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Dissociation of leptin secretion and adiposity during prehibernatory fattening in little brown bats

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Kronfeld-Schor, Noga, Christopher Richardson, Brian A. Silvia, Thomas H. Kunz, and Eric P. Widmaier. Dissociation of leptin secretion and adiposity during prehibernatory fattening in little brown bats. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R1277–R1281, 2000.—Hibernating animals deposit adipose tissue before hibernation to withstand long periods of reduced energy intake. Normally, adiposity is positively correlated with increased secretion from adipose tissue of the satiety hormone, leptin. During the prehibernatory phase of the little brown bat, *Myotis lucifugus*, body mass and adiposity increased to a maximum within 12 days. Leptin secretion from adipose tissue in vitro and plasma leptin, however, increased before the increase in adiposity, then significantly decreased when adiposity increased. Basal metabolic rate (BMR) decreased when plasma leptin was increasing. This was followed by an increase in nonshivering thermogenic capacity and brown adipose tissue mass. We conclude that in the early prehibernatory phase, BMR decreases despite increasing plasma leptin levels, suggesting a state of relative leptin resistance at that time. At later stages, adiposity increases as BMR continues to decrease, and plasma leptin becomes dissociated from adiposity. Thus, in *M. lucifugus*, hibernation may be achieved partly by removing the metabolic signal of leptin during the fattening period of prehibernation.

white adipose tissue; basal metabolic rate; nonshivering thermogenesis; brown adipose tissue; prehibernation

HIBERNATION IS A CHIEF STRATEGY used by some mammals during periods of climatic challenges associated with reduced food availability. During hibernation, metabolism and body temperature are greatly reduced, as are basic behaviors such as eating and defecating. To survive extended periods without eating, energy must be stored as fat during a brief prehibernatory period, often leading to a doubling of body mass (2, 18, 21, 31).

To accumulate fat, it appears that the metabolic effects of the satiety hormone, leptin, must be circumvented. Leptin acts as a feedback controller of energy balance (1) by signaling the hypothalamus with information about body fat and energy balance. It is primarily produced and secreted by adipose cells, and its concentration in the circulation is normally correlated with body adiposity (4, 5, 19). Because adiposity is expected to increase before hibernation, it follows that circulating concentrations of leptin would also increase as a consequence. High circulating levels of leptin, however, should decrease food intake and increase energy expenditure, preventing the increase in body mass and adiposity. Thus it appears that prehibernatory animals somehow overcome the satiety and metabolic signals associated with leptin to deposit large fat stores.

Administration of recombinant murine leptin to captive Arctic ground squirrels (*Spermophilis parryii*) during prehibernation reduced food intake, body mass, and adiposity, but only after exposure to high concentrations of leptin for a period of weeks (22). This finding suggests that during prehibernatory fattening, the hypothalamus retains at least some sensitivity to leptin in this species. If true, one would predict that circulating levels of leptin must be suppressed during this time. Plasma levels of leptin and leptin secretion rates in free-ranging, prehibernatory mammals have yet to be quantified, however. We have determined the circulating profile of leptin and the rates of adipose tissue leptin secretion in vitro in a free-ranging hibernator, the little brown bat (*Myotis lucifugus*). The little brown bat is a small insectivorous species (7–11 g) with a range covering most of North America (7). In winter, this species forms large hibernating colonies in caves and mines. In New England, the bat begins to arrive at swarming sites in July and enters hibernation in early to mid-October (6, 15). Postlactational females begin arriving at the site in mid-late July (6, 15). During the intervening (prehibernatory) period between leaving maternity roosts and entering hibernation, bats continue to feed nightly and usually enter torpor during the day. Changes in leptin secretion have been compared with changes in body mass, adiposity, and metabolic rate to test the hypothesis that leptin and adiposity become dissociated during prehibernatory fattening in *M. lucifugus*.

METHODS

Study species. Prehibernatory bats were collected from a cave in southern Vermont at biweekly intervals from July 23

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through September 27, 1998. Adult female bats were captured at each date by using a harp trap (27), 2–3 h after dark on their return from the first nightly foraging. Animals were transported to Boston University in a simulated roost (17) without food.

Determination of basal metabolic rate. Basal metabolic rate (BMR) was measured from 1300 to 1600 on postabsorptive bats (*M. lucifugus* is generally postabsorptive by 1200, with a pronounced lull in metabolic activity between 1300 and 1700). The metabolic chambers were designed to simulate natural roost conditions (17). Each chamber was carved from a wooden beam taken from a barn and mounted inside the lid of a 1-gallon paint can with the opening to the chamber facing the bottom of the can. Perforated (to ensure adequate mixing of air inside the chamber) inlet and outlet tubes were inserted into the lid and secured with silicone sealant to create an airtight seal. Bats were allowed to crawl into the wooden chamber, and then a hardware cloth screen was placed over the opening to contain them within the chamber. An airtight seal was created in the can by fitting the lid with silicone lubricant.

Six bats, each in a separate chamber, were sequentially monitored for oxygen consumption for 5 min every 35 min. An empty metabolic chamber was also monitored; values obtained from this chamber were subtracted from experimental chambers. Oxygen consumption (ml/h) was measured at 30°C in a positive-pressure, open-circuit respirometry system, using calibrated upstream flowmeters [Brooks Sho-Rate; flow rate at 225–260 ml/min standard temperature and pressure, dry (STPD)] and an Amtek Applied Electrochemistry S-3A/I oxygen analyzer interfaced to an analog-to-digital converter and microcomputer (24). Oxygen concentration was measured and recorded by using Sable Systems data-acquisition program. The lowest value obtained during the 3-h testing period was considered the BMR.

Determination of regulatory nonshivering thermogenesis capacity. Regulatory nonshivering thermogenesis (NST) is the additional (nonshivering) heat production above BMR that occurs at ambient temperatures below the thermoneutral zone (13) and is derived primarily from metabolic activity of brown adipose tissue (BAT). Essentially all BAT in *M. lucifugus* occurs in the bilobed midscapular pads (12). An index of maximal NST (basal plus regulatory) is determined by measuring oxygen consumption before and after an injection of norepinephrine, an activator of BAT activity (3, 8). Animals used for determination of BMR were subsequently injected subcutaneously in the midscapular region with 7 μg/g of freshly prepared norepinephrine [(-)-norepinephrine bitartrate salt (Sigma) in 0.9% saline]. This dose was chosen on the basis of previously published response curves (24). NST trials were conducted at 20°C to minimize risk of hyperthermia. Preliminary observations confirmed that rectal temperature did not exceed 37°C before and after NST trials. Oxygen consumption was determined as for BMR but with a flow rate of 710–720 ml/min (STPD), because of the increased oxygen consumption that occurs during NST. The potential contribution of locomotor activity to oxygen consumption, based on behavioral observations, is likely to be low. At the conclusion of the experiment, the animals were killed by decapitation and the interscapular brown fat pads were exposed and removed. One pad was frozen for future biochemical analyses; the other was weighed to determine BAT mass.

Determination of leptin secretion in vitro. Bats from each collection date were decapitated, and trunk blood was collected and processed for RIA of leptin. Subcutaneous fat was dissected, weighed, minced, and washed to remove residual extracellular fluid. Adipose tissue (~50 mg wet wt/tube) from each animal was aliquoted into glass test tubes in 1 ml serum-free Krebs buffer supplemented with 40 g/l BSA. Adipose tissue was incubated for 120 min at 37°C in a humidified O_2-CO_2 chamber with gentle shaking. The infranatants were collected, dehydrated with a Speed-Vac, and frozen for future RIA. Adipose tissue from each tube was collected and dried at 60°C for 3 days to obtain dry fat mass for normalizing secretion data.

Leptin RIA. Leptin concentrations in plasma and medium were quantified by using a commercial RIA kit (Linco) for human leptin. Dried media samples were first reconstituted to 10% original volume with assay buffer. In our experience, chiropteran peptide hormones, including leptin, exhibit greatest cross-reactivity with antibodies generated against homologous human peptides than for peptides of other species (16, 28–30), which may reflect the taxonomic relationship between bats and primates. Authentic human leptin and leptin-like immunoreactivity in bat plasma diluted in parallel in the human leptin RIA, and recovery of exogenous human leptin in bat serum, was ~100%. The intra- and interassay coefficients of variation were ~12 and 13–15%, respectively.

Determination of fat content. Carcasses were subjected to destructive analysis for body composition. Carcasses were minced and desiccated in a drying oven at 60°C until constant mass was achieved. Body fat was extracted from the desiccated subjects by using a Soxhlet apparatus with petroleum-ether and ethanol (1:3) as organic solvent (23). Each sample was fluxed with the organic solvent for ~12 cycles (45 min each) per day, for 2 days. The samples were bathed in fresh organic solvent between extraction days. The samples were dried again for at least 24 h at 60°C after extraction and reweighed to generate a value for lean dry mass. Fat mass was considered the difference between dry mass (including the dry mass of the adipose tissue removed for analysis of in vitro leptin secretion) and lean dry mass. Stomachs were assumed to be void of food because the bats were held overnight in captivity without food. Thus stomach contents were not removed before extraction.

Data were analyzed by analysis of variance followed by Neuman-Keuls or Bonferroni's post hoc tests for individual differences, using a criterion of $P < 0.05$.

RESULTS

Body mass significantly increased by 38% in the 2 wk between mid- and late August (Fig. 1). A similar pattern was observed for body adiposity, which nearly

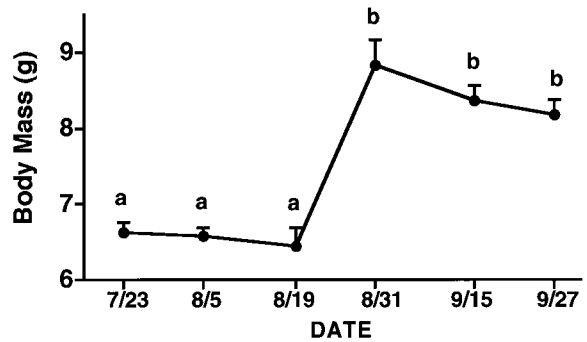

Fig. 1. Body mass of *Myotis lucifugus* during the prehibernatory period. Each point represents the mean and SE of 12–18 animals. Numbers on the *x*-axis in this and subsequent figures represent the date of capture and are scaled according to time. Values with identical letters (*a*, *b*) are not statistically different from each other.

tripled during the same interval (Fig. 2). Plasma leptin significantly increased before the increase in body mass and adiposity. Subsequently, plasma leptin decreased to levels below those found in animals that arrived at the swarming site, despite the sustained increase in mass and adiposity (Fig. 3). Leptin secretion rate from fat in vitro increased around the time that plasma leptin increased and then significantly decreased during the later prehibernatory period (Fig. 4).

BMR decreased during the entire period of the study, before any detectable change in body mass or adiposity (Fig. 5). Regulatory NST capacity (maximal NST minus BMR) was significantly ($P < 0.03$) increased between August 19 and September 27 (Fig. 6), and BAT mass was significantly increased from August 31 onward (Fig. 7).

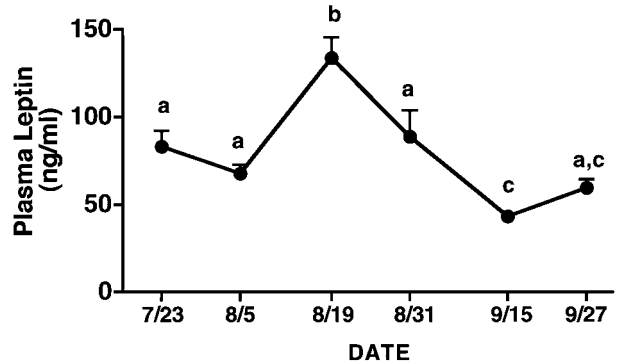

Fig. 3. Plasma leptin in *M. lucifugus* during the prehibernatory period. Trunk blood was obtained at the time of death, after overnight fasting, and processed for leptin radioimmunoassay. Each point is the mean and SE of 10–12 animals. Values with identical letters (*a*, *b*) are not significantly different from each other.

DISCUSSION

The regulatory controls that determine seasonal adiposity in mammals are presently unclear. Leptin is a hormone produced by adipocytes, which signal the hypothalamus to suppress appetite and accelerate metabolism. In the absence of leptin, animals overeat, reduce their metabolic rates, and consequently gain fat mass. We tested the hypothesis that for prehibernatory fattening to occur, plasma levels of leptin should decrease. Alternatively, sensitivity of target organs to leptin should decrease. The results of this study suggest that both phenomena may occur in a common hibernating species, *M. lucifugus*.

Changes in total body mass in *M. lucifugus* during the postmigratory, prehibernatory period were consistent with the pattern reported by Kunz et al. (18). The rapid and significant increase in adiposity and body mass in late August represents a period of marked increase in fat reserves between the energy-demanding periods of migration and mating, when the majority of sustained autumn fattening occurs (18).

Interestingly, both plasma leptin and leptin secretion from adipose tissue in vitro increased before the increase in adiposity. Because secretion of leptin is normally highly correlated with adiposity in mammals (e.g., 4, 5, 19), this observation suggests that leptin and adiposity are dissociated in the early prehibernatory period. This hypothesis is supported by our observations that both plasma leptin and leptin secretion decreased during the later prehibernatory period, when adiposity was maximal.

The dissociation between leptin secretion and adiposity raises several questions. For example, the initial increase in leptin secretion should lead to a decrease in feeding, an increase in BMR, and a decrease in body mass. We do not have a quantitative measure of feeding in free-ranging or captive *M. lucifugus* during the prehibernatory period, but body mass was stable early in this period and then increased, whereas BMR decreased. This suggests that a state of relative leptin resistance may develop early in the prehibernatory period, although we have no direct data at this time to support this hypothesis. Although the mechanism of this putative resistance, if it exists, is unknown, it is possible that plasma leptin may be sequestered by circulating leptin binding-proteins, as appears to be the case in mice during the hyperleptinemic period of pregnancy (9). Whether such binding proteins exist in bats is presently unknown. Notwithstanding, a period

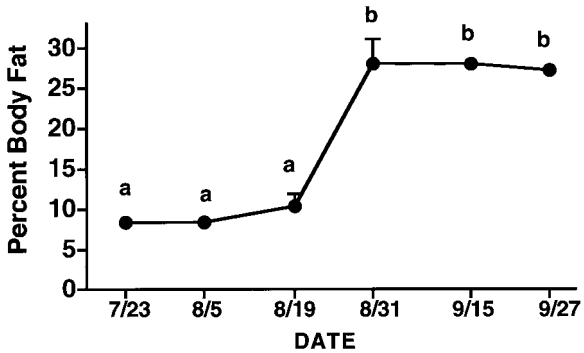

Fig. 2. Changes in adiposity in *M. lucifugus* during the prehibernatory period. Each point represents the mean and SE of 12 (7/23), 18 (8/5), 17 (8/19), and 6 (all other dates) animals. At some dates, carcasses of the animals whose data are represented in Fig. 1 were not available for fat composition analysis. Values with identical letters (*a*, *b*) are not statistically different from each other.

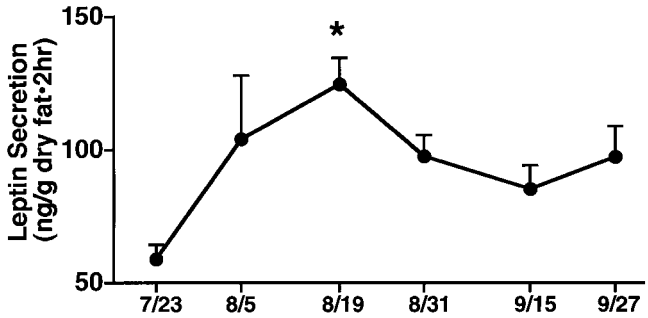

Fig. 4. Secretion of leptin from isolated adipose tissue in vitro. Each point is the mean and SE of 9–10 animals. Adipose tissue was incubated for 2 h in buffer at 37°C. The medium was collected and processed for leptin radioimmunoassay. The fat was collected, dried, and weighed to normalize leptin secretion. *Significantly different from all other dates, $P < 0.05$.

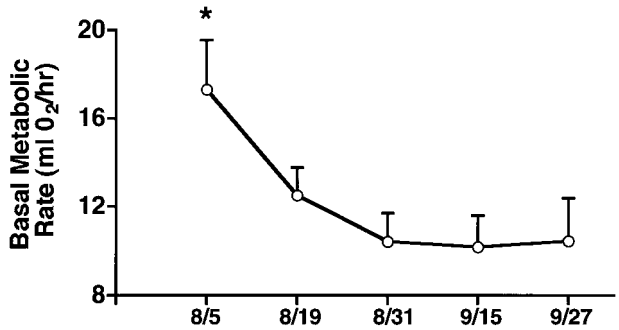

Fig. 5. Basal metabolic rate in *M. lucifugus* during the prehibernatory period. Each point is the mean and SE of 6 animals. These animals were not used for determination of leptin secretion or plasma leptin but are included in the data in Figs. 1 and 2. Note that the first data point is at 8/5, not 7/23. * Significantly different from all other dates, $P < 0.05$.

of relative leptin insensitivity should remove an inhibitory signal from hypothalamic orexigenic centers, thus permitting fat deposition to occur despite increasing leptin levels. In this context, Ormseth et al. (22) demonstrated that prehibernatory Arctic ground squirrels reduce their food intake and body mass when chronically infused with leptin, suggesting that in this species leptin sensitivity is retained during this time. However, in ground squirrels, the effects of leptin required weeks and occurred at what may have been pharmacological plasma concentrations. Thus prehibernatory Arctic ground squirrels may, in fact, be partially resistant to physiological levels of leptin.

The progressive decrease in BMR over the prehibernatory period is consistent with deposition of fat reserves in *M. lucifugus* (18; this study). The decrease in plasma leptin (after an initial increase) to levels at or below those at the onset of the prehibernatory period is also consistent with these results. This suggests a second mechanism for maintaining fat mass, once it is achieved, before hibernation. Downregulation of leptin secretion would be expected to sustain appetite and maintain a reduced BMR. However, the signals responsible for downregulation of leptin secretion are presently unknown. One possibility is a reduction in gonadal or adrenal steroid hormones, both of which are known to upregulate leptin secretion in other mammals (e.g., 14, 20, 26). The annual profile of plasma estradiol concentrations in *M. lucifugus* is unknown, however, but circulating cortisol concentrations in *M. lucifugus* decrease in August (10).

Like other hibernators, bats rely on white fat and BAT and nonshivering thermogenesis to provide heat needed for arousals during and at the end of hibernation. BAT-mediated NST is a major source of thermogenesis during arousal in the big brown bat (12) and *M. lucifugus* (11, 12). The present results document that NST capacity increases during the prehibernatory period when adiposity increases. In addition, BAT mass increases during the same interval. Although not surprising, these results confirm that during the prehibernatory period in *M. lucifugus*, energy in the form of white adipose tissue is deposited at a time when BAT mass and potential NST activity are increased. Moreover, these changes are temporally coordinated with a steady decrease in metabolic rate. In light of the dissociation between plasma leptin and adiposity, it is unknown what role, if any, leptin may play in prehibernatory BAT accumulation in *Myotis lucifugus*. Nonetheless, leptin has been reported to stimulate BAT activity in other species (25), and thus we cannot rule out the possibility that leptin may also modulate seasonal BAT activity in hibernators.

In summary, these results suggest that seasonal regulation of circulating leptin concentrations and, possibly, leptin sensitivity is closely linked with annual changes in adiposity that precede hibernation in *M. lucifugus*. Seasonal changes in body mass and adiposity in prehibernatory animals are of interest not only from an ecological and evolutionary point of view, but also as a unique model of reversible regulation of body mass, adiposity, and metabolism in a free-ranging animal. Further investigation of the mechanisms of downregulation of leptin secretion and the possibility

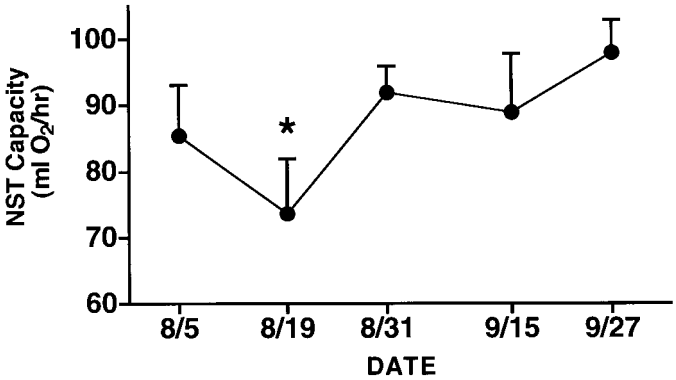

Fig. 6. Nonshivering thermogenic (NST) capacity in *M. lucifugus*. Each point is the mean and SE of 6 animals (the same individuals used for basal metabolic rate in Fig. 5). Note that the first data point is at 8/5. Values represent regulatory NST (maximal oxygen consumption after norepinephrine minus basal metabolic rate in the same individuals). *$P < 0.03$ vs. 9/27 by unpaired *t*-test.

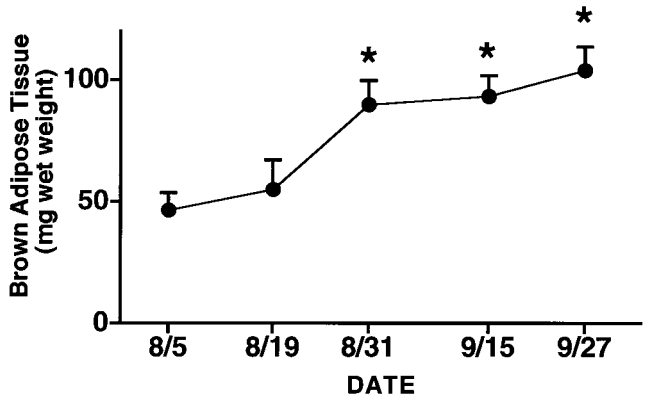

Fig. 7. Brown adipose tissue mass in *M. lucifugus*. An entire brown fat pad was removed from the interscapular region of 6 animals at each date (mean and SE, $n = 6$). The results represent wet weights of the single pad (the other pad was immediately frozen for future molecular analyses). *$P < 0.05$ vs. 8/5.

of reversible leptin sensitivity may have broader implications for pathological disorders involving dysregulation of adiposity in other animals, including humans.

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