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Prolonged inhibition of presynaptic catecholamine synthesis does not alter leptin secretion in normal-weight men and women

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Leptin has been called a hormone of reproduction, and seems to link fat and fertility. It has been speculated that the neurotransmitter norepinephrine (NE) (noradrenaline), possibly via the sympathetic nervous system, may represent the afferent signal which modulates leptin release from adipocytes. The purpose of this study was to produce a state of decreased sympathetic output by using the catecholamine synthesis inhibitor alpha-methyl-para-tyrosine (AMPT), in order to study the effect of this compound on the secretion of leptin from fat cells. Ten subjects (five women and five men) received a total of 5 × 1 g doses of AMPT or 5 × 50 mg promethazine (active placebo) over a 26 h period, separated by 4–6 weeks using a randomized, double-blind, placebo-controlled, cross-over design. Blood samples for hormone measurements were obtained over 24 h (18 time points) on day 2 of each experiment. Urinary measurement of the NE metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) on study day 2 served as a marker of the effectiveness of AMPT as an inhibitor of NE synthesis. The daily excretion of this metabolite decreased from 1.56 ± 0.22 mg in the placebo experiment to 0.53 ± 0.1 mg in the active experiment ($P < 0.05$). Plasma leptin concentrations measured in the control group in women and men were similar to those reported previously in lean subjects with a body mass index < 27.5 kg/m^2. Leptin concentrations in women were 3-fold higher than in men. Leptin is secreted in a circadian rhythm in both sexes with an increase of nocturnal concentrations by ~50%. Two-way analysis of variance reveals no significant difference in leptin secretion between the control and active groups in women and men. In summary, preliminary results do not support the hypothesis that NE represents the afferent signal from the central nervous system which modulates leptin release from adipocytes in the human. Further studies are needed to define the role of the sympathetic nervous system as well as NE in the regulation of leptin secretion and its involvement in obesity and reproduction.

Key words: leptin/norepinephrine/sympathetic nervous system

Introduction

Leptin is the product of the *ob* gene in mice and has been cloned in humans (Zhang *et al.*, 1994). The gene is expressed in white fat cells, and its role has been described as a fat 'reporter' (Wurtman, 1996). Leptin concentrations are low in women suffering from hypothalamic amenorrhoea (Laughlin and Yen, 1997) and anorexia nervosa (Nakai *et al.*, 1997), and are elevated in patients suffering from obesity (Ostlund *et al.*, 1996), a condition associated with infertility and in a subgroup of women diagnosed with polycystic ovary syndrome (Carmina *et al.*, 1997). Based on these associations leptin has been called a hormone of reproduction, and seems to link fat and fertility (Conway and Jacobs, 1997).

To understand the physiology and pathophysiology of leptin it is important to study the regulation of leptin production and secretion from adipocytes. It is known that expression of the *ob* gene in white fat cells is stimulated by insulin (Gettys *et al.*, 1996) and glucocorticoids (Wabitsch *et al.*, 1996), whereas its production and secretion are decreased by norepinephrine (NE) (noradrenaline) and β_3 agonists (Gettys *et al.*, 1996). Reduced sympathetic activity predisposes to body weight gain and obesity (Bray, 1991), which are associated with increased blood leptin concentrations and at times with infertility (Gettys *et al.*, 1996). It has been speculated that the neurotransmitter NE, possibly via the sympathetic nervous system, may represent the afferent signal from the central nervous system which modulates leptin release from adipocytes (Gettys *et al.*, 1996). The purpose of this study was to produce a state of decreased sympathetic output by using the catecholamine synthesis inhibitor alpha-methyl-para-tyrosine (AMPT), which blocks the production of NE (Zimmermann *et al.*, 1994), and to study the effect of this compound on the secretion of leptin from fat cells. Based on the present knowledge available, we postulated that AMPT would stimulate leptin release from fat cells due to its effect of blocking NE production.

Materials and methods

Subjects

The study was reviewed and approved by the Institutional Review Board. Ten healthy subjects, five women and five men, participated in the study. Their mean age (±SE) was 25±1.5 years and 24±2.0 years respectively. Body weights were 62±3.0 kg in females and 73±4.0 kg in males. The body mass index (BMI, kg/m^2) was

22.3±0.8 for women and 24.5±0.9 for men. All subjects were free of medical and psychiatric illness, based on a detailed physical examination, semistructured psychiatric interview using the *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, 1987) and routine screening tests of blood and urine. The women reported normal menstrual cycles of 27–32 days duration. None of the subjects had taken any medication during the 4 weeks prior to the study, including non-steroidal anti-inflammatory drugs. All subjects gave voluntary written informed consent to participate in the study.

Study design

The study used a randomized, placebo-controlled, double-blind, cross-over design with active and sham tests separated by 4 weeks. In women, both tests were performed on day 3–7 of the menstrual cycle. Each subject received a standardized diet as described previously over the 48 h observation period (Zimmermann *et al.*, 1994). A total of five doses of 1 g of AMPT or five doses of 50 mg of promethazine (active placebo) was given to each subject at the following time points: day 1 at 08:00 h, 12:00 h, 18:00 h, and day 2 at 08:00 h and 12:00 h. To maintain the double-blind design, an active rather than an inactive placebo was chosen. Promethazine causes drowsiness, which is also a common side effect of AMPT, and this helped to maintain the double-blind design. Promethazine is an H1 antagonist (Pontiroli *et al.*, 1981), and its mechanism of action is unlikely to alter leptin secretion. To validate this assumption 24 h leptin secretion was measured in a subset of four subjects (two women and two men) while on no medication, and no significant difference was detected when compared with patients taking promethazine (Figure 1a and b). As promethazine did not alter leptin secretion its use as a placebo was considered appropriate.

The subjects were admitted to the Clinical Research Center at 07:30 h on study day 1. Urine was collected at 12 h intervals over a 48 h period starting at 08:00 h on day 1 and urinary 3-methoxy-4-hydroxyphenylglycol (MHPG), which reflects NE secretion, was measured on study day 2. At 07:30 h on study day 2, an i.v. cannula with a heparin lock was placed into an antecubital or forearm vein for blood sampling. Blood samples (5 ml) were drawn at the following time points: 08:00 h, 10:00 h, 12:00 h, 14:00 h, 16:00 h, 18:00 h, 20:00 h, 21:00 h, 22:00 h, 23:00 h 24:00 h, 01:00 h, 02:00 h, 03:00 h, 04:00 h, 05:00 h, 06:00 h and 08:00 h. From 18:00 h onwards the subjects stayed in a room until 08:00 h the following morning to control for light exposure, which was kept below 200 lux. The subjects were asked to stay awake until 23:00 h (verified by nursing observation), and asked to go to sleep at 23:00 h, at which time the light was turned off; they were awakened at 07:00 h the following morning. Normal sleep patterns were verified by EEG monitoring. Vital signs including blood pressure, pulse and temperature were monitored three times per day at 08:00 h, 14:00 h and 18:00 h. There were no differences between the two study periods regarding vital signs. As AMPT can cause crystal formation in the urine all subjects were asked to drink at least 2 l of fluid per 24 h period, and the collected urine was strained for crystals. No crystal formation was detected in the urine of any of the subjects studied.

Assays

Leptin was measured using radioimmunoassay (Linco Research, St Charles, MO, USA) as described previously (Laughlin and Yen, 1997). The intra- and interassay coefficients of variation were 3.7% and 10.4%, respectively.

Analysis of MHPG was based on quantification of the native fluorescence of the molecule after its isolation by liquid chromatography (Moyer *et al.*, 1982).

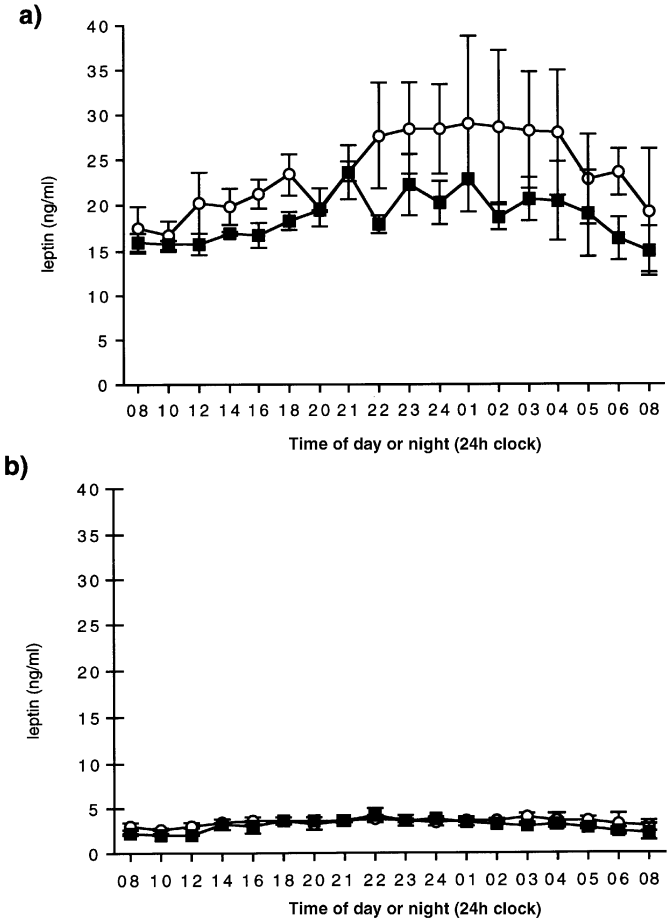

Figure 1. Mean (±SE) leptin concentrations in (**a**) two female subjects and (**b**) two male subjects on no medication (○) or taking promethazine (■).

Data analysis

Mean leptin and MHPG concentrations were calculated by averaging the values from all blood or urine samples for each time point or time period obtained from all subjects within each condition (active versus sham). Two-way analysis of variance (ANOVA) with repeated measurements was used to evaluate the interaction between test day (active versus sham) and time using the Greenhouse–Geisser (GG) correction procedure, and post-hoc paired *t*-tests were performed when appropriate. To demonstrate diurnal variations a one-way ANOVA was performed using the GG correction procedure and post-hoc paired *t*-tests were performed when appropriate. Differences in MHPG excretion were analysed by paired *t*-test. Statview (Abacus Concepts Inc., CA, USA) and SuperANOVA (Abacus Concepts) computer programs were used to perform data analysis and graphic presentation.

Results

Influence of AMPT on MHPG excretion

Measurement of urinary MHPG served as a marker of the effectiveness of AMPT as an inhibitor of NE synthesis. Daily MHPG excretion decreased from 1.56±0.22 mg in the placebo experiment to 0.53±0.1 mg in the active experiment ($P < 0.01$) on study day 2.

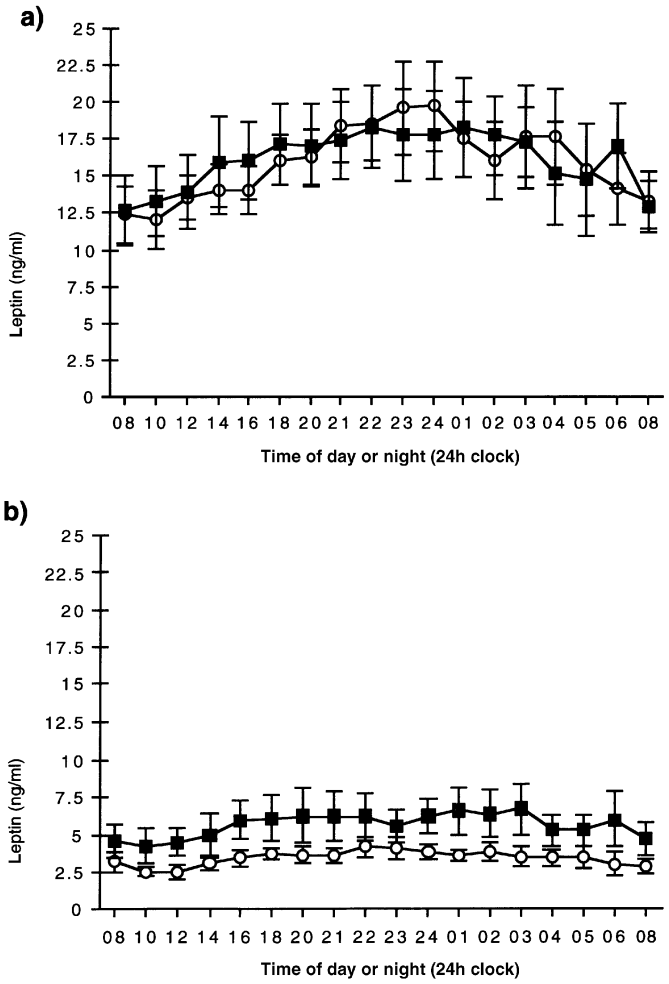

Figure 2. Mean (±SE) leptin concentrations in (**a**) five female subjects and (**b**) five male subjects on active placebo (○) or taking AMPT (■).

Influence of AMPT on leptin secretion in women and men

Women

During day 2 of the control experiment (promethazine placebo), leptin secretion showed the typical circadian secretion pattern in the five women studied, as described by Laughlin and Yen (1997). Mean blood concentrations of leptin increased from 11 ng/ml at 08:00 h to 14 ng/ml at 14:00 h, and to 16 ng at 18:00 h. This concentration was maintained until 04:00 h and then fell to 11 ng/ml at 08:00 h the following morning (Figure 2a; one-way ANOVA: d.f. = 17; $F = 7.29$; $P < 0.01$; GG = 0.013) with leptin concentrations at the following time points being different from those at 08:00 h on study day 2: 14:00 h, 16:00 h, 18:00 h, 20:00 h, 21:00 h, 22:00 h, 23:00 h, 24:00 h, 01:00 h, 02:00 h, 03:00 h and 04:00 h. During the AMPT experiment leptin concentrations were similar to those of the control experiment. Two-way ANOVA revealed no significant difference in leptin secretion between the control and active groups ($F = 1.6$; d.f. = 17,68; $P > 0.05$; GG = 0.25).

Men

During the control experiment (promethazine) on study day 2, leptin secretion showed the typical circadian secretion pattern in the five men studied as described by Sinha *et al.* (1996). Leptin concentrations increased from ~2.5 ng/ml at 10:00 h to ~3.5 ng/ml at 16:00 h; this was maintained until 04:00 h and then fell to 2.5 ng/ml at 08:00 h the following morning (Figure 2b; one-way ANOVA: $F = 5.1$, $P < 0.01$; GG = 0.021) with leptin concentrations at the following time points being different from those at 08:00 h on study day 2: 16:00 h, 18:00 h, 22:00 h, 23:00 h, 24:00 h, 01:00 h, 02:00 h, 03:00 h. Although throughout the AMPT experiment leptin concentrations were typically ~1–1.5 ng/ml higher than in control subjects (the circadian rhythm being similar in both groups), two-way ANOVA revealed this inter-group difference not to be statistically significant ($F = 1.16$, d.f. = 17,68, $P > 0.05$; GG = 0.36).

Comparison of women with men

Comparison of leptin secretion between men and women after promethazine or AMPT treatment revealed significant differences ($P < 0.05$) for all time points studied (promethazine: $F = 3.7$; $P < 0.01$; GG = 0.05; AMPT: $F = 3.8$, $P < 0.01$, GG = 0.05).

Discussion

Plasma leptin concentrations measured in the control group of women and men in this study were similar to those reported previously in lean subjects with a BMI < 27.5 (Ostlund *et al.*, 1996). In agreement with previous studies, leptin concentrations in women were 3-fold higher than in men (Ostlund *et al.*, 1996). Leptin is secreted in a circadian rhythm in men and women with an increase of nocturnal concentrations by ~50%, similar to the results of Sinha *et al.* (1996) and Laughlin and Yen (1997). As the results reported for the control group agree well with data reported in the literature, as well as from our preliminary data (Figure 1), it can be concluded that the promethazine group served as a valid control.

As expected, AMPT induced a significant reduction in excretion of MHPG (Engelman *et al.*, 1968). Based on the results of in-vitro experiments which showed that NE reduces leptin release from isolated rat adipocytes (Gettys *et al.*, 1996), and that propranolol partially reverses the inhibitory effect of NE on leptin production at the level of mRNA (Kosaki *et al.*, 1996), we expected that the AMPT-induced decrease in NE would induce an increase in leptin secretion. We were surprised to detect no change in leptin secretion by men or women exposed to AMPT. One might speculate that the AMPT-induced reduction of NE in the synapse was insufficient to induce postsynaptic changes related to the functioning of receptors which are regulated by NE. Therefore, the question has to be asked whether the AMPT-induced decrease in NE production is biologically relevant with respect to leptin. We have shown previously, using the same paradigm and a similar set of patients (eight subjects participated in both studies), that AMPT reduces nocturnal melatonin secretion to daytime values (Zimmermann *et al.*, 1996). Melatonin production is regulated by noradrenergic neurons in the sympathetic superior cervical ganglion via postsynaptic β-adrenergic receptors in the pineal gland (Moore, 1978). Therefore, the lowering of melatonin secretion by AMPT is indirect evidence that the presynaptic NE output of the sympathetic nervous system at the level of the pineal gland can be decreased to a level which does not

allow stimulation of the β-adrenergic receptor to induce production and secretion of this hormone.

The feasibility of the use of AMPT as a pharmacological clamp of catecholamine activity was confirmed by others (Plosker *et al.*, 1995). Based on these observations it can be concluded that AMPT can induce consistently a biologically significant decrease in catecholamine production and release. Thus, the results of our study make it unlikely that a state of decreased concentration of NE can alter leptin production and secretion from adipocytes in humans in a significant way. This might be clinically relevant for obese patients as it has been shown that leptin plasma concentrations in men and women with a BMI > 27.5 are at least twice those in subjects with a BMI < 27.5. Even though we did not study obese patients, our results suggest that the increase in leptin secretion in this group might not be caused by a decreased concentration of NE at the level of the synapse that innervates fat cells. It is more likely that the increased concentrations of leptin are attributed to the greater mass of fat cells. In summary, the preliminary results of this study do not support the hypothesis by Gettys *et al.* (1996) that NE represents the afferent signal from the central nervous system which modulates leptin release from human adipocytes. Further studies are needed to define the role played by the sympathetic nervous system and NE in the regulation of leptin secretion and its involvement in obesity and reproduction.

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