The Effects of Central or Peripheral Leptin Administration on Norepinephrine Turnover in Defined Fat Depots

Dawn M. Penn, Lisa C. Jordan, Emily W. Kelso, Jessica E. Davenport, and Ruth B.S. Harris

Department of Foods and Nutrition,
Dawson Hall,
University of Georgia,
Athens, GA, 30602

Running Title: Leptin and adipose tissue norepinephrine turnover

Corresponding author:
Dawn M. Penn,
Department of Foods and Nutrition,
University of Georgia,
Dawson Hall,
Athens, GA, 30602
Tel: 706-583-0817; Fax: 706-583-0658; e-mail: dawnpenn@uga.edu
ABSTRACT

Leptin preserves lean tissue, but decreases adipose tissue by increasing lipolysis and/or inhibiting lipogenesis. The sympathetic nervous system (SNS) is a primary regulator of lipolysis but it is not known if leptin increases norepinephrine turnover (NETO) in white adipose tissue. In this study we examined the effect of leptin administered either as a chronic physiologic dose, 40 µg/d for 4 days (from intraperitoneal miniosmotic pumps) or as an acute injection into the third ventricle (1.5 µg injected twice daily for 2 days) on NETO and the size of brown and white fat depots in male Sprague-Dawley rats. NETO was determined from the decline in tissue NE during 4 hours following administration of the NE synthesis inhibitor, α-methyl-para-tryrosine. The centrally injected leptin-treated animals demonstrated more dramatic reductions in food intake, body weight, fat pad size, and increase in NETO compared to the peripherally infused animals. Neither route of leptin administration caused a uniform increase in NETO across all fat pads tested and in both treatment conditions leptin decreased the size of certain fat pads independent of an increase in NETO. Similar discrepancies in white fat NETO were found for rats pair-fed to leptin treated animals. These results demonstrate that leptin acting either centrally or peripherally selectively increases sympathetic outflow to white fat depots and that a leptin-induced change in fat pad weight does not require an increase in NETO.

Key words: white adipose tissue, brown adipose tissue, sympathetic nervous system
INTRODUCTION

Leptin, the product of the ob gene that is expressed predominantly in adipose tissue, was originally proposed to function as a negative feedback signal in the regulation of energy balance (47), but it is now clear that the hormone influences multiple physiological systems including reproduction, immunity and angiogenesis (18). Although the primary function of leptin remains undefined, it is well established that administration of exogenous leptin causes a specific reduction in body fat mass while protecting lean tissue (19). Injections of leptin into the cerebral ventricles (icv) inhibit food intake and increase energy expenditure (16). Peripheral infusions of leptin produce less dramatic changes in body fat that are not necessarily associated with an inhibition of energy intake (4). The metabolic and mechanistic basis of the reduction in body fat mass under the two experimental conditions has not been clearly elucidated.

The amount of energy that is available for storage in fat depots is determined both by the energy balance status of the animal and by the partitioning of nutrients between different tissue types. The loss of body fat in leptin-treated animals could be associated with changes in a number of different pathways, including those that reduce the number of preadipocytes (40), those that promote lipid mobilization out of adipocytes (14), and those that inhibit accumulation of triglyceride in fat cells (23). In vivo and in vitro studies indicate that leptin has the potential to influence each of these pathways (13, 17, 40, 41).

All of these changes in adipose metabolism can be activated in response to peripheral infusions of leptin, however, it is not clear whether the in vivo response is due to a direct effect of leptin on adipocytes or whether leptin crosses the blood brain barrier and acts centrally to initiate the changes in metabolism. Adipocytes express leptin receptors (3), making it reasonable to assume that an increase in circulating leptin could directly reduce the size of fat depots, however, adipose tissue also is
innervated by the sympathetic nervous system (SNS) (44), providing the opportunity for central regulation of adipose tissue mass. Because sympathetic nerves in white adipose tissue regulate both lipolysis and the number of cells present, and because some of the nuclei that have been identified as sites of initiation of sympathetic outflow to white fat (2) also express leptin receptors (11), this provides an obvious pathway by which leptin might influence body fat content. Haynes et al (22) have clearly demonstrated that icv leptin can activate renal and lumbar sympathetic outflow, but it is not clear that leptin activates the sympathetic supply to white adipose tissue. Physiological stimuli promote SNS activity in a tissue-specific manner, therefore activation of SNS outflow to one organ does not necessarily represent outflow to all organs (29). Measurements of norepinephrine turnover (NETO) in specific tissues has been used as a measure of site-specific sympathetic activity in conditions such as fasting (28), and seasonal changes in energy balance in hibernating animals (27). Collins et al (7) found only a small non-significant increase in norepinephrine turnover (NETO) to retroperitoneal (RP) white adipose tissue of mice given an intraperitoneal injection of leptin, despite a significant increase in intrascapular brown adipose tissue (IBAT) NETO.

Previously we attempted to determine the importance of sympathetic innervation of white fat in mediating leptin-induced changes in body fat mass by chemically sympathectomizing one fat pad in leptin-treated mice or rats (34). The outcome of this study was complicated by the finding that sympathectomy of one fat pad influenced both the norepinephrine (NE) content and leptin responsiveness of other neurally intact fat pads in the same animal. Therefore, the studies described here measured NETO in different fat depots of rats that received either central injections or peripheral infusions of leptin to test whether there was a direct association between a leptin-induced increase in NETO and a reduction in fat pad mass. Due to the high probability that peripheral and central leptin activate different subpopulations of leptin receptors and because it is impossible to calculate how much
of the peripheral leptin would reach hypothalamic sites activated by third ventricle infusions of leptin, we did not attempt to match the treatments for degree of response in the animals but compared the effects of peripheral versus central leptin administration on a qualitative rather than a quantitative level. Measurements in multiple fat depots allowed us to determine whether the relative differences in leptin-responsiveness of individual fat depots was associated with differences in NETO in these depots and whether peripheral leptin produced significant changes in adipose NETO, which would imply that some, or all, of the metabolic response to peripheral leptin is mediated by centrally located receptors.

**METHODS**

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia and were in accordance with the principles of the American Physiological Society (1).

**Experiment 1: Effect of varying peripheral doses of leptin on food intake, body weight, fat pad weight, and serum leptin.**

The objective of this study was to identify a dose of leptin that produced a significant change in body composition after 13 days of peripheral infusion. Twenty-five male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) with body weights of approximately 250 g were individually housed in hanging wire cages in a humidity- and temperature-controlled room on a 12:12 hour light:dark cycle with lights on at 0700. Animals had free access to tap water and standard rat chow (LabDiet 5012, PMI Nutrition International, Brentwood, MO). After 1 week of adaptation to the environment baseline daily food intakes, corrected for spillage, and body weights were recorded for 6 days before rats were divided into 3 weight-matched groups. Under isofluorane anesthesia, each rat was then fitted with an intraperitoneal (i.p.) Alzet miniosmotic pump (Model 2002; Durect, Curpertino, CA) that delivered either 40 µg leptin (recombinant rat leptin R&D Systems, Minneapolis, MN), 75 µg
leptin or an equal volume of 0.01 M phosphate buffered saline (PBS) per day for 13 days. Daily body weights and food intakes were measured and on day 13, trunk blood was collected for measurement of serum leptin (Leptin RIA: Linco Research, St. Charles, MO). Epididymal (EPI), retroperitoneal (RP), mesenteric (MES), and intrascapular brown adipose (IBAT) were excised and weighed. The results of this experiment demonstrated that 13 day infusions of 40 or 75 µg of leptin were both capable of reducing fat pad size to a similar degree even though 40 µg levels of leptin did not significantly increase serum leptin concentration above control levels. Therefore the lower dose of leptin was used in Experiment 2.

**Experiment 2: Effect of peripherally-infused leptin on NETO in specific fat depots.**

Fifty-five male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) with body weights of approximately 300 g were housed in similar conditions as described in Experiment 1. After 3 days of adaptation to the environment baseline daily food intakes, corrected for spillage, and body weights were recorded for 7 days before rats were divided into 3 weight-matched groups: leptin (n=19), pairfed (n=18), and control (n=18). Rats were fitted with i.p. miniosmotic pumps (Model 1007D) on the seventh day that delivered either 40 µg of leptin or an equal volume of 0.01 M PBS per day. Daily food intakes and body weights were measured for 4 days. Pair-fed rats were fed the average amount of food that the leptin rats had eaten on the previous day. Food was given to the pair-fed rats in two meals: one at the start of the light cycle and one at the start of the dark cycle.

On day 4 of infusion food was removed from the cages at 0800 hours. Starting at 1000 hours half of the rats in each treatment group were decapitated (Time 0) while the remaining rats were given the first of two i.p. injections of alpha-methyl-para-tyrosine (αMPT; Sigma, St Louis MO), a tyrosine hydroxylase inhibitor that blocks NE synthesis. αMPT was first dissolved in glacial acetic acid (0.5 µL/mg αMPT) and then diluted to a final concentration of 300 mg/mL in sterile PBS. The solution was
kept in the dark and on ice. At Time 0 the rats were injected with 300 mg αMPT/kg body weight and 2 hours later they were given a second injection of 150 mg αMPT/kg body weight. Exactly 4 hours after the first injection of αMPT the rats were decapitated (Time 4). Only two time points were used for the measurement of turnover as previous publications have shown linearity of the logarithmic decline in NE content over periods of 6 or 8 hours (28).

Trunk blood was collected for measurement of serum leptin. IBAT, MES, and combined left and right RP, inguinal (ING), and EPI fat pads were weighed. Approximately 50 mg of IBAT, 100 mg of MES, and 200 mg of each of the other pads were snap frozen for measurement of tissue norepinephrine (NE) by reverse phase HPLC, as described below. For bilateral depots approximately equal quantities of fat were taken from the same anatomical location of each pad and combined for analysis in order to control for differences in cellularity and morphology within a depot. Remaining tissue was snap frozen for RNA extraction.

**Experiment 3: Effect of centrally-infused leptin on NETO in specific fat depots.**

Fifty-seven male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) with body weights of approximately 185 g were housed as described for Experiment 1. After 1 week of adaptation animals were fitted with 3rd ventricle cannulas. They were anesthetized by i.p. injections of ketamine (90 mg/kg) and xylazine (10 mg/kg). Guide cannulas (22 gauge, 15mm long) were placed using the stereotaxic coordinates applied to a flat skull: anteroposterior -2.8, lateral 0.0, ventral -8.1 from bregma (38). Injection cannulas (28 gauge) were designed to project 1 mm beyond the tip of the guide cannula. The rats were given an injection of analgesic (Ketophen, 2 mg/Kg) immediately after surgery and on the day after surgery. Proper cannula placement was confirmed 10 days later by monitoring water intake during the 5 minutes following infusion of angiotensin II (10 ng delivered over 2 minutes). Three days later, baseline body weights and food intakes, corrected for spillage, were
recorded for 5 days and then animals were divided into 3 weight-matched groups: leptin (n=19),
pairfed (n=19), and control (n=19). Rats in the leptin group received twice daily 3rd ventricle infusions
of 1.5 µg of leptin, in a volume of 2 µl infused over 2 minutes. Infusions were given at 0900 and 1700
hours for 2 days and then a final infusion was given on the third day two hours before Time 0. The
control and pair-fed animals were infused with an equivalent volume of 0.01 M PBS. On the third day
animals were injected with αMPT and tissue samples were collected exactly as described for
Experiment 2. Tissue NE concentration was measured as described below.

NE Measurements

NE content of white fat depots and IBAT was measured by reverse-phase high pressure liquid
chromatography (HPLC) with electrochemical detection. EPI, MES, or RP fat was sonicated on ice 3
times for 30 sec in 800 µl 0.2 M perchloric acid containing 3 µg/ml ascorbic acid and 25 ng/ml 3,4-
dihydroxybenzylamine hydromide (DHBA) as an internal standard. ING and IBAT were processed in
an identical manner except that they were sonicated in 1 ml of DHBA. The sonicated fat mixture was
centrifuged for 15 min at 9,000 rpm at 4ºC. Following centrifugation the infranate was filtered through
a 0.2 µm Nylon Sterile 25-mm syringe filter. NE was assayed using an ESA HPLC system (Bedford,
MA) that consisted of a model 582 Solvent Delivery Module, a model 542 auto sampler maintained at
6ºC, and a model 5600A CoulArray detector at 350 mV. The column was a Phenomenex (150 x 4.6
mm) SYNERGI, 4 u, Max RP-80A and the mobile phase consisted of 0.1 M sodium phosphate
monobasic, 0.1 mM disodium EDTA, 0.3 mM 1-octanesulfonic acid, and 4% acetonitrile, pH of 3.1.
Chromatograms were analyzed with CoulArray for Windows, v.1.04, and NE content was calculated
from a standard curve. Data are expressed as ng of NE per fat depot.
**Calculation of NETO**

Fractional turnover rates of NE were measured based on the rate of decline in tissue NE content after inhibition of synthesis. This method has been widely used in measuring NE fractional degradation rate and turnover time and is an application of the principles of steady state kinetics to the change with time of NE concentration (5). NETO was calculated using the following formula:

\[
\text{NETO} = k\left[\text{NE}\right]_0
\]

\[
k = \left( \log[\text{mean NE}]_0 - \log[\text{mean NE}]_4 \right) / (0.434 \times 4)
\]

\[
k = \text{the fractional turnover rate}
\]

\[
[\text{NE}]_0 = \text{NE concentration at Time 0}
\]

\[
[\text{NE}]_4 = \text{NE concentration at Time 4}
\]

**Real Time PCR**

MES and ING tissue from Time 0 rats in each experiment were used to measure hormone sensitive lipase (HSL) and leptin mRNA because these were the white fat depots that were the least and the most responsive to leptin, respectively, in terms of increased NETO. IBAT was used to measure uncoupling protein 1 (UCP 1) mRNA. Total RNA was extracted using Trizol reagent according to the manufacturer’s protocol (Invitrogen; Carlsbad, CA). RNA concentration and quality were determined by the spectrophotometric 260:280 ratio and by agarose gel electrophoresis.

Following extraction, 1µg of RNA was transcribed for cDNA as described previously (20). Primers used to amplify leptin, UCP1, HSL mRNA, and 18S rRNA are described in Table 1. The SYBR green reaction was prepared according to the manufacturer’s protocol (BioRad, Hercules, CA) and PCR was performed using the Bio-Rad iCycler system. Amplification conditions were as follows: 95 °C for 3 minutes; 40 cycles of 95 °C for 30s, 58°C for 30s, and 72 °C for 30s. Immediately following Q-PCR amplification, a melt curve analysis was performed to ensure the specificity of the
amplification. Delta Ct (dCt) values, or difference in threshold cycles for the mRNA of interest and 18S rRNA were determined for each sample and relative mRNA expression was expressed as 1/dCt.

**Statistical Analysis**

Body weight and food intake comparisons were made for each day of treatment using repeated measures analysis of variance (Statistica Software, Statsoft, OK). Measures at specific time points were analyzed by post-hoc Duncan’s Multiple Range Test. Baseline and experimental cumulative food intake comparisons, tissue weights, NETO for each tissue, serum leptin concentrations, HSL, UCP1, and leptin mRNA expression were all compared between groups using one-way ANOVA. Duncan’s Multiple Range Test was used for all post hoc comparisons. Comparisons of tissue weights for rats killed at Time 0 and Time 4 of each treatment group were made using Student’s t-test. Differences between means were considered statistically significant at P<0.05 for all tests.

**RESULTS**

*Experiment 1.*

Both doses of leptin caused weight loss compared with controls although there were no effects on food intake during either the first 6 days of the infusion (Table 2) or the complete 13 day experimental period (data not shown). Both doses of leptin also reduced the size of EPI and RP fat pads (Figure 1). This inhibition was significant for RP pads in rats receiving 40 or 75 µg leptin/day. EPI fat was significantly reduced in rats infused with 75 ug leptin/day but the difference did not reach significance (P<0.08) for rats infused with 40 ug leptin/day (Figure 1). Leptin did not influence MES or IBAT pad weight. Serum leptin was significantly higher in the 75 µg infused animals than the control or 40 µg leptin infused rats (Table 2).
**Experiment 2.**

Body weight was reduced in all of the rats on the day after placement of Alzet pumps but control rats returned to baseline weight by the second day after surgery and the leptin-treated rats returned to baseline weight by the third day after surgery (data not shown). After 4 days of infusion there was no difference in the weights of control and leptin-treated groups but the pair-fed rats weighed significantly less than either of the two other groups (Table 3). Total food intake during the four days of infusion was inhibited in leptin-treated rats compared with controls and reduced even further in pair-fed rats due to spillage (Table 3). As expected, serum leptin concentrations were significantly higher in leptin-treated rats than in either control or pair-fed rats (Table 3).

Unexpectedly, the weights of EPI and IBAT pads from control rats, EPI pads from leptin-treated rats and EPI and MES pads from pair-fed rats were all significantly greater at Time 4 than at Time 0 (data not shown), but tissue weights from both time points were combined for each fat depot to keep data consistent between experiments. The increase in weight of the pads meant that detection of leptin-induced changes in fat pad weight was based on a conservative comparison of data. The weights of all of the fat pads measured were lower in leptin-treated and pair-fed rats than in controls. The difference was significant for ING, EPI, RP, and MES of pair-fed rats, but only for the MES pad in leptin-infused rats (Figure 2A). There was no significant effect of leptin infusion on NETO in any of the white fat pads (Figure 3A). NETO was higher in ING fat than any other white fat pad and was higher in pair-fed than control or leptin-treated rats due to an increase in fractional turnover rate, rather than a change in ING Time 0 NE concentration (Table 4). IBAT NETO was about three-fold greater than in ING white fat and was significantly higher in pair-fed rats than in either control or leptin-treated animals (Figure 4A) due to an increase in $k$ (Table 4). The fractional turnover rate ($k$) was negative for some fat pads due to slightly higher NE concentrations in fat from rats killed at Time 4.
than at Time 0. These results were interpreted as negligible rates of NETO and were due to the unavoidable requirement of using different animals for measures at the two time points.

There was no effect of treatment or of fat depot on HSL mRNA expression in rats that received peripheral infusions of leptin (Figure 5A). Leptin mRNA expression was significantly higher in ING than MES fat (P<0.04), but there was no effect of treatment (Figure 5C). One way ANOVA showed no significant difference in IBAT UCP1 expression between treatment groups, but post-hoc analysis revealed that pair-feeding significantly inhibited UCP1 mRNA expression compared with that in control rats (P<0.05) (Figure 6A).

*Experiment 3*

Leptin and pair-fed rats lost weight on all days of central infusion and weighed significantly less than controls at the end of the experiment (Table 3). Similarly, leptin and pair-fed groups ate less than controls during the 2 days of infusion (Table 3). Although leptin was infused into the 3rd ventricle, the leptin treated group had significantly higher serum leptin levels than control or pair-fed animals (Table 3). At the end of the experiment all fat pads weighed less in leptin-treated and pair-fed rats than in controls. These differences were significant for ING, EPI, RP, and IBAT, but not for MES fat (Figure 2B).

The changes in white fat NETO were depot specific and different from those induced by peripheral leptin infusions. In contrast to Experiment 2, ING NETO was not different between treatment groups, but leptin significantly increased NETO in EPI and MES fat (Figure 3B). Pair-feeding increased NETO in EPI, MES, and IBAT, but dramatically inhibited NETO in RP fat (Figure 3B and 4B). NETO was higher in MES fat than in other white fat depots (Figure 3B). Differences in NETO between fat depots was due to difference in both tissue NE content and fractional turnover rate
(k). In contrast, when there was a treatment effect on NETO for a specific depot it was due to a change in k rather than in tissue NE concentration at Time 0 (Table 4).

In contrast to Experiment 2 we found significant effects of leptin and of pair-feeding on white fat mRNA expression. Pair-feeding stimulated HSL mRNA expression in ING fat (Figure 5B), and both leptin and pair-feeding inhibited leptin mRNA expression in ING fat (Figure 5D). HSL and leptin mRNA expression were greater in the ING than the MES fat depot (Figure 5B). There were no significant differences in IBAT UCP1 mRNA expression (Figure 6B).

**DISCUSSION**

In these experiments leptin was infused either peripherally or centrally to determine whether the resulting decrease in body fat was dependent on activation of the SNS in white adipose tissue in each of the two conditions and whether the response of individual pads differed with route of leptin administration. Few *in vivo* studies have examined the effect of leptin on the activity of sympathetic nerves in white fat depots (7, 30, 34) and, to our knowledge, a comparison of the effects of peripheral and central leptin infusion on NETO in individual fat depots has not previously been reported. We found that, regardless of the route of administration, leptin did not cause a uniform NETO response among the fat depots measured and that leptin decreased fat pad size independently of changes in NETO in several fat depots. We also observed a difference in the degree and pattern of NETO stimulation among the fat pads depending on the route of leptin administration.

The measures of NETO in Experiments 2 and 3 cannot be compared quantitatively because the pattern of receptor activation is likely to be different between peripheral and central leptin administration, NE measurements for each of the studies were made at different times and the duration of leptin treatment was different for Experiments 2 and 3. NETO varied between white fat depots in both experiments but the pattern of response was different. This could not simply be attributed to a
generalized response to the more energetically stressful condition produced by central leptin infusions and the resulting more extreme food restriction of the pair-fed controls in Experiment 3, because differences between the two experiments were not consistent among white fat depots. NETO was relatively low in ING fat from centrally infused rats but high in ING from peripherally infused rats, whereas MES and RP fat NETO were relatively low in peripherally infused animals but high in centrally infused rats. In addition, leptin and pair-feeding had opposing effects on NETO in RP fat from the centrally infused animals. Because the size of all white fat depots except MES decreased in both the centrally leptin-infused and pair-fed animals in Experiment 3 these results indicate that leptin does not depend on the SNS to reduce the size of all fat depots, consistent with the results of a study in which rats with one sympathetically denervated fat pad were infused with leptin (34). The results from Experiment 2 also support this conclusion because peripherally infused leptin caused a significant decrease in the size of the MES tissue whereas ING fat was the only depot to show even a tendency for an increase in NETO. The results from Experiment 1 demonstrated that a 13 day infusion of 40 µg of leptin significantly reduced fat pad weight, therefore, we can conclude that the absence of a significant decrease in size of the majority of fat pads in rats in Experiment 2 was due to the short duration of treatment, which was selected to ensure that measurements were made during the dynamic phase of leptin-induced SNS activation.

The variability in NETO across different fat depots confirms previous findings that physiological stimuli promote sympathetic activity in a tissue-specific manner (43) and suggests either a variation in the degree of innervation, or a difference in the concentration and ratio of adrenergic receptors in the different tissues (24). Others have reported variations in the degree of innervation of different white fat pads (45), and that catecholamine concentrations (33,34), oxidative capacity (9), and cellularity (10) are different in different fat depots. These region-specific differences imply that
the response to endocrine and neural factors varies between depots and that the variable responses of
the different fat pads were not unique to the experiments reported here.

How leptin caused a decrease in pad size without increasing NETO is not clearly understood.
We have reported that the size of sympathetically denervated EPI pad of mice and RP pad of rats were
reduced in response to peripheral infusion of physiological doses of leptin (34), and others reported
that transplanted EPI fat, with no apparent re-innervation, decreased in size in rats expressing
adenovirus-leptin (42). Additionally, in vitro studies have shown that direct administration of leptin
stimulates lipolysis in isolated fat pads (15, 37, 41, 42), and that the sensitivity of fat pads to the
lipolytic effects of leptin appear to be depot specific (15). In contrast, adipocyte lipogenesis has been
shown to be inhibited in mice infused (17) or injected (6) with leptin but leptin does not directly inhibit
adipocyte lipogenesis in vitro (17). The direct actions of leptin on adipose tissue require a functional
long-form leptin receptor (Ob-Rb) as several in vitro studies have shown that, in contrast to fat from
lean rats, white fat from obese Zucker rats (fa/fa), which have a mutation in the extracellular domain of
the leptin receptor, fails to respond to doses of leptin ranging from physiological to supraphysiological
(15, 37, 41).

We measured white fat HSL mRNA expression in the studies reported here because the SNS is
thought to be a primary regulatory of lipolysis (25). Neither peripheral nor central infusions of leptin
influenced MES or ING HSL mRNA expression even though centrally infused leptin caused a
substantial increase in MES NETO and produced a significant decrease in the weight of the ING pad.
These observations do not account for post-translational changes in HSL activity or for regulation of
lipolysis by lipases other than HSL (12), both of which could have a significant impact on adipose
tissue lipolysis. Leptin mRNA expression was measured because both circulating leptin (8) and
activation of β-adrenoreceptors (39) inhibit adipose tissue leptin mRNA expression. The results
reported here are consistent with previous reports that subcutaneous fat has greater levels of leptin expression than visceral fat (31, 36) but we did not find a specific effect of either peripheral or central leptin on white fat leptin mRNA expression. In Experiment 3, ING expression of leptin mRNA was inhibited in both the leptin and pair-fed groups probably due to the reduction in fat depot size as there was no treatment effect on NETO in this pad and the two groups of rats had very different circulating levels of leptin.

NETO in IBAT was higher than in any of the white fat pads but was similar across the two experiments. Leptin did not increase IBAT NETO in either peripherally or centrally infused rats. These results do not exclude the possibility that leptin increased thermogenesis in this tissue independent of an increase in the transcription of the protein. An unexpected finding in both experiments was the significant increase in IBAT NETO of only the pair-fed animals despite them being food restricted during the experimental period and not receiving any food for over 15 hours before sacrifice. Neither peripherally nor centrally infused pair-fed animals showed an increase in UCP1 mRNA expression to correspond with the increased NETO. It has been reported that the thermoregulatory processes of IBAT are depressed in response to 10 days of food restriction (35) but also that there is an increase in expenditure of lean subjects during an 84 hr fast (46). Because we did not measure energy expenditure of the rats in this study it is unclear whether the increase in NETO was associated with an increase in thermoregulation or was associated with an unrelated aspect of IBAT metabolism.

A surprising finding in the centrally infused animals was the elevation of serum leptin above control levels. This most likely resulted from the icv leptin infusion leaking into the periphery because there was no increase in leptin expression in the ING or MES tissues of leptin infused animals. Others have reported that serum leptin is increased following a central leptin infusion (26, 32). The half-life
of leptin is estimated to be 36.3 minutes (21). With the rats having an average weight of 272 g, administration of 1.5 µg leptin 2 hours before sacrifice, and the average serum leptin concentration for control rats at 1.5 ng/mL, it can be calculated that serum leptin would rise to ~8.4 ng/mL and our actual serum leptin was measured at 7.6 ng/mL.

In summary, the results of the experiments reported here indicate that leptin can reduce body fat independent of stimulation of SNS activity in white fat depots. Experiment 1 demonstrates that chronic peripheral infusions of physiological doses of leptin reduce body fat content but Experiment 2 shows that these doses of leptin do not increase NETO in any white fat depot. Infusion of leptin into the third ventricle, an area adjacent to nuclei that express leptin receptors and also initiate sympathetic outflow to white fat, reduced the size of most white fat depots but produce selective increases in NETO. While leptin increased NETO in some fat depots, we did not determine whether this directly increased lipolysis or inhibited lipogenesis in these tissues. Further studies are needed to identify the metabolic pathways that lead to a reduction in fat mass in normal weight animals treated with leptin, how the individual white fat depots respond to leptin and which aspects of leptin action go awry in obesity or other conditions of leptin resistance.
**FIGURE LEGENDS**

**Figure 1:** Fat pad weights of rats infused for 13 days with 40 or 75 µg leptin from an intraperitoneal pump in Experiment 1. Data are means ± SE for groups of 8 to 10 rats. Values for a specific fat pad that do not share a common superscript are significantly different (P<0.05).

**Figure 2:** Fat pad weights of rats peripherally infused with leptin for 4 days (A), or centrally infused with leptin for 3 days (B). Data are means ± SE for groups of 18 to 19 rats. Values for a specific fat pad that do not share a common superscript are significantly different (P<0.05).

**Figure 3:** NETO in white adipose tissue of rats infused peripherally with leptin (A) and rats infused centrally with leptin (B). Negative values of NETO were interpreted to mean NETO was zero. Data are means ± SE for groups of 9 to 10 rats. Values for a specific fat pad that do not share a common superscript are significantly different (P<0.05).

**Figure 4:** NETO in IBAT of rats infused peripherally with leptin (A) and rats infused centrally with leptin (B). Data are means ± SE for groups of 9 to 10 rats. Values for a specific treatment condition that do not share a common superscript are significantly different (P<0.05).

**Figure 5:** HSL mRNA expression in the MES or ING fat depots of rats peripherally infused with leptin (A) and rats centrally infused with leptin (B). Leptin mRNA expression in the MES or ING fat depots of rats peripherally infused with leptin (C) and rats centrally infused with leptin (D). Data are means ± SE for groups of 9 to 10 rats from Time 0 in each study. Values within each fat pad and treatment condition that do not share a common superscript are significantly different (P<0.05).
Figure 6: UCP1 mRNA expression in the IBAT of rats peripherally infused with leptin (A) and rats centrally infused with leptin (B). Data are means ± SE for groups of 9 to 10 rats from Time 0 in each study. Values within each fat pad and treatment condition that do not share a common superscript are significantly different (P<0.05).
ACKNOWLEDGEMENT

This work was supported by National Institutes of Health Grant R01DK53903 and by Georgia Agricultural Experiment Station grant CSREES/GEO00932 awarded to R.B.S. Harris. The authors appreciate the helpful comments provided by Dr Renato Migliorini during the preparation of this manuscript.
Table 1: Leptin, UCP 1, HSL and 18S primer pairs used for PCR

<table>
<thead>
<tr>
<th></th>
<th>5’ Primer (5’-3’)</th>
<th>3’ Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>TTG TCA CCA GGA TCA ATG ACA TTT</td>
<td>GAC AAA CTC AGA ATG GGG TGA AG</td>
</tr>
<tr>
<td>UCP 1</td>
<td>TCC CTC AGG ATT GGC CTC TAC</td>
<td>GTC ATC AAG CCA GCC GAG AT</td>
</tr>
<tr>
<td>HSL</td>
<td>CAA CAT GGC ATC AAC CAC TG</td>
<td>GCC TGG GAT CAG AGG TGA TG</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>ACG GAA GGG CAC CAC CAG GA</td>
<td>CAC CAC CAC CCA CGG AAT CG</td>
</tr>
</tbody>
</table>
Table 2: Changes in body weight during the experimental period, cumulative baseline and experimental food intake, and serum leptin for rats in Experiment 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial Body Weight (g)</th>
<th>Final Body Weight (g)</th>
<th>Wt Change (g/13 d)</th>
<th>6 day Cumulative Food Intake (g)</th>
<th>Serum Baseline (ng/ml)</th>
<th>Serum Experimental (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>289 ± 5</td>
<td>323 ± 6</td>
<td>35 ± 3a</td>
<td>134 ± 3</td>
<td>141 ± 2</td>
<td>2.9 ± 0.2b</td>
</tr>
<tr>
<td>40 µg leptin/day</td>
<td>293 ± 6</td>
<td>315 ± 10</td>
<td>23 ± 4b</td>
<td>136 ± 5</td>
<td>133 ± 5</td>
<td>4.8 ± 0.5b</td>
</tr>
<tr>
<td>70 µg leptin/day</td>
<td>287 ± 5</td>
<td>308 ± 7</td>
<td>21 ± 3b</td>
<td>131 ± 4</td>
<td>131 ± 3</td>
<td>8.2 ± 2a</td>
</tr>
</tbody>
</table>

Data are means ± SE for groups of 8 to 9 rats infused with leptin for 13 days from an intraperitoneal pump. Values for a specific parameter that do not share a common superscript are significantly different at P<0.05
Table 3: Changes in body weight during the experimental period, cumulative baseline and experimental food intake, and serum leptin for rats in Experiments 2 and 3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial Body Weight (g)</th>
<th>Final Body Weight (g)</th>
<th>Wt Change (g/13 d)</th>
<th>Baseline Food intake (g)</th>
<th>Experimental Food Intake (g)</th>
<th>Serum leptin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>309 ± 3</td>
<td>321 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86 ± 1</td>
<td>81 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leptin</td>
<td>310 ± 2</td>
<td>316 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86 ± 2</td>
<td>75 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.7 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>311 ± 2</td>
<td>302 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-9 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88 ± 1</td>
<td>63 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Experiment 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial Body Weight (g)</th>
<th>Final Body Weight (g)</th>
<th>Wt Change (g/13 d)</th>
<th>Baseline Food intake (g)</th>
<th>Experimental Food Intake (g)</th>
<th>Serum leptin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>298 ± 6</td>
<td>293 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-5 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44 ± 1</td>
<td>33 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leptin</td>
<td>301 ± 6</td>
<td>272 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-28 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44 ± 1</td>
<td>15 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.6 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>301 ± 6</td>
<td>274 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-27 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44 ± 1</td>
<td>15 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are means ± SE for groups of 9 to 14 rats. Values for a specific parameter within an experiment that do not share a common superscript are significantly different at P<0.05. The duration of Experiment 2 was 4 days and of Experiment 3 was 3 days.
Table 4: Time 0 NE concentration and fractional rate of NE turnover ($k$) for NE in white and brown fat pads in Experiments 2 and 3.

<table>
<thead>
<tr>
<th>Fat Pad</th>
<th>Experiment 2 (peripheral leptin)</th>
<th></th>
<th>Experiment 3 (central leptin)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time 0 [NE] (ng/pad)</td>
<td>$k$ %/hour</td>
<td>Time 0 [NE] (ng/pad)</td>
<td>$k$ %/hour</td>
</tr>
<tr>
<td>ING</td>
<td>Control</td>
<td>1014 ± 80</td>
<td>1.98</td>
<td>371 ± 50</td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td>1034 ± 32</td>
<td>3.04</td>
<td>339 ± 46</td>
</tr>
<tr>
<td></td>
<td>Pairfed</td>
<td>1013 ± 12</td>
<td>9.11</td>
<td>315 ± 49</td>
</tr>
<tr>
<td>EPI</td>
<td>Control</td>
<td>166 ± 34</td>
<td>-2.17</td>
<td>223 ± 45</td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td>187 ± 31</td>
<td>-3.41</td>
<td>183 ± 56</td>
</tr>
<tr>
<td></td>
<td>Pairfed</td>
<td>184 ± 36</td>
<td>1.70</td>
<td>173 ± 30</td>
</tr>
<tr>
<td>RP</td>
<td>Control</td>
<td>144 ± 7</td>
<td>9.42</td>
<td>354 ± 44</td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td>128 ± 16</td>
<td>-3.20</td>
<td>338 ± 40</td>
</tr>
<tr>
<td></td>
<td>Pairfed</td>
<td>121 ± 11</td>
<td>5.52</td>
<td>283 ± 24</td>
</tr>
<tr>
<td>MES</td>
<td>Control</td>
<td>457 ± 54</td>
<td>1.40</td>
<td>1876 ± 283</td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td>400 ± 58</td>
<td>-3.29</td>
<td>3236 ± 685</td>
</tr>
<tr>
<td></td>
<td>Pairfed</td>
<td>449 ± 63</td>
<td>0.76</td>
<td>3387 ± 651</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Leptin</td>
<td>Pairfed</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
<td>--------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>IBAT</td>
<td>888 ± 88</td>
<td>795 ± 79</td>
<td>1023 ± 178</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.19</td>
<td>4.34</td>
<td>15.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1849 ± 107</td>
<td>1610 ± 125</td>
<td>1825 ± 114</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.74</td>
<td>8.45</td>
<td>11.58</td>
<td></td>
</tr>
</tbody>
</table>

Values for tissue NE concentration are means ± SE for groups of 8 to 9 rats. There were no significant effects of leptin or pair-feeding on NE content of any tissue within the peripherally infused or centrally infused experiments. $k$ is the fractional turnover rate calculated for each tissue and only one value was obtained for each treatment group.
REFERENCES

1. **American Physiological Society.** Guiding principles for research involving animals and

2. **Bamshad M, Song CK, and Bartness TJ.** CNS origins of the sympathetic nervous system

3. **Bjorbaek C and Kahn BB.** Leptin signaling in the central nervous system and the periphery.

4. **Bowen H, Mitchell TD, and Harris RBS.** Method of leptin dosing, strain, and group housing

5. **Brodie BB, Costa E, Dlabac A, Neff NH, and Smookler HH.** Application of steady state
   kinetics to the estimation of synthesis rate and turnover time of tissue catecholamines. *J Pharmacol

6. **Bryson JM, Phuyal JL, Swan V, and Caterson ID.** Leptin has acute effects on glucose and

7. **Collins S, Kuhn CM, Petro AE, Swick AG, Chrunyk BA, and Surwit RS.** Role of leptin in

8. **Commins SP, Watson PM, Levin N, Beiler RJ, and Gettys TW.** Central leptin regulates the
   UCP1 and ob genes in brown and white adipose tissue via different beta-adrenoceptor subtypes. *J Biol

9. **Deveaud C, Beauvoit B, Salin B, Schaeffer J, and Rigoulet M.** Regional differences in
   oxidative capacity of rat white adipose tissue are linked to the mitochondrial content of mature


A  Peripherally Infused Rats

B  Centrally Infused Rats
A  Peripherally Infused Rats

B  Centrally Infused Rats
A  IBAT of Peripherally Infused Rats

B  IBAT of Centrally Infused Rats
A  UCP1 mRNA Expression of Peripherally Infused Rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Leptin</th>
<th>Pairfed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/dCt</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

B  UCP1 mRNA Expression of Centrally Infused Rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Leptin</th>
<th>Pairfed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/dCt</td>
<td>0.8</td>
<td>1.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>