although values for the leptin group seem consistently higher (Fig. 2B). Blood glucose remained at constant levels for both groups throughout the study, and no difference between the two groups was observed (Fig. 2C). Differences in plasma insulin were not statistically significant (Fig. 2D).

DISCUSSION

The lipostat hypothesis is an attractive explanation for the maintenance of long-term body weight in mammals, and there is ample evidence to support the role of leptin as an adipose tissue-secreted satiety factor that is involved in the control of adiposity. Conservation of the *ob* gene sequence in a number of mammals suggests that the function of leptin may also be conserved. Our results demonstrate that mouse recombinant leptin reduces food intake and body weight during prehibernation fattening in arctic ground squirrels.

The initial decrease in food intake and body weight observed in both groups may reflect the stress of surgery or settling into the availability of ad libitum rodent chow (introduced 2 wk before the beginning of the study). Alternatively, the initial drop may represent a seasonal reduction in food intake in preparation for hibernation. Our experiment took place at the end of

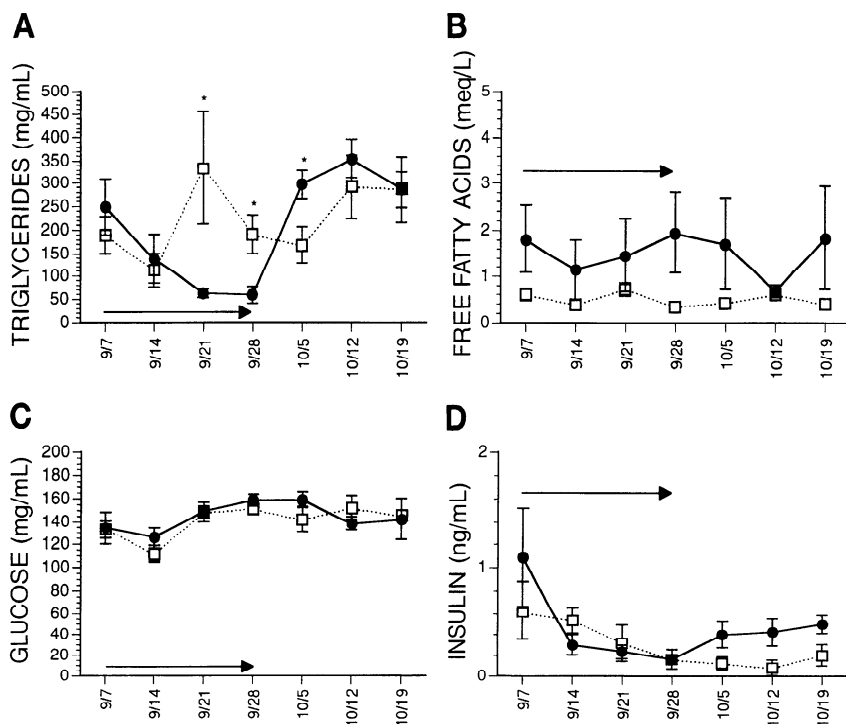


Fig. 2. Plasma concentrations of triglycerides (A), free fatty acids (B), glucose (C), and insulin (D) in groups of squirrels receiving leptin (●) or saline (□) miniosmotic pumps. Arrows indicate period of leptin administration. Calendar date is displayed on x-axis. Values are means \pm SE; $n = 6$ (except in leptin group; after 9/21, $n = 4$). *Significant difference between data points ($P < 0.05$, 2-sample t -test).

seasonal hyperphagia, when squirrels gradually reduce food intake and body weight (15). Reduction and recovery of food intake and body weight in the squirrels receiving leptin was not immediate. This implies that the removal of leptin may be slow or that the effect of leptin is long lasting. A slow removal rate is supported by the steady increase in circulating leptin observed in these animals with each new pump implantation throughout the administration period.

Food intake (per kg body wt^{0.75}) for the leptin group increased after pump removal and exceeded the food intake of the control animals. These results support the concept that individuals regulate weight at a preferred level via changes in food intake or energy utilization (10). Interestingly, rebound hyperphagia was not observed in *ob/ob* mice after leptin administration (20), even when we attempted to normalize the food intake to body weight from the published data.

It is possible that reduction of food intake is not the only means by which leptin influences body weight. For example, leptin increases metabolic rate and body temperature in *ob/ob* mice (16), and pair-feeding experiments support the hypothesis that leptin exerts adipose-reducing effects beyond what can be accounted for by reductions in food intake (14). To determine how reductions in food intake and weight were related in the current study, we divided weight loss by average food intake during the leptin administration period. This gave us a crude measure of how efficiently the squirrels stored energy, similar to the metabolic efficiency index used for animals in positive energy balance (2, 8). The ratio for squirrels receiving leptin was -7.8 , whereas it was -2.1 for the control group. This suggests that the squirrels receiving leptin lost more weight per unit of food than did the control animals and implies that energy expenditure was higher for the leptin group during this period.

The decreased body weight associated with the administration of leptin to *ob/ob* mice has been shown to be primarily the result of a decrease in body fat (9, 16). This study is the first to compare body fat reserves at weekly intervals during administration of recombinant leptin. TOBEC allows for the noninvasive estimation of adipose tissue mass, and our analysis of TOBEC measurements indicated a significant reduction in fat mass between the leptin and control groups during the fourth and fifth week of the study. This delayed reduction in fat mass is consistent with a stable leptin effect. Although we cannot explain the increase in body fat during the first week of leptin administration, the apparent steady decline in control group percent body fat values may be explained by the observation that squirrels lose weight immediately before hibernation (15). Squirrels receiving leptin increased their body fat in the last 2 wk of the study and converged to the same level as the controls, possibly as a result of the animals attempting to achieve a set seasonal level of adiposity.

Circulating levels of triglycerides decreased during leptin administration and recovered after leptin removal. Mobilization of triglycerides might account for this decrease because an increase in free fatty acids

was observed in several, but not all, of the animals receiving leptin. Blood glucose homeostasis was maintained throughout the experiment for both groups, even though food intake was dramatically reduced in the leptin group during this time. Leptin reduces circulating glucose and insulin in *ob/ob* mice and is thought to normalize pancreatic function (16). Our data suggest that physiological mechanisms defending blood glucose levels during seasonal hyperphagia in *S. parryii* may not be significantly affected by leptin.

Do hibernators seasonally adjust a lipostat mechanism to allow for massive weight gain before hibernation? In midsummer, *S. parryii* spontaneously increase food intake almost twofold and remain hyperphagic until the end of September, when food intake gradually drops to the level of starvation and the squirrels enter hibernation (15). In addition, body weight nearly doubles during this period of prehibernation fattening. Hibernators maintain seasonally appropriate body weight in the absence of environmental cues involving light, temperature, and food availability (17, 21). Furthermore, after starvation (11) or lipectomy (6, 8), adipose tissue mass is restored to seasonally appropriate levels. These observations, as well as the metabolic similarities of hyperphagic *S. parryii* to genetically obese mice, suggest that a feedback loop involving a lipostat, and possibly leptin, is utilized by hibernators in the regulation of body weight. Changes in metabolic rate and body temperature may also contribute to their ability to regulate adiposity.

Additional components of this system have yet to be elucidated. Recent cloning of the leptin receptor (18) and the discovery that a nonfunctional receptor accounts for the phenotype of the *db* mutation (4, 13, 18) indicate that the receptor may also play an important role in the seasonal regulation of adiposity.

Perspectives

Genetically obese mice and rats are commonly used for investigating appetite and body weight regulation. In this paper, we have used a natural model of seasonal obesity to characterize the effect of mouse recombinant leptin during prehibernation hyperphagia, the arctic ground squirrel. Our results indicate that pharmacological doses of leptin reduce food intake and body weight during prehibernation fattening and provide further support for the conservation of leptin function in mammals. We are now faced with more complex questions. Is endogenous squirrel leptin involved in the control of seasonal changes in appetite and body weight?

Understanding leptin's involvement in the annual body weight cycle in hibernating mammals will require analysis of circulating leptin and leptin receptor density changes during the phases of hyperphagia (prehibernation fattening) versus hypophagia or anorexia (hibernation) and determining whether such changes cause or are caused by altered body weight, adipose tissue mass, or other factors. The spontaneous and wide swings in appetite and the seasonal nature of body weight regulation observed in hibernating species

should provide meaningful clues to advance our understanding of the physiological control mechanisms regulating adiposity and energy balance in mammals.

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