Leptin antagonist reveals that the normalization of caloric intake and the thermic effect of food after high-fat feeding are leptin dependent


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Submitted 27 March 2006; accepted in final form 27 September 2006

Consumption of a high-fat diet leads to obesity and severe metabolic consequences (7, 19). When rodents are fed a high-fat diet ad libitum, the immediate response is an increase in caloric intake and a corresponding increase in energy expenditure. In most circumstances, the increase in caloric intake is transient and returns to control or nearly control levels within several weeks (21). The exact mechanism underlying this homeostatic normalization of caloric intake is unknown but, presumably, it involves leptin. Leptin is one factor that mitigates food consumption while promoting energy expenditure (2, 4–6, 17). Because leptin levels rise after food consumption (3), it is reasonable to conjecture that this hormone mediates normalization of caloric intake after high-fat feeding. This is supported by our recent study indicating that when lean leptin-resistant rats are fed a high-fat diet, they respond with an unabated increase in caloric intake, indicating that leptin-resistant rats are unable to homeostatically downregulate caloric intake after high-fat feeding (15).

In addition to decreasing food intake, leptin enhances energy expenditure and may mediate the well-described thermic effect of food consumption, which in rodents is manifested by elevated thermogenesis in brown adipose tissue (BAT) (12, 13). In contrast to the transient nature of the increased caloric intake, the temporal relation of the elevated energy expenditure with high-fat feeding is less well documented but, likely, dissipates as obesity and leptin resistance emerge.

Although circumstantial evidence indicates a role for leptin in the normalization of the caloric intake and the enhanced energy expenditure after high-fat feeding, direct evidence is lacking. In the present study, we used a leptin antagonist [a triple mutant of rat leptin resulting in a L39A/D40A/F41A mutant (Protein Laboratories, Rehovot, Israel)] to examine the role of leptin in these biological processes. To this end, we first established the effectiveness of the leptin antagonist in blocking centrally introduced leptin-mediated signal transducer and activator of transcription-3 (STAT3) phosphorylation in the hypothalamus and the anorexie response to leptin, as well as the consequences of a short infusion of the antagonist on food consumption and body weight. We then fed rats chow or a high-fat diet and simultaneously infused the leptin antagonist or vehicle while assessing caloric intake, hypothalamic leptin signaling, and uncoupling protein-1 (UCP1) levels in BAT.

Materials and Methods

Experimental Animals

Four-month-old male Fischer 344 × Brown Norway rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). On arrival, rats were examined and remained in quarantine for 1 wk. Animals were housed individually with a 12:12-h light-dark cycle (lights on from 0700 to 1900) and cared for in accordance with the principles of the National Institutes of Health Guide to the Care and Use of Experimental Animals. Protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Experimental Design

This study consisted of four experiments. Experiment 1. Murine leptin (100 ng), leptin (100 ng) + rat leptin antagonist (0.3, 1, 3.5, 10, or 20 μg; Protein Laboratories Rehovot), or

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vehicle was injected intracerebroventricularly into the third ventricle. Rats were killed 1 h later, and hypothalamic leptin signaling was assessed by STAT3 phosphorylation levels. In a separate group, after central administration of 20 µg of rat leptin antagonist, leptin signaling was compared with that in vehicle-injected controls.

Experiment 2. Rats fed standard chow were infused with leptin antagonist (25 µg/day into the lateral ventricle) or vehicle (control) by minipump for 7 days. A separate group of rats was fed standard chow and infused with leptin (1 µg/day into the lateral ventricle), leptin + leptin antagonist (200 µg/day), or vehicle (control) by minipump for 7 days. Food intake and body weight were recorded daily, and animals were euthanized on day 7 for tissue analysis.

Experiment 3. Rats were fed a high-fat diet (60% fat, 5.2 kcal/g; diet D12492, Research Diets, New Brunswick, NJ) and simultaneously infused with leptin antagonist (25 µg/day into the lateral ventricle) or vehicle by minipump for 7 days (control-high-fat and antagonist-high-fat groups, respectively). Rats fed standard chow (4.4% fat, 3.1 kcal/g; diet 8604, Harlan Teklad, Madison, WI) were also infused with vehicle (control-chow group). Caloric intake and body weight were recorded daily. Animals were euthanized 7 days after infusion for tissue analysis.

Experiment 4. Rats were fed chow or a high-fat diet for 2 days and intracerebroventricularly injected with 250 ng of leptin or vehicle as control into the third ventricle (chow-control, chow-leptin, high-fat-control, and high-fat-leptin groups, respectively). Rats were killed 1 h later, and hypothalamic leptin signaling was assessed by STAT3 phosphorylation levels. Serum was collected from control animals for assessment of leptin levels.

Acute Leptin and Antagonist Administration

Under anesthesia with ketamine (75 mg/kg) and xylazine (7 mg/kg), the animal’s head was prepared for surgery, and the animal was placed in a stereotaxic frame. A small (1.5-cm) incision was made over the midline of the skull to expose the landmarks of the cranium (bregma and lambda). The coordinates for injection into the third cerebral ventricle are 1.3 mm anterior to bregma and 9.6 mm ventral from the skull surface, at an angle of 20° anterior to posterior. A small hole was drilled through the skull, and insertion of a 23-gauge stainless steel guide cannula was followed by placement of an injection cannula. A 10-µl syringe was used to deliver a 5-µl volume containing 100 ng of leptin, 100 ng of leptin + antagonist (0.3, 1, 3.5, 10, or 20 µg), or vehicle (artificial cerebrospinal fluid).

Leptin Antagonist Infusion

Rats were anesthetized with 5% isoflurane by inhalation and maintained on 2.5% isoflurane after a surgical plane of anesthesia had been reached. After the animal’s head was prepared for surgery and the animal was placed in a stereotaxic frame, a small (1.5-cm) incision was made over the midline of the skull to expose the landmarks of the cranium (bregma and lambda). A cannula (Durect, Cupertino, CA) was placed into the lateral ventricle: 1.3 mm posterior to bregma and 1.9 mm lateral to the mid sagittal suture, to a depth of 3.5 mm. Acrylic dental cement was used to anchor the cannula to the skull. A subcutaneous pocket on the dorsal surface was created using blunt dissection, and an osmotic minipump (Durect) was inserted. The pumps (model 2001, Alzet) infuse 1 µl of fluid per hour for a minimum of 7 days and have a total capacity of 200 µl. In these infusion studies, leptin and the antagonist were dissolved in an NaHCO3 solution (0.4% wt/vol), which was used as vehicle. All prefilled minipumps were incubated in sterile saline at 37°C for 24 h before implantation. A catheter tube was employed to connect the cannula to the osmotic minipump flow moderator. The incision for the minipump was then closed with sutures. Rats were kept warm during the manipulation and until fully recovered.

Tissue Harvesting and Preparation

Rats were killed by thoracotomy under pentobarbital sodium anesthesia (150 mg/kg). Blood samples were collected by heart puncture, and serum was harvested by 10 min of centrifugation in serum separator tubes. The circulatory system was perfused with 20 ml of cold saline. Perirenal, retroperitoneal, and epididymal white adipose tissues, interscapular BAT, and the hypothalamus were excised. For removal of the hypothalamus, an incision was made medial to the pitiform lobes, caudal to the optic chiasm, and anterior to the cerebral crus, to a depth of 2–3 mm. The hypothalamus was sonicated in 10 mM Tris·HCl (pH 6.8), 2% SDS, and 0.08 µg/ml okadaic acid + protease inhibitors. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA). BAT samples prepared similarly were filtered through a 0.45-µm syringe filter (Whatman, Clifton, NJ) to remove lipid particles before protein measurement.

STAT3 and Phosphorylated STAT3 Assay

The methods for STAT3 and phosphorylated STAT3 assay were described in detail previously (8). Briefly, samples were boiled and separated on a 10% agarose-Tris·HCl gel (Bio-Rad) and electrotransferred to a nitrocellulose membrane. Immunoreactivity was assessed with an antibody specific to phosphorylated STAT3 (Cell Signaling, Danvers, MA). Immunoreactivity was visualized by the ECL Plus detection system (Amersham, Piscataway, NJ) and quantified by ImageQuant TL (Amersham). After phosphorylated STAT3 quantification, membranes were stripped of antibody with Immunopure (Pierce, Rockford, IL), and immunoreactivity was reassessed using a total STAT3 antibody.

UCP1 in BAT

The BAT homogenate prepared as described above was separated on an agarose-Tris·HCl gel and electrotransferred to a nitrocellulose membrane. Immunoreactivity was assessed with antibodies specific to UCP1 (Linco Research). Immunoreactivity was visualized by the ECL Plus detection system and quantified by ImageQuant TL.

Serum Leptin

Serum leptin levels were measured using a rat radioimmunoassay kit (Linco Research).

Statistical Analysis

Values are means ± SE and were analyzed by one- or two-way ANOVA. When the main effect was significant, a post hoc test was applied to determine individual differences between means. P < 0.05 was considered significant.

RESULTS

Leptin Signaling After Antagonist Blockade

Hypothalamic leptin signaling was measured as leptin-induced STAT3 phosphorylation using 100 ng of leptin, a dose previously determined to evoke maximal signaling (14). This dose of leptin, in combination with increasing doses of antagonist (0.3, 1, 3.5, 10, or 20 µg), or vehicle was centrally administered, and hypothalamic leptin signaling was assessed 1 h later. As expected, phosphorylated STAT3 was increased fourfold in leptin- compared with vehicle-injected rats. The leptin antagonist inhibited this leptin-mediated signaling in a dose-response manner (Fig. 1). The highest dose of the antagonist representing a 200-fold higher concentration than the administered leptin mostly inhibited the leptin-mediated signaling (Fig. 1). Administration of higher doses of the leptin antagonist was limited by solubility of the compound. To
verify that the compound does not have partial agonist characteristics, a separate group of rats was injected with the highest dose of antagonist (20 μg), and the STAT3 phosphorylation levels were compared with those of vehicle-injected rats (Fig. 1, inset).

Leptin Antagonist Infusion

To characterize the physiological effects of the leptin antagonist, we infused chow-fed rats with antagonist (25 μg/day into the lateral ventricle) or vehicle for 7 days and assessed caloric intake and body weight. The caloric intake was moderately, but significantly, higher in rats infused with antagonist than in control rats throughout most of the infusion period, with a peak increase of 16.1 kcal/rat (equal to 5.2 g of chow diet) on day 2 (Fig. 2A). However, over time, the increase waned to <20% and, by day 6, was no longer statistically significant. Over the 7-day period, the antagonist-infused rats not only consumed 32% more energy but also gained more weight than the control rats: 12.7 ± 10.36 vs. 8.74 ± 2.40 g (Fig. 2B). At death, leptin signaling was assessed by hypothalamic STAT3 phosphorylation, yet there were no significant differences between the groups (data not shown).

Leptin Antagonist Blocks the Leptin-Induced Anorexic Effects

To further demonstrate the blockade of leptin by the antagonist, we infused chow-fed rats with leptin alone (1 μg/day into the lateral ventricle), the same dose of leptin + leptin antagonist (200 μg/day), or vehicle for 7 days. In the leptin-infused rats, daily food intake was significantly lower than in control rats starting at day 2, whereas body weight loss was significant from day 5 (Fig. 3). In contrast, food intake and body weight in rats infused with leptin + antagonist were not different from controls. At death, leptin infusion resulted in a 70% increase in hypothalamic STAT3 phosphorylation, which was completely blocked in the leptin + antagonist-treated group (Fig. 4A). Leptin infusion elevated UCP1 levels in the BAT nearly sixfold. Antagonist infusion completely prevented the increase in UCP1 protein (Fig. 4B).

High-Fat Feeding

Rats were fed a high-fat diet (60% kcal as fat) and simultaneously infused with antagonist (25 μg/day into the lateral ventricle) or vehicle by minipump for 7 days. Rats fed standard chow were also infused with vehicle. Daily caloric intake in antagonist-high-fat and control-high-fat groups peaked on day 2, with increases of 90.2% and 66.7%, respectively, over
control-chow rats (Fig. 5A). In control-high-fat animals, the increased caloric intake rapidly declined, with a nearly complete attenuation by day 7, whereas that of antagonist-high-fat rats remained elevated and was significantly greater throughout the remainder of the experiment. Over the 7-day period, the control-high-fat group consumed 42% more energy than the control-chow group, whereas the antagonist-high-fat group consumed 90% more than control-chow group and 33% more than the control-high-fat group (Table 1). Moreover, the peak antagonist-induced increase in caloric intake in animals fed the high-fat diet was similar to the increase observed in chow-fed rats in Fig. 2. For example, on day 2, the antagonist evoked a 15.2 kcal/day increase in animals fed the high-fat diet (antagonist-high-fat vs. control-high-fat group; Fig. 5) compared with a 16.2 kcal/day increase (calculated from an increase of 5.21 g/day) in chow-fed rats also at day 2 (antagonist-chow vs. control-chow group; Fig. 2).

Body weight in the control-chow group was relatively stable throughout the 7-day experiment, whereas body weight increased in both high-fat groups. Body weight increased more rapidly in the antagonist-high-fat than in control-high-fat group, and weight gains between the two groups significantly diverged by day 3. By the end of the experiment, the control-high-fat group gained ~6-fold more weight than the control-chow group, whereas the antagonist-high-fat group gained 14-fold more weight than the control-chow group and 2.4-fold more than the control-high-fat group (Fig. 5B).

Adiposity Levels

The high-fat diet significantly elevated white adipose tissue and BAT weights in antagonist-high-fat and control-high-fat groups (Table 1), but the increase was not significantly greater in the antagonist-high-fat than in the control-high-fat group (P > 0.05; Table 1).

UCP1 Protein in BAT

Animals were killed 7 days after antagonist or vehicle infusion, and UCP1 protein levels in BAT were assessed. Consistent with our previous findings (21), the high-fat diet increased UCP1 protein levels in BAT (Fig. 6). Antagonist infusion completely blocked the effect of the high-fat diet,

![Graph](https://example.com/graph1.png)

Fig. 3. Daily food intake (A) and body weight (B) in chow-fed rats after 7 days of leptin (1 μg/day), leptin (1 μg/day) + antagonist (200 μg/day), or vehicle infusion. Infusions commenced on day 0. Values are means ± SE of 6 rats in each group. Significantly different from control (⁎P < 0.05; ⁎⁎P < 0.01). Neither food intake nor body weight in the leptin + antagonist group was different from the control group during the infusion.

![Graph](https://example.com/graph2.png)

Fig. 4. Hypothalamic STAT3 phosphorylation (A) and uncoupled protein (UCP1) protein levels in brown adipose tissue (BAT, B) after 7 days of leptin (1 μg/day), leptin (1 μg/day) + antagonist (200 μg/day), or vehicle infusion. STAT3 phosphorylation was normalized to total STAT3, and levels in control rats were set to 100, with SE adjusted proportionally. **P < 0.001 vs. control and leptin + antagonist.
inasmuch as UCP1 levels were nearly identical in the antagonist-high-fat and control-chow groups (Fig. 6). When expressed as UCP1 per milligram of BAT protein, the high-fat diet induced a similar 2.6-fold elevation in UCP1 (258 ± 110 vs. 32.4 ± 11.9 arbitrary units in control-high-fat and control-chow groups, respectively, \(P < 0.01\)) that was also blocked by the antagonist infusion (94.3 ± 16.7 arbitrary units).

**Leptin Signal Transduction in the Hypothalamus**

Unexpectedly, STAT3 phosphorylation levels were not significantly different between control-chow, control-high-fat, and antagonist-high-fat groups (Table 1). Leptin Challenge After High-Fat Feeding

The lack of an increase in phosphorylated STAT3 levels 7 days after high-fat feeding suggests that leptin signaling may already have returned to baseline level in parallel with the normalization of caloric intake. For this reason, we examined leptin signaling at the peak of high-fat-induced caloric intake. At this time, 2 days after initiation of high-fat feeding, serum leptin levels were elevated threefold in the high-fat-fed compared with the chow-fed rats (Fig. 7A). However, despite this elevated serum leptin, basal levels of STAT3 phosphorylation were similar in the high-fat- and chow-fed rats (Fig. 7B, chow-control vs. high-fat-control group). We then examined maximal leptin signaling by intracerebroventricular administration of leptin to chow- and high-fat-fed rats. Leptin elevated STAT3 phosphorylation more than threefold in the chow-fed rat but just over twofold in the high-fat-fed rats, with the maximal level of leptin signaling significantly reduced in high-fat- compared with the chow-fed rats (Fig. 7B).

**DISCUSSION**

The importance of leptin in long-term homeostatic body weight regulation is well established (5, 16), but its exact role in the short-term regulation of food intake and energy expenditure is less certain. In the present study, we employed a leptin
antagonist to ascertain the role of leptin in the normalization of caloric intake after high-fat feeding and in the thermic effect of increased caloric intake. We first characterized the antagonist properties of this compound. This antagonist fully blocked, in a dose-response manner, the leptin-mediated signaling after an acute dose of centrally administered leptin. A 1-wk central infusion of the antagonist resulted in the predicted increase in food consumption and body weight gain. Moreover, when infused for 1 wk simultaneously with leptin, the antagonist prevented the anorexic and weight-reducing responses to leptin as well as the increase in leptin signaling. Additionally, the action of this agent appears to be that of a pure antagonist: the compound neither stimulated STAT3 phosphorylation nor reduced basal levels of phosphorylated STAT3. Finally, the antagonist blocked the leptin-mediated elevation of UCP1 protein in BAT. These data indicate that this compound is a pharmacologically and physiologically active antagonist of rat leptin receptor.

When administered simultaneously with the initiation of high-fat feeding, the antagonist prevented the normalization of caloric intake that ordinarily occurs, demonstrating the importance of endogenous leptin in everyday weight control. Moreover, these results support our previous findings in rats that were made leptin resistant by chronic central overexpression of leptin. In those studies, the leptin-resistant rats displayed the same response to high-fat feeding as did the leptin antagonist-treated rats in the present study; i.e., they failed to downregulate the high-fat-induced increase in caloric intake, thus resulting in an exacerbated weight gain (15, 21). These data further support our contention that leptin resistance is not only a consequence of obesity but also one cause of obesity.

Increased caloric intake, whether by high-fat feeding or otherwise, often results in elevated energy expenditure (1). High-fat feeding stimulates the sympathetic outflow to BAT and the subsequent activation of thermogenesis, the putative thermic effect of food (9, 10, 18). Although thermogenesis was not assessed in the present report, UCP1 protein, a reasonable marker for BAT thermogenesis, was elevated by nearly two-fold after high-fat feeding. Moreover, this increase in UCP1 protein levels was completely prevented by the antagonist infusion, indicating that the BAT thermogenic effect of food in response to high-fat feeding is leptin dependent.

Despite the overt detrimental outcomes of the antagonist treatment on body weight, we were unable to relate the failure of caloric restoration to a specific inhibition of leptin signaling. This was mainly because the high-fat-fed rats did not express elevated hypothalamic leptin signaling on day 7 after high-fat feeding or on day 2, during the peak increase in caloric intake. This lack of an endogenous increase in leptin signaling occurred, despite a threefold elevation in serum leptin at the peak of caloric intake. These data suggest that any leptin-mediated signaling event triggered by increased caloric intake is below detection by our method of examining whole hypothalamic STAT3 phosphorylation, that leptin signaling has already returned to basal levels by day 2, or that this physiological response of leptin is mediated by another signaling pathway, such as the phosphatidylinositol 3-kinase pathway (11, 22). Nevertheless, it is apparent that the normalization of caloric intake after high-fat feeding is a leptin-mediated event.

Even though we were unable to detect an increase in leptin signaling associated with high-fat feeding, when exogenous leptin was centrally administered on day 2, at the peak caloric intake, there was a robust increase in hypothalamic leptin signaling, confirming pharmacological responsiveness to leptin. However, maximal leptin signaling during this period of peak caloric intake was diminished, suggesting that desensitization may have occurred, potentially in response to the high-fat diet-induced elevated leptin. We previously demonstrated that high-fat feeding reduces the maximal signaling capacity and that this is associated with a similar reduction in leptin receptor expression. In our previous study, however, the high-fat feeding was prolonged (115 days) and the result was leptin-resistant animals (20). The present study suggests that this putative desensitization occurs rapidly and is likely not related to the development of leptin resistance.

In summary, the present report describes the physiological responses to the central infusion of a leptin antagonist in chow- and high-fat-fed rats. The antagonist was able to block hypothalamic leptin signaling in response to an acute exogenous

Fig. 7. Serum leptin levels (A) and STAT3 phosphorylation after central leptin administration (B) after 2 days of high-fat feeding. Values are means ± SE of 5–6 rats in each group. STAT3 phosphorylation was normalized to total STAT3, and levels in chow-control (Ctrl) rats were set to 100, with SE adjusted proportionally. *P < 0.01 between HF-leptin and chow-leptin groups; P < 0.001 between HF-leptin and both control groups.
central challenge with leptin or a 1-wk central infusion of leptin. Infusion of the antagonist resulted in the predicted increase in food consumption and weight gain, and the antagonist prevented the anorexic response to a leptin infusion. The homeostatic normalization of elevated caloric intake after high-fat feeding and the increase in UCP1 protein in BAT were prevented by the leptin antagonist, indicating that these processes are leptin dependent. These data demonstrate an important role for leptin in the homeostatic response to high-fat feeding.

**REFERENCES**

This work was supported by the Medical Research Service of the Department of Veterans Affairs and National Institute on Aging Grants AG-20985 and AG-26159.

**GRANTS**