Leptin Antagonist Reveals an Uncoupling between Leptin Receptor Signal Transducer and Activator of Transcription 3 Signaling and Metabolic Responses with Central Leptin Resistance

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ABSTRACT
Leptin-resistant rats have reduced leptin receptors and signaling and are refractory to exogenous leptin. However, it is unclear how leptin-mediated hypothalamic signal transducer and activator of transcription 3 (STAT3) signaling relates to the loss of physiological responsiveness. We hypothesized that if leptin resistance is associated with leptin receptors that are no longer functionally coupled to leptin responses, then a leptin antagonist should be less effective in leptin-resistant compared with leptin-responsive rats. Hypothalamic leptin resistance was induced in lean rats with a recombinant adeno-associated viral (rAAV) vector encoding leptin by intracerebroventricular injection. Following development of leptin resistance, at day 153, these rats and control rats were infused centrally either with vehicle or a rat leptin antagonist for 14 days. Food intake, body weight, adiposity, and uncoupling protein 1 expression increased with antagonist infusion in controls but elevated only marginally in leptin-resistant rats. Basal hypothalamic STAT3 signaling remained unchanged with antagonist infusion in control rats despite the pronounced orexigenic response in these animals. STAT3 phosphorylation in rats pretreated with rAAV-leptin to induce leptin resistance was elevated 2-fold. Paradoxically, in these leptin-resistant rats, the antagonist fully reversed the 2-fold elevated phosphorylated STAT3, but it evoked minimal physiological responses. These data reveal an uncoupling between leptin receptor activation and metabolic responses with central leptin resistance.

Peripherally produced leptin acts within the central nervous system to regulate energy homeostasis by curtailing food consumption and boosting energy expenditure (Elmquist et al., 1998; Friedman and Halaas, 1998). These central effects of leptin are often diminished or fully lost in humans and animals with genetic, diet-induced, or adult-onset obesity (Caro et al., 1996; Considine et al., 1996; Halaas et al., 1997; Scarpace et al., 2000), a phenomenon identified as leptin resistance. Despite intensive investigation, the nature of leptin resistance remains elusive. Central leptin resistance is characterized by reduced leptin receptors and diminished leptin signaling through both the STAT3 phosphorylation and PI3 kinase pathways (Scarpace et al., 2001; Sahu and Metlakunta, 2005). Stimulation of the central melanocortin system downstream of the leptin receptor with α-melanocyte-stimulating hormone agonists circumvents leptin resistance associated with diet-induced obesity (Hansen et al., 2001), adult-onset obesity (Zhang et al., 2004), or chronic exposure to high levels of leptin (Scarpace et al., 2003). These data suggest that leptin resistance lies primarily within the first-order, leptin-receptor-containing neurons, and they argue that leptin resistance is a result of diminished leptin receptor activity.

However, the reductions in leptin receptors and hypothalamic STAT3 signaling apparently do not match either the magnitude or temporal loss of physiological leptin responses. For example, in diet-induced obese (DIO) rodents with leptin resistance, basal STAT3 phosphorylation (p-STAT3) in the
hypothalamus is elevated in continuously high-fat-fed rats compared with chow-fed lean animals (Wilsey et al., 2003). When stimulated with exogenous leptin, p-STAT3 is increased in both types of rats, but maximal signaling capacity is diminished in the rats with leptin resistance due to diet-induced obesity (Wilsey and Scarpace, 2004). A similar phenomenon is observed in our leptin-resistant model of chronic hypothalamic overexpression of leptin via recombinant adeno-associated viral-mediated central leptin (rAAV-leptin) delivery in young lean rats. In this model, food consumption and body weight decrease significantly soon after the leptin gene delivery (Scarpace et al., 2002a,b, 2003, 2005). Gradually, these leptin responses wane over time despite continued leptin transgene expression, and eventually, the decreased food consumption and weight loss are reversed (Scarpace et al., 2003, 2005). Furthermore, central infusion of exogenous leptin fails to initiate any physiological response in these rAAV-leptin-treated rats, indicating development of leptin resistance (Scarpace et al., 2003). In such rats, the central overexpression of leptin produces a 2-fold increase in hypothalamic p-STAT3, similar to that observed in the DIO leptin-resistant model (Zhang and Scarpace, 2006). However, this level of STAT3 signaling remains constant in spite of the diminishing physiological responses over time (Zhang and Scarpace, 2006). Administration of exogenous leptin, similar to the observations in DIO leptin-resistant rodents, augments hypothalamic p-STAT3 albeit maximal signaling is diminished (Scarpace et al., 2003, 2005). A schematic representation of this phenomenon is represented in Fig. 1.

These observations question the relationship between hypothalamic leptin-mediated STAT3 signaling and leptin responses under conditions of leptin resistance. In particular, is the increased hypothalamic p-STAT3 due to leptin overexpression (or high-fat feeding) functionally coupled to metabolic regulation of energy homeostasis despite the leptin-resistant state? Additionally, is the ability of exogenous leptin to elevate leptin-mediated STAT3 signaling in the leptin-resistant state imply that endogenous leptin still modulates leptin signaling, and does that signaling have a role in mediating metabolic responses?

The present study uses a rat leptin antagonist to address these questions. We previously demonstrated that this antagonist, in vivo, results in the predicted increase in food consumption and body weight gain. Moreover, this antagonist blocks leptin-mediated anorexic and weight-reducing responses, and it prevents, in a dose-response manner, the leptin-induced hypothalamic STAT3 phosphorylation in response to exogenously administered central leptin (Zhang et al., 2007). Thus, this compound seems to be both a pharmacologically and physiologically active antagonist of rat leptin. We predict that if the leptin receptor signaling activity that is still present with leptin resistance remains functionally coupled to metabolic control, then a leptin receptor antagonist should be effective in both leptin-resistant and leptin-responsive rats. Conversely, if leptin resistance is associated with leptin receptors that are no longer functionally coupled to leptin responses, then the leptin antagonist should be less effective in leptin-resistant compared with leptin-responsive rats.

To test our hypothesis, we induced hypothalamic leptin resistance in lean rats through intracerebroventricular (i.c.v.) injection of rAAV-leptin. Upon development of leptin resistance, these and control rats were centrally infused either with vehicle or a rat leptin antagonist, and parameters of energy homeostasis and hypothalamic leptin signaling were assessed.

**Materials and Methods**

**Experimental Animals.** Three-month-old male F344 × Brown Norway (F344xBN) rats were obtained from Harlan (Indianapolis, IN). Upon arrival, rats were examined and remained in quarantine for 1 week. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals. Rats were housed individually with a 12:12-h light/dark cycle (7:00 AM–7:00 PM).

**Experimental Design.** This study consisted of two groups of rats, those administered control vector and those administered rAAV-leptin by i.c.v. injection. Control and rAAV-leptin rats were allowed free access to food and water, ad libitum, and food consumption and body weight were recorded daily to weekly. During the 152-day pretreatment period with control vector or rAAV-leptin, oxygen consumption was assessed at day 99, and blood was drawn for assessment of serum leptin at day 105. At day 153, half of the rats in each group were infused with either artificial cerebrospinal fluid or 25 µg/day leptin antagonist into the lateral ventricle for 14 days.

**rAAV-Leptin and Control Vector Administration.** Preparation of the rAAV vector (pTR-β-OhW) encoding rat leptin cDNA (a generous gift from Roger Unger, University of Texas, Southwestern Medical Center, Dallas, TX; Chen et al., 1996) or control vector encoding green fluorescent protein under the control of a chicken β-actin promoter linked to cytomegalovirus enhancer was described previously (Scarpace et al., 2002b). Vectors were packaged, purified, concentrated, and titered as described previously (Zolotukhin et al., 1999). Rats were administered a single dose of 3.5 × 10^9 physical particles/rat (6.6 × 10^2 infectious particles/rat) in 4 µl of either control vector or rAAV-leptin by i.c.v. injection into the third cerebral ventricle as described previously (Scarpace et al., 2002b).

**Leptin Antagonist Infusion.** The leptin antagonist is a triple mutant (L39A/D40A/F41A) of the full-length rat leptin polypeptide (Protein Laboratories Rehovot, Israel). It was infused (25 µg/day) by osmotic mini pump (Durect Corporation, Cupertino, CA) into the lateral ventricle via an implanted cannula as described previously (Zhang et al., 2007).

**Acute Leptin and Antagonist Administration.** Under pentobarbital anesthesia (60 mg/kg), the drugs were injected into the third
cerebral ventricle, using the coordinates 1.3 mm anterior to bregma, 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 a 23-gauge stainless steel guide cannula inserted followed by an injection cannula. Using a 10-μl syringe, a 4-μl volume was delivered containing 100 ng of leptin, 100 ng of leptin plus 5 or 20 μg of antagonist, or vehicle (NaHCO₃).

**O₂ Consumption.** O₂ consumption was assessed in up to seven rats simultaneously with an oxygen analyzer (model 5-3A; AEI Technologies, Naperville, IL). Flow rates were 150 ml/min with a 1-min sampling time at 8-min intervals. The rats were acclimated to the chambers for approximately 1 h. During this period, oxygen consumption was monitored until the values settled to a consistent value. Oxygen consumption was then assessed for the next hour. By observation, nearly all rats were asleep or at rest without movement during the 1-h period when oxygen consumption values were collected. Food was not available. Results were expressed as mass adjusted consumption (milliliter ⋅ minute⁻¹ ⋅ kilogram (kg)⁻¹) and as oxygen consumption per rat (milliliter ⋅ minute⁻¹).

**Tissue Harvesting and Preparation.** Rats were sacrificed by cervical dislocation under 150 mg/kg pentobarbital anesthetic. Blood samples were collected by heart puncture, and serum was harvested by a 10-min centrifugation in serum separator tubes. The circulatory system was perfused with 20 ml of ice-cold saline, and perirenal and retroperitoneal white adipose tissues (PWAT and RTWAT, respectively), brown adipose tissue (BAT), and hypothalamus were excised. The hypothalamus was removed by making an incision medial to the crus, to a depth of 2 to 3 mm. The hypothalamus was sonicated in 10 mM Tris-HCl, pH 6.8, 2% SDS, and 0.08 μg/ml okadaic acid plus 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/Phosphorylated-STAT3 Assay.** These methods were described in detail previously (Scarpace et al., 2001). In brief, samples were boiled and separated on a 10% agarose Tris-HCl gel (Bio-Rad) and electrotransferred to nitrocellulose membrane. Immunoreactivity was assessed with an antibody specific to phosphorylated-STAT3 (Cell Signaling Technology Inc., Danvers, MA). Immunoreactivity was visualized by ECL Plus detection system (GE Healthcare, Piscataway, NJ) and quantified by ImageQuant TL (GE Healthcare). Following phosphorylated-STAT3 quantification, membranes were stripped of antibody with Immunopure (Pierce Chemical, Rockford, IL), and immunoreactivity was reassessed using a total STAT3 antibody.

**Protein Tyrosine Phosphatase 1B and Phosphorylated-AMP Kinase.** Protein levels were determined as described for STAT3, except immunoreactivity was assessed with antibodies specific to protein tyrosine phosphatase 1B (1B; EMD Biosciences, San Diego, CA) and phosphorylated (Thr172) AMP kinase (Cell Signaling Technology Inc.) in hypothalamic homogenates.

**Leptin Levels.** Serum leptin levels were measured using a radioimmunoassay kit (Linco Research, St. Charles, MO).

**Statistical Analysis.** Data were analyzed by one- or two-way ANOVA. When the main effect was significant, a Bonferroni post hoc test was applied to determine individual differences between means. A value of P < 0.05 was considered significant.

**Results**

**Pretreatment Period (152 Days)**

**Food Consumption and Body Weight.** Following rAAV-leptin administration, food consumption decreased rapidly and became significantly different from control rats by day 4. The reduction in food intake reached a nadir at day 9, amounting to a 36% decrease compared with rats adminis-

**Serum Leptin Levels.** Serum level, one marker of adiposity, was examined at day 105 during the pretreatment period. By day 105, food intake was nearly the same between the control and rAAV-leptin rats, but body weight was still significantly lower in the latter. At this time point, serum leptin level was considerably less in the rAAV-leptin-treated rats, amounting to only 5% of the value in control rats, indicating these rats were nearly devoid of white adipose tissue (Table 1).

**Whole-Body Oxygen Consumption.** Just before assessment of serum leptin, whole body oxygen consumption was

**Fig. 2.** Daily food consumption (top) and body mass (bottom) in rats following administration of control vector (open squares) or rAAV-leptin (closed circles). The rAAV-leptin or control vectors were administered at day 0. Values represent the mean ± S.E. of seven control and eight to nine rAAV-leptin rats. Food consumption differs between control and rAAV-leptin-treated rats beginning day 4 and continuing through day 130 (P < 0.05 to 0.001 by ANOVA). Body mass in the rAAV-leptin-treated rats was significantly different from that of control rats beginning at day 8 through day 152 (P < 0.01 to 0.0001 by ANOVA).
TABLE 1

Serum leptin levels (day 105) and whole-body oxygen consumption (day 99) following control vector or rAAV-leptin gene delivery

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<thead>
<tr>
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<th>Control Virus</th>
<th>rAAV-Leptin</th>
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<tr>
<td>Serum leptin (ng/ml)</td>
<td>7.99 ± 0.72</td>
<td>0.39 ± 0.08***</td>
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<tr>
<td>Oxygen consumption (ml/rat)</td>
<td>2.31 ± 0.07</td>
<td>2.01 ± 0.06**</td>
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***P < 0.0001 for difference from control rats.
**P = 0.007 for difference from control rats.

Determined. Oxygen consumption, expressed as consumption per rat, demonstrated a 13% decrease in the rAAV-leptin-treated compared with the control rats (Table 1). However, when oxygen consumption was expressed per lean body mass, there were no differences between groups (data not shown).

Leptin Antagonist Infusion

Food Consumption. Following 152 days of pretreatment with rAAV-leptin or control vector, half of the rats in each group were infused with a leptin antagonist (25 μg/d, lateral ventricle) and the other half of the rats with vehicle for 14 days, and food intake and body weight were assessed. In the two groups of rats infused with vehicle, i.e., those pretreated with either rAAV-leptin or control vector, daily food consumption was unchanged from food intake before the antagonist treatment (Fig. 3, top). In contrast, food consumption in control-vector rats infused with the antagonist was significantly greater compared with the vehicle-infused control-vector rats beginning at the third day of infusion and throughout the remainder of the infusion period (Fig. 3, top). However, food intake in the rats pretreated with rAAV-leptin and infused with the leptin antagonist was only marginally elevated compared with respective vehicle-infused rats, and intake was significantly less than the increased food intake in the rats pretreated with control vector and infused with leptin antagonist (Fig. 3, top). When the cumulative food intake during the 14-day infusion period was considered, there was a 47% increase in food consumption in the antagonist-infused rats pretreated with control virus compared with only a 15% increase in the rats pretreated with rAAV-leptin (Fig. 3, bottom).

Body Weight. Parallel to the food consumption data, body weight significantly increased following antagonist infusion in the rats pretreated with control vector (Fig. 4). The gain in body weight was significantly different beginning at day 3 (+3.88 ± 1.20 g versus -0.18 ± 1.24 g; P = 0.034) and continuing throughout the infusion period, ending with a weight gain of 31.2 ± 4.36 versus 2.17 ± 0.87 g (P < 0.001) in the antagonist infused compared with the vehicle-infused rats pretreated with the control vector. In contrast, in the rats pretreated with rAAV-leptin, body weight was not significantly different from vehicle-infused rats (Fig. 4), and weight gain was unchanged with antagonist infusion for the first 12 days of infusion.

Adiposity Levels. The amount of white adipose tissue (WAT) was extremely low in the rats pretreated with rAAV-leptin and vehicle-infused (Table 2). In seven of nine rats, we were unable to remove any PWAT or RTWAT. Following antagonist infusion, WAT increased in both groups (Table 2), but the gain in adiposity (sum of PWAT plus RTWAT) was 5-fold greater in the rats pretreated with control virus (+4.5 g) compared with the leptin-resistant rats (+0.78 g).

UCP1 Expression in Brown Adipose Tissue. Similar to changes in WAT, total BAT tissue weight decreased in rats pretreated with rAAV-leptin (Table 2). In contrast, there was a substantial increase in BAT weight following antagonist infusion in both those pretreated with control or rAAV-leptin (Table 2). At sacrifice, UCP1 expression level in BAT was unchanged with rAAV-leptin treatment (Table 2). Antagonist infusion reduced UCP1 expression by nearly 40% in rats pretreated with control virus, but there was no significant change in UCP1 expression level in the rats pretreated with rAAV-leptin (Table 2).

Leptin Signal Transduction in Hypothalamus. In a separate group of rats, the ability of the leptin antagonist to block exogenous leptin activation of p-STAT3 was examined. Hypothalamic leptin signaling was stimulated with 100 ng of leptin. Two doses of antagonist (5 and 20 μg) or vehicle in conjunction with this dose of leptin were centrally administered, and hypothalamic leptin signaling was assessed 1 h later. As expected, leptin increased p-STAT3 by greater than 4-fold over vehicle-injected rats. The increase in p-STAT3 was fully blocked by coadministration of either 5 or 20 μg of antagonist (Fig. 5).
p-STAT3 signaling also was assessed in the hypothalamus in the rats infused with the leptin antagonist or vehicle pretreated with either rAAV-control or rAAV-leptin for 152 days. Similar to our previous findings (Scarpace et al., 2002a,b), rAAV-leptin induced a submaximal 2-fold increase in p-STAT3 (comparison between vehicle-infused, rAAV-leptin-pretreated versus vehicle-infused, control vector-pretreated; Fig. 6). Infusion of the leptin antagonist did not significantly decrease basal p-STAT3 levels (comparison between antagonist and vehicle in rats pretreated with rAAV-leptin for 152 days; Fig. 6). As expected, the antagonist infusion in the rats pretreated with rAAV-leptin reversed the elevated p-STAT3 level to basal level (Fig. 6). Also, as observed previously (Scarpace et al., 2002b), total STAT3 increased by 22% in the rats pretreated with rAAV-leptin for 152 days (100 ± 3.15

### TABLE 2

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<tr>
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<th>Pretreated with Control Virus</th>
<th>Pretreated with rAAV-Leptin</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Leptin Antagonist</td>
</tr>
<tr>
<td>PWAT (g)</td>
<td>0.76 ± 0.04</td>
<td>1.46 ± 0.13**</td>
</tr>
<tr>
<td>RTWAT (g)</td>
<td>2.76 ± 0.28</td>
<td>6.14 ± 0.48**</td>
</tr>
<tr>
<td>BAT (g)</td>
<td>477 ± 25</td>
<td>748 ± 51**</td>
</tr>
<tr>
<td>UCP1 mRNA U/μg RNA</td>
<td>100 ± 4.7</td>
<td>618 ± 8.4**</td>
</tr>
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P < 0.0001 for main difference with pretreatment and with antagonist infusion for all parameters (except P < 0.001 for UCP1 mRNA) by two-way ANOVA.

*** P < 0.0001 for difference from control rats pretreated with control virus for all parameters except there is no significant difference for UCP1 protein.

** P < 0.01 for difference from respective control rats for all parameters.

**Fig. 4.** Body weight following leptin antagonist infusion (dashed line) or vehicle (solid line) in rats pretreated for 152 days with control vector (open circles) or rAAV-leptin (closed circles). Values represent the mean ± S.E. of seven to nine rats per group. Gain in body weight in leptin antagonist infused in rats pretreated with control virus was significantly greater than respective control beginning at day 3 and throughout the infusion period (P < 0.001 by one-way ANOVA).

**Fig. 5.** Hypothalamic STAT3 phosphorylation 1 h after i.c.v. administration of vehicle (open bar), leptin (100 ng; closed bar), and leptin (100 ng) plus antagonist (5 μg; striped bar or 20 μg, checkered bar). Results are expressed in arbitrary units per microgram of hypothalamic protein. STAT3 phosphorylation was normalized to total STAT3, and levels of STAT3 phosphorylation in the vehicle-injected rats were set to 100 and the S.E.M. was adjusted proportionally. Values represent the mean ± S.E.M. of five rats per group. **, P < 0.0001 for difference between leptin and all other groups by one-way ANOVA.

**Fig. 6.** STAT3 phosphorylation following vehicle (open bars) or antagonist (filled bars) infusion in rats pretreated with control vector or with rAAV-leptin. Values represent mean ± S.E.M. of seven to nine rats in each group. STAT3 phosphorylation levels in the vehicle control rats were set to 100 with the S.E.M. adjusted proportionally. P < 0.0001 for main difference with pretreatment and P = 0.0025 for main difference with antagonist infusion by two-way ANOVA. ***, P < 0.001 for difference between vehicle rAAV-leptin and vehicle control groups.
versus 122.17 ± 8.50; P = 0.028). Thus, p-STAT3 was not normalized to total STAT3.

Another modulator of leptin signaling, protein tyrosine phosphatase 1B protein level was also examined in the hypothalamus. There was no change with either rAAV-leptin or antagonist infusion (data not shown). In addition, phosphorylation of hypothalamic AMP kinase, an enzyme downstream in the leptin activation pathway, was also unchanged under all conditions (data not shown).

Discussion

A common model used to investigate leptin resistance is that of diet-induced obesity. However, in this model, two confounding factors, both obesity and elevated leptin, could contribute to the leptin resistance and hence complicate the investigations. In addition, there are at least two separate components to diet-induced leptin resistance, a peripheral component related to an inability of leptin synthesized in white adipose tissue to reach target sites in the brain (Caro et al., 1996; Banks et al., 1999), and a central component, purportedly related to the inability of central leptin to activate the leptin pathway at and distal to leptin receptors (El-Haschimi et al., 2000; Scarpace et al., 2001; Levin and Dunn-Meynell, 2002). The current study focuses on the central component of leptin resistance and uses a leptin-resistant model of chronic hypothalamic overexpression of leptin. This model of central leptin resistance shares many common characteristics with the leptin resistance induced by diet-induced obesity. Moreover, most of the consequences of diet-induced obesity on central leptin resistance can be mimicked by elevation of central leptin, suggesting that the central component of leptin resistance may be mainly due to elevated leptin secondary to obesity (Scarpace et al., 2002b, 2003, 2005; Wilsey et al., 2002). Therefore, mechanistic studies with our overexpression model should aid in understanding the central leptin resistance associated with diet-induced obesity.

In the present study, 152 days after gene delivery of leptin, there is evidence of apparent leptin resistance. Food consumption returned to nearly control level, and energy expenditure was no longer elevated as evidenced by the lack of increases in either whole body oxygen consumption (day 99) or BAT UCP1 mRNA. Our previous studies have provided temporal details on energy expenditure following rAAV-leptin gene delivery in similarly treated rodents—both oxygen consumption and BAT UCP1 are initially elevated but gradually diminish until there are no longer differences from controls (Scarpace et al., 2002a). Furthermore, we previously demonstrated that the anorexic and weight-reducing responses to centrally administered exogenous leptin are absent in rAAV-leptin-treated rats, confirming the leptin-resistant state (Scarpace et al., 2003). Such leptin-resistant rats eventually not only regain all the weight initially lost in response to central leptin gene delivery but also draw level with the control rats, who steadily gain weight (Scarpace et al., 2003). It is noteworthy that, in the present study, leptin antagonist treatment was initiated after regain of the body weight initially lost but before catching up with the body weight of controls. During the former period, when the rats were losing weight, they were in a state of negative energy balance, whereas during the later period, they were in a state of positive energy balance, further suggestive of leptin resistance. Moreover, previous studies indicate that even prior to achieving parity in body weight with controls, such rAAV-leptin-treated rats are unresponsive to exogenous leptin (Scarpace et al., 2003).

The central infusion of the leptin antagonist provided us with two important findings. First, the physiological responses with respect to food consumption and body weight were clearly different between the rAAV-leptin-induced leptin-resistant rats and control (leptin-responsive) rats. Whereas the rats pretreated with control virus had increased food consumption over the duration of the antagonist infusion, the leptin resistant rats ate only slightly more. There were greater gains in body weight and adiposity in the control compared with the leptin-resistant rats. Additionally, UCP1 expression in BAT, a reasonable marker of leptin-mediated sympatho-activation of BAT was diminished following antagonist infusion in the control rats, but it was unchanged in the rats pretreated with rAAV-leptin. These data indicate that leptin receptor activation is virtually uncoupled from metabolic responses in rats with rAAV-leptin-induced leptin resistance. We consider elevated central leptin causal to central leptin resistance in both our rat model and DIO rats. Therefore, our data predict that hypothalamic leptin receptor activation is also functionally disconnected from metabolic responses with diet-induced obesity.

The second finding concerns leptin-related STAT3 signaling in the hypothalamus. In our previous and current studies, central rAAV-leptin gene delivery results in a persistent 2-fold increase in hypothalamic p-STAT3 levels despite blunted leptin responses (Zhang and Scarpace, 2006). This 2-fold elevation in p-STAT3 is theoretically submaximal relative to the nearly 6-fold stimulation normally produced by an acute dose of central leptin injected into lean and leptin-sensitive rats (Scarpace et al., 2001). If the full extent or a threshold level of STAT3 signaling was necessary to induce physiological responses, then the subthreshold increase in p-STAT3 brought about by rAAV-leptin (or persistent high-fat feeding per se) would not be sufficient to evoke a metabolic response. Consequently, a leptin antagonist that blocks this submaximal increase in leptin signaling would have minimal effect on metabolic consequences. In fact, this is what was observed in the present study: the leptin antagonist reversed the 2-fold increase in rAAV-leptin-mediated p-STAT3, yet produced little metabolic consequences.

The lack of metabolic consequences with the antagonist infusion is apparently not due to insufficient blockade. In a previous study, we demonstrated that rAAV-leptin increases cerebral spinal fluid leptin level only by 75% (100 to 175 pg/ml) (Scarpace et al., 2002b); thus, with less than 2-fold change in leptin levels, it is likely similar degrees of blockade were achieved. Moreover, the complete reversal of the 2-fold elevation in p-STAT3 in the rAAV-leptin animals supports this contention.

Paradoxically, we were unable to relate endogenous STAT3 signaling to physiological consequences of the antagonist infusion. The rats pretreated with the control virus demonstrated a marked orexigenic response to the leptin antagonist, yet endogenous basal p-STAT3 was unchanged. In contrast, the rats pretreated with rAAV-leptin displayed only a slight increase in food consumption, but the 2-fold augmentation in p-STAT3 was fully reversed by the antagonist. These seemingly puzzling results, however, fall within the known complexities of in vivo pharmacological assessments.
The kinetics, potency, and efficacy of leptin-mediated signaling all contribute to phosphorylated STAT3 levels. For example, the unchanged basal p-STAT3 in the control rats with the leptin antagonist could be the consequence of "snapshot" methodology. It is probable that the leptin receptor system, as with most receptor systems, is not always activated, but subjected to highly regulated bursts of activity. Thus, examination at a single random time point will likely assess resting (basal) rather than endogenously activated receptor signaling. A pure antagonist (as opposed to an inverse agonist) should not alter basal activity, especially when this basal activity may involve contributions from partial STAT3-linked cytokine receptors other than the leptin receptor. In contrast, the rAAV-leptin-mediated overexpression provides a continuous stimulation of the receptor, as evidenced by the persistent submaximal p-STAT3 level. This receptor activation would be subject to reversal by the antagonist, and this is exactly what was observed. Alternatively, another leptin signaling system, such as the PI3 kinase pathway, may be more tightly coupled to leptin-mediated physiological responses. A recent report indicated that PI3 kinase activity seems to correlate leptin responses with the development of leptin resistance (Pal and Sahu, 2003; Sahu and Metlakunta, 2005). Conceivably, the leptin antagonist evoked orexigenic response in the control rats may be due to blocking of PI3 kinase-mediated signaling. Additionally, our assessment of signaling within the whole hypothalamus could mask selective leptin resistance within regions of the hypothalamus (Ladyman and Grattan, 2004; Munzberg et al., 2004; Ladyman and Grattan, 2005). The residual STAT3 signaling following antagonist treatment in the control rats could be in a specific hypothalamic region that is not coupled to the physiological responses. Further investigations are necessary to fully elucidate these possibilities.

In aggregate, our data seem to be consistent with the following interpretation. The leptin antagonist prevents the choreographed periodic stimulation of the leptin receptor in the control rats, blocking endogenous normal functions of leptin in homeostatic energy regulation and leading to increases in food intake and body weight gain. In the rAAV-leptin-treated rats, the leptin overexpression produces a persistent submaximal level of receptor signaling but limits maximal receptor signaling and disrupts the normal endogenous leptin control of energy balance, resulting in the central leptin resistance. Although the leptin antagonist reversed hypothalamic p-STAT3 to the control level, it exerts little effect on food intake and body weight in the animals with impairments in both maximal leptin signaling capacity (see Fig. 1) and the usual endogenous leptin regulation of energy homeostasis.

In summary, the present study demonstrates that the central infusion of a leptin antagonist increases body weight gain, adiposity, and food consumption in normal, lean, leptin-responsive rats, but that these physiological consequences are nearly absent in rats made leptin resistant by central overexpression of leptin. Our data reveal a disconnection between leptin receptor activation and metabolic responses in the leptin resistant state and uncover disarray in both leptin receptor signaling regulation and homeostatic control of energy balance. Even though the observations are made specifically from our leptin-induced leptin-resistant model, they should also provide important insights into most forms of leptin resistance associated with elevated central leptin.

References

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