CLINICAL STUDY

Plasma soluble tumor necrosis factor- α receptors circulate in proportion to leptin levels during the menstrual cycle in lean but not in obese women

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Abstract

Objective: In recent studies serum leptin levels were significantly higher in the luteal phase than in the follicular phase, but the mechanism of changing leptin levels are unknown. Several research lines indicate a potential role for tumor necrosis factor (TNF- α) in ovulation and reproductive events. As TNF- α appears to regulate leptin secretion, we speculated that TNF- α might be involved in leptin variations during the menstrual cycle.

Design and methods: Nine healthy never obese and ten overweight normally cycling women were studied. TNF- α action – through the plasma levels of the soluble fraction of the tumor necrosis factor receptors 1 and 2 (sTNFR1 and sTNFR2) – and leptin concentrations were measured in the follicular (F), peri-ovulatory (PO) and luteal phases (L) of their menstrual cycles.

Results: Circulating leptin levels were significantly associated with the stage of the menstrual cycle ($P < 0.001$), being higher in PO and L phases. However, only three of ten overweight subjects vs eight of nine lean women (Chi square $P = 0.014$ after Fisher's exact test) showed significantly higher leptin levels in the PO and L than in the F phase (95% confidence interval (95% CI) of the differences, 3.7 to 10.2 ng/ml, paired t -test $P = 0.001$). In these women (group 1), the changes in leptin levels paralleled the variations observed in plasma sTNFR1 (2.50 ± 0.1 vs 2.11 ± 0.05 ng/ml, $P < 0.0001$, 95% CI, 0.21 to 0.56) and sTNFR2 levels (5.19 ± 0.28 vs 4.55 ± 0.25 ng/ml, $P < 0.0001$, 95% CI, 0.47 to 0.81). In the remaining women (group 2), leptin (95% CI, -1 to 9.2 ng/ml, $P =$ not significant (NS)), sTNFR1 (95% CI, -0.3 to 0.14 ng/ml, $P =$ NS) and sTNFR2 levels (95% CI, -0.95 to 0.39 ng/ml, $P =$ NS) were essentially unaltered throughout the menstrual cycle. Group 2 women were similar in age (36.1 ± 2.9 vs 37.3 ± 1.4 years) and significantly overweight (body mass index 31 ± 2.9 vs 23.9 ± 1.2 kg/m²) compared with group 1 women. A negative correlation was observed between leptin levels in the follicular phase and the change in plasma leptin from F to L phase in all subjects ($r = -0.67$, $P = 0.002$).

Conclusions: Circulating leptin and sTNFRs levels change significantly during the menstrual cycle of most lean women. In contrast, the levels of these molecules remain essentially unaltered during the F, PO and L phases in the majority of overweight women. Obesity might be associated not only with blunted diurnal excursions and dampened pulsatility, but also with blunted excursions during the menstrual cycle.

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Introduction

There is increasing evidence that leptin is involved in reproductive events. It has been demonstrated that the administration of recombinant leptin restores full fertility in leptin-deficient female (*ob/ob*) mice, one of the genetic models of obesity (1–3). Leptin stimulated luteinizing hormone (LH) production and increased primary and Graafian follicle numbers (2). Leptin also

produced a dose-related increase in LH-releasing hormone, follicle-stimulating hormone (FSH) and LH release in *in vitro* and *in vivo* studies (4). In addition, the administration of leptin to normal prepubertal mice results in earlier maturation of the reproductive tract and earlier reproduction, despite the inhibition of growth rate (3). In humans, leptin levels were found to rise by approximately 50% just before the onset of puberty in boys (5). The onset of puberty has

long been recognized to involve adipose tissue mass, once a critical body weight is reached (6–8). Menarche is thought to be triggered by the attainment of a critical percentage of fat, and the maintenance of menstrual cycles requires the persistence of a minimal level of fat (7, 8).

In recent studies serum leptin levels were found to be significantly higher in premenopausal than postmenopausal women (9), and higher in the luteal phase than in the follicular phase (9–12), with one exception (13), suggesting that estrogen regulates leptin production, although, *a priori*, progesterone cannot be excluded (10). Given that leptin changes were not related to changes in sex hormones (12), other factors should be investigated.

Several research lines indicate a potential role for tumor necrosis factor (TNF- α) in ovulation (14–17) and in the molecular control of the implantation window (18). Immunoreactive TNF- α has been localized in the human granulosa cell layer of healthy antral follicles, in the ooplasm of the oocyte, and can be measured in human follicular fluid (14, 15). Granulosa cells collected from patients undergoing *in vitro* fertilization released TNF- α into the culture medium over a 15-h incubation period (15). Furthermore, TNF- α has been consistently detected in uterine secretion in a recent study (19). TNF- α was found in quite low concentrations at the beginning of the cycle, rose sharply in the mid- to late proliferative phase and decreased towards the end of the cycle using immunohistochemistry, enzyme-linked immunosorbent analysis and reverse transcription-polymerase chain reaction (19).

TNF- α actions appear to occur via an autocrine–paracrine mechanism. Circulating cytokine molecules are seldom found in the unbound state and are usually not informative. They are almost always bound to binding or carriers proteins, autoantibodies and soluble receptors. The usual sandwich-format immunoassays recover free and some predictably bound cytokine, but miss other cytokines bound by unpredictable binding entities. Neither the mechanisms that control this restricted activity nor the receptor systems that are involved in TNF- α signaling are clearly understood (20, 21). TNF signals through at least two known cell surface receptors (21) (tumor necrosis factor receptors; TNFRs): TNFR1 (p60) and TNFR2 (p80), that are present in virtually all cells of higher mammals. It appears that TNFR1 can signal for virtually all known activities of TNF, including apoptosis, differentiation, and proliferation. Both receptors for TNF exist also in soluble forms, apparently derived by proteolytic cleavage from the cell surface forms (22, 23). It has been suggested that soluble TNFR1 (sTNFR1) and sTNFR2 represent a buffer system that prolongs the biologic effects of TNF- α by forming a ‘slow release reservoir’ and impeding spontaneous denaturation of the cytokine (23). In this way, plasma sTNFRs might indicate a surrogate of previous TNF action because sTNFR1

and sTNFR2 levels remain elevated for longer periods of time after TNF administration (23).

TNF- α appears to regulate leptin secretion *in vitro* (24) and *in vivo* in mice (25), hamsters (26) and humans (27–30). Hence, we speculated that TNF- α , if secreted into the plasma in parallel with ovary and uterine secretions, might be involved in leptin variations during the menstrual cycle. We therefore measured TNF- α action – through the levels of sTNFR1 and sTNFR2 – and leptin concentrations in follicular, peri-ovulatory and luteal phases in healthy women.

Subjects and methods

Study population

Nine never obese and ten obese normally cycling women (cycle length 27–32 days) were studied as healthy volunteers (staff from our hospital). All subjects were at their maximal lifetime weight and had maintained this weight within a 2 kg range for at least 3 months prior to enrollment. The early follicular phase blood extraction was performed within 3 or 4 days of the onset of menstrual bleeding. The mid-cycle and mid-luteal blood extractions were performed at days 12–15 and 17–22 respectively. A chart was given to all women to document their menstrual cycles.

Inclusion criteria included the following. (i) No consumption of drugs or alcohol >20 g a day; (ii) absence of any systemic or metabolic disease other than obesity; (iii) no hormone use for 3 months, including oral contraceptive agents; (iv) absence of any infections in the previous month. None of the women had vasomotor symptoms or evidence of the perimenopause or of overt androgen excess (visible acne, abnormal facial hair or temporal hair recession). Appropriate informed consent was obtained prior to the study.

Procedures

Women with body mass index (BMI; (weight (kg))/(height (m))²) >25 kg/m² were considered as overweight and subjects with BMI <25 kg/m² were classified as lean.

Percentage of body fat and fat-free mass were measured using bioelectric impedance (Holtain, Cambridge, UK). The subject's waist was measured with a soft tape midway between the lowest rib and the iliac crest. The hip circumference was measured at the widest part of the gluteal region.

Each woman had three times of serum sampling across one cycle. Fasting glucose and insulin were measured only at the follicular phase.

Laboratory analysis

The serum insulin level was measured in duplicate by monoclonal immunoradiometric assay (Medgenix Diagnostics, Fleunes, Belgium). The lowest limit of detection was 4.0 mU/l. The intra- and interassay coefficients of

variation were 5.2% at a concentration of 10 mU/l and 6.9% at 14 mU/l respectively.

Immunoreactive serum leptin concentrations were measured in samples obtained after an overnight fast, between 0800 and 0830 h (Linco Research Inc., St Charles, MO, USA). The lower limit of detection was 0.5 ng/ml. Intra- and interassay coefficients of variation were <7% and <8% respectively.

Plasma sTNFR1 and sTNFR2 levels were measured as previously described (30). In brief, the MEDGENIX sTNF-R1 and sTNF-R2 EASIA (BioSource Europe S.A., Fleunes, Belgium) are solid-phase enzyme amplified sensitivity immunoassays (EASIA) performed on micro-titer plates. The minimum detectable concentration was estimated to be 0.1 ng/ml and was defined as the sTNFR1 or sTNFR2 concentration corresponding to the average optical density of 20 replicates of the zero standard+2 standard deviations. The intra- and inter-assay coefficients of variation were <7% and <9%. sTNFR1 EASIA does not cross-react with sTNFR2. TNF- α does not interfere with the assay.

LH and FSH were measured by immunoenzymatic assay with two monoclonal antibodies, one of them labeled with alkaline phosphatase (Technicon-Bayer, Tarrytown, NY, USA). Data are expressed in terms of the Second International Reference Preparation of Pituitary FSH (78/549) and the First International Standard for Pituitary LH (68/40). The sensitivity was 0.1 IU/l for FSH and 0.3 IU/l for LH, with coefficients of variation of 2.7 and 3.3% respectively. Estradiol was determined by a competitive immunoenzymatic assay (Technicon-Bayer) with a detection limit of 10 pg/ml and a 5% coefficient of variation. Progesterone was measured by a competitive immunoenzymatic assay (Immulite-DPC, Los Angeles, CA, USA). The sensitivity was 0.2 ng/ml and the coefficient of variation 6.7%. The progesterone level was considered to be ovulatory if greater than 6 ng/ml.

Statistical analyses

Descriptive results are presented as means $\pm$ s.d. Non-Gaussian distributed variables were $\log_{10}$ transformed

to achieve normality. This applied to leptin, estradiol, progesterone, sTNFR1 and sTNFR2. Relationships between variables were sought by Pearson's correlation coefficient, and between proportions by χ^2 using Fisher's exact test. Comparison of variables across lean and obese women was performed by two-way ANOVA, with specific differences between stages of the menstrual cycle tested by the Bonferroni–Dunn post hoc test. Levels of statistical significance were set at $P < 0.05$. All these analyses were performed with the BMDP statistical package (BMDP Statistical Software, Cork, Republic of Ireland).

Results

Table 1 summarizes the demographical and biochemical characteristics of the subjects. Three women in the lean group and two in the overweight group were current smokers. Any of the findings described below did not change substantially when these women were excluded from the analysis. Follicular leptin levels correlated with sTNFR1 in all subjects ($r = 0.50$, $P = 0.028$), as recently demonstrated (28). When the menstrual cycle was subdivided into functional stages, circulating leptin levels were significantly associated with the stage of the menstrual cycle ($P < 0.001$), being higher in the peri-ovulatory and luteal phases. However, only three of ten obese subjects versus eight of nine lean women (Chi square $P = 0.014$ after Fisher's exact test) showed significantly higher leptin levels in the mid-cycle and luteal phases than in the follicular phase (95% confidence interval (95% CI) of the differences, 3.7 to 10.2 ng/ml, paired t -test $P = 0.001$, Table 2 and Fig. 1). These changes in leptin levels paralleled the variations observed in plasma sTNFR1 (2.50 ± 0.1 vs 2.11 ± 0.05 ng/ml, $P < 0.0001$, 95% CI, 0.21 to 0.56) and sTNFR2 levels (5.19 ± 0.28 vs 4.55 ± 0.25 ng/ml, $P < 0.0001$, 95% CI, 0.47 to 0.81) (Fig. 2).

In the other women (group 2), both leptin (95% CI, -1 to 9.2 , $P =$ not significant (NS)), sTNFR1 (95% CI, -0.3 to 0.14 , $P =$ NS) and sTNFR2 levels (95% CI, -0.95 to 0.39 , $P =$ NS) were essentially unaltered throughout the menstrual cycle (Table 2 and Fig. 1).

Table 1 Demographic and biochemical characteristics. Data are expressed as means $\pm$ s.d.

Variable	Lean women	Overweight women	P
<i>n</i>	9	10	
Age (years)	35.8 ± 2	37.7 ± 2.1	NS
BMI (kg/m^2)	24.2 ± 1.7	32.6 ± 2.1	<0.0001
Fat mass (kg)	7.6 ± 1.6	28 ± 4.2	0.001
Fat-free mass (kg)	48.9 ± 1.9	56.7 ± 2.3	0.02
Waist-to-hip ratio	0.77 ± 0.018	0.79 ± 0.013	NS
Fasting glucose (mmol/l)	5.25 ± 0.51	5.52 ± 0.62	NS
Fasting insulin (mU/l)	7.4 ± 1.1	5.52 ± 0.62	NS
Leptin (ng/ml)	7.6 ± 1	28 ± 6.3	0.01

NS, not significant.

Table 2 Comparison between subjects with changing (Group 1) versus unaltered (Group 2) leptin levels. Data are expressed as means $\pm$ s.d.

Variable	Group 1	Group 2	P
Lean/overweight	8/3	1/7	0.014
Age (years)	37.3 $\pm$ 1.4	36.1 $\pm$ 2.9	NS
BMI (kg/m ²)	23.9 $\pm$ 1.2	31 $\pm$ 2.9	0.025
Fat mass (kg)	12 $\pm$ 2.7	26.9 $\pm$ 6.2	0.024
Leptin ff (ng/ml)	9 $\pm$ 1.3	31.3 $\pm$ 7.4	0.02
Leptin max (ng/ml)	15.9 $\pm$ 2.5*	35.3 $\pm$ 8.5	0.06
Leptin (% change)	81.5 $\pm$ 15	22 $\pm$ 10.9	0.009
sTNFR1 ff (ng/ml)	2.11 $\pm$ 0.1	2.56 $\pm$ 0.18	0.045
sTNFR1 max (ng/ml)	2.5 $\pm$ 0.05*	2.47 $\pm$ 0.20	NS
sTNFR1 (% change)	18.4 $\pm$ 3.6	-3.8 $\pm$ 3.4	0.001
sTNFR2 ff (ng/ml)	4.55 $\pm$ 0.25	5.01 $\pm$ 0.52	NS
sTNFR2 max (ng/ml)	5.19 $\pm$ 0.28*	4.73 $\pm$ 0.58	NS
sTNFR2 (% change)	14.3 $\pm$ 1.6	-5.5 $\pm$ 6.1	0.01

ff, follicular phase; max, maximal level reached in the mid-cycle and luteal phases.

* $P < 0.0001$ vs the same parameter in the follicular phase; NS, not significant.

The latter women were similar in age and significantly more obese (see Table 2). A negative correlation was observed between leptin levels in the follicular phase and the change in plasma leptin from follicular to luteal phase in all subjects ($r = -0.67$, $P = 0.002$).

Steroid hormones and gonadotropins exhibited characteristic secretion profiles throughout the menstrual cycle (Table 3 and Fig. 3). FSH, LH, and estradiol levels peaked at ovulation ($P < 0.05$) and subsided thereafter in all subjects. A peak in progesterone concentration ($P < 0.05$) occurred during the luteal phase of the cycle. There were no differences between group 1 and group 2 women in relation to these parameters (Table 3). LH in the peri-ovulatory phase was found to correlate with leptin in the peri-ovulatory and luteal phases ($r = 0.67$ and $r = 0.68$, $P = 0.045$ and $P = 0.042$ respectively) only in lean women. In contrast,

leptin levels during the reproductive cycle were not found to correlate with either estradiol or progesterone.

Discussion

We here confirm recent findings concerning changes in plasma leptin during the menstrual cycle (9–12). However, these variations occurred mainly in lean women. In previous studies, leptin levels were found to change during the menstrual cycle in six women with a BMI of 21.6 ± 0.5 (10), in 15 women with a BMI of 22.9 ± 3.1 (11), in 13 women with a fat mass of 14.27 ± 1.2 kg (similar to that in our group 1 subjects) (9), and in nine women with a BMI of 23.9 ± 1.8 (12). Only one study did not find changes in leptin during the menstrual cycle in eight women with a BMI of 21.2 ± 1.6 kg/m² (13). We have observed leptin

Phase of the cycle

Figure 1 Percentual change in serum leptin levels during the menstrual cycle in group 1 (○) and group 2 (■) subjects. The data are grouped according to the main stages of the ovarian cycle: F, follicular phase; P-O, peri-ovulatory period; L, luteal phase.

Phase of the cycle

Figure 2 Percentual change in plasma sTNFRs levels observed between the follicular phase and the maximal levels in the peri-ovulatory period or the luteal phase (Peak). Group 1 sTNFR1 (○), group 2 sTNFR1 (●), group 1 sTNFR2 (□) and group 2 sTNFR2 (■).

Table 3 Comparison between group 1 and group 2 subjects in gonadotropins and steroid hormones. Data are expressed as means $\pm$ S.D.

Variable	Group 1	Group 2	P
LH ff (IU/l)	7.3 $\pm$ 1	5.6 $\pm$ 0.6	NS
LH max (IU/l)	23.7 $\pm$ 6.9†	15.7 $\pm$ 3.7†	NS
Estradiol ff (pg/ml)	57.7 $\pm$ 18.4	54.2 $\pm$ 4.9	NS
Estradiol max (pg/ml)	193.1 $\pm$ 25*	183.9 $\pm$ 57.8†	NS
Progesterone ff (ng/ml)	0.65 $\pm$ 0.07	0.73 $\pm$ 0.09	NS
Progesterone max (ng/ml)	9.18 $\pm$ 1.3*	6.73 $\pm$ 0.75*	NS

ff, follicular phase; max, maximum level reached in the mid-cycle and luteal phases.

* $P < 0.01$ vs the same parameter in the follicular phase; † $P < 0.05$ vs the same parameter in the follicular phase; NS, not significant.

variations only in three out of ten overweight women. Although the circadian variation in leptin levels was controlled for, the pulsatile nature of leptin secretion was not in our study. However, recent studies involving more frequent sampling have demonstrated increases in plasma leptin in women with regular menstrual cycles and not in postmenopausal women (12). Furthermore, sTNFRs are quite stable proteins and their plasma concentrations changed significantly only in women in whom leptin had also changed.

Figure 3 Changes in circulating LH (■), FSH (○) (top panel), estradiol (■) and progesterone (○) (lower panel) in spontaneously cycling women. The data are grouped according to the main stages of the ovarian cycle: F, follicular phase; P-O, peri-ovulatory period; L, luteal phase. Those points with the same initial are significantly different at $P < 0.05$.

Obesity has been associated not only with higher leptin levels but also with blunted diurnal excursions and dampened pulsatility (31). In fact, a negative correlation between leptin levels in the follicular phase and their changes throughout the ovarian cycle ($r = -0.67$, $P = 0.002$) was found, suggesting a flattened variability during the menstrual cycle. As abnormal rhythmicity has been hypothesized to contribute to leptin resistance in obesity (31), we speculate that the absence of significant changes in leptin levels during the menstrual cycle of overweight women might also contribute to their leptin resistance. Basal temperature levels are higher, metabolic rates are higher and caloric intakes are higher in the luteal phase of the normal menstrual cycle (32). Hence, increased leptin levels, as a reflection of leptin resistance, might have a role in preparing the body for the metabolic demands of pregnancy. Furthermore, in our experience, plasma levels of sTNFR2 are associated with insulin resistance (30), and the latter is well described in the luteal phase of healthy women (33), just the phase in which sTNFR2 increased (this report).

Leptin regulates the minute-to-minute oscillations in the levels of LH and estradiol, and the nocturnal rise in leptin determines the change in the nocturnal LH profile in the mid-to-late follicular phase that precedes ovulation, as demonstrated in recent studies (34). That leptin influences ovary function had already been suggested by the finding of a high level of expression of the near identical receptor for the obesity gene product B219/ob-r in the human ovary and granulosa cells of the preovulatory follicle (35). The absence of significant oscillations in leptin concentrations during the menstrual cycle of obese women might be the cause of their increased prevalence of anovulatory cycles (36). In our overweight women, we have found unaltered levels of sTNFR1 and sTNFR2 coupled with flattened leptin concentrations during the ovarian cycle, which suggests that these cycles were anovulatory.

In agreement with Riad-Gabriel *et al.* (12) we found that changes in plasma leptin during the menstrual cycle were not related to changes in sex hormones. This means that additional factors might be involved. We provide here some data compatible with the idea that the increase in leptin is perhaps due to enhanced TNF- α action as a result of ovulation. As increased TNF- α concentration has been demonstrated to occur in uterine secretion in the mid-to-late proliferative phase (19), it is tempting to speculate that increased plasma sTNFR1 and sTNFR2 levels are reflecting the increased TNF- α action secreted into the plasma in parallel with its exogenous release. Gonadotropins have been found to induce the release of TNF- α from the human preovulatory follicle and, simultaneously, to correlate with circulating leptin during the menstrual cycle (4, 13, present findings).

After the submission of this manuscript, another work has confirmed that leptin fluctuates during the

menstrual cycle, increasing after a peri-ovulatory peak (37). In addition, endometrium from the proliferative phase has been found to express higher levels of TNF- α mRNA than that from secretory phase or during menses (38). Interestingly, TNFR1 expression was higher in endometrium from the secretory phase compared with the proliferative phase or with the menses (38), a finding that is extended with our observation of increased circulating sTNFRs in the luteal phase and its relation with leptin.

In summary, circulating leptin and sTNFRs levels change in parallel during the menstrual cycle of most lean women. In contrast, the levels of these molecules remain essentially unaltered during the follicular, peri-ovulatory and luteal phases of overweight women. Whether leptin and sTNFRs oscillations are inter-correlated with ovulation rates merits further research.

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