Aging-related sex-dependent loss of the circulating leptin 24-h rhythm in the rhesus monkey

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Abstract

The adipocyte-derived hormone leptin plays a pivotal role in the regulation of body weight and energy homeostasis. Many studies have indicated that the circulating levels of leptin show a 24-h rhythm, but the exact cause and nature of this rhythm is still unclear. In the present study, we remotely collected blood samples every hour from young and old, male and female rhesus monkeys, and examined their 24-h plasma leptin profiles. In both the young males (10–11 years) and females (7–13 years), a clear 24-h plasma leptin rhythm was evident with a peak occurring ~ 4 h into the night and a nadir occurring ~ 1 h into the day (lights on from 0700 to 1900 h). A 24-h plasma leptin rhythm was also observed in

the old males (23–30 years), even when they were maintained under constant lighting conditions (continuous dim illumination of ~ 100 lx). In marked contrast, plasma leptin concentrations were relatively constant across the day and night in old peri- and post-menopausal females (17–24 years), regardless of the lighting schedule. These data establish that rhesus monkeys, like humans, show a daily nocturnal rise in plasma leptin, and the magnitude of this rhythm undergoes a sex-specific aging-dependent attenuation. Furthermore, they suggest that the underlying endocrine mechanism may be driven in part by a circadian clock mechanism.

Journal of Endocrinology (2006) **190**, 117–127

Introduction

Leptin, the adipocyte-derived hormone product of the obese (*ob*) gene, provides feedback information about the status of peripheral energy stores to the brain, and thereby plays an important role in regulating energy homeostasis and body weight (Zhang *et al.* 1994, Campfield *et al.* 1995, Halaas *et al.* 1995, Pellemounter *et al.* 1995, Stephens *et al.* 1995, Weigle *et al.* 1995). Many factors are involved in determining the concentration of leptin in the circulation, including: fat mass, caloric intake, meal timing and frequency, circulating glucose and insulin (Considine 2001, Havel 2002), sex and the sex steroids estradiol and testosterone (Casabiell *et al.* 2001, Mayes & Watson 2004), and adrenal steroids (Casabiell *et al.* 2001, Considine 2001, Leal-Cerro *et al.* 2001). Although the level of leptin in the circulation generally correlates well with the level of stored fat, there is also evidence to indicate that leptin levels vary markedly during the day. For example, a nocturnal rise in circulating leptin concentrations has been observed both in lean and obese humans, as well as in individuals with type II diabetes (Sinha *et al.* 1996a, Langendonk *et al.* 1998, Simon *et al.* 1998). In addition, a detailed examination of 24-h leptin concentrations revealed a superimposed pulsatile secretory pattern of leptin in lean and obese individuals (Sinha *et al.* 1996b, Licinio *et al.* 1997, Simon *et al.* 1998). Non-human primate data

supporting a diurnal variation in circulating leptin are less clear. This variation has only been observed utilizing two sampling points over the 24-h day in male rhesus monkeys (*Macaca mulatta*) during late pre-puberty (Suter *et al.* 2000) and has yet to be determined in adult monkeys. Rodent studies have demonstrated a nocturnal rise in circulating leptin levels as well as in leptin mRNA expression in adipose tissue (Saladin *et al.* 1995). So far, however, neither the genesis of the 24-h leptin rhythm has been elucidated nor is it clear what the physiological significance of a diurnal variation in circulating leptin may be. A disruption of this rhythm may contribute to body weight dysregulation and obesity (Laughlin & Yen 1997, Matkovic *et al.* 1997, Balligand *et al.* 1998, Støving *et al.* 1998) and an interference in the maintenance of bone mass (Ducy *et al.* 2000, Eleftheriou *et al.* 2005). This could be particularly evident during aging, as loss of circadian rhythmicity or an attenuation of the amplitude of many circadian rhythms appears to be a characteristic of aging (Schwartz 1993, Touitou *et al.* 1997, Touitou & Haus 2000, Downs *et al.* 2001, Pandi-Perumal *et al.* 2002, Urbanski *et al.* 2004). Little is currently known about the effect of aging on the 24-h pattern of circulating leptin. However, moderate dampening of nocturnal leptin levels has been reported in old men, and with advancing age the acrophase of the leptin rhythm appears to occur earlier in the day (Franceschini *et al.* 1999, Zhao *et al.* 2002).

Possible endocrine candidates influencing the 24-h release of leptin include cortisol and dehydroepiandrosterone sulfate (DHEAS). Cortisol and DHEAS are released from the adrenal cortex in a circadian fashion, i.e. antiphase to the release pattern of leptin (Urbanski *et al.* 2004). However, cortisol and DHEAS appear to exert opposing effects on leptin release. Cortisol acts directly on adipose tissue to stimulate leptin synthesis and secretion in both sexes, while DHEAS inhibits leptin release from adipose tissue from female donors (Casabiell *et al.* 2001, Considine 2001, Leal-Cerro *et al.* 2001). Although glucocorticoid administration stimulates leptin production, they appear to play a modulatory role and are not necessary in generating a leptin diurnal rhythm. Patients lacking endogenous cortisol secretion retained a normal 24-h leptin rhythm when injected with saline only and when given replacement hydrocortisone infusions with normal or reversed diurnal patterns (Purnell & Samuels 1999). Additionally, we have previously reported in rhesus monkeys, a marked aging-related decline in plasma DHEAS concentrations and an associated attenuation of its diurnal variation. In contrast, plasma cortisol concentrations did not significantly differ with age (Urbanski *et al.* 2004, Aghazadeh-Sanai *et al.* 2005). Further, the decline in plasma DHEAS concentrations was observed at an earlier age in females than in males (Urbanski *et al.* 2004, Aghazadeh-Sanai *et al.* 2005). Given these observations, we chose to focus our investigation on the possible correlation between plasma DHEAS, rather than cortisol, and leptin concentrations during aging.

The aims of the present study were twofold: (1) to evaluate the effect of aging on the 24-h pattern of circulating leptin in the male and female non-human primate, and (2) to determine if the circulating 24-h leptin rhythm persists under constant environmental conditions (lighting, temperature, and masked auditory cues). To address these issues, we chose to utilize the rhesus monkey as our animal model. This model closely resembles the human in terms of its physiology, circadian rhythmicity, menopause, and aging. Moreover, rhesus monkeys can be maintained under tightly controlled environmental conditions and can have serial blood samples collected remotely. Preliminary data from this study have previously been published in abstract form (Downs & Urbanski 2003).

Materials and methods

Animals and experimental groups

To evaluate sex-specific aging-related effects on the 24-h circulating leptin rhythm in the non-human primate, 28 adult rhesus monkeys were divided into five groups based upon sex, age, and reproductive status (Tables 1 and 2). The animals were housed in a temperature-controlled environment (24 °C), under a 12 h light:12 h darkness photoperiod, with meal times at 0800 and 1500 h (Purina High Protein Monkey Chow No. 5045; Purina Mills, Inc., St Louis, MO, USA). Chow consumed per meal consisted of 100–125 g for males and 45–50 g for females regardless of age group, and the duration

Table 1 Analysis of male rhesus monkey 24-h plasma hormone profiles

	Young (n = 10)	Old (n = 7)
Body weight (kg)	9.5 ± 0.6	8.5 ± 0.8
Mean leptin (ng/ml)	1.3 ± 0.2	1.5 ± 0.3 ^b
Peak leptin (ng/ml)	1.8 ± 0.3	2.7 ± 0.3
Nadir leptin (ng/ml)	0.6 ± 0.2	0.5 ± 0.2
Fasting leptin (ng/ml)	0.8 ± 0.2	1.1 ± 0.5
Fasting insulin (μU/ml)	11.4 ± 1.8	18.0 ± 4.3
Fasting DHEAS (ng/ml)	217.0 ± 24.4	52.7 ± 12.3 ^a
Mean testosterone (ng/ml)	5.8 ± 0.47	3.4 ± 0.54 ^c

Values represent mean ± S.E.M. Blood samples to determine fasting plasma leptin, insulin, and DHEAS were collected at 0700 h after an overnight fast and before the AM meal. Young = 10–11 years, old = 23–30 years. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.0001 vs young males (Student's *t*-test).

of each meal was less than 2 h for each group. Remote monitoring of their physical activity, using Actiwatch recorders (Mini Mitter Company, Inc., Sunriver, OR, USA) showed that their activity occurred while lights were on (0700–1900 h). One milliliter of blood sample was collected hourly for 25 h using a remote blood sampling system (see below) from all 28 animals. Samples were collected from young (10.6 ± 0.08 years; *n* = 10) and old (26.3 ± 0.89 years; *n* = 7) males starting at 1900 h and samples were collected from young (9.3 ± 1.40; *n* = 4), old pre-menopausal (20.5 ± 1.67 years; *n* = 3), and old peri- and post-menopausal (21.7 ± 0.67 years; *n* = 8) females starting at 0700 h. The purpose of the different collection start times was to eliminate the possibility that any plasma leptin rhythm found was an artifact of the blood collection paradigm.

To determine if the 24-h circulating leptin rhythm may be driven by an underlying circadian clock, five old males (26.4 ± 1.18 years) and seven old peri- and post-menopausal females (22.3 ± 0.24 years) were maintained under the following constant environmental conditions: constant temperature (24 °C), continuous dim illumination (~100 lx), and masking of vocalized social cues of other nearby animals by playing a 2-h loop of recorded 'background' sounds made by monkeys within the housing area. The animals were maintained under constant conditions for 2 or more weeks to allow for their endogenous circadian rhythms to 'free-run'. During this time, the animals continued to have consolidated sleep/wake cycles. These were monitored using Actiwatch recorders, which were housed in a pocket on the back of a catheter-protection jacket. After the 2-week period, 1 ml of blood sample was collected hourly for 25 h using the remote blood sampling system (see below). Sample collection started at 1900 h for males and at 0700 h for females.

Remote blood collection

Each animal was surgically fitted with an indwelling sub-clavian catheter as previously described (Urbanski *et al.* 1997) to enable i.v. blood sampling from an adjacent room without disturbing the animal. Briefly, this consisted of a swivel apparatus at the top of the cage and a protective stainless-steel

Table 2 Analysis of female rhesus monkey 24-h plasma hormone profiles

	Pre-menopause		Peri/post-menopause
	Young (n=4)*	Old (n=3)	Old (n=8)
Body weight (kg)	5.9±0.4 ^b	6.1±0.5 ^b	7.5±0.3
Mean leptin (ng/ml)	1.7±0.5	2.5±1.4	5.1±0.7 ^a
Peak leptin (ng/ml)	2.4±0.6	3.0±1.2	6.2±0.9 ^a
Nadir leptin (ng/ml)	1.1±0.5 ^c	1.2±0.6 ^c	3.9±0.4
Fasting leptin (ng/ml)	1.4±0.4 ^c	2.0±1.6 ^b	5.0±0.6
Fasting insulin (μU/ml)	23.9±4.7	32.8±5.0	36.1±8.9
Fasting DHEAS (ng/ml)	86.4±15.0	23.2±3.9 ^a	34.6±6.5 ^a

Values represent mean±S.E.M. Blood samples to determine fasting plasma leptin were collected after an overnight fast and before the AM meal. Young = 7–13 years, old pre-menopausal = 17–22 years, old peri/post-menopausal = 17–24 years. ^a*P*<0.05 vs young females, ^b*P*<0.05 vs old peri/post-menopausal females, ^c*P*<0.01 vs old peri/post-menopausal females (one-way randomized ANOVA, Newman–Keuls post-analysis).

*Fasting insulin values were determined for three young females rather than four due to the unavailability of plasma.

tether (Lomir Biomedical, Inc., Malone, NY, USA); to maintain catheter patency, heparinized saline (5 IU/ml) was continuously infused (~1 ml/h) using a peristaltic pump. Reproductive status of the young and old females was determined by detailed menstruation records and analysis RIA of blood samples for 17β-estradiol and progesterone (Resko *et al.* 1974, 1975, Gundlah *et al.* 2000). All the young and old pre-menopausal females were sampled from and during the mid-late follicular phase (days 5–12 of a ~28-day cycle) of their menstrual cycles.

This study was approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center (ONPRC), and animal care was provided by the ONPRC in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Hormone assays

Serial blood samples were remotely collected in EDTA-coated tubes at 1-h intervals across the day and night for 25 h and then centrifuged at 4 °C; the plasma supernatant was removed and stored at –20 °C. Plasma leptin concentrations were determined by RIA (Linco Research, Inc., St Charles, MO, USA) with a detection limit of 0.25 ng/ml. The intra-assay coefficients of variation were 5.6% at 3.5 ng/ml and 6.0% at 21.1 ng/ml. The corresponding inter-assay coefficients of variation were 7.9 and 7.0% respectively. Plasma insulin concentrations were determined by electrochemiluminescence using the Elecsys 2010 System (Roche); the assay limit of sensitivity was 0.2 μU/ml and the intra-assay coefficient of variation was 4.5%. Plasma testosterone and DHEAS concentrations were determined by RIA as previously described (Resko *et al.* 1973, Urbanski *et al.* 2004).

Statistical analysis

Twenty-four-hour plasma leptin concentrations were assessed both as raw values (ng/ml) as well as percent variability (% of mean). Student's *t*-test was used to evaluate group differences in body weight, as well as mean, fasting (after an overnight fast

and prior to the AM meal), peak, and nadir raw plasma leptin concentrations between young and old males. One-way randomized ANOVA followed by the Newman–Keuls multiple range test was used to evaluate group differences in body weight, as well as mean, fasting (after an overnight fast and prior to the AM meal), peak, and nadir raw plasma leptin concentrations between young, old pre-menopausal, and old peri- and old post-menopausal females. Variability was defined as a percentage of the individual 24-h mean using the formula:

$$\text{Variability at time } t = \left(\frac{\text{hormone concentration at time } t}{24\text{-h individual mean concentration}} \right) \times 100$$

Plasma leptin variability was plotted against time for each group, and was analyzed using one-way repeated measures ANOVA. Values significantly different from the nadir value were then determined by the Dunnett's-test, and a *P*-value of <0.05 was used to indicate statistical significance.

Group differences in 24-h mean testosterone, fasted morning plasma insulin, and DHEAS concentrations were determined by Student's *t*-test between young and old males. Group differences in fasted morning plasma insulin and DHEAS concentrations were determined by one-way randomized ANOVA followed by the Newman–Keuls multiple range test between young, old pre-menopausal, and old peri- and old post-menopausal females. The relationships between age and fasted morning plasma leptin, insulin, and DHEAS concentrations and between 24-h mean plasma leptin and fasted morning DHEAS concentrations were determined by Pearson's correlation analysis.

Results

Comparison of 24-h leptin profiles in young and old male rhesus monkeys

Significant diurnal variation of plasma leptin was evident in both the young (*P*<0.01) and old (*P*<0.05) males (Fig. 1). However, some quantitative and qualitative differences were

Figure 1 24-h plasma leptin concentrations in young adult male rhesus monkeys (■; $n=10$) and old male rhesus monkeys (□; $n=7$). The horizontal black and white bar on the abscissa corresponds to the 12 h light:12 h darkness lighting schedule. The monkeys were fed two meals daily at 0800 and 1500 h. Although the blood samples were collected over 24 h, from 1900 to 1900 h, the data have been double plotted to aid in the visualization of the leptin circadian rhythm. The plasma leptin concentrations are expressed as a percentage of the 24-h mean ($=100\%$). Data points represent mean values ($\pm$ S.E.M.). Significance values represent a Dunnett's test comparing each plasma leptin concentration with the nadir concentration over 24 h within a group. Young and old males had a nocturnal elevation in plasma leptin (indicated by thick and thin arrows, respectively). A schematic of activity and rest (indicated by vertical and dashed lines, respectively) and the relationship to the timing and duration of meals (indicated by two square symbols) is shown above the lighting schedule.

also apparent. The young males had a longer period of significant nocturnal leptin elevation relative to the morning nadir (1700–0400 h, $P<0.01$) compared to the old males (1700–0100 h, $P<0.05$), whose leptin rhythm was more variable.

Male body weight and plasma hormone characteristics

The body weight, 24-h plasma leptin characteristics, morning plasma insulin and DHEAS concentrations, and 24-h mean testosterone concentrations of the males are shown in Table 1. The young males had a significantly higher mean body weight ($P<0.05$), but lower mean plasma leptin concentration ($P<0.01$) compared to young females (Student's *t*-test; 9.5 ± 0.6 kg and 1.3 ± 0.2 ng/ml, vs 5.9 ± 0.4 kg and 1.7 ± 0.5 ng/ml). Additionally, although there was no difference in fasted morning plasma insulin concentrations between the young and old males, the young males had a lower mean plasma leptin concentration ($P<0.01$) compared to the old males, which probably stems from differences in body fat content rather

Figure 2 24-h plasma leptin concentrations in young adult (●; $n=4$) female rhesus monkeys and old peri- and post-menopausal (○; $n=8$) female rhesus monkeys. The horizontal black and white bar on the abscissa corresponds to the 12 h light:12 h darkness lighting schedule. The monkeys were fed two meals daily at 0800 and 1500 h. Although the blood samples were collected over 24 h, from 0700 to 0700 h, the data have been double plotted to aid in the visualization of the leptin circadian rhythm. The plasma leptin concentrations are expressed as percentage of the 24-h mean ($=100\%$). Data points represent mean values ($\pm$ S.E.M.). Significant values represent a Dunnett's test comparing each plasma leptin concentration with the nadir concentration over 24 h within a group. Only the young females had a significant nocturnal elevation in plasma leptin (indicated by horizontal arrows). A schematic of activity and rest (indicated by vertical and dashed lines, respectively) and the relationship to the timing and duration of meals (indicated by two square symbols) is shown above the lighting schedule.

than body weight, which was well matched for the two groups. Young males had significantly higher levels of fasted morning plasma DHEAS ($P<0.05$) and 24-h mean plasma testosterone ($P<0.0001$).

Comparison of 24-h leptin profiles in young and old female rhesus monkeys

Significant diurnal variation of plasma leptin was also evident in the young pre-menopausal females ($P<0.01$), with a nocturnal elevation from 2000 to 0300 h (Fig. 2). Unlike their old male counterparts and young females, the old peri- and post-menopausal females had relatively constant levels of plasma leptin across the day and night. However, this aging-related sexual dimorphism was not evident in all of the old females. The old pre-menopausal females in the mid-late follicular phase of their menstrual cycles (i.e. prior to ovulation, when plasma 17β -estradiol concentrations are rising) showed a significant elevation in plasma leptin relative to the morning nadir ($P<0.01$) from 1900 to 0300 h (Fig. 3), which was qualitatively indistinguishable from the 24-h leptin rhythm of the young females.

Figure 3 24-h plasma leptin concentrations in old pre-menopausal female rhesus monkeys ($n=3$). The horizontal black and white bar on the abscissa corresponds to the 12 h light:12 h darkness lighting schedule. The monkeys were fed two meals daily at 0800 and 1500 h. Although the blood samples were collected over 24 h, from 0700 to 0700 h, the data have been double plotted to aid in the visualization of the leptin circadian rhythm. The plasma leptin concentrations are expressed as a percentage of the 24-h mean ($=100\%$). Data points represent mean values ($\pm$ S.E.M.). Significant values represent a Dunnett's test comparing each plasma leptin concentration with the nadir concentration over 24 h within a group. Old premenopausal females had a significant nocturnal elevation in plasma leptin (indicated by horizontal arrows). A schematic of activity and rest (indicated by vertical and dashed lines, respectively) and the relationship to the timing and duration of meals (indicated by two square symbols) is shown above the lighting schedule.

Female body weight and plasma hormone characteristics

The body weight, 24-h plasma leptin characteristics, and morning plasma insulin and DHEAS concentrations of the females are shown in Table 2. An age-associated difference in plasma leptin levels and body weights was apparent in the females despite no differences in fasted morning plasma insulin levels between female groups. Compared to the young adult females, the old peri- and post-menopausal females had a significantly higher body weight ($P<0.05$) as well as significantly higher mean plasma leptin ($P<0.01$), peak ($P<0.05$), nadir ($P<0.01$), and fasting plasma leptin ($P<0.01$) concentrations. The old pre-menopausal females showed no difference in mean or peak plasma leptin compared to the old peri- and post-menopausal females. However, compared to the old pre-menopausal females, the old peri- and post-menopausal females had a significantly higher body weight ($P<0.05$) as well as higher nadir ($P<0.01$), and fasting plasma leptin ($P<0.05$) concentrations. The higher morning concentrations of plasma leptin in the old peri- and post-menopausal females compared to the pre-menopausal females caused a blunting of the 24-h plasma leptin variation to a constant concentration of plasma leptin throughout the 24-h day in the old peri- and

Figure 4 Circadian rhythm of plasma leptin concentrations in old male rhesus monkeys ($\square$; $n=5$) and relatively constant 24-h plasma leptin concentrations in old peri- and post-menopausal female rhesus monkeys ($\circ$; $n=7$) under continuous dim illumination (~ 100 lu) indicated by the hatched horizontal bar on the abscissa. The monkeys were fed two meals daily at 0800 and 1500 h. Although the blood samples were collected over 24 h, from 1900 to 1900 h and from 0700 to 0700 h from males and females, respectively, the data have been double plotted starting at 1900 h to aid in the visualization of the leptin circadian rhythm. The plasma leptin concentrations are expressed as a percentage of the 24-h mean ($=100\%$). Data points represent mean values ($\pm$ S.E.M.). Significant values represent a Dunnett's test comparing each plasma leptin concentration with the nadir concentration over 24 h within a group. Only the old males had a significant nocturnal elevation in plasma leptin during their subjective night (indicated by horizontal arrows). A schematic of activity and rest (indicated by vertical and dashed lines, respectively) and the relationship to the timing and duration of meals (indicated by two square symbols) is shown above the lighting schedule.

post-menopausal females. A similar blunting occurred with age in morning plasma DHEAS ($P<0.05$) concentrations, when plasma concentrations of DHEAS typically peak, in both the old pre-menopausal and the old peri- and post-menopausal females compared to the young pre-menopausal females.

Circadian nature of circulating leptin under continuous dim illumination

To demonstrate the circadian nature of the 24-h plasma leptin rhythm, a group of old males and old peri- and post-menopausal females were maintained under constant environmental conditions. The old males showed persistence in the elevation of plasma leptin during their subjective night, from 1600 to 0200 h (Fig. 4). Interestingly, this elevation was more significant ($P<0.01$) than the one previously observed when the animals were maintained under the 12 h light:12 h darkness lighting schedule. In contrast to the males, the old peri- and post-menopausal females failed to show a significant ($P>0.05$) diurnal variation in their plasma leptin

Figure 5 Correlations between plasma leptin concentrations and plasma DHEAS concentrations in young and old male and female monkeys ($n=28$). A significant negative linear correlation is present between leptin and DHEAS. Data points represent 24-h mean leptin values and early morning (0700 h) pre-prandial DHEAS values for each individual monkey.

concentrations while maintained under constant environmental conditions (Fig. 4).

Age and plasma hormone correlations

A significant negative correlation ($P<0.05$) was present between 24-h mean plasma leptin concentrations and morning pre-prandial plasma DHEAS concentrations (Fig. 5). To better understand if the aging-related decline in plasma DHEAS concentrations could explain the elevation in plasma leptin levels in the old males and old peri- and post-menopausal females compared to their young counterparts, we conducted a correlation comparison with age and plasma leptin, insulin and DHEAS (Fig. 6). A significant positive linear correlation ($P<0.001$) was present between leptin and age in old peri- and post-menopausal females. A significant negative correlation between DHEAS and age was present in both males ($P<0.001$), and old peri- and post-menopausal females ($P<0.05$). We found no significant correlation with age and insulin in any group.

Discussion

The present study clearly demonstrates the existence of a 24-h circulating leptin rhythm in a non-human primate. This leptin rhythm is characterized by a peak at or near 23 00 h (4 h after lights out) and a nadir near 07 00 h (at light on-set). Overall, therefore, these findings confirm the results from previous studies in humans (Sinha *et al.* 1996a, Langendonk *et al.* 1998, Simon *et al.* 1998), which showed a clear nocturnal rise in circulating leptin, while bringing new insights to the sex-specific aging of this hormonal rhythm.

Additionally, our data concur with previous non-human primate studies, in that we observed similar plasma concentrations of leptin that increased with age (Urbanski & Pau 1998, Muehlenbein *et al.* 2005). However, this work advances the paucity of data regarding circulating leptin in rhesus monkeys in several ways. Unlike previous works utilizing rhesus monkeys, we were able to determine the 24-h pattern of release by utilizing a remote blood-sampling system that allowed for frequent sampling (hourly for 25 h) rather than a single daytime only, or a single day and nighttime sample (Plant & Durrant 1997, Urbanski *et al.* 1998, Suter *et al.* 2000, Muehlenbein *et al.* 2005), and this remote sampling technique also eliminated the potential for a stress response that may confound the observed levels of leptin. Much of the previous data on circulating leptin in the non-human primate pertained to the relationship between circulating leptin levels and the initiation of puberty (Plant & Durrant 1997, Suter *et al.* 2000). These studies utilized young animals only and they focused exclusively on males. In contrast, we studied adult animals of different age groups as well as different sexes. Additionally, in an effort to better understand the circadian-like nature of circulating leptin, we endeavored to sample from animals in the absence of photoperiodic cues and other potential zeitgebers.

Much is already known about the humoral factors that contribute to the synthesis and release of leptin. These include glucose and insulin (Considine 2001, Havel 2002), adrenal steroids (Casabiell *et al.* 2001, Considine 2001, Leal-Cerro *et al.* 2001), and the sex steroids estradiol and testosterone (Casabiell *et al.* 2001, Mayes & Watson 2004). On the other hand, the underlying mechanism by which these factors exert their influence on leptin is unclear, especially the mechanism that controls leptin's 24-h rhythm. The sex-specific effects on plasma leptin we observed in rhesus monkeys were consistent with those observed in human studies (Casabiell *et al.* 2001, Mayes & Watson 2004). Overall, mean values of plasma leptin in the adult female monkeys were higher than those found in males despite having lower body weights. In part, this may be due to a higher body fat content in females than in males. However, it is likely that body fat differences are not the solitary factor contributing to this sexual dimorphism. Spontaneous leptin secretion *in vitro* from identical adipose tissue samples produce more leptin from samples obtained from female donors than those from male donors (Casabiell *et al.* 1998, Casabiell *et al.* 2001). Gonadal sex steroids are also likely to play a role in the sex differences that are observed in plasma leptin concentrations. Studies of human omental adipose tissue *in vitro* demonstrated the stimulatory effect of estrogen on the release of leptin from adipose tissue of women but not men (Casabiell *et al.* 1998). Gender differences in circulating leptin concentrations *in vivo* are reversed in transsexuals receiving cross-sex steroid hormone treatment (Elbers *et al.* 1997), and the inappropriately high leptin levels that are observed in hypogonadal men are attenuated by testosterone replacement (Jockenhovel *et al.* 1997). Similarly, we observed significantly higher plasma leptin concentrations and significantly lower

Figure 6 Correlations between plasma leptin (A–C), insulin (D–F) and DHEAS (G–I) levels with age in male (■, young; □, old adults) and female (●, young adults; ○, old pre-menopausal; ×, old peri- and post-menopausal) monkeys. A significant positive linear correlation is present between leptin and age only in peri/post-menopausal females, whereas DHEAS is negatively correlated with age in both males and peri-post-menopausal females. There is no significant correlation between insulin and age in any of the groups. Data points represent individual early morning (0700 h) pre-prandial values for each monkey.

testosterone concentrations in old males compared to young male monkeys.

In the present study, we observed a 24-h pattern of circulating leptin in male monkeys, even when they were maintained under long-term constant environmental conditions (consisting of continuous dim illumination and constant temperature while masking the vocalized social cues of other nearby animals). Although not all potential zeitgebers, or time-of-day cues, such as meal timing, could be completely ruled out in this study, the data represent the first evidence in a primate that the plasma leptin rhythm may be truly 'circadian' in nature (i.e. maintains a ~24-h period even in the absence of external time-of-day cues). In the present study, it is possible that meal timing acts as the primary zeitgeber for the 24-h leptin rhythm. The variable of meal

timing is difficult to bypass, as even under feeding *ad libitum* the rhesus monkey self-imposes consolidated meal times. To minimize this variability, we chose to keep meal timing at 0800 and 1500 h with all other external cues being held constant. Previous efforts to determine if the 24-h circulating leptin rhythm is truly circadian did not allow enough time under 'constant conditions' for subjects' endogenous circadian rhythms to be viewed as 'free-running'; instead, they showed a leptin rhythm shift after a short photoperiod change, where any circadian control of leptin release would have been masked by a shift in activity and behavior (Schoeller *et al.* 1997). Future studies will be needed to address the issue of 'free-running' conditions and continuous feeding or meal timing shifts to determine the primary driving force behind leptin's temporal release pattern.

In the previous studies, Franceschini *et al.* (1999) and Zhao *et al.* (2002) sought to identify aging-related changes in circulating 24-h leptin concentrations and found that there were minor changes in amplitude and overall mean levels respectively, but in both cases the 24-h leptin rhythm was generally preserved with old age. We were able to advance these previous observations in several ways. First, we performed more frequent blood samples to generate a more detailed leptin profile than the previous two studies. Secondly, we chose animals of appropriately young ages for our control groups to compare with appropriately old-aged monkeys. In the Franceschini *et al.* (1999) study, it is possible that more significant changes in the 24-h leptin rhythms would have been observed if the old group (72–87 years) would have been compared to a younger control group rather than middle-aged men (35–50 years). Additionally, in the Zhao *et al.* (2002) study, the men varied in age from 31 to 63 years. It is possible that this span in ages was insufficient to disclose changes in the 24-h rhythmicity of leptin other than the typical age-associated increases in overall leptin concentrations and increases in body fat. Furthermore, both the two previous studies utilized male subjects exclusively. Age-related changes to endocrine rhythms are commonly different in males compared to females, and thereby warrant sex-specific investigation especially with respect to the endocrine changes occurring around the time of menopause for women. The effect of age on plasma leptin concentrations in women remains controversial (Mayes & Watson 2004). Parts of the discrepancies in leptin concentrations found in aging women are likely due to the complications of their variable hormonal milieus associated with the transition into menopause and hormone therapies. For this reason, we chose to divide our old female group into a pre-menopausal group and a peri-/post-menopausal group.

An unexpected key finding in the present study was that the circadian leptin rhythm was preserved in old males, but not in old peri- and post-menopausal females. While being exposed to constant environmental conditions and during the 12 h light:12 h darkness lighting schedule, these old females displayed a relatively constant level of plasma leptin over 24 h with no clear evidence for a nocturnal peak. The elevation in morning plasma leptin levels in peri- and post-menopausal females could not be accounted for by any differences in food intake or plasma insulin levels. This aging-associated sexual dimorphism of the 24-h circulating leptin pattern may be indicative of differences in the aging of the circadian system *per se*, being more pronounced or advanced in old females compared to old males. Evidence for the dampening of other circadian hormone rhythms with advancing age indicates that this may indeed be the case (Touitou *et al.* 1997, Gundlach *et al.* 2000, Touitou & Harris 2000, Downs *et al.* 2001, Pandi-Perumal *et al.* 2002, Urbanski *et al.* 2004). For example, we have previously shown that in female monkeys, there is a dramatic attenuation in the amplitude of the DHEAS circadian rhythm around the time of menopause (Downs *et al.* 2001), whereas the circadian pattern (and overall mean

plasma levels) of this adrenal hormone are maintained to a much later age in males (Urbanski *et al.* 2004). Although the old males did not appear to lose their nocturnal plasma leptin peak, their rhythms did differ slightly from those of young males; the plasma levels were more variable and the duration of the nocturnal peak was shorter, which is consistent with previous observations (Franceschini *et al.* 1999, Zhao *et al.* 2002).

The reason for the disappearance of the 24-h plasma leptin rhythm in many of the old females is unclear, but it may be related to aging-associated changes in the sex-steroid environment. In support of this view is the observation that the nocturnal plasma leptin peak persisted in the old pre-menopausal females, but not in the old peri- and post-menopausal females despite identical meal timing, food intake, and fasted plasma insulin levels. Although an aging-related decrease in sex-steroid secretion from the ovaries may play a causal role in the suppression of a 24-h variation in plasma leptin, one cannot rule out the possibility that the aging-associated decrease in DHEA, and its sulfated form DHEAS, from the adrenal gland is also involved. In monkeys, DHEA(S) has a circadian rhythm, characterized by a morning peak, which is in antiphase with the circadian plasma leptin rhythm (Downs *et al.* 2001, Urbanski *et al.* 2004). Furthermore, DHEA(S) is a precursor to testosterone, and previous work has shown that androgens such as testosterone can inhibit the release of leptin (Elbers *et al.* 1997, Jockenhovel *et al.* 1997, Wabitsch *et al.* 1997, Machinal-Qulin *et al.* 2002). Therefore, it is plausible that the absence of a circadian leptin rhythm in the old peri- and post-menopausal females, stems from a loss of androgen-mediated leptin inhibition via DHEA(S). In support of this view, we found a marked attenuation in plasma DHEAS in old females. Although old males also displayed a significant attenuation in plasma DHEAS concentrations, overall they were twice that of the old females. Additionally, an aging-related decline in plasma testosterone concentrations in the males may help to maintain a pronounced 24-h leptin rhythm, as the nighttime peak of testosterone declines in old males so may the testosterone-mediated inhibition of peak plasma leptin levels. Further, elevated plasma testosterone in males compared to females may help to explain the commonly seen differences in plasma leptin concentrations between males and females even after correction for differences in body fat content (Havel *et al.* 1996, Ostlund *et al.* 1996, Rosenbaum *et al.* 1996, Kennedy *et al.* 1997, Lahlou *et al.* 1997, Licinio *et al.* 1998, Saad *et al.* 1998). However, the lower mean plasma leptin concentration observed in the young males (compared to the young females and old males) is likely to be at least partially attributable to their lower body fat content.

At present, the pathophysiological significance of a reduced diurnal variation in plasma leptin during old age is largely unknown, but such changes have the potential to contribute to disrupted eating patterns and body weight regulation and bone remodeling, as well as to perturbation of consolidated

night-time sleep. The loss of bone mass in post-menopausal women leading to osteoporosis is of great concern and a deeper understanding of the causal factors is especially important. Although gonadal failure and the following loss of sex-steroids are considered to be the primary reason for the loss of bone mass in post-menopausal women; recent findings demonstrate that leptin, acting separately from its associated feeding and reproductive neurocircuitry, operates upon the sympathetic nervous system to regulate bone remodeling (Ducy *et al.* 2000, Eleftheriou *et al.* 2005). When leptin was replaced in leptin deficient mice, their elevated bone mass declined (Ducy *et al.* 2000). Leptin exerted its effects through the sympathetic nervous system to increase the production of receptor activator nuclear factor-kappaB (RANK) ligand, which in turn stimulated the formation of osteoclasts (Eleftheriou *et al.* 2005). Moreover, an intact circadian clock mechanism was required for leptin's effect on bone (Fu *et al.* 2005). These new insights emphasize the potential pathophysiological significance of the elevated baseline levels and the loss of leptin rhythmicity we find in old peri- and post-menopausal female rhesus monkeys. Additionally, it has been suggested that a nocturnal rise in leptin curbs nighttime hunger, and thereby may help to maintain consolidated sleep (Sinha *et al.* 1996a, 1996b). However, this explanation does not account for the observation that nocturnal rodents also have elevated circulating leptin concentrations at night, even though they are awake (Saladin *et al.* 1995). Several factors that influence leptin release (Casabiell *et al.* 2001, Considine 2001, Leal-Cerro *et al.* 2001, Havel 2002, Mayes & Watson 2004) are known to change with age (Forbes 1987, Rosenbaum *et al.* 1996, Touitou *et al.* 1997, Moller *et al.* 1998, Touitou & Haus 2000, Pandi-Perumal *et al.* 2002, Urbanski *et al.* 2004). It has been demonstrated that an increase in body fat content in humans is associated with a dampened diurnal variation and pulsatility in plasma leptin secretion (Matkovic *et al.* 1997). However, an aging-related gain in body fat is unlikely to be the basis for the loss of diurnal variation in plasma leptin that was observed in the old peri- and post-menopausal females, because a strong diurnal variation in plasma leptin was preserved in age and body weight-matched old premenopausal females. Despite this match in age and body weight, old peri- and post-menopausal females had significantly higher baseline plasma leptin concentrations, which is consistent with previous reports (Havel *et al.* 1996, Rosenbaum *et al.* 1996, Licinio *et al.* 1998, Saad *et al.* 1998). While the cause of the aging-related loss of a circadian leptin rhythm and an elevation in baseline leptin in old peri- and post-menopausal females is uncertain, it is likely that the higher baseline concentration of plasma leptin in old peri- and post-menopausal females is responsible for masking its circadian rhythm and possibly contributing to an aging-related loss of bone.

In summary, this study demonstrates that the 24-h variation of plasma leptin persists under long-term constant environmental conditions, suggesting that the leptin rhythm may truly be circadian in nature. Additionally, the study shows an

aging-related sexually dimorphic loss of the circulating leptin circadian rhythm in the female rhesus monkey, which may be related to loss of circulating steroid concentrations around the time of menopause.

Acknowledgements

We wish to thank Vasilios T. Garyfallou and Laura L. James at the ONPRC for their work in maintaining catheter patency, and the ONPRC Division of Animal Resources for their animal care.

Funding

This work was supported by NIH grants: AG-023477, AG-19100, AG-19914, HD-29186, RR-00163, and RR-14451. The authors declare that there was no conflict of interest that would prejudice the impartiality of the research reported.

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Received 22 February 2006

Accepted 29 March 2006

Made available online as an Accepted Preprint

13 April 2006