Seasonal changes in serum leptin, food intake, and body weight in photoentrained woodchucks

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Concannon, P., K. Levac, R. Rawson, B. Tennant, and A. Bensadoun. Seasonal changes in serum leptin, food intake, and body weight in photoentrained woodchucks. Am J Physiol Regulatory Integrative Comp Physiol 281: R951–R959, 2001.—Male woodchucks (Marmota monax) were maintained in northern vs. southern hemisphere photoperiods, provided feed and water ad libitum, and evaluated every 2 wk for 23 mo for body weight, absolute and relative food intake, body temperature, serum testosterone, and serum concentrations of leptin measured using an anti-mouse leptin enzyme-linked immunosassay. During late spring and summer, body weight increased 56 ± 4% above winter nadirs, and during the autumn and early winter weights decreased 27 to 45% below midsummer maxima. Serum leptin initially increased during increases in body weight, in the late spring, reached peak values (490 ± 32 pg/ml) in summer during the initial decline in body weight, and later decreased along with body weight to reach basal values (20 ± 5 pg/ml) in late winter. Spontaneous declines in food intake in summer began 2–6 wk before resulting declines in body weight and occurred during increases in leptin > 100 pg/ml. The rate of decline in food intake was greatest when serum leptin was at or near peak values. Food intake increased in late winter when leptin was low and 7–10 wk before resulting increases in body weight. Testis recrudescence occurred when leptin was declining to near basal levels. The results suggest that leptin is involved in the hormonal regulation of the circannual cycle in the drive for voluntary food intake in this species.

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In the arctic ground squirrel, another marmotine species of sciurid rodent that also hibernates, administration of recombinant leptin has been observed to inhibit seasonal hyperphagia and reduce body weight (36) without affecting energy expenditure (5). The objective of the present study in woodchucks was to determine whether the anorectic hormone leptin is present in detectable amounts in woodchucks and whether there are seasonal changes in leptin that might suggest that leptin participates in the physiological regulation of food intake in this species.

METHODS

Animals. Two groups of five to six adult male woodchucks each were studied beginning at 2.4–3.6 yr of age. All woodchucks were born in the laboratory of animals that had been previously entrained to either northern hemisphere (boreal, \( n = 5 \)) or southern hemisphere (austral, \( n = 6 \)) photoperiods (14) and were maintained in their original photoperiods. Photoperiods continued to simulate those of 42°N and 42°S, through 25–30°C filter paper. Ni-NTA resin (Qiagen) was equilibrated with 5 column volumes of loading buffer (3 M guanidine hydrochloride, 0.1 M NaH₂PO₄, and 0.01 M Tris-HCl, pH 8.0) and centrifuged at 9,500 rpm for 15 min. The supernatant was diluted 1:2 with 0.1 M NaH₂PO₄ and 0.01 M Tris-HCl, pH 8.0, and reconstituted as above. The supernatant was decanted and filtered through 25-μm filter paper. Ni-NTA resin (Qiagen) was equilibrated with 5 column volumes of loading buffer (3 M guanidine hydrochloride, 0.1 M NaH₂PO₄, and 0.01 M Tris-HCl, pH 8.0). The cell lysate was loaded onto the column at 1 ml/min. The column was washed at 1 ml/min until the optical density at 280 nm was stable (5–10 column volumes) with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH 8.0). The 6-His-leptin was eluted at a flow of 0.5 ml/min with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole, pH 8.0). Fractions were screened for the presence of the eluted 6-His-leptin by SDS-PAGE (17.5% acrylamide). Positive fractions were pooled, concentrated, and applied to 17.5% SDS-PAGE preparative gels. The 6-His-leptin was electroeluted from the gels into 0.3 M Tris and 0.2 M 3-(cyclohexylamino)-1-propane sulfonic acid, pH 9.4, using a Bio-Rad (Hercules, CA) Whole Gel Eluter (200 mA, 35 min). Eluter fractions were screened by 17.5% SDS-PAGE, positive fractions were pooled, protein concentration was determined by a modified Lowry method (3), and the highly purified leptin (>95%) was then concentrated, typically using an Amicon stirred cell and Amicon PM10 DIAFLO_Ultrafiltration membrane (Millipore, Bedford, MA).

Polyclonal antibody production and purification. Antibodies were raised in a rabbit against purified recombinant 6-His-leptin. The rabbit was first immunized with ~150 μg leptin protein in complete Freund’s adjuvant (GIBCO-BRL, Rockville, MD), injected subcutaneously. Further injections began 5 mo later with 3 injections of ~500 μg protein each, followed by 13 injections of ~300 μg each, all delivered subcutaneously in incomplete Freund’s adjuvant, at intervals of 3–4 wk. Blood was collected from the medial ear artery in the presence of 4 mM EDTA at intervals of 6–8 wk. After centrifugation, the immune plasma was stored at ~20°C until affinity purification on a leptin-affinity column (AffiPrep 10, Bio-Rad) prepared according to manufacturer’s instructions.

Measurement of serum leptin. Leptin protein was measured by ELISA in 96-well microtiter plates (Costar, Corning, NY) coated with 0.5 μg/well polyclonal anti-leptin antibody.
Antibodies were diluted to the appropriate concentration in coating buffer (15 mM Na2CO3, 35 mM NaHCO3, and 0.02% sodium azide, pH 9.6) and dispensed at 200 μl per well. Plates were incubated at 4°C at least overnight. Plates were washed three times with PBS-Tween in a plate washer (Dynex Technologies, Chantilly, VA) and blocked with 300 μl 1% BSA-PBS-Tween (15.5 mM Na2HPO4·H2O, 17.4 mM NaH2PO4·H2O, 150 mM NaCl, 0.05% Tween 20, and 1% ELISA-grade BSA; Sigma Chemical, St. Louis, MO) for 2–4 h at 37°C in a humidified environment. Blocking buffer was removed, and standards and samples were added in 200-μl volumes. The standard was highly purified recombinant 6-His-leptin, which was stored in 1% BSA-PBS-Tween in small single-use volumes at ~80°C. The standard curve ranged from 0.005 to 0.20 ng leptin (25–1,000 pg/ml) per well, and the leptin was added to the wells in 1% BSA-PBS-Tween. Woodchuck serum samples were centrifuged to remove insoluble material, and Tween 20 was added to a final concentration of 0.05%. All ELISA assays employed 200 μl serum per well, and each sample was assayed in duplicate. Plates were incubated overnight at 4°C. Plates were washed six times with PBS-Tween, and biotinylated anti-leptin antibody was added at 200 μl per well (1:20,000 dilution of an −3 mg/ml stock), diluted in 1% BSA-PBS-Tween. Plates were incubated overnight at 4°C. Plates were washed six times with PBS-Tween, and horseradish peroxidase-conjugated streptavidin was added in 200 μl volumes (1:3,000 dilution of 1 mg/ml stock), diluted in 1% BSA-PBS-Tween. Plates were incubated for 2 h at 37°C in a humidified environment. Plates were washed 6 times with PBS-Tween and then developed by the timed addition of 200 μl substrate buffer (6.0 ml 0.1 M citric acid, 6.425 ml 0.2 M Na2HPO4, 12.5 ml H2O, 10 mg o-phenylenediamine, and 10 μl 30% H2O2). Development was stopped after 15–20 min by the timed addition of 50 μl 2.5 M H2SO4. The plate was read at 490 nm and the results quantified using Revelation software (plate reader and software from Dynex Technologies).

Optical densities significantly different from those of background were typically associated with 0.0020–0.0050 ng leptin (10–25 pg/ml). The coefficients of variation in assays averaged 4%. The coefficient of variation between assays averaged 13%. All samples from any one animal were run in the same two plates of an assay “run,” and samples from one or more animals in both groups were assayed at the same time. All samples were assayed at the single volume of 200 μl per well to ensure that any sample volume effect on the assay did not affect detection of accurate changes within animals. Assay development and evaluation involved assay and analysis of serial dilutions of woodchuck serum samples. The concentration of leptin in woodchuck serum was very low, with peak values of ~600 ng/ml, as determined by assaying 200 μl of undiluted serum in the ELISA. Characterization of the ELISA signal from a range of different volumes revealed a significant and consistent serum effect. Specifically, increasing the volume of serum added to the well decreased the apparent concentration. However, to reach the limit of detection in serum samples with low concentrations of leptin, it was necessary to assay a 200-μl volume. To eliminate the serum effect as a variable, all serum samples were therefore assayed using only volumes of 200 μl. This resulted in an underestimation of the absolute concentration of woodchuck serum leptin, but the relative differences within and between animals are accurately reflected. Analysis of the serum titration curve indicated that absolute values are underestimated by a factor of two (data not shown). Hence, all reported values can be multiplied by this correction factor to obtain an estimate of the true leptin concentration. The magnitude of the correction factor was found to be identical using samples of rat serum, indicating that the serum effect is not specific to woodchuck serum (data not shown).

**RESULTS**

Changes in body weight and in absolute and relative food intake (Fig. 1) were similar in magnitude and timing to those reported for other woodchucks maintained in similar photoperiods (14), with changes in austral animals occurring ~6 mo earlier than in boreal animals. Nadir body weights ranged from 2.6 to 3.5 kg and averaged 3.1 ± 0.1 kg. This represented declines of 27–43% (35 ± 2%) from the previous maxima. Peak body weight ranged from 4.3 to 5.6 kg and averaged 4.8 ± 0.1 kg. This represented weight gains of 37–76% (56 ± 4%) above the previous nadir. Mean body weights in both groups were the same twice a year, at or shortly after the equinoxes (Fig. 1), and were not different at the equinoxes (P > 0.14–0.25). Absolute food intake ranged from nadirs of 6 ± 1 g/day to peaks of 208 ± 6 g/day. In many instances, estimated food intake was negligible, because apparent intake could be accounted for on the basis of dehydration of food and because measured intake was, therefore, no different from no intake based on the method used. Relative food intake ranged from nadirs of 1 ± 1 g·kg−1·day−1 to peaks of 49 ± 9 g·kg−1·day−1. Body weights were at nadir in early winter, increased slowly in late winter, increased rapidly in early or midspring, reached peak values in early or midsummer, and then declined throughout the autumn. A winter period of very negative energy balance was confirmed in both groups, in that mean food intake decreased 20% or more below peak values 3–5 wk before the decrease in mean body weight.

Changes in serum testosterone (Fig. 1) and testis size (data not shown) were typical of those observed in laboratory woodchucks. Testosterone increased in the winter, reached peak values in late winter, and then declined to reach near nadir values in midsummer (Fig. 1). Rectal temperature profiles demonstrated seasonal declines in body temperature. Nadir temperature in individual animals ranged from 20 to 34°C, averaged 26.8 ± 6.7°C, and typically occurred in autumn in both groups (Fig. 1).

Serum leptin was lowest in late winter and early spring. Leptin was nondetectable for 1–8 wk in six animals (4 boreal, 2 austral), in 1 yr or more, and
ranged from 20 to 50 pg/ml during the seasonal nadir in the remaining five animals. Nadirs, with nondetectable leptin assigned a value of 10 pg/ml, averaged 21 ± 5 pg/ml. Serum leptin increased two- to fivefold to near or above 100 pg/ml in late spring and reached 100 pg/ml while body weight was increasing to 11–62% (27 ± 4%) above nadir. Leptin concentrations in individual males rose above 100 pg/ml at 2–12 wk (6.5 ± 1.4 wk) before food intake began to decline and at 0–4 wk before relative food intake began to decline. The largest declines in absolute food intake, measured as the midpoints of the steepest rates of change, typically occurred when serum leptin was approaching peak values (Fig. 2). The largest decline in mean absolute food intake in both groups, in each season studied, occurred when mean leptin was at or near peak value (Fig. 1). The date of the greatest decline in food intake in individual animals occurred an average of 37 ± 4 days before \( (P < 0.05) \) the peak in serum leptin.

Leptin typically increased rapidly to near-peak concentrations with little or no further increase in body weight in late spring or summer. Peak leptin concentrations in individual males ranged from 290 to 675 pg/ml, averaged 490 ± 32 pg/ml, and occurred in middle to late summer, at August 28 ± 3 days in boreal males and February 2 ± 4 days in austral males. Serum leptin increased to peak concentrations during the initial declines in body weight, and the mean times of leptin peaks were later \( (P, 0.05) \) than those of peaks in body weight. Peak leptin concentrations occurred 2–7 wk after the peaks in body weight for 12 of 14 seasonal excursions in leptin with no missing data points during the months before and months after the peak. When data for all animals were aligned to a common time of peak leptin, the peak in mean leptin occurred 4 wk after the peak in mean body weight and while body weight was decreasing (Fig. 2).
Leptin continued to decline in the autumn and early winter (Fig. 1). Spontaneous increases in food intake typically became apparent and were considered to be obvious as food intake increased from near nadir amounts of 5–30 g/day to progressively higher amounts of 20–110 g/day within a 2-wk period. These increases in food intake occurred as serum leptin declined to near-nadir values of 20–105 pg/ml in late autumn or early winter. Absolute food intake increased most rapidly in the winter while serum leptin was at nadir values.

DISCUSSION

The present results provide the first detailed description in any species of the pattern and extent of circannual changes in serum leptin concentrations in relation to large spontaneous seasonal changes in food intake and body weight. The changes in body weight, food intake, and serum testosterone were similar to those observed in other woodchucks maintained in similar photoperiods (11, 13, 15). The finding that the changes in austral-photoperiod animals were phase shifted 6 mo from those of the boreal animals was in agreement with the fact that they were born 6 mo earlier to animals previously entrained to the austral photoperiod. It also confirms the ability of daily changes in photoperiod to entrain the woodchuck circannual cycle in the absence of changes in food availability or temperature (11, 13). The observed timing of some changes associated with these photoentrained endogenous cycles probably differs to some extent from what occurs in the wild (11, 13). The late-winter increase in temperature and rise in serum testosterone would likely occur ~6 wk later in the wild (13). In the wild, food is not available during hibernation, and the extent of hypothermia is far greater, and thus emergence out of the hibernation period is slower and later in the wild than in laboratory woodchucks kept at room temperature.
The observed 37–76% increases in body weights are assumed to be almost entirely due to increases in adipose tissue (18). The factors regulating white adipose tissue seasonal changes in lipogenesis and lipolysis in these and other hibernating rodent species are not known, but the timing of seasonal changes in the circulating concentrations of free thyroxine and prolactin has suggested that those hormones might be involved (11).

The extent and pattern of changes in body temperature in laboratory-maintained woodchucks have not been previously reported. Autumnal declines in temperature occurred at a time when woodchucks in the wild would be entering hibernation. The occurrence in individual animals of temperatures as low as 20°C and often only 1–3°C above ambient temperature is to some extent similar to the situation during normal or laboratory-induced hibernation, in which rectal temperature is a few degrees above the low temperature of the hibernaculum during bouts of torpor (29). Presumably, the more moderate depths of physiological hypothermia in the present animals involve the same hypothalamic mechanisms that allow more complete hibernation to occur. These results therefore suggest that the signal for and the process of initiating hibernation in woodchucks are not dependent on a change in ambient temperature, or on removal of food, as previously suggested (28). Rather, seasonal torpor in woodchucks, as in some other species of hibernating rodents, is obligatory and not facultative (19).

The observation that serum leptin increased after increases in body weight and eventually decreased after decreases in body weight was not unexpected. Leptin has been reported in several species to be produced primarily, although not exclusively, by white adipose tissue and to be present in serum in concentrations relative to body weight or body fat content (25, 31, 44).

The assay used in the present study was able to demonstrate physiological changes in serum leptin in woodchucks. The concentrations of leptin in woodchuck serum in the present study were low compared with those reported in mice (45) but were similar to or only slightly less than those reported for rats (8, 40). Leptin concentrations increased severalfold to ~100 pg/ml before spontaneous seasonal decreases in absolute as well as relative food intake were evident, and leptin was at the highest concentrations when the rate of decline in food intake was maximal. Those observations suggest that leptin may play a role in the regulation of appetite and spontaneous food intake in woodchucks, just as it does in other species (8, 25). Such a role is also suggested by the fact that leptin administration suppresses food intake in another hibernating species of marmotine, sciurrid rodent, the arctic ground squirrel (36). It is not known whether or not the hypothalamic drive for food intake in woodchucks is equally sensitive to leptin effects throughout the year or whether there are seasonal changes in receptors for, or sensitivity to, leptin. However, studies in the Djungarian hamster suggest that there is a seasonal modulation in the sensitivity to the anorectic effects of leptin in that species (26). Although the stimulus for spontaneous decreases in food intake during the summer may involve the observed rise in serum leptin concentrations, the fact that thyroid hormone and prolactin are concomitantly decreased from previously high concentrations may also be involved, because these other hormones can have anorectic effects when elevated in other species, as previously reviewed (11).

Absolute and relative food intakes were calculated from a series of daily measurements of food weights every 2 wk. Aspects of feeding behavior such as meal frequency and meal size were not measured in this study, and whether they might also change in relation to seasonal changes in leptin is not known. Water
intake was also not measured in this study. However, preliminary observations on other similarly housed woodchucks suggest that the pattern of seasonal changes in water intake is similar to that for food intake (P. Concannon, unpublished observation).

The small increase in food intake in late autumn and early winter may be related to the fact that leptin concentrations were reduced to very low levels. However, such increases in food intake would not occur under natural conditions at this time, during the hibernation period, because in the wild the extent of torpor is far deeper than in laboratory woodchucks maintained at room temperature and because woodchucks do not store food caches. Leptin levels were at nadir when food intake increased rapidly in late winter, and the decline in leptin to very low concentrations may be important in promoting that food intake. However, in late winter there are rapid increases in serum concentrations of prolactin and free thyroxine that have been suggested as stimuli for the concurrent increases in food intake (11).

Although the changes in serum leptin showed an obvious relationship to body weight, there were further increases in serum leptin after the attainment of peak body weight and during the initial decline in weight. Such continued increases in leptin unrelated to body weight suggest that the extent of leptin secretion may be regulated by factors other than total adipocyte fat mass. Alternatively, there may be a delay of up to several weeks between the attainment of maximum cell lipid content and the increased leptin synthesis that is the direct result of that final increase in adipocyte fat content. Factors reported to affect leptin synthesis and/or secretion by adipocytes include corticosteroids and insulin, which are both stimulatory (6). Catecholamines, drugs that activate peroxisome proliferator-activated receptor-γ, and β-adrenergic agonists are inhibitory (21). Thyroid hormone also appears to be potentially inhibitory. In humans, serum leptin concentrations have been reported to be low in hyperthyroid patients and high in hypothyroid patients, and treatment of hypothyroid patients with thyroxine can decrease a decrease in leptin (37). In rats, both thyroxine and triiodothyronine cause a decrease in serum leptin and in the ratio of leptin to body weight (22). These apparent negative effects of thyroid hormone on leptin expression may be indirect via effects on lipolysis or other pathways but may also involve a direct effect, because triiodothyronine but not thyroxine stimulated leptin expression and secretion by 3T3-L1 adipocytes in vitro (47). If thyroid hormone has an inhibitory effect on leptin production in woodchucks, then the observation that the greatest increase in serum leptin concentration occurs when serum concentrations of total thyroxine, total triiodothyronine, and free thyroxine as well as prolactin all decline to near nadir levels in the late spring and summer (11) may be important in this regard. The potential for the concomitant decline in prolactin to be involved in the increased secretion of leptin can also be considered. States of reduced dopaminergic tone or hyperprolactinemia have been associated with an increase in body weight or a reduction in serum leptin, both in humans (23) and in lactating rats (7).

Gonadal regression and the decline in testosterone occurred throughout the period of increasing serum leptin. It is possible, then, that the seasonal decline in androgen played a role in the timing or extent of the seasonal increase in leptin. In rats, androgen can reduce in vitro leptin expression and castration can increase leptin expression in perirenal fat (30). Conversely, it is possible that the increase in leptin plays a role in the timing or rate of testis regression and decline in testosterone secretion, because leptin has been reported to inhibit testosterone secretion by the adult rat testis in vitro (43). High concentrations of leptin can also have an inhibitory effect on gonadotropin secretion, as opposed to the stimulatory effect it has at low concentrations (49). However, the present results do not seem to support any direct stimulatory action of leptin in the annual gonadal cycle in woodchucks. The major increase in body weight occurred during a decline in testosterone, and testosterone can be lipolytic and antilipogenic (20). However, any role of testosterone and testosterone removal in body weight gain may be small, because female woodchucks undergo similar changes in body weight (13).

Leptin declined before and during gonadal recrudescence in fall and winter. In woodchucks, the earliest evidence of seasonal rejuvenation of the hypothalamic-pituitary-gonadal axis, represented by increased responsiveness to gonadotropin-releasing hormone challenge, occurs in the autumn (15), at a time when serum concentrations of leptin were very low in the present study. Furthermore, peak gonadal activity occurred in middle to late winter, when serum leptin was at or near nadir. Although leptin can have a stimulatory effect on gonadotropin secretion, its role in the onset of puberty is unclear (33, 35). Stimulatory effects of leptin apparently do not play a significant role in the initiation or progression of seasonal gonadal recrudescence in woodchucks. The same may be the case in the male rat, in which serum leptin increases after puberty onset (35).

Although leptin has been reported to stimulate thermogenesis and sympathetic outflow to brown fat (39), the annual increases in body temperature occurred before any detectable increase in serum leptin, and peak leptin concentrations occurred at a time when body temperature showed evidence of declining. Thus leptin does not appear to play a major role in acutely stimulating thermogenesis in woodchucks. However, the nadir in body temperature occurred during the decline in leptin to low levels, and thus leptin withdrawal might facilitate the extent and/or duration of periods of hypothermia in laboratory woodchucks if it has some chronic thermogenic effect. On the other hand, whether ambient or body temperature plays a role in regulating serum leptin is not known. The reduction in body temperature at this time presumably reduces many metabolic processes, possibly including the level of leptin synthesis compared with the eu-
thermic state. In eutherian rats, cold exposure can reduce plasma leptin (4). Thus, in the wild, the seasonal reduction in serum leptin in autumn and winter may be enhanced by lower ambient temperatures even when the animals are not in torpor.

In conclusion, the present study demonstrates that the seasonal changes in serum leptin concentrations in photoentrained laboratory woodchucks involve increases during increases in body weight and fat mass and the occurrence of peak levels 2–7 wk after peak body weight at the time of the most rapid decline in food intake. These data suggest that leptin is likely to be an anorectic adipocyte hormone in woodchucks as in other species and that leptin participates in the regulation of the timing and extent of spontaneous decreases in food intake during endogenous circannual cycles in this species.

**Perspectives**

Obesity is considered maladaptive in most species, and the homeostatic control of body weight is intensely investigated in efforts to understand obesity and its occurrence in humans. Leptin appears to be a major regulatory component, with plasma leptin increasing in relation to the level of adiposity. Increases in leptin effect a decrease in food intake, allowing body weight to be tightly regulated in healthy individuals. In contrast, the endogenous circannual cycle of hibernators like the woodchuck under natural conditions includes adaptive transitions from a lean to an obese state. The cycle appears to be entrained primarily by photoperiod and is additionally synchronized by the timing of spontaneous immergence into and subsequent emergence from the hypothermia and light deprivation of hibernation. Most if not all of the associated biochemical changes appear to be qualitatively if not quantitatively the same in photoentrained laboratory woodchucks held in conditions of constant temperature and food availability. The change in the hormonal milieu during the cycle includes coordinated and predictable changes in concentrations of free thyroxine, prolactin, and leptin and potentially includes changes in tissue sensitivity to one or more of these hormones. Although other factors are undoubtedly involved, changes in these three hormones alone appear sufficient to explain the occurrence, and in most cases the timing, of the large seasonal changes in food intake, basal metabolism, locomotor activity, body temperature, adiposity, and body weight. Woodchucks and other fat-accumulating hibernators apparently go from a state of leptin resistance to one of leptin sensitivity, which allows for a time delay in the feedback effect of adiposity on food intake and a resulting extended period of fat deposition. Experiments involving the premature elevation of serum leptin to different degrees and at different times, or the inhibition of leptin activity by active or passive immunization, could help define the role of leptin in spontaneous cessation of food intake as well as any role in the onset of hibernation in this species. Knowledge about the regulation of leptin secretion and efficacy during the annual cycle in woodchucks or other hibernators could provide information useful for understanding normal and abnormal regulation of adiposity in humans and other nonhibernating species.

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