Peptides that Regulate Food Intake

Central leptin gene therapy fails to overcome leptin resistance associated with diet-induced obesity

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Submitted 11 April 2003; accepted in final form 11 August 2003

Wilsey, Jared, Sergei Zolotukhin, Victor Prima, and Philip J. Scarpace. Central leptin gene therapy fails to overcome leptin resistance associated with diet-induced obesity. Am J Physiol Regul Integr Comp Physiol 285: R1011–R1020, 2003; 10.1152/ajpregu.00193.2003.—The objective of this study was to determine if central overexpression of leptin could overcome the leptin resistance caused by 100 days of high-fat feeding. Three-month old-F344xBN male rats were fed either control low fat chow (Chow), which provides 15% of energy as fat, or a high-fat/high-sucrose diet (HF), which provides 59% of energy as fat. Over several weeks, the HF-fed animals spontaneously split into two groups of animals: those that became obese on the HF diet (DIO) and those that did not gain extra weight on the HF diet [diet resistant (DR)]. After 100 days of HF feeding, animals were given a single intracerebroventricular injection containing 5.75E10 particles of rAAV encoding leptin (rAAV-leptin) or control virus (rAAV-con). Chow animals responded robustly to rAAV-leptin, including significant anorexia, weight loss, and lipopenia. In contrast, DIO were completely unresponsive to rAAV-leptin. DR rats responded to rAAV-leptin, but in a more variable fashion than Chow. Unlike what was observed in Chow, the anorectic response to rAAV-leptin rapidly attenuated and was no longer significant by day 14 postvector delivery. Both DIO and DR animals were found to have reduced long-form leptin receptor expression and enhanced basal P-STAT-3 in the hypothalamus with respect to Chow. rAAV-leptin caused an increase in STAT3 phosphorylation and proopiomelanocortin expression in the hypothalamus and an increase in uncoupling protein-1 in brown adipose tissue in both Chow and DR animals, but failed to do so in DIO. This suggests that central overexpression of leptin is not a viable strategy to reverse diet-induced obesity.

STAT3; neuropeptide Y; proopiomelanocortin; uncoupling protein-1; adeno-associated virus

When a few injections of leptin were found to rapidly reverse obesity in the ob/ob mouse, excitement soared over a potential cure for the growing obesity epidemic. Unfortunately, the ob/ob mouse did not turn out to be a model of common obesity. Typical obese humans and animals are hyperleptinemic and resistant to exogenous leptin. Clinical trials with leptin have been disappointing due to this phenomenon of leptin resistance in the obese state, and interest in pharmacological leptin to treat obesity has waned. Since several obese, leptin-resistant models respond better to central vs. peripheral administration of recombinant leptin (11, 18, 24), it has been reasoned that leptin is not reaching its hypothalamic targets. Transport of leptin across the blood-brain barrier appears to be saturated at serum leptin levels observed in obesity (1), suggesting that deficient blood-brain barrier transport may play a role in leptin resistance. Thus, central delivery of leptin may be effective in cases of peripheral leptin resistance in obese humans and animal models.

A single intracerebroventricular injection of rAAV encoding leptin (rAAV-leptin) has been shown to cause a rapid and complete disappearance of white adipose tissue in genetically normal young adult Fischer 344 (F344) × Brown Norway (BN) male rats (22). In a recent report, a single injection of rAAV-leptin prevented diet-induced obesity in young male Sprague-Dawley rats when administered before commencing high-fat feeding (7). In the clinical treatment of obesity, however, a more typical objective is to reverse obesity in an already obese subject. The purpose of the present investigation was to determine if a single intracerebroventricular injection of rAAV-leptin could reverse the obesity caused by 100 days of high-fat feeding. To this end, we administered rAAV-leptin or control vector to high-fat-fed obese and diet-resistant animals as well as lean, chow-fed animals. Physiological responses to the rAAV-leptin vs. control vector were measured in all dietary groups, including anorexia, weight loss, and whole body energy expenditure via indirect calorimetry. After death, biochemical responses to rAAV-leptin were evaluated, including leptin signal transduction and neuropeptide expression in the hypothalamus, and uncoupling protein (UCP) in brown adipose tissue (BAT).
Regulatory element was placed downstream to enhance the transcription rate, as described by Unger (4) and green fluorescent protein (GFP) reporter gene expression was assessed using the nonesterified fatty acids measurement. All measurements were made between 0900 and 1400. Results are expressed as O2 consumption relative to metabolic body size (mL/min·kg0.75).

Animals

Three-month-old male F344 × BN rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). On arrival, rats were examined and remained quarantined for 1 wk. Animals were individually caged with a 12:12-h light-dark cycle (0700 to 1900). Animals were cared for in accordance with the guidelines of the National Institutes of Health Guide to the Care and Use of Experimental Animals.

Experimental Design

All animals were maintained on standard rat chow (Chow; diet 2018 from Harlan Teklad, Madison, WI) from weaning until 2 wk after arriving in our laboratory, at which point animals were ~3 mo old. This Chow diet provides 3.3 kcal/g of digestible energy and 15% of energy as fat. At this point, 22 animals were switched to a high-fat/high-sucrose (HF) diet (F3282 from Bioserv, Frenchtown, NJ), whereas 10 continued to receive chow. This HF diet provides 5.3 kcal/g and 59.4% of energy as fat. Animals were subsequently analyzed separately. After ~100 days of HF feeding, all animals (including Chow fed) were prepared for stereotaxic surgery as described below. Animals were given a single injection into the third cerebroventricle of rAAV-leptin or control virus encoding green fluorescent protein (rAAV-con). This yielded six groups of animals: an rAAV-leptin and an rAAV-con subgroup in each original dietary group (Chow, DIO, and DR). These six groups are abbreviated Chow-con, Chow-leptin, DIO-con, DIO-leptin, DR-con, and DR-leptin. Animals were monitored for another 30 days and then killed for tissue analysis.

Oxygen Consumption

O2 consumption was assessed in up to four rats simultaneously with an Oxycube analyzer (OX5-4; Omnitech Electronics, Columbus, OH) as described previously (20). Flow rates were 2 l/min with a 30-s sampling time at 5-min intervals. The rats were placed into the chamber for 120 min with the lowest six consecutive O2 consumption values during this period used in the calculations. By visual inspection, animals in all dietary groups were observed to be inactive during the sustained monoxenic experiment. Food was withdrawn a minimum of 2 h before commencing V02 measurements. All measurements were made between 0900 and 1400. Results are expressed as O2 consumption relative to metabolic body size (mL·min−1·kg0.75).

Construction of rAAV Vector Plasmid

pTR-Ob encodes rat leptin cDNA (a kind gift from R. Unger (4)) and green fluorescent protein (GFP) reporter gene cDNA. The woodchuck hepatitis virus posttranscriptional regulatory element was placed downstream to enhance the expression of the transgenes (16). The control vector encodes GFP driven by a chicken β-actin (CBA) promoter. Vectors contain AAV terminal repeats at both sides of the cassette to mediate replication and packaging of the vector (3).

Packaging of rAAV Vectors

Vectors were packaged, purified, concentrated, and titered as described previously (6). The titer of rAAV-Ob was 2.3E13 physical particles per milliliter. A mini-adenovirus helper plasmid (pDG) (9) was used to produce rAAV vectors with no detectable adenovirus or wild-type AAV contamination. rAAV vectors were purified using isodixanol gradient/heparin-affinity chromatography and were >99% pure as judged by PAAG/silver-stained gel electrophoresis (not shown).

Vector Administration

Rats were anesthetized with 60 mg/kg pentobarbital sodium and heads were prepared for surgery. Animals were placed into a stereotaxic frame, and a small incision (1.5 cm) was made over the midline of the skull to expose the landmarks of the cranium (bregma and lambda). The following coordinates were used for injection into third cerebroventricle: 1.3 mm posterior to bregma and 9.4 mm ventral from the skull surface on the midline (medial fissure), with the nose bar set at 3.3 mm below the ear bars (below zero) and the cannula set at 20° posterior from vertical. A small hole was drilled through the skull and a 23-gauge stainless steel guide cannula was lowered to the third cerebroventricle. This was followed by an injection cannula attached to a 10-μl syringe. We injected 2.5 μl of viral particles dissolved in Ringer solution at ~0.25 μl/min. Animals received either the rAAV-leptin (n = 5 Chow, n = 5 DIO, and n = 5 DR) or control virus encoding GFP (n = 5 Chow, n = 4 DIO, and n = 8 DR).

Blood Collection

Blood was harvested from all animals on day 75. Animals were placed under temporary anesthesia by enflurane inhalation. With the use of a sterile, razor-sharp scapula, a small piece (~2 mm) of the tip of the tail was excised. Then, with the use of a gentle “milking” motion, 0.5 ml of blood was collected from the tail into a sterile microfuge tube. The blood was allowed to clot, and then was immediately spun at 1,300 g for 10 min. The top serum layer was placed in a fresh tube and stored at −20°C. Blood was withdrawn at death by cardiac puncture (below).

Tissue Harvesting

Rats were anesthetized with 85 mg/kg pentobarbital sodium and killed by cervical dislocation. Blood was collected by cardiac puncture, and serum was harvested by 10-min centrifugation in serum separator tubes. The circulatory system was perfused with 20 ml of cold saline. Inguinal, perirenal, and retroperitoneal white adipose tissue, brown adipose tissue, and hypothalamus were excised, weighed, and immediately frozen in liquid nitrogen. The hypothalamus was removed by making an incision medial to piriform lobes, caudal to the optic chiasm, and anterior to the cerebral crus to a depth of 2–3 mm. Tissues were stored at −80°C until analysis.

Serum Leptin and Free Fatty Acids

Serum leptin was measured using a rat radioimmunoassay kit (Linco Research, St. Charles, MO). Serum free fatty acids were measured using the nonesterified fatty acid C colorimetric kit from WAKO Chemicals (Neuss, Germany).
**RT-PCR**

Leptin transgene expression and long-form leptin receptor (ObRb) expression were evaluated by using relative quantitative RT-PCR through the use of QuantumRNA 18S Internal Standards kit (Ambion, Austin, TX). Total RNA (3 µg) was treated with RNase-free DNase using a DNA-free kit (Ambion, Austin, TX). Total RNA (3 µg) was denatured, and isopropanol precipitated using a modified protocol. The midpoints of the linear range for signal intensity vs. number of cycles. The optimum ratio of 18s primer to competitor was 1:9. PCR was performed at 94°C denaturation for 60 s, 59°C annealing temperature for 45 s, and 72°C elongation temperature for 45 s, and 72°C elongation temperature for 120 s. The PCR product was electrophoresed on a 5% acrylamide Tris borate EDTA gel and stained with SYBR green (Molecular Probes, Eugene, OR). Similarly, the number of cycles found to be at the midpoints of the linear range was 26 cycles for ObRb. The sequence for ObRb primers were sense: GGGAACTGTGAGGATGAGTG; antisense: TAGC-CCTTGGCTTTCACTAG. Gels were scanned using a STORM fluorescent scanner, and digitized data were analyzed using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

**STAT3/Phospho-STAT3 Assay**

These methods were described in detail previously (21). Briefly, the hypothalamus was sonicated in 10 mM Tris·HCl, pH 6.8, 2% SDS, and 0.08 µg/ml okadaic acid plus protease inhibitors (PMFS, benzamidine, and leupeptin; an aliquot of this sonicate was frozen for RNA analysis). Sonicate was diluted and quantified for protein using a detergent compatible Bradford assay. Samples were boiled and separated on an SDS-PAGE gel and electrotransferred to nitrocellulose membrane. Immunoreactivity was assessed with an antibody specific to phosphorylated-STAT3 (antibody kit from New England Biolabs, Beverly, MA). Immunoreactivity was visualized by chemiluminescence detection (Amersham Life Sciences, Piscataway, NJ) and quantified by video densitometry (BioRad, Hercules, CA). After P-STAT3 quantification, membranes were stripped of antibody (Pierce, Rockford, IL) and immunoreactivity was reassessed using a total STAT3 antibody.

**Probes**

POMC mRNA is detected using a 24-mer antisense oligonucleotide probe (5′-CGGACGCCAAGCCGCTTCCGCGAGG-3′). The oligonucleotide probe was end labeled by terminal deoxynucleotidyl transferase (Promega). The rat preproNPY cDNA was provided by J. Allen (University of Glasgow, UK). The cDNA NPY probe was labeled using a random primer kit (Prime-a-Gene, Promega, Madison, WI). Both probes were purified with Nick columns (Pharmacia). The NPY cDNA probe was heat denatured for 2 min before hybridization. All probes have been verified to hybridize to the corresponding specific mRNA by Northern analysis before use in dot blot assay (see mRNA Levels).

**mRNA Levels**

Tissue was sonicated in guanidine buffer, phenol extracted, and isopropanol precipitated using a modification of the method of Chomczynski and Sacchi (5). Isolated RNA was quantified by spectrophotometry and integrity was verified using 1% agarose gels stained with ethidium bromide. For dot blot analysis, multiple concentrations of RNA was immobilized on nylon membranes using a dot blot apparatus (Bio-Rad, Richmond, CA). Membranes were baked in an ultraviolet cross-linking apparatus. Membranes were then prehybridized in 10 ml Quickhyb (Stratagene, LaJolla, CA) for 30 min followed by hybridization in the presence of a labeled probe and 100 µg salmon sperm DNA. After hybridization for 2 h at 65°C, the membranes were washed and exposed to a phosphor imaging screen for 24–72 h (depending on anticipated strength of signal). The screen was then scanned using a Phosphor Image (Molecular Dynamics) and analyzed by Image Quant software (Molecular Dynamics).

**Statistical Analysis**

All data are expressed as means ± SE. Body mass, food intake, and VO2 during the HF-feeding period were compared by a repeated-measures two-way ANOVA, with dietary group and time serving as factors. Total weight gain through day 75 was compared by one-way ANOVA with Tukey’s post hoc. Comparisons in cumulative caloric intake (through day 5) and average daily caloric intake (days 6–100) were made by one-way ANOVA. A one-way ANOVA was used to compare serum leptin, serum free fatty acids, and oxygen consumption in the three dietary groups (Chow, DIO, and DR) before vector delivery. When one-way ANOVA was significant, a Tukey’s post hoc was used to evaluate pairwise comparisons. After vector delivery, comparisons were made by two-way ANOVA on the now six groups (each dietary group was divided into rAAV-leptin and control subgroups) with dietary group and vector as factors. When only two-way ANOVA main effects were significant, relevant pairwise comparisons were made using the Bonferroni multiple comparison method with the error rate corrected for the number of contrasts. When there was an interaction, factors were separated and a further one-way ANOVA was applied with a Bonferroni multiple comparison post hoc. When separation of factors resulted in only two population means to compare, the one-way ANOVA was replaced with Student’s t-test. α-Level was set at 0.05 for all analyses. The correlation between endpoint

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Body mass (BW) during high-fat (HF) feeding (prevector delivery). Values represent mean ± SE of chow-fed (Chow; n = 10), diet-induced obesity (DIO; n = 9), and diet-resistant (DR; n = 13) animals. By 2-way ANOVA, significance was found for effects of dietary group (F = 1350.7, P < 0.0001), time (F = 19.97, P < 0.0001), and the interaction between group and time (F = 9.97, P < 0.0001). P < 0.001 for difference in body mass gained in DIO and Chow during first 75 days of HF-feeding.
serum leptin and visceral adiposity was evaluated by a Pearson coefficient and a two-tailed test of significance. GraphPad Prism software (San Diego, CA) was used for all statistical analysis and graphing. Post hoc analysis of two-way ANOVAs was done using GraphPad QuickCalc (GraphPad.com).

RESULTS

HF Feeding

Food consumption and body mass. Male F344 × BN rats, age 3 mo, were either maintained on standard chow diet (Chow) or an HF diet as described in METHODS. The HF-fed animals spontaneously divided into two distinct groups: those that were becoming obese on the HF diet (DIO) and those that were not gaining extra weight on the HF diet (DR). The body weights of DIO animals appeared to diverge from both Chow and DR (Fig. 1), and their body weights became significantly greater by day 25. By day 75, DIO animals had gained over 25% more mass than both Chow and DR (mass gain through 75 days of HF feeding was 151.6 ± 5.80 g in Chow, 189.7 ± 6.05 g in DIO, and 148.4 ± 3.90 in DR; by Tukey’s post hoc analysis, DIO gained greater mass than both Chow and DR (P < 0.001), whereas Chow and DR did not differ). During the first 5 days of HF feeding, acute hyperphagia was observed in all HF-fed animals (both DIO and DR). Cumulative caloric intake was nearly 30% greater in HF-fed animals than Chow-fed animals during the first 5 days of HF feeding (327.9 ± 8.57 kcal in Chow, 434.7 ± 17.69 kcal in DIO, and 399.5 ± 15.35 kcal in DR), but this acute hyperphagia attenuated by day 7 (Fig. 2). After this acute phase, caloric intake was similar in DIO (66.38 ± 0.87 kcal/day) and Chow (65.22 ± 1.08), whereas DR animals consumed significantly less calories (60.99 ± 1.12) than both DIO and Chow during this phase of the experiment (P < 0.01 by Tukey’s post hoc; data refer to caloric intake days 7–91 after commencing HF feeding in DIO and DR).

Oxygen consumption. VO₂ was first measured 30 days after beginning HF feeding. At this point, oxygen consumption (ml·min⁻¹·kg⁻²3) was significantly reduced in DIO compared with both Chow and DR (P < 0.01), suggesting that reduced energy expenditure in DIO contributes to their accelerated rate of weight gain during this phase. By day 70, there was no longer a difference in VO₂ in the three groups (Fig. 3).

![Image](https://example.com/image1.png)

**Fig. 2.** Caloric intake during HF feeding (prevector delivery). Values represent mean ± SE of Chow (n = 10), DIO (n = 9), and DR (n = 13). By 2-way ANOVA, significance was found for effects of dietary group (F = 5.83, P < 0.01) and the interaction between group and time (F = 7.96, P < 0.001). ***P < 0.01 for difference between DIO and Chow on day 30 and difference between DIO and DR on day 30.

![Image](https://example.com/image2.png)

**Fig. 3.** Oxygen consumption on 30 and 70 days after commencing HF feeding (prevector delivery). Values represent mean percent of control ± SE of Chow (n = 10), DIO (n = 9), and DR (n = 13). By 2-way ANOVA, significance was found for effects of dietary group (F = 5.83, P < 0.01) and the interaction between group and time (F = 7.96, P < 0.001). ***P < 0.01 for difference between DIO and Chow on day 30 and difference between DIO and DR on day 30.

![Image](https://example.com/image3.png)

**Fig. 4.** Changes in body mass during 29 days postvector delivery. Values represent mean percent change ± SE of Chow-con (n = 5), Chow-lep (n = 5), DIO-con (n = 4), DIO-leptin (n = 5), DR-con (n = 8), and DR-leptin (n = 5). By 2-way ANOVA, significance was found for effects of dietary group (F = 3.74, P < 0.05), vector (F = 19.86, P < 0.001), and the interaction between group and vector (F = 4.22, P < 0.05). ***P < 0.0001 for effect of rAAV-leptin on body mass in Chow; **P < 0.01 for effect of rAAV-leptin in DR.

Table 1. Serum leptin and FFAs after 75 days of high-fat feeding (DIO and DR)

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>DIO</th>
<th>DR</th>
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<tbody>
<tr>
<td>Serum leptin, ng/ml</td>
<td>4.90 ± 0.52</td>
<td>13.29 ± 1.43</td>
<td>9.05 ± 0.58</td>
</tr>
<tr>
<td>Serum FFA, meq/l</td>
<td>0.55 ± 0.059</td>
<td>0.44 ± 0.045</td>
<td>0.64 ± 0.055</td>
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</table>

Data represent mean ± SE of Chow-fed, Chow; **P < 0.0001 for effect of rAAV-leptin on body mass in Chow; **P < 0.01 vs. Chow, P < 0.01 vs. DR, †P < 0.01 vs. Chow and DIO.
Response to rAAV-leptin during the second week post-vector delivery (Fig. 4). DR animals had a significant anorexia rapidly attenuated and was no longer significant by day 14 (Fig. 4). Chow-leptin (n = 5) and DR-leptin (n = 5) animals lost an average of 8.4% of their body mass, whereas Chow-con animals increased their body mass by 3.2% during the same 29 days of observation (P < 0.0001, F = 4.03, P < 0.0001). *P < 0.0001 for effect of rAAV-leptin on mean food intake in Chow, days 7–29; *P < 0.05 for effect in DR, days 8–12.

Serum leptin and free fatty acids. At day 75, serum leptin was significantly greater in DIO compared with DR (P < 0.01, Table 1). Chow animals had lower serum leptin than both DIO and DR (P < 0.001, P < 0.01, respectively). Serum free fatty acids did not differ across dietary groups.

Post-rAAV-Leptin Delivery

Food consumption and body mass. After ~100 days of Chow or HF feeding, animals were given a single intracerebroventricular injection of 5.75E10 physical particles of rAAV-leptin or control virus (con). Chow-leptin animals had a robust response to rAAV-leptin, losing 6.6% of their body mass, whereas Chow-con animals increased their body mass by 3.2% during the same 29 days of observation (P < 0.0001) (Fig. 4). Chow-leptin animals also had a significant (P < 0.001) anorectic response to rAAV-leptin, consuming an average of 20.6% less calories than controls from day 7 through 29 postvector delivery (Fig. 5). In contrast, DIO were completely unresponsive to the weight-reducing (Fig. 4) and anorectic effects of rAAV-leptin (Fig. 5). DR rats responded to rAAV-leptin, but in a more variable fashion than Chow animals. DR-leptin animals lost an average of 8.4% of their body mass, whereas DR-con animals increased their body mass by 4.7% during the 29 days postvector delivery (P < 0.01) (Fig. 4). DR animals had a significant anorectic response to rAAV-leptin during the second week post-gene delivery (P < 0.05), but unlike in Chow animals, this anorexia rapidly attenuated and was no longer significant by day 14 (Fig. 5).

Oxygen consumption. rAAV-leptin did not significantly increase oxygen consumption 7 days after vector delivery in any of the three dietary groups (Table 2). This was not a surprise as it has been previously demonstrated that leptin prevents the decrease in energy expenditure normally observed after reduced caloric intake, yet does not necessarily increase energy expenditure above that of untreated, ad libitum-fed controls (20, 22). At this point in the present study, significant anorexia was recorded in Chow-leptin and DR-leptin animals but not in DIO-leptin animals (compared with animals in the respective dietary group given control vector) (Fig. 5).

Adiposity. Animals were killed 30 days after vector delivery. At this point, visceral adiposity (sum of retroperitoneal and perirenal white adipose tissue) of Chow-leptin was 19.5% of that in Chow-con (P < 0.0001, Fig. 6). Although DIO animals had nearly threefold greater visceral adiposity compared with Chow-con animals, there was no effect of rAAV-leptin on adiposity in DIO (Fig. 6). This confirms that DIO animals were completely unresponsive to a dose of rAAV-leptin that causes a near complete disappearance of white fat in age-matched chow-fed animals. DR-con had more than double the visceral adiposity of Chow-con despite similar body masses (Fig. 6). Visceral adiposity was reduced by 57% in DR-leptin vs. DR-con (P < 0.01), suggesting these animals were indeed leptin responsive despite high initial adiposity. Unexpectedly, visceral adiposities were not statistically different between DIO and DR control rats despite significant differences in total body mass and rates of weight gain and despite different leptin responsiveness.

Table 2. Whole body oxygen consumption on day 7 after rAAV-leptin or control vector delivery

<table>
<thead>
<tr>
<th></th>
<th>Chow-Con</th>
<th>Chow-Lep</th>
<th>DIO-Con</th>
<th>DIO-Lep</th>
<th>DR-Con</th>
<th>DR-Lep</th>
</tr>
</thead>
<tbody>
<tr>
<td>VO2, ml/kg ·min ·1</td>
<td>11.29 ± 0.44</td>
<td>12.38 ± 0.82</td>
<td>11.44 ± 0.22</td>
<td>11.91 ± 0.31</td>
<td>12.54 ± 0.43</td>
<td>12.07 ± 0.19</td>
</tr>
</tbody>
</table>

Values represent means ± SE of Chow-con (n = 5), Chow-lep (n = 5), DIO-con (n = 4), DIO-lep (n = 5), DR-con (n = 8), and DR-lep (n = 5).
between end-point serum leptin (Table 3) and the adiposity data shown in Fig. 6 was calculated. There was a significant correlation between these variables ($r = 0.9493$, $P < 0.0001$). There was no effect of dietary group or rAAV-leptin on serum free fatty acids (Table 3).

**Leptin transgene expression in the hypothalamus.** Primers were designed to specifically detect leptin expressed by rAAV-leptin transgene via RT-PCR. mRNA for the leptin transgene was detected in the hypothalami of all animals administered rAAV-leptin, but not in the animals administered rAAV-con (Fig. 7). No differences in transgene expression were found across dietary groups.

**Leptin receptor expression and signal transduction in the hypothalamus.** Leptin receptor (ObRb) expression was significantly reduced in both DIO-con and DR-con compared with Chow-con animals ($P < 0.05$) (Fig. 8). rAAV-leptin treatment tended to reduce ObRb expression in Chow animals, but this failed to reach statistical significance ($P = 0.07$). rAAV-leptin did not affect the already suppressed ObRb expression in DIO and DR animals (Fig. 8).

Basal P-STAT3 was significantly elevated in both DIO-con ($P < 0.01$) and DR-con animals ($P < 0.05$) compared with Chow-con. rAAV-leptin treatment caused a significant increase in STAT-3 phosphorylation in Chow ($P < 0.01$) and DR animals ($P < 0.05$), but not in DIO animals (Fig. 9). Total STAT levels were not affected by diet or rAAV-leptin in any group (Chow-con: $1.00 \pm 0.21$ arbitrary units; Chow-leptin: $1.27 \pm 0.15$; DIO-con: $1.07 \pm 0.12$; DIO-leptin: $1.26 \pm 0.16$; DR-con: $1.06 \pm 0.05$; DR-leptin: $1.03 \pm 0.04$).

**POMC and NPY expression in the hypothalamus.** Leptin normally causes an increase in POMC (the precursor to α-melanocyte stimulating hormone (α-MSH)), and this is one downstream component of the leptin signaling cascade that ultimately results in anorexia and increased energy expenditure (8). In the present study, rAAV-leptin treatment caused a significant increase in POMC expression in Chow animals ($P < 0.01$) and in DR animals ($P < 0.05$), but not in DIO (Table 4). There was no effect of diet on POMC expression.

NPY is an orexigenic signal whose expression may be downregulated by leptin (8, 23). rAAV-leptin treatment significantly reduced NPY expression in Chow animals ($P < 0.05$), but not in DIO or DR (Table 4).

Table 3. Serum leptin and FFA at death

<table>
<thead>
<tr>
<th></th>
<th>Chow-Con</th>
<th>Chow-Lep</th>
<th>DIO-Con</th>
<th>DIO-Lep</th>
<th>DR-Con</th>
<th>DR-Lep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum leptin, ng/ml</td>
<td>2.33 ± 0.148</td>
<td>0.37 ± 0.114*</td>
<td>7.34 ± 0.497*</td>
<td>6.56 ± 0.643</td>
<td>5.76 ± 0.945†</td>
<td>1.93 ± 0.830‡</td>
</tr>
<tr>
<td>Serum FFA, meq/l</td>
<td>0.59 ± 0.015</td>
<td>0.49 ± 0.033</td>
<td>0.64 ± 0.037</td>
<td>0.79 ± 0.079</td>
<td>0.68 ± 0.066</td>
<td>0.59 ± 0.051</td>
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</table>

*Values represent means ± SE of Chow-con ($n = 5$), Chow-lep ($n = 5$), DIO-con ($n = 4$), DIO-lep ($n = 5$), DR-con ($n = 8$), and DR-lep ($n = 5$). $P$ values are results of post hoc analysis of rAAV-leptin effect in each dietary group after 2-way ANOVA with dietary group and vector as factors. $*P < 0.0001$ vs. Chow-Con; $†P < 0.001$ vs. Chow-Con; $‡P < 0.01$ vs. DR-Con.
Expression of NPY did not differ across the three dietary groups (Table 4).

**UCP-1 in brown adipose tissue.** The increase in energy expenditure in leptin-treated compared with paired-fed animals is mediated in part by UCP-1 (19, 22). UCP-1 uncouples mitochondrial respiration from ATP production, thus decreasing the efficiency of metabolism and increasing heat production. In the present study, both total UCP-1 protein in the interscapular brown adipose tissue (IBAT) and UCP-1 per gram total protein in IBAT was significantly increased by HF feeding (Table 5). HF feeding increased total IBAT UCP-1 protein by approximately fivefold in both DIO and DR (Table 5). rAAV-leptin treatment caused a significant threefold increase in UCP-1 levels in Chow animals, but had no effect on the already elevated UCP-1 levels in DIO and DR animals (Table 5). Although UCP-1 was elevated in DR-con to the same degree as in DIO-con animals, rAAV-leptin caused a significant 2.4-fold further increase in UCP-1 in DR-leptin animals (Table 5).

**DISCUSSION**

The major objective of the present study was to compare the effects of leptin gene therapy in Chow-fed and DIO animals with hopes of gaining new insight into the mechanisms of obesity-associated leptin resistance. To our knowledge, this is the first study to measure responses to leptin gene delivery after a period of chronic high-fat feeding. During the initial 100 days of high-fat feeding, 40% of HF-fed animals became obese (DIO), gaining significantly more body mass and adiposity than Chow-fed controls. The remaining HF-fed animals were “diet resistant,” gaining body weight at a similar rate to Chow-fed controls. Levin’s group (13–15) previously described this phenomenon of DIO and DR in detail using Sprague-Dawley rats. According to a recent publication by Levin and Dunn-Meynell (12), there are preexisting differences between DIO and DR Sprague-Dawley rats before commencing high-fat feeding. For example, DIO-prone animals have elevated expression of the orexigenic signal NPY in the arcuate nucleus compared with DR-prone animals despite similar leptin levels (before HF feeding) (12). This disparity in NPY expression is not eliminated until a considerable gain in adiposity and serum leptin (~70% increase with respect to DR) is allowed to occur in DIO by provision of a high-fat diet (12). Because it is possible to selectively breed for DIO and DR (13), it appears that these phenotypes can be accounted for by genetic differences. In our F344 × BN model, we recorded increased visceral adiposity in DR animals with respect to Chow-fed controls despite similar body masses. Thus DR animals apparently do not completely escape the negative effects of chronic high-fat/high-sucrose feeding despite their resistance to weight gain.

It was recently reported that an intracerebroventricular injection of rAAV-leptin can prevent diet-induced obesity for 9 wk when administered before the start of HF feeding (7). However, in that study animals were not leptin resistant at the time that rAAV-leptin was administered. Therefore, the ability of rAAV-leptin to reestablish normal body weight and adiposity regulation could not be properly evaluated. The present study demonstrates that if animals are allowed to become obese first, rAAV-leptin is ineffective. Because central overexpression of leptin did not overcome this leptin resistance, it is clear that central insufficiency of leptin is not the only factor in DIO-related leptin resistance. Rather, there is a central nervous system component to leptin resistance. This conclusion is consistent with the observations of Levin and Dunn-Meynell (12), who recorded reduced central sensitivity to pharmacological leptin peptide in DIO Sprague-Dawley rats.

Phosphorylated STAT3 is a second messenger for the long-form leptin receptor, which is widely expressed in the hypothalamus (10). Signaling through STAT3 appears to be an absolute requirement for leptin’s effects
on energy homeostasis and the anorexic/thermogenic melanocortin system (2). rAAV-leptin caused a twofold increase in hypothalamic P-STAT3 in Chow animals, yet had no effect in DIO animals. However, basal levels of STAT3 activation in the obese state appear equivalent to the activation achieved as a result of rAAV-leptin transgene in the highly responsive Chow animals. This suggests that the signaling defect in DIO animals lies, at least in part, downstream of leptin receptor signal transduction. Hypothalamic expression of anorectic and orexigenic signals were appropriately increased and decreased, respectively, by rAAV-leptin in Chow animals but not DIO. Expression of POMC, a propeptide for the anorexic signal α-MSH, was increased in Chow but not in DIO animals in response to rAAV-leptin. Similarly, rAAV-leptin reduced hypothalamic expression of the orexigenic signal NPY in Chow but not DIO animals. End-organs responses to rAAV-leptin mirrored hypothalamic leptin signal transduction responses. Namely, rAAV-leptin increased UCP-1 in BAT threefold in Chow but had no effect in DIO animals. We conclude that DIO animals were completely unresponsive to a dose of rAAV-leptin that caused an 80% reduction in visceral adiposity in Chow-fed controls over 30 days.

In contrast to both Chow and DIO animals, the DR animals were partially responsive to rAAV-leptin, experiencing a 57% reduction in adiposity and significant anorexia and weight loss. Because the DR controls had twofold greater adiposity than Chow controls, the magnitude of visceral fat loss with rAAV-leptin was actually slightly greater in the DR group (Fig. 6). Basal STAT3 phosphorylation was increased in DR with respect to CHOW, yet still was less than DIO (Fig. 9). rAAV-leptin resulted in a significant increase in P-STAT3 in DR, suggesting that an induction in P-STAT3 is a predictor of physiological responsiveness to exogenous leptin. The magnitude of P-STAT3 induction was certainly less in DR than in Chow animals, consistent with the overall reduced rAAV-leptin responsiveness in DR with respect to Chow. POMC expression was enhanced in response to rAAV-leptin in DR animals despite the relatively small increase in P-STAT3. However, we did not observe a significant decrease in NPY in the rAAV-leptin-treated DR animals. This latter finding may not be surprising in light of the food intake data. Whereas rAAV-leptin initially caused anorexia in DR, this response rapidly attenuated and was no longer significant by day 14 postvector delivery. Such an attenuation was not observed in Chow animals, and only Chow animals had reduced NPY expression in response to rAAV-leptin on day 30 postvector delivery. Thus we may conclude that DR animals are prone to a faster onset of leptin resistance compared with Chow animals. The reason for this rapid onset of leptin resistance may be a function of greater starting adiposity, serum leptin, or both. This warrants further investigation.

In addition to impairments downstream of leptin signal transduction, the reduced leptin receptor (ObRb) expression in the hypothalamus of all HF-fed animals with respect to Chow-fed controls may be another contributor to the leptin resistance/reduced responsiveness in these animals. At death, both DIO and DR animals were hyperleptinemic and had significantly reduced ObRb expression with respect to Chow-

### Table 4. Hypothalamic POMC and NPY expression at death

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow-Con</th>
<th>Chow-Lep</th>
<th>DIO-Con</th>
<th>DIO-Lep</th>
<th>DR-Con</th>
<th>DR-Lep</th>
</tr>
</thead>
<tbody>
<tr>
<td>POMC</td>
<td>100 ± 5.5</td>
<td>123 ± 4.2</td>
<td>119 ± 13.2</td>
<td>98.0 ± 2.2</td>
<td>110 ± 9.1</td>
<td>143 ± 14.3</td>
</tr>
<tr>
<td>NPY</td>
<td>100 ± 10.1</td>
<td>74.5 ± 4.4</td>
<td>74.5 ± 9.9</td>
<td>95.9 ± 8.0</td>
<td>85.4 ± 4.2</td>
<td>91.4 ± 4.4</td>
</tr>
</tbody>
</table>

Food was withdrawn a minimum of 2 h before collecting tissue samples. Samples were collected between 1000 and 1400. Data represent the mean ± SE of Chow-con; (n = 5), Chow-lep (n = 5), DIO-con (n = 4), DIO-lep (n = 5), DR-con (n = 8), and DR-lep (n = 5). All mRNA data are expressed as arbitrary units and were standardized so that the mean value in Chow-Con animals was set at 100. POMC and NPY mRNA were measured by RNA dot blot. P values are results of post hoc analysis of rAAV-leptin effect in each dietary group after 2-way ANOVA with dietary group and vector as factors. POMC, proopiomelanocortin; NPY, neuropeptide Y. *P < 0.01 vs. Chow-con; †P < 0.05 vs. DR-Con; ‡P < 0.01 for difference in both DIO-con and DR-con with respect to Chow-con.

### Table 5. Brown adipose tissue parameters death

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chow-Con</th>
<th>Chow-Lep</th>
<th>DIO-Con</th>
<th>DIO-Lep</th>
<th>DR-Con</th>
<th>DR-Lep</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBAT mass, g</td>
<td>0.25 ± 0.015</td>
<td>0.15 ± 0.024</td>
<td>0.59 ± 0.027</td>
<td>0.59 ± 0.061</td>
<td>0.58 ± 0.048</td>
<td>0.36 ± 0.066</td>
</tr>
<tr>
<td>Total protein/IBAT pad, mg</td>
<td>26.3 ± 1.27</td>
<td>23.3 ± 2.63</td>
<td>61.6 ± 3.17</td>
<td>65.1 ± 5.60</td>
<td>62.7 ± 5.33</td>
<td>52.2 ± 6.1</td>
</tr>
<tr>
<td>UCP-1, arbitrary units per g IBAT protein</td>
<td>1.0 ± 0.18</td>
<td>3.0 ± 0.49</td>
<td>2.2 ± 0.47</td>
<td>2.9 ± 0.23</td>
<td>2.7 ± 0.63</td>
<td>6.3 ± 1.75</td>
</tr>
<tr>
<td>UCP-1, arbitrary units/total IBAT pad</td>
<td>1.0 ± 0.20</td>
<td>2.9 ± 0.79</td>
<td>5.0 ± 0.97</td>
<td>6.7 ± 1.04</td>
<td>5.4 ± 1.56</td>
<td>12.8 ± 3.90</td>
</tr>
</tbody>
</table>

Food was withdrawn a minimum of 2 h before collecting tissue samples. Samples were collected between 1000 and 1400. Data represent means ± SE of Chow-con (n = 5), Chow-lep (n = 5), DIO-con (n = 4), DIO-lep (n = 5), DR-con (n = 8), and DR-lep (n = 5). Uncoupling protein (UCP)-1 data are expressed in arbitrary units either per mg IBAT protein or per IBAT pad. P values are results of post hoc analysis after 2-way ANOVA with dietary group and vector as factors. Effect of rAAV-leptin vs. rAAV-con on parameters: Chow; *P < 0.001 for effect on IBAT mass, UCP-1 per unit protein, and UCP-1 per total IBAT; DR, †P < 0.01 for effect on IBAT mass, DR-con and DR-lep with respect to Chow-con; ‡P < 0.05 for difference in both DIO-con and DR-con with respect to Chow-con.
fied controls. rAAV-leptin tended to suppress ObRb expression in Chow-fed rats, but this effect did not reach statistical significance. The sum of these findings suggests that leptin, either in the form of obesity-related chronic hyperleptinemia or centrally overexpressed transgene, may be a negative regulator of leptin receptor expression. We believe that central overexpression of leptin in Chow-fed animals via rAAV-leptin caused elevated leptin in the vicinity of the hypothalamic receptors and this was sufficient to cause the observed trend of leptin receptor expression downregulation. However, we speculate with caution given the statistical nonsignificance of rAAV-leptin’s effect in Chow animals. Nevertheless, the effect of high-fat feeding on ObRb expression was significant, including a 50% reduction in hypothalamic ObRb mRNA in DIO animals (Fig. 8). This receptor downregulation may limit the ceiling of ObRb-mediated STAT3 phosphorylation in response to exogenous leptin, thereby preventing an increase in P-STAT3 over the already elevated basal levels in the DIO animals. Indeed, P-STAT3 induction by rAAV-leptin was absent in DIO and reduced in magnitude in DR. Future experiments are required to better understand the relationship between hyperleptinemia, leptin receptor expression, receptor signaling capacity, and leptin resistance.

The BAT UCP-1 protein data were particularly interesting. Basal BAT UCP-1 protein was increased in both DIO-con and DR-con approximately fivefold compared with Chow-con animals. rAAV-leptin caused a further 2.4-fold increase in BAT UCP-1 in DR, yet had no effect in DIO animals (Table 5). Thus it appears that an UCP-1-inducing signal is released in response to rAAV-leptin in DR but not in DIO animals. This signal is likely enhanced sympathetic nerve activity, because this is known to be the primary inducer of UCP-1 expression (19). Indeed, DIO animals have been shown to have impaired induction of lumbar sympathetic nerve activity in response to leptin (17). Moreover, obese animals are known to retain sensitivity to the effects of selective β3-agonists, including the stimulatory effects of these compounds on brown fat UCP-1 and thermogenesis (25). These results imply that DIO rats still have the capacity to increase UCP-1 levels if they receive the appropriate peripheral signal. This prompts us to speculate that leptin resistance in DIO animals is primarily a phenomenon of the central nervous system, not one of reduced end organ responsiveness.

In summary, these results show that central overexpression of leptin cannot overcome leptin resistance in a DIO model. Thus we conclude that central delivery of leptin gene is not a viable strategy for overcoming leptin resistance. Leptin resistance in DIO is characterized by impaired central nervous system response to leptin, and this central nervous system defect must be corrected or bypassed to harness the power of leptin (or leptin-mediated signals) to reverse obesity.

DISCLOSURES

This work was supported by the Medical Research Service of the Department of Veterans Affairs and National Institutes of Health Grant AG-17047.

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AJP-Regul Integr Comp Physiol • VOL 285 • NOVEMBER 2003 • www.aipregu.org


