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Determination of the half life of circulating leptin in the mouse

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Abstract

Background—The adipokine hormone, leptin, is a major component of body weight homeostasis. Numerous studies have been performed administering recombinant mouse leptin as an experimental reagent; however, the half life of circulating leptin following exogenous administration of recombinant mouse leptin has not been carefully evaluated.

Methods—Exogenous leptin was administered (3 mg leptin/kg body weight) to ten week old fasted non-obese male mice and plasma was serially collected at seven time points; plasma leptin concentration was measured by ELISA at each time point to estimate the circulating half life of mouse leptin.

Results—Under the physiological circumstances tested, the half life of mouse leptin was 40.2 (+/− 2.2) minutes. Circulating leptin concentrations up to one hour following exogenous leptin administration were 170-fold higher than endogenous levels at fasting.

Conclusions—The half life of mouse leptin was determined to be 40.2 minutes. These results should be useful in planning and interpreting experiments employing exogenous leptin. The unphysiological elevations in circulating leptin resulting from widely used dosing regimens for

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exogenous leptin are likely to confound inferences regarding some aspects of the hormone's clinical biology.

Keywords

Leptin; half life; obesity

Introduction

Leptin (*Lep)* is a peptide hormone produced in adipocytes that signals via isoforms of the leptin receptor (*LepR*) (1). A major function of leptin is to signal the levels of peripheral energy stores in adipose tissue to the central nervous system (2). Mice and humans hypomorphic for *LEP* or *LEPR* develop severe, hyperphagic obesity (3–6). Circulating leptin concentrations are closely correlated with adipose tissue mass in both humans and mice (7–9).

The major site of leptin synthesis is the adipocyte; leptin synthesis is regulated by nutritional status, insulin, glucocorticoids, catecholamines, cold exposure, and growth hormone, amongst other signals (10). Long term nutritional status regulates leptin at the transcriptional level; leptin mRNA levels are elevated in obesity and decreased by calorie restriction (11). Circulating signals such as insulin regulate leptin posttranscriptionally; *ex vivo* insulin stimulation of adipose tissue explants increases the association of leptin mRNA with translationally active polysomes (12). Leptin is secreted from adipocytes via small intracellular vesicles in both constituitive and regulated pathways (13). Leptin secretion in humans is diurnal, with a nighttime zenith and morning nadir (14). In the circulation, leptin exists in both bound and free forms and is mainly cleared by the kidneys (14).

Leptin signals through its receptor, LepRb, in the arcuate nucleus of the hypothalamus primarily through JAK/STAT signaling, but also through PI3K, SHP2/ERK, and p38/MAPK signaling pathways (15). Hypothalamic levels of phosphorylated STAT3 following recombinant leptin administration in mice are commonly used as a read out of leptin activity at this receptor isoform (16). The response of other downstream signaling in the leptin-melanocortin pathway is also studied in response to leptin administration (17). Despite a multitude of studies utilizing the experimental paradigm of exogenous leptin stimulation, there have been limited studies investigating the pharmacokinetic properties of leptin. While the circulating half life of leptin has been determined in adult human subjects and female rats, the circulating half life of leptin in mice, the most commonly used model in obesity-related research, has not been rigorously calculated (18, 19). Here, we report the circulating half life of recombinant leptin in mice and compare the blood levels of commonly used concentrations of injected recombinant leptin to endogenous levels during fasting and refeeding in mice.

Materials and Methods

Mouse anthropometric, blood glucose, and food intake measurements

All animal studies were conducted in accordance with IACUC and CUMC standards and were IACUC approved under protocol AC-AAAH1203. Ten week old male C57BL/6J mice from Jackson Laboratories were used (stock #000664). Body weights, fat mass, and lean mass were measured at 8 and 10 weeks of age. Fat mass and lean mass was measured using the Bruker Minispec TD NMR. Mice were fed Research Diets PicoLab Rodent Diet 20 (#5053) with a fat content of 4.5% of calories. Blood was collected by submandibular bleeding and used for both blood glucose and plasma leptin measurements. Blood glucose was measured following an overnight (16 hours) fast and following 4 hours of *ad lib* re-feeding using a FreeStyle Lite blood glucose meter and strips (accurate range 30–372 mg/dL). Food intake during the 4-hour refeeding period was measured on a per-cage basis. Using an Acculab Vicon VIC212 scale; five similarly sized chow pellets were weighed and placed in the cage for the feeding period; and were weighed again at its completion; the difference in these numbers is the cumulative cage food intake for the 4 hour period. Statistical analysis was done using Prism 7 from GraphPad Software, Inc.

Measurement of circulating leptin at fasting and refeeding

Plasma leptin concentrations were measured from plasma following an overnight fast (16 hours), after 4 hours of re-feeding, and subsequent to injections of recombinant mouse leptin in fasted animals. Blood was collected in heparinized tubes and immediately placed on ice then centrifuged at 2,000 RCF for 15 minutes at 4 degrees Celsius. The R&D systems Mouse/Rat Leptin Quantikine ELISA Kit (SMOB00) was used to measure plasma leptin concentrations. Statistical analysis was performed using Prism 7 from GraphPad Software, Inc. Variance is reported as the standard error of the mean.

Measurement and calculation of circulating leptin half life

Mouse recombinant leptin was purchased from the National Hormone and Peptide Program (www.humc.edu); lyophilized leptin was dissolved in 1X PBS, pH 8.0 (1X PBS = 137 mM NaCl, 2.7 mM KCl; 10 mM Phosphate buffer). (Note that mouse recombinant leptin must be dissolved into PBS with a slightly basic pH, i.e. pH 8.0). Forty-five mice were arbitrarily separated into nine groups containing five mice each. Seven groups received intraperitoneal leptin injected at 3 mg/kg body weight at 7:45 AM following an overnight (16 hours) fast. Of the seven groups receiving recombinant leptin, circulating leptin concentrations were measured at the following time points post-injection: 0.25 hours, 0.5 hours, 1 hour, 2 hours, 4 hours, 6 hours, and 8 hours (naming convention as follows: 0.25 Hr L, 0.5 Hr L, 1 Hr L or 1HL, 2 Hr L, 4 Hr L or 4HL, 6 Hr L or 6HL, and 8 Hr L, respectively). All mice remained fasting for the duration of the experiment. Two groups of mice were injected with vehicle (saline) only, and circulating leptin concentrations were measured at 0.5 hours and 4 hours post-injection (naming convention: 0.5 Hr S, 4 Hr S, respectively). Blood collection and measurement of plasma leptin concentrations were carried out as described above. The half life of leptin was estimated by modeling the data with a single exponential decay process. Modeling the half life of leptin using a double exponential decay process did not

significantly improve curve fitting. Half life modeling was carried out in Origin8 software from OriginLab.

Results

Modeling the plasma concentrations post-injection over time with a single exponential decay model yielded an estimate of the half life of circulating leptin of 40.2 minutes (+/− 2.2 minutes) (Figure 1). We also modeled the half life of circulating leptin using a double exponential decay process which did not improve the curve fit; thus we used the single exponential decay model. This estimate is similar to that reported by others for injected leptin in human subjects (24.9 +/− 4.4 minutes) and for *ad lib* fed female rats (71 minutes) (18, 19).

We then compared plasma concentrations of leptin following 3 mg/kg injection to those of mice administered saline. Plasma leptin concentrations of fasted mice administered saline were not different from fasted mice that were not injected. At 1 hour following leptin administration, the concentration of circulating leptin was 170-fold higher than endogenous fasted leptin levels, and 13-fold higher than endogenous fed leptin concentrations in mice that were not injected with exogenous leptin (Figure 2). Four hours following injection, circulating leptin levels were 76-fold higher than endogenous leptin concentrations in fasted animals and 4.75-fold higher than endogenous leptin concentrations in fed animals. Six hours following the intraperitoneal injection of exogenous leptin into fasted mice, the concentrations of leptin were not different from the endogenous concentrations of leptin in non-fasted mice. There were no significant differences in body weights, fat or lean mass, food intake, or blood glucose or leptin concentrations among the groups of animals (Figure 3A–G). All fasted and fed blood glucose concentrations were in the normal physiological range (Figure 3 C, F). Blood glucose concentrations increased for all groups from fasting to re-feeding (Figure 3C, F). Circulating leptin concentrations also increased for all groups from fasting to re-feeding and were similar among the designated groups (Figure 3 D, G).

Discussion

We measured plasma leptin concentrations in fasted, non-obese, male mice at successive time points following injection of mouse recombinant leptin. From these data we estimated that the half life of circulating mouse leptin is 40.2 (+/− 2.2) minutes (Figure 1). The half life was calculated by fitting the serial plasma leptin concentrations to a single exponential decay process. Modeling the data with a double exponential decay process did not significantly improve the fit. The half life for mice is consistent with those reported for humans (24.9 minutes) and rats (71 minutes) (18, 19). These data are also consistent with those reported by Ahima *et al.*, with regard to the decay of circulating leptin concentrations following administration of recombinant mouse leptin to fasted mice at 1 mg/kg body weight (20).

Leptin is secreted from the adipocyte in small intracellular vesicles; ~60% of cellular leptin is co-secreted bound to soluble leptin receptor (13, 21). Soluble leptin receptor is the main carrier protein for leptin; however, c-reactive protein and other proteins can also bind

circulating leptin (22, 23). The main sites of soluble leptin receptor production are adipose tissue and liver; fasting upregulates hepatic soluble leptin receptor expression (24, 25). Mouse soluble leptin receptor can be produced from alternative splicing of the *LepR* gene to LepRe; other splice forms can also be posttranslationally modified to soluble leptin receptor by ectodomain shedding (26, 27). In humans, *LEPR* transcript is not spliced to LepRe; the soluble leptin receptor is produced exclusively by ectodomain shedding (26, 27). Free circulating leptin is the bioactive form of leptin and bound circulating leptin has greater stability (longer circulating half life) than that of free circulating leptin (28, 29). The KD of the leptin-leptin receptor complex has been reported to be 0.23+/−0.08 nM, suggesting high affinity ligand binding (30). In fasted, lean animals the majority of circulating leptin is bound and this correlates with a minimal pSTAT3 signal in arcuate LepRb neurons at fasting (31). The proportion of free to bound leptin decreases in the fed state, thus resulting in higher concentrations of circulating free leptin, which likely results in a shorter half life for total circulating leptin in the fed state than in the fasted state. In states of chronic hyperleptinemia, such as mice in which leptin is continuously perfused by minipumps or transgenic mice overexpressing leptin, the half life of circulating leptin may differ from what is reported here circumstances (32, 33). Although it is unclear what fraction of leptin is free or bound in these circumstances, it is likely that the proportion of free leptin is increased thus resulting in a decreased half life of circulating leptin as compared non-hyperleptinemic adiposity-matched animals (32, 33).

Plasma leptin accesses the cerebrospinal fluid by active transport mediated at least partially by LepRa, LepRc, and LRP2 expression in median eminence tanycytes and ependymal cells of the choroid plexus (34–36). Upon leptin binding to LepRb or LepRa homodimers, leptin is internalized by clathrin coated vesicles (37). The percentage of leptin receptors at the cell surface at any given time is small; however in the presence of leptin at the cell surface, leptin receptors may cluster, resulting in enhanced ligand-receptor complexing and endocytosis of leptin (38–41). Phosphorylated STAT3 in response to intraperitoneal leptin injection (1–6 mg leptin/kg body weight) in non-obese, fasted mice can be observed within 30 minutes following injection (42–45). We found that 30 minutes following injection of recombinant mouse leptin (3mg/kg body weight) plasma leptin concentrations were 4,976-fold higher than endogenous fasted plasma leptin concentrations and 391-fold higher than endogenous re-fed plasma leptin concentrations (Figures 1, 2). The highest plasma leptin concentrations were measured at the first time point of blood collection, 15 minutes following injection, at which point plasma leptin concentrations of injected mice were 6,481-fold higher than endogenous fasted plasma leptin concentrations and 509-fold higher than endogenous fed plasma leptin concentrations (Figures 1, 2). Reduced leptin signaling through LepRb as well as decreased plasma concnetrations of soluble leptin receptor in obesity, may play roles in determining both the physical and biological half life of leptin in the obese state, which may differ from those of fasted, lean mice receiving exogenous leptin.

The results reported here have important implications for data obtained in the many studies intended to understand the systemic and neuro-molecular physiology of leptin. As we show, the widely applied doses of 2–4 mg/kg in mice clearly produce un-physiological elevations in circulating leptin, with consequences for leptin response pathways that could confound

inferences regarding the biology of leptin in body weight homeostasis, by altering leptin sensitivity.

For example, among the most pressing clinical questions regarding regulation of body weight are those relating to whether the apparent “set point” for body fat can be increased by environmentally-induced chronic weight gain (46); and whether the compensatory behavioral and metabolic responses to maintenance of reduced body fat (hence reduced circulating leptin) can be mitigated by the administration of “replacement” exogenous leptin (47–49). With regard to the former, experimental parsing of the relative contributions of elevated dietary fat - and the elevations of circulating leptin consequent to secondary gains in body fat – requires careful titration of administered leptin to levels consistent with those resulting from increments in fat mass (32, 50). For the latter, use of high doses of leptin in the induction of weight loss, or in the maintenance of reduced body weight, can change central leptin responses in ways that will confound interpretation of the efficacy of the interventions (51). In short, many of the relevant studies conducted to date have used doses of leptin that might obscure important aspects of clinical biology.

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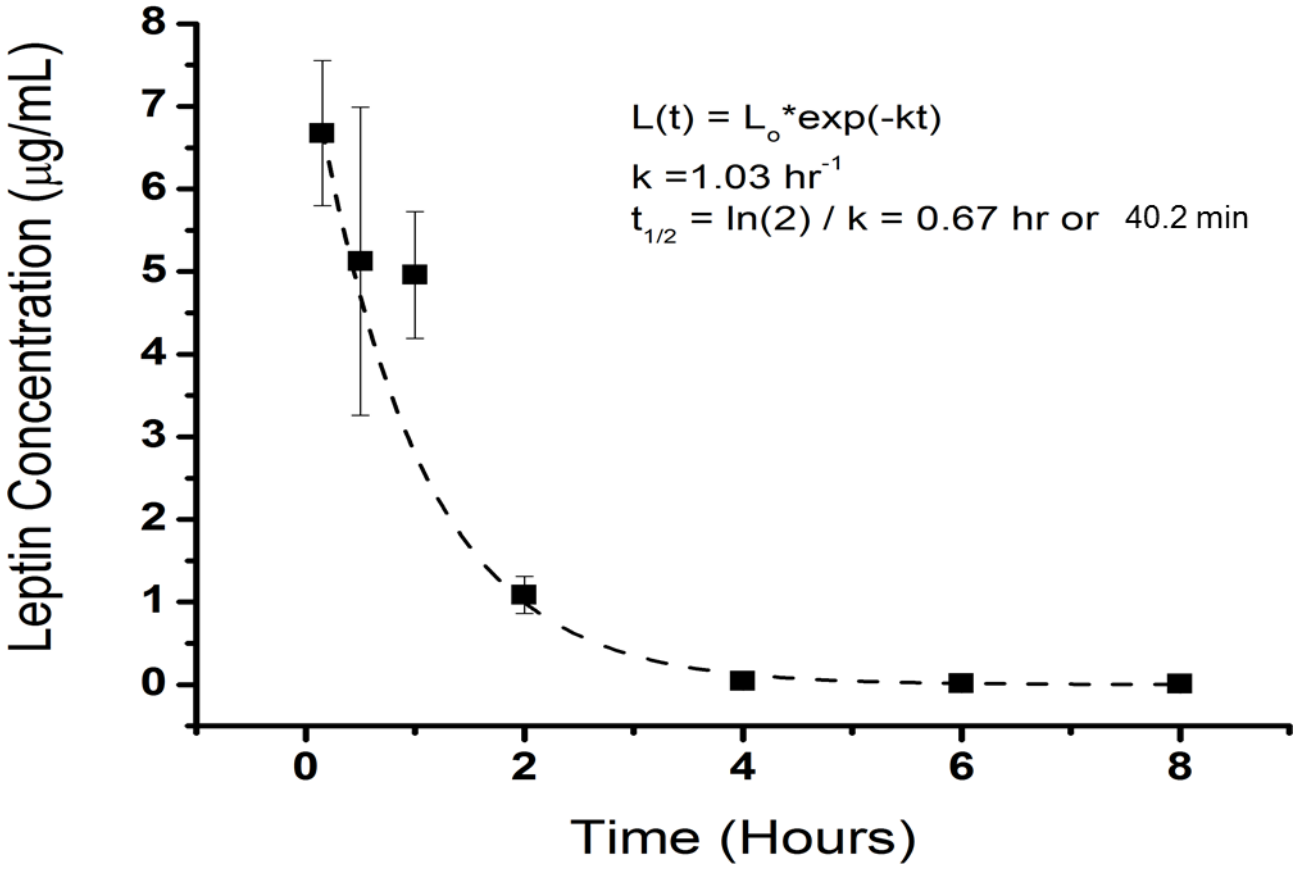

Figure 1. The half life of mouse recombinant leptin is (40.2 +/−2.2) minutes

The half life of leptin was estimated by modeling the data with a single exponential decay process. (n=5 WT, 10 week old male mice/group, error bars are SEM; fasted mice were administered recombinant leptin at 3 mg/kg body weight).

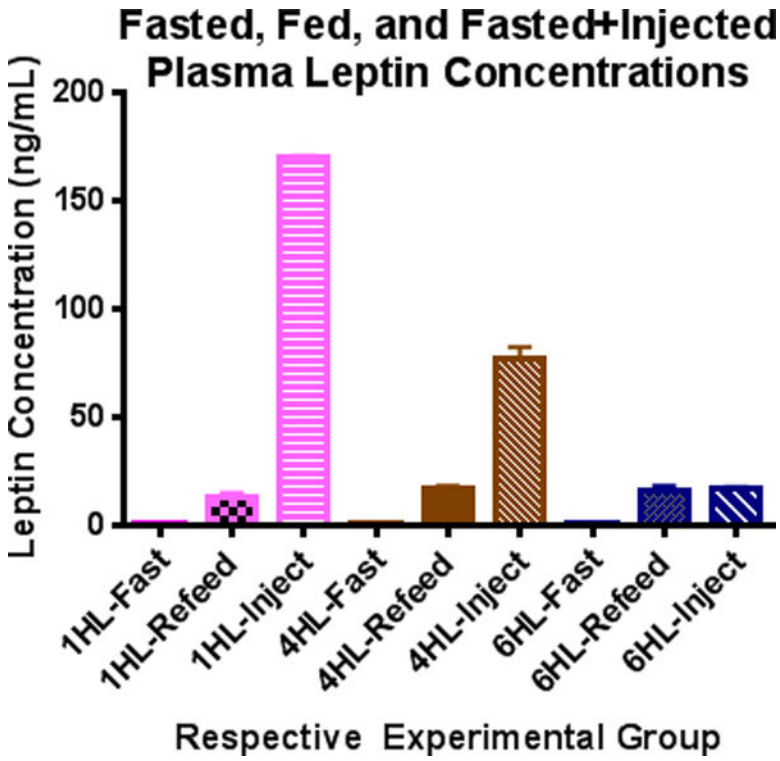

Figure 2. Commonly used recombinant leptin injection concentrations are supraphysiological

Plasma leptin concentrations were measured in the same three groups of wild type (WT) mice at fasting, refeeding (5 hours), and following recombinant mouse leptin injection into fasted animals. 1HL, 4HL, and 6HL refer to three separate groups of mice in which circulating leptin concentrations were measured 1, 4, or 6 hours following leptin injection. One hour following injection of recombinant mouse leptin to fasted mice (dose = 3 mg/kg body weight) plasma leptin concentrations were 170-fold higher than those at fasting and 13-fold higher than those at refeeding. By six hours following leptin injection, plasma leptin concentrations were not different from those at refeeding (n=5 WT, 10 week old male mice/ group, error bars are SEM).

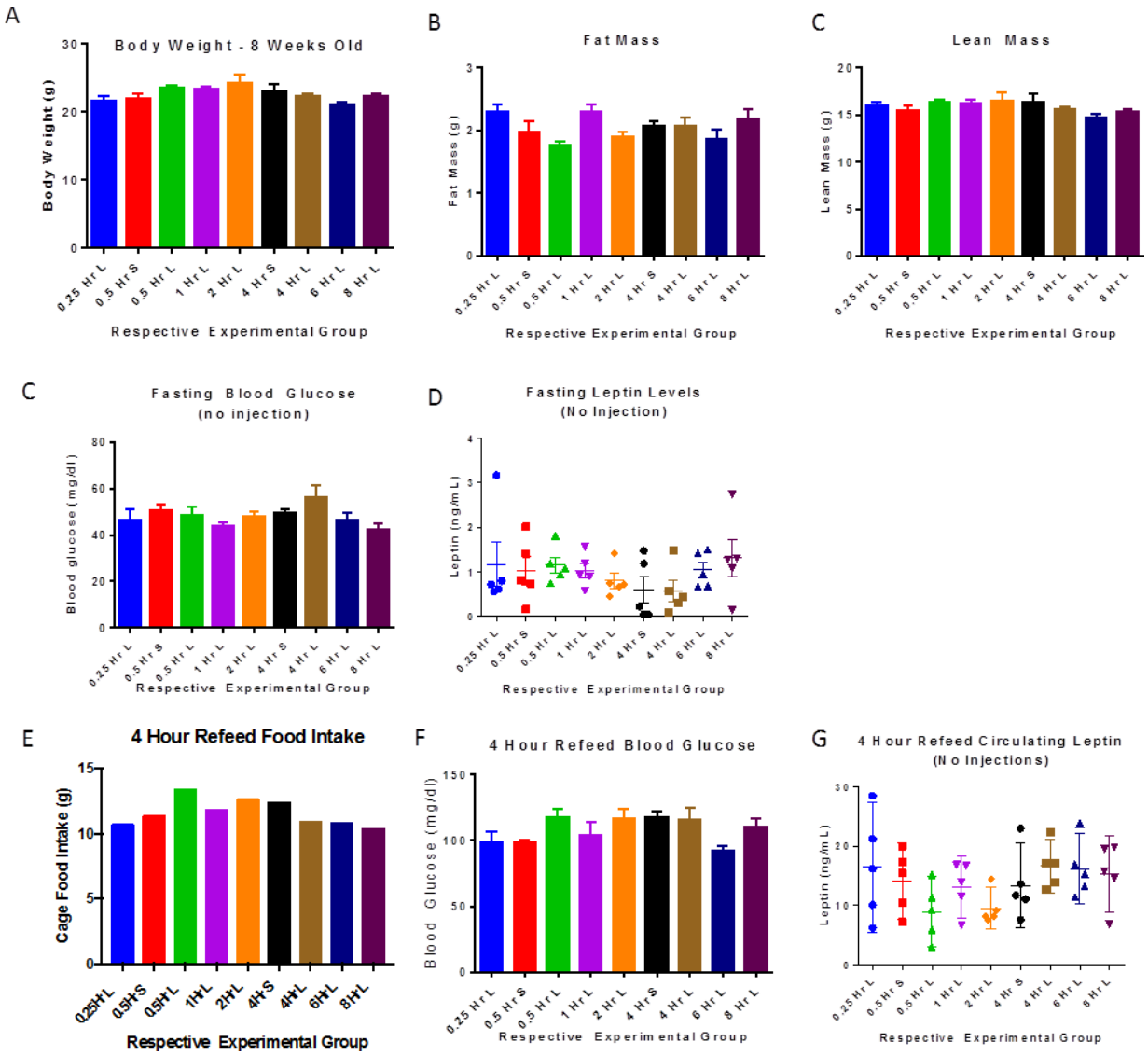

Figure 3. Body weight/compositions, blood glucose, food intake, and plasma leptin concentrations of wild type mice

There is no difference in body mass or composition measures, blood glucose levels, or leptin values for animals used in the study. Naming convention follows that described in Figure 2 and Methods, S=saline, L=leptin (n=5 WT 10 week old male mice/group, error bars are SEM; food intake measurement in panel E is of each cage containing 5 mice).