Leptin does not fully account for the satiety activity of adipose tissue-conditioned medium

DAVID S. WEIGLE,1 AMY M. HUTSON,1 JANET M. KRAMER,2 MARGARET G. M. FALLON,2 JOYCE M. LEHNTER,2 SI LOK,2 AND JOSEPH L. KUIJPER2

1Department of Medicine, University of Washington School of Medicine, Seattle 98195; and 2ZymoGenetics Corporation, Seattle, Washington 98102

Weigle, David S., Amy M. Hutson, Janet M. Kramer, Margaret G. M. Fallon, Joyce M. Lehnert, Si Lok, and Joseph L. Kuijper. Leptin does not fully account for the satiety activity of adipose tissue-conditioned medium. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R976–R985, 1998.—To determine whether leptin alone accounts for the satiety activity secreted by native adipose tissue, we prepared culture media conditioned by microdissected adipose tissue from overfed Long-Evans rats, fa/fa rats, or db/db mice (media A, B, and C, respectively). Medium A significantly suppressed food intake following intracerebroventricular delivery to Long-Evans rats (2-h chow intake = 68 ± 5% of baseline, P < 0.001). Media B and C significantly suppressed food intake following intraperitoneal delivery to ob/ob mice (24-h chow intake = 56 ± 7% of baseline for medium B, P = 0.001; 4-day chow intake = 78 ± 3% of baseline for medium C, P = 0.004). Using a leptin receptor-based bioassay, we determined that the leptin concentration of medium C was 392 ± 18 ng/ml. This concentration was 20-fold lower than the concentration of recombinant murine leptin required to produce a similar degree of feeding suppression following 5 days of administration to ob/ob mice. Neither medium conditioned by adipose tissue from ob/ob mice nor medium conditioned by adipose tissue from fa/fa rats and subsequently immunodepleted of leptin had significant satiety activity. We conclude that leptin is necessary but not sufficient to account for the satiety activity of native adipose tissue, perhaps due to the production by adipocytes of a cofactor that augments the effect of leptin to suppress feeding.

obesity; appetite; body composition; energy balance; lipostatic factor

The existence of a satiety factor secreted by adipose tissue was first postulated by Kennedy in 1953 (19). We (18) and others (9, 17) had obtained evidence for an adipose-derived protein with satiety activity before the discovery of leptin; however, the difficulty of a purification guided only by a feeding bioassay prevented the isolation of the responsible molecule(s). Leptin appears to fulfill all of the predictions of the Kennedy lipostatic hypothesis in that it is produced only by adipocytes in the adult animal, circulates in proportion to total adipose tissue mass, and interacts specifically with a hypothalamic receptor to reduce food intake and promote weight loss (4). Following the demonstration that recombinant leptin behaves as a prototype adipose satiety factor (3, 12, 24, 34), there have been no subsequently published studies attempting to characterize the satiety activity of native adipose tissue.

Several observations suggest that the role of leptin in regulating adipose mass is more complex than predicted by the original lipostatic hypothesis. The majority of animal (22) and human (22, 35) obesities are associated with elevated circulating leptin levels, large doses of recombinant leptin are required to suppress food intake by lean mice and mice with obesity syndromes unrelated to mutation of the obese gene (3, 11, 12, 24), and circulating leptin levels in humans with identical body fat contents vary as much as 10-fold (22, 35). Collectively, these observations have been interpreted to reflect the existence of variable resistance to leptin action in the central nervous system (22, 27). An alternative possibility is that adipose-derived satiety activity is not fully explained by leptin. Native adipose tissue could secrete other factors that augment leptin action or secrete leptin in a form that has greater biological activity than recombinant protein. Variations in these additional factors or processing pathways could be as important as the measured circulating leptin level in regulating adipose mass.

We reported previously that medium conditioned by adipose tissue from db/db mice contains immunoreactive leptin and can suppress food intake for a 24-h period following intraperitoneal injection in ob/ob mice (34). In the present study we present a more complete biological characterization of adipose-derived satiety activity and the leptin content of adipose-conditioned media. Our results suggest that native adipose tissue has the capacity to enhance the effect of leptin on feeding and energy balance in vivo.

METHODS

Preparation of conditioned media. All procedures involving animals were approved by the University of Washington Animal Care Committee. Donors of adipose tissue for preparation of conditioned media included Long-Evans rats (Simonson Laboratories, Gilroy, CA) that had been chronically overfed a mixture of powdered rat chow and corn oil by gastric infusion to produce obesity as described previously (36), lean Long-Evans rats that had been fasted for 48 h, nonfasted Zucker fa/fa rats (Harlan Sprague Dawley, Indianapolis, IN), and nonfasted ob/ob and db/db mice (both from Jackson Laboratories, Bar Harbor, ME). Quadriceps muscle was obtained from Sprague-Dawley rats (Harlan Sprague Dawley) for preparation of control conditioned media. All animals were males and were 10–14 wk old at the time of tissue collection. Muscle or pooled epididymal, inguinal, dorsal, and retroperitoneal adipose tissue pads were removed under Metofane anesthesia, placed into room-temperature medium,
and immediately dissected with sharp scissors into 10- to 15-mg fragments. Long-Evans rat adipose tissue was processed in Medium 199 plus 15 mM HEPES, 1 µg/ml leupeptin, and 1% penicillin-streptomycin solution (Life Technologies, Grand Island, NY). Tissues from all other animals were processed in DMEM containing 4.5 g/l glucose, 20 mM HEPES, and 1% penicillin-streptomycin solution. Tissue fragments were washed twice in medium and then incubated in 150-mm culture dishes for 3–5 h at 37°C in a 5% CO₂ atmosphere (2.5 ml medium/g tissue). The dishes were rotated gently for 1 min every 0.5 h. Following incubation, tissue was removed by centrifugation, and the aqueous layer was filtered through a 0.2-µm membrane. The medium was concentrated 16- to 80-fold at a 1-kDa cutoff in a sterile ultrafiltration cell (YM-1 membrane; Amicon, Beverly, MA), and final protein concentration was determined using a bicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin standards. The concentration of tumor necrosis factor (TNF)-α in conditioned media was measured by L929 cell cytotoxicity assay (1), and recombinant murine TNF-α was from Genzyme (Cambridge, MA). The endotoxin concentration of conditioned media was measured by an automated limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA), and purified endotoxin (Escherichia coli O113:H10) was from Associates of Cape Cod. Assay of satiety activity of conditioned media. The satiety activity of media conditioned by adipose tissue from overnight and fasted Long-Evans rats was assayed by intracerebroventricular injection into six 300-g male Long-Evans rats that had stainless steel 22-gauge guide cannulas stereotaxically placed into the third ventricles. Cannula placement was verified by an immediate drinking response to a 100-ng injection of angiotensin II. After recovery from surgery, the assay rats were kept on a 10:14-h light-dark cycle and were trained to consume a 2-h test meal of powdered chow at the onset of darkness. Chow was also presented for 6 h at the end of the dark phase to assure normal 24-h caloric intake and growth. Conditioned media were given as 15-µl injections immediately before the beginning of the dark phase, and chow was weighed 2 h later. The feeding response to an injection of conditioned medium was expressed as a percentage of mean of 2-h Chow intake following 15-µl injections of unconditioned medium on the day preceding and following the test day.

To assay the effect of peripherally administered conditioned media on feeding, male 6- to 8-wk-old ob/ob mice were caged individually and accustomed to daily 1-ml intraperitoneal injections of PBS given between 1000 and 1100. Animals had continuous access to water and standard rodent chow and were trained to consume a 2-h test meal of powdered chow at the onset of darkness. Chow was also presented for 6 h at the end of the dark phase to assure normal 24-h caloric intake and growth. Conditioned media were given as 15-µl injections immediately before the beginning of the dark phase, and chow was weighed 2 h later. The feeding response to an injection of conditioned medium was expressed as a percentage of mean of 2-h Chow intake following 15-µl injections of unconditioned medium on the day preceding and following the test day.

Conditioned taste aversion testing. Overnight-fasted ob/ob test and control mice (n = 5 per group) were trained over 4 days to drink flavor 1 liquid diet (vanilla Ensure; Abbott Labs, Columbus, OH) for 2 h following a 1-ml intraperitoneal injection of PBS. On day 5, test mice received a 1-ml injection of adipose-conditioned medium, control mice received a 1-ml injection of PBS, and both groups were offered flavor 2 diet (chocolate Ensure). On day 6, all mice were injected with 1 ml of PBS, both flavor 1 and flavor 2 were presented, and the consumption of each diet was quantified. As a positive control on day 7, test mice received 1 ml of 0.15 M lithium chloride, control mice received 1 ml of PBS, and both groups were offered flavor 3 diet (egg nog Ensure). On day 8, all mice were injected with 1 ml of PBS, both flavor 1 and flavor 3 were presented, and the consumption of each diet was quantified.

Production of recombinant mouse leptin. Mouse glutamine (+)-leptin was expressed as a secreted protein in Saccharomyces cerevisiae. The mature NH₂-terminal of the murine cDNA was fused to the S. cerevisiae α-factor prepro segment, and the fusion construct was expressed behind the triose phosphate isomerase promoter as previously described (32). Cells were grown in shake flask cultures to a final density of 2 g/l total cell protein, and Western analysis demonstrated the presence of free leptin protein in culture medium at a final concentration of ~4 mg/l. Medium was adjusted to pH 5.7 in 20 mM MES buffer and brought to 30% saturation in ammonium sulfate, and leptin was bound to a high-substituted phenyl Sepharose column (Pharmacia, Piscataway, NJ) at 4°C. The column was washed with MES buffer, and leptin was eluted in 10 mM borate buffer, pH 8.8. The eluate was adjusted to pH 7.0 and concentrated by ultrafiltration, and leptin was quantified by reverse-phase HPLC against standards that had been measured by mass spectrophotometry. Immunoassay of leptin in conditioned media. Quantification of immunoreactive leptin in conditioned media was accomplished by one of two assays. The first was a commercial radioimmunoassay based on a polygonal antiserum and standards of either rat or mouse recombinant leptin (Linco, St. Charles, MO). The second assay, an ELISA developed for precise quantification of murine leptin, was based on a previously described rabbit polyclonal antibody to leptin (34) and a monoclonal antibody generated by immunizing mice to a maltose-binding/leptin fusion protein (34) and screening hybridomas prepared from immune spleen cells for leptin-specific binding activity. The wells of microtiter plates were coated with the monoclonal antibody at 2.5 µg/ml, murine leptin standards and unknowns were added to triplicate wells, and plates were incubated at 37°C for 2 h. Plates were then washed, polyclonal rabbit anti-leptin antibody was added at 2.5 µg/ml, plates were incubated at 37°C for 1 h. Plates were again washed, goat anti-rabbit IgG conjugated to horseradish peroxidase (BioSource, Camarillo, CA) was added at a 1:2,000 dilution, o-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) was added, and the optical density of the wells was read at 490 nm. The detection limit of this assay was 1 ng/ml, and the intra-assay coefficient of variation was 5.8%.

Receptor-based BaF3 cell proliferative assay of leptin in conditioned media. A CDNA encoding a chimeric receptor was constructed using the extracellular domain [amino acid (aa) 1–838] of the human leptin receptor (31) fused to the transmembrane and cytoplasmic domains (aa 483-830) of the mouse thrombopoietin receptor (29). This construct was cloned into a mammalian expression vector designated pHZ-1. The pHZ-1 vector was identical to the previously described pHZ-200 vector (34), except that a neomycin resistance gene
was substituted for the dihydrofolate reductase gene as a selectable marker. Recombinant plasmid (30 µg) was transfected by electroporation into 5 × 10^6 cells of an interleukin-3 (IL-3)-dependent murine lymphocytic line designated BaF3 (21). Transfected cells were grown for 10 days in selective medium consisting of DMEM plus 10% fetal bovine serum (FBS), 5 ng/ml murine IL-3 (R & D Systems, Minneapolis, MN), and 0.5 mg/ml neomycin. Before an assay was set up, cells were washed four times with assay medium (DMEM plus 5% FBS and 0.5 mg/ml neomycin) to remove IL-3. Standards and unknown samples were serially diluted into assay medium and mixed with 5 × 10^5 cells per well in a standard microtiter plate. After the assay was incubated for 3 days at 37°C in a 5% CO₂ atmosphere, cell number was quantified by absorbance at 570 nm using the metabolic dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide as previously described (21). To control for nonspecific mitogens in adipose tissue-conditioned media, recombinant murine leptin standards were diluted into matched leptin-free conditioned media prepared from the adipose tissue of ob/ob mice. Additionally, all samples were tested in an assay using nontransfected BaF3 cells, and no mitogenic response was detected. The detection limit of this assay was 50 pg/ml, and the intra-assay coefficient of variation was 7.6%.

Immunodepletion of leptin from conditioned media. Approximately 30 mg each of monoclonal antibody to either leptin or thrombopoietin (control) were dissolved in 0.1 M NaHCO₃-0.5 M NaCl, pH 8.3, and coupled to 1.5 g dry weight of cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer’s instructions. Columns of each affinity resin were poured and washed extensively with PBS at 4°C. Fifteen milliliters of conditioned medium prepared from fa/fa rat adipose tissue were then recirculated at a rate of 0.65 ml/min over each column for 14 h at 4°C. Aliquots of immunodepleted medium were saved for leptin and endotoxin assays.

Statistics. In all versions of the feeding assay, variability was reduced by expressing the response to a test substance as a percentage of the response to control injections given to the same animal over a comparable time period. Statview 4.01 software was used to perform analysis of variance of mean feeding data, and Fisher’s protected least-significant difference test was used to perform post hoc comparisons between groups. All results are expressed as means ± SE, unless noted otherwise, with a significance level of 0.05.

RESULTS

Characterization of adipose-derived satiety activity. We demonstrated previously that induction of obesity in rats by chronic overfeeding resulted in a nearly complete suppression of voluntary food intake (36). Accordingly, our first efforts to detect a satiety activity in adipose-conditioned media were based on tissue collected from this animal model. Feeding was assayed following intracerebroventricular injections into test animals to make optimal use of limited volumes of concentrated adipose-conditioned media and to minimize the possibility of satiety factor degradation in the circulation. As shown in Fig. 1, injections of medium conditioned by adipose tissue from overfed donor animals suppressed 2-h chow intake to 68 ± 5% of baseline, whereas mean 2-h chow intake following injections of unconditioned medium was 99 ± 3% of baseline (P < 0.001). As a control, medium was conditioned using adipose tissue collected from hyperphagic 48-h-fasted rats and concentrated to the same final protein content as medium prepared from overfed rats. Mean 2-h chow intake following injections of fasted donor adipose-conditioned medium was 87 ± 7% of baseline (P < 0.05 vs. overfed donor adipose-conditioned medium, P = NS vs. unconditioned medium). Thus the satiety activity of conditioned media measured in assay animals following intracerebroventricular injection reflected the feeding status of the adipose tissue donor animal. Heating overfed donor adipose tissue-conditioned medium to 90°C for 5 min destroyed its satiety activity, suggesting that the responsible factor could be a protein.

On the basis of the hypothesis that adipose-derived satiety factor was transported to the brain in the circulation (19), we administered conditioned media by intraperitoneal injection in all subsequent feeding assays. We chose ob/ob mice as assay animals and fa/fa rats as adipose tissue donors because parabiosis studies published before the discovery of leptin suggested that the fa/fa rat, like the db/db mouse, had high levels of circulating satiety activity, whereas ob/ob mice were deficient in this activity (6, 7, 13, 33). As shown in Fig. 2, fa/fa rat adipose tissue-conditioned media suppressed 24-h food intake by ob/ob mice in proportion to the relative concentration of the media achieved by ultrafiltration (maximal suppression = 56 ± 7% of baseline intake, P = 0.001). Administration of muscle-conditioned medium at a protein concentration comparable to the most concentrated adipose tissue-conditioned medium had no effect on feeding. Repeated daily administration of fa/fa rat adipose tissue-conditioned medium for 12 days produced a sustained reduction in
food consumption accompanied by a progressive decrease in body weight (Fig. 3). Food consumption and body weight returned to control values after conditioned media injections were stopped.

Satiety activity could be recovered from a 66% saturated ammonium sulfate precipitate of adipose tissue-conditioned medium but was destroyed by heating to 90°C for 5 min or treating with a denaturing agent, 6 M guanidine hydrochloride (data not shown). These observations suggested that the satiety activity of adipose tissue-conditioned medium resided in a protein. TNF-α is a known anorectic protein that is produced by adipose tissue (15); however, the concentration of TNF-α in adipose tissue-conditioned media was only 1.79 ± 0.70 ng/ml. We observed significant feeding suppression in our assay only with injections of more than 300 ng of recombinant mouse TNF-α (24-h food intake 598% of baseline with 200 ng, 92% with 300 ng, 71% with 500 ng, and 65% with 1,000 ng of TNF-α). Similarly, endotoxin levels in adipose tissue-conditioned media ranged from unmeasurable to 6 ng/ml. We observed significant feeding suppression in our assay only with injections of more than 50 ng of purified endotoxin.

To establish that the satiety activity measured in our assay did not represent a nonspecific toxic effect, we administered adipose tissue-conditioned medium at a dose of 0.4 mg/g to five db/db mice. This dose reduced 24-h food intake only to 89 ± 5% of baseline in db/db animals as opposed to 58 ± 6% in three ob/ob mice (P = 0.01). The diminished effect in db/db animals agreed with the insensitivity of this strain to the parabiotic satiety factor (6, 7) and argued against nonspecific toxicity, which should have affected both strains equally. We performed a two-bottle conditioned taste aversion test as a final check on the specificity of adipose-derived satiety factor. This test is a standard paradigm for distinguishing physiological regulators of feeding from substances that reduce feeding through the induction of malaise (30). As summarized in Table 1, a taste aversion could not be produced in ob/ob mice by pairing injections of adipose tissue-conditioned medium with the presentation of a novel flavor (day 6 data). In contrast, a taste aversion was easily produced by pairing injections of lithium chloride, an established nauseant (23), with a novel flavor (day 8 data).

Comparison of the satiety activity of leptin and adipose tissue-conditioned medium. Following the sequencing and Northern analysis of the obese gene transcript
gave comparable leptin concentrations of 503 and 455 ng/ml in conditioned medium. This totally distinct assay radioimmunoassay to repeat the measurement of leptin by our assay. Accordingly, we used a commercial kit contained in adipose tissue-conditioned medium was assayed to determine the logical activity of rat and mouse leptin. Recombinant leptin at this dose failed to suppress 4-day food consumption of assay animals (ob/ob mice). We used db/db mice rather than fa/ob rats as adipose tissue donors for this experiment to avoid any confusion caused by differences in immunological activity of rat and mouse leptin. Recombinant murine leptin at a dose of 250 ng or 1 ml of db/db mouse adipose tissue-conditioned medium was then given to four ob/ob mice each, following the 4-day feeding assay protocol. Recombinant leptin at this dose failed to suppress 4-day food consumption of assay animals (100 ± 9% of baseline), whereas conditioned medium suppressed 4-day food consumption to 78 ± 3% of baseline (P = 0.004). We used the multiple-day feeding assay format in this and all subsequent experiments, both to minimize assay variability and to allow the detection of feeding effects that might take longer than 24 h to develop.

It was possible that the apparent difference in satiety activity between recombinant leptin and the leptin contained in adipose tissue-conditioned medium was due to underestimation of the native leptin concentration by our assay. Accordingly, we used a commercial radioimmunoassay to repeat the measurement of leptin in conditioned medium. This totally distinct assay gave comparable leptin concentrations of 503 ± 27 ng/ml in db/db mouse adipose-tissue conditioned medium and 455 ± 53 ng/ml in fa/ob rat adipose tissue-conditioned medium.

It remained possible that differences in the immunoreactivity of native leptin and the two different recombinant leptins used as immunoassay standards caused an underestimation of the leptin content of adipose tissue-conditioned medium. To address this possibility, we developed an assay in which BaF3 cells were transfected with a chimeric receptor construct composed of the extracellular domain of the leptin receptor fused to the transmembrane and intracellular domains of the thrombopoietin receptor. Administration of leptin to these cells led to a proliferative response that could be used to measure picogram quantities of leptin. With the use of recombinant murine leptin standards, the leptin concentration of db/db mouse adipose tissue-conditioned medium was found to be 392 ± 18 ng/ml when measured in the linear range of the receptor-based bioassay. The same stock of leptin used to standardize the bioassay was then used to construct a feeding dose-response curve in ob/ob mice (Fig. 4). Most importantly, this assay was sensitive only to the active receptor-binding region of the leptin molecule. With the use of recombinant murine leptin standards, the leptin concentration of db/db mouse adipose tissue-conditioned medium was found to be 392 ± 18 ng/ml when measured in the linear range of the receptor-based bioassay. The same stock of leptin used to standardize the bioassay was then used to construct a feeding dose-response curve in ob/ob mice.

Table 1. Two-bottle conditioned taste aversion test of fa/ob rat adipose tissue-conditioned medium

<table>
<thead>
<tr>
<th>Prior Stimulus</th>
<th>Flavor 1</th>
<th>Flavor 2</th>
<th>Flavor 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conditioned medium</td>
<td>5.9±1.8</td>
<td>2.1±1.8</td>
<td></td>
<td>8.0±0.4</td>
</tr>
<tr>
<td>PBS</td>
<td>4.1±2.3</td>
<td>3.8±2.5</td>
<td></td>
<td>7.9±0.5</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Day 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>7.6±0.8</td>
<td></td>
<td>0.2±0.1</td>
<td>7.8±0.9</td>
</tr>
<tr>
<td>PBS</td>
<td>5.1±2.3</td>
<td>2.4±1.8</td>
<td></td>
<td>7.5±0.6</td>
</tr>
<tr>
<td>P</td>
<td>0.05</td>
<td>0.03</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD. P values are from unpaired 2-tailed t-test with a significance level of 0.05.

Fig. 4. Standard curve for chimeric leptin/thrombopoietin receptor-based BaF3 cell proliferative leptin bioassay. Recombinant murine leptin standards were quantified by mass spectrometry. Line represents best linear fit to data points (r^2 = 0.962).

Fig. 5. Suppression of cumulative chow intake of db/db mice following 5 daily 1-ml intraperitoneal injections of recombinant murine leptin at the indicated doses (○) or 4 daily 1-ml intraperitoneal injections of 26-fold concentrated db/db mouse adipose tissue-conditioned medium containing 392 ± 18 ng/ml leptin (□). Data are expressed as percentage of cumulative chow intake following daily 1-ml injections of saline for an equal period of time immediately preceding the test days. Points represent means ± SE for 2–6 mice studied at each dose of recombinant leptin or 4 mice given adipose tissue-conditioned medium. Line represents best logarithmic fit to the recombinant leptin data points (r^2 = 0.886).
using the 5-day feeding assay protocol. As shown in Fig. 5, the satiety activity of db/db adipose tissue-conditioned medium was equivalent to a dose of leptin 20-fold greater than that measured in the medium.

Leptin dependence of the satiety activity of adipose tissue-conditioned medium. Although our data confirmed a greater satiety effect of adipose tissue-conditioned medium than an equivalent amount of recombinant leptin, it was unclear whether this additional activity resided in one or more molecules acting totally independently of the leptin signaling pathway. We performed two further experiments to address this issue. In the first experiment, we made conditioned medium from rat muscle, ob/ob mouse adipose tissue, and db/db mouse adipose tissue. All media were concentrated by ultrafiltration to a protein content of 15–20 mg/ml and assayed in ob/ob mice according to our long protocol. As shown in Fig. 6, db/db mouse adipose tissue-conditioned medium suppressed cumulative chow intake to a significantly greater degree than the suppression produced by muscle-conditioned medium. The feeding suppression produced by ob/ob mouse adipose tissue-conditioned medium did not differ significantly from that observed in response to muscle-conditioned medium. We interpreted this experiment to indicate that some amount of active native leptin is required to manifest the full satiety effect of adipose tissue-conditioned medium. To confirm this result, we produced additional adipose tissue-conditioned medium from fa/fa rat donors. The leptin concentration of this medium was determined by commercial radioimmunoassay to be 455 ± 53 ng/ml. We recirculated one-half of this medium over a Sepharose column to which a monoclonal antibody directed against thrombopoietin was conjugated. The other one-half of the medium was recirculated over a column to which a monoclonal antibody directed against leptin was conjugated. The final leptin concentrations in the two processed media were 379 ± 43 and 13 ± 1 ng/ml, respectively. As shown in Fig. 7, the control thrombopoietin-immunodepleted conditioned medium produced the expected degree of feeding suppression in our 5-day assay. The feeding suppression produced by leptin-immunodepleted conditioned medium did not differ significantly from the response to muscle-conditioned control media.

The results of these two experiments would be consistent with the production by adipose tissue of a cofactor that physically associates with leptin and augments its effect on feeding. If this hypothesis were true, it might be possible to reconstitute the full satiety activity observed in db/db mouse adipose tissue-conditioned medium by adding leptin at the concentration measured in this medium to ob/ob mouse adipose tissue-conditioned medium. Accordingly, we added recombinant leptin at a concentration of 500 ng/ml to both 100% ob/ob mouse adipose tissue-conditioned medium and 1% ob/ob mouse adipose tissue-conditioned medium in saline. These mixtures were prepared daily and incubated at 37°C for 1 h immediately before injection into 10 assay animals for 3 consecutive days. The effects of these mixtures on feeding were compared with the effect of concurrent control injections in 10 additional animals of 100% ob/ob mouse adipose tissue-conditioned medium without added leptin. As shown in Fig. 8, no significant suppression of feeding was produced by any of these injectates over the course of 3 days. We concluded from this result that if there were
physically associated leptin cofactor produced by adipose tissue, it could not be demonstrated by a simple in vitro mixing experiment.

As discussed earlier, neither TNF-α nor endotoxin, which causes the release of TNF-α and other proinflammatory cytokines in vivo, significantly suppressed feeding when injected in saline at the concentrations measured in adipose tissue-conditioned media. It remained possible, however, that even low concentrations of these substances could potentiate the effect of leptin in conditioned media and account for the suppression of feeding observed in our assay. To test this hypothesis, we measured daily food intake of ob/ob mice injected with either 4 µg/day of recombinant leptin, 100 ng/day of purified endotoxin, or a combination 4 µg/day of leptin and 100 ng/day of endotoxin. After baseline food intake data was collected for 4 days, three groups of five mice each received these injections for a period of 6 days. As shown in Fig. 9, endotoxin caused a suppression of daily food intake that characteristically resolved after 3 days despite continued daily injections. Leptin caused the expected degree of feeding suppression that was sustained over the entire course of the experiment. The combination of endotoxin and leptin was no more effective in suppressing feeding than either agent alone on each day of the experiment. This lack of synergy suggested that cytokines produced by adipose tissue were not responsible for the enhanced satiety effect of adipose tissue-conditioned media relative to leptin.

DISCUSSION

Our data establish that native adipose tissue secretes a satiety activity that conforms both to the predictions of the original lipostatic hypothesis (19) and to more recent results obtained by administering leptin to animals. The adipose-derived satiety factor acts specifically by either peripheral administration or direct delivery into the central nervous system, and it causes weight loss with repeated administration. Hulsey and Martin (17) and Goodner and Goodner (9) have detected similar activity following either aqueous or acid-ethanol extraction of adipose tissue from rats. The unexpected outcome of this work was that the leptin content of adipose tissue-conditioned medium was 20-fold lower than the dose of recombinant leptin required to produce a similar suppression of feeding in assay animals. Recombinant leptin administered at a dose equivalent to that delivered by injections of conditioned medium had absolutely no effect on feeding. The recombinant murine leptin used to establish the feeding dose-response relationship was the same leptin used to standardize the receptor-based leptin bioassay in which conditioned medium was analyzed. This experimental design eliminated the possibility that an error in quantifying our recombinant leptin could have altered the apparent relative potency of native and recombinant molecules. Furthermore, the use of a receptor-based assay corrected for possible structural differences between recombinant and native leptin that...
could have affected binding in the central nervous system.

There are two possible explanations for our findings. The first is that the recombinant leptin we used differs from native leptin at a site other than that required for receptor binding, and that this difference leads to reduced activity either by impairing entry into the central nervous system or by increasing clearance in the circulation. We feel that a critical structural abnormality of recombinant leptin is unlikely, in that the material we used was produced in the secretory pathway of yeast according to a protocol that has produced other mammalian proteins in fully active form (32). Mature leptin is not known to be glycosylated, and there is only one possible site for intramolecular disulfide bond formation. We have obtained similar feeding dose-response relationships using leptin produced both in baby hamster kidney cells (34) and in the cytosolic pathway of yeast followed by in vitro refolding. Finally, our dose-response data agree with studies published by other groups in which recombinant leptin was given by intraperitoneal bolus injection (3, 12, 24). Even when delivered by the much more physiological methods of continuous intraperitoneal (14) or continuous subcutaneous (11) infusion, the minimally effective daily dose of leptin ranges from 2 to 4.8 µg/day. These doses are greater by a factor of 5–12 than the amount of leptin delivered in a single daily bolus injection of adipose tissue-conditioned medium.

The second possible explanation for our findings is that native adipose tissue has the capacity to enhance the effect of leptin on feeding and body weight in vivo. This augmentation could be through posttranslational processing of leptin in a manner that is unique to adipose tissue, through the production of molecules with a satiety action that is completely independent of leptin, or through the production of molecules that interact with leptin and potentiate its effect. Although our data do not allow us to address the possibility of a unique adipocyte processing pathway for leptin, there is no evidence for an unusual glycosylation or cleavage pattern of the mature leptin molecule. We have looked for satiety factors that act independently of leptin by measuring the effect of ob/ob mouse adipose tissue-conditioned medium and leptin-immunodepleted fa/fa rat adipose tissue-conditioned medium on feeding. Neither of these preparations demonstrated significant satiety activity, arguing against the production of an independently acting factor. Therefore, we favor the hypothesis that adipose tissue produces a factor that potentiates leptin action, possibly through a direct physical interaction that reduces the degradation of leptin in the circulation or enhances the ability of leptin to cross the blood-brain barrier. In support of this hypothesis, there is evidence both that leptin circulates in association with several binding proteins (16, 28) and that adipose tissue produces a soluble form of the leptin receptor (20). We were unable to reconstitute the full satiety activity seen in fa/fa rat or db/db mouse adipose tissue-conditioned media by adding leptin at the concentration measured in these media to ob/ob mouse adipose tissue-conditioned media. This negative result might be explained by inadequate in vitro incubation conditions, a requirement for leptin and its putative cofactor to associate within the protein secretory pathway of adipocytes, or a requirement for functional leptin acting in vivo as an autocrine or paracrine agent to enable production of the putative cofactor by adipocytes.

It has been established that adipose tissue produces TNF-α, a potent anorectic cytokine and a possible mediator of the insulin resistance that accompanies obesity (15). It has also been demonstrated that bacterial endotoxin causes the secretion of leptin by adipose tissue as well as the release of proinflammatory cytokines from mononuclear phagocytes (10, 26). These observations raise the interesting possibility that leptin and cytokines might interact to reduce appetite both in obesity and in chronic inflammatory disorders. Our failure to find a synergistic, or even an additive, effect of endotoxin and leptin on food intake speaks against this hypothesis and suggests that an adipocyte-derived cytokine is not responsible for the enhanced satiety effect of adipose tissue-conditioned medium relative to leptin. These results are consistent with the finding of Faggioni and co-workers (8) that endotoxin is able to elicit a nearly normal anorectic response in leptin-resistant db/db mice and an exaggerated anorectic response in leptin-deficient ob/ob mice.

The elevated leptin levels that characterize animal and human obesity may reflect central resistance to leptin action, which, if present in the preobese state, could be the proximate cause of excess fat deposition (22, 27). Alternatively, if the physiological role of leptin is only to deactivate neuroendocrine adaptations to fasting when energy reserves are adequate (2), then hyperleptinemia may be irrelevant to the development and maintenance of obesity. Our data suggest a third possibility: leptin may be only one component of the endocrine signal that communicates the status of the body’s energy reserves from adipose tissue to the brain. This situation would be analogous to the complex interaction between insulin-like growth factor (IGF)-1 and the IGF-binding proteins in mediating the somatotropic effects of growth hormone (5). Variability or relative deficiency of the putative leptin cofactor could account for a variable or diminished ability of leptin to elicit satiety and curtail weight gain. The presence of an adipocyte-derived molecule that augments leptin action has obvious implications for the use of leptin in the treatment of human obesity.

Perspectives

The observation that adipose tissue secretes a satiety factor predated the discovery of leptin, and several groups attempted to isolate this factor using conventional biochemical techniques (6, 7, 9, 17, 18). Although it is unclear whether any of these attempts would have ultimately been successful, we feel that even in the age of molecular biotechnology there is a role for physiological experimentation in the discovery of important new regulatory and effector molecules. Our results suggest
that molecules in addition to leptin and insulin (25) may be involved in the feedback loop that communicates the status of the body's energy reserves to the brain. A leptin cofactor might be produced constitutively by adipocytes and secreted as a complex with leptin, or production of the putative leptin cofactor might be regulated by a variety of hormonal and fuel molecules. Modulation of the leptin signal by another molecule could explain why individuals of identical body fat content may remain in energy balance despite having circulating leptin levels that vary by as much as 10-fold (22, 35). In view of the striking redundancy of molecules involved in the feedback loop be-


