Dihydrotestosterone, stanozolol, androstenedione and dehydroepiandrosterone sulphate inhibit leptin secretion in female but not in male samples of omental adipose tissue \textit{in vitro}: lack of effect of testosterone

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Abstract

Leptin, the product of the Ob gene, is a polypeptide hormone expressed in adipocytes which acts as a signalling factor from the adipose tissue to the central nervous system, regulating food intake and energy expenditure. It has been reported that circulating leptin levels are higher in women than in men, even after correction for body fat. This gender-based difference may be conditioned by differences in the levels of androgenic hormones.

To explore this possibility, a systematic \textit{in vitro} study with organ cultures from human omental adipose tissue, either stimulated or not with androgens (1 µM), was undertaken in samples obtained from surgery on 44 non-obese donors (21 women and 23 men). The assay was standardized in periods of 24 h, ending at 96 h, with no apparent tissue damage. Leptin results are expressed as the mean ± S.E.M. of the integrated secretion into the medium, expressed as ng leptin/g tissue per 48 h.

Spontaneous leptin secretion in samples from female donors (4149 ± 301) was significantly higher ($P<0.01$) than that from male donors (2456 ± 428). Testosterone did not exert any significant effect on \textit{in vitro} leptin secretion in either gender (4856 ± 366 in women, 3322 ± 505 in men). Coincubation of adipose tissue with dihydrotestosterone (DHT) induced a significant ($P<0.05$) leptin decrease in samples taken from women (3119 ± 322) but not in those taken from men (2042 ± 430). Stanozolol, a non-aromatizable androgen, decreased ($P<0.05$) leptin secretion in female samples (2809 ± 383) but not in male (1553 ± 671). Dehydroepiandrosterone sulphate (DHEA-S) induced a significant ($P<0.01$) leptin decrease in female samples (2996 ± 473), with no modifications in samples derived from males (1596 ± 528). Exposure to androstenedione also resulted in a significant reduction ($P<0.01$) of leptin secretion in samples taken from women (2231 ± 264), with no effect on male adipose tissue (1605 ± 544).

In conclusion, DHT, stanozolol, DHEA-S and androstenedione induced a significant inhibition of \textit{in vitro} leptin secretion in samples from female donors, without affecting the secretion in samples from men. Testosterone was devoid of activity in either gender.


Introduction

Leptin, the 167-amino acid peptide product of the Ob gene (Zhang et al. 1994) is produced in adipose tissue (Lonnqvist et al. 1995, Masuzaki et al. 1995). After its release into the bloodstream, leptin acts by signalling the amount of body fat stores to hypothalamic centres regulating appetite and energy expenditure (Ahima et al. 1996, Caro et al. 1996). Besides its main role in metabolism and in the neuroendocrine adaptation to fasting (Ahima et al. 1996), the participation of leptin in new functions has been described, such as in the regulation of growth hormone secretion (Carro et al. 1997), gonadal function and gestation (Ahima et al. 1997, Butte et al. 1997, García-Mayor et al. 1997, Mantzoros et al. 1997, Pombo et al. 1997) and possibly also placental function (Señaris et al. 1997). Furthermore, the presence of leptin in both placental tissue (Señaris et al. 1997) and human milk (Casabiell et al. 1997) would suggest further unexpected functions for this hormone.

It is widely assumed that circulating leptin levels are determined by the size of the body fat stores (Maffei et al. 1995, Havel et al. 1996). However, the large variations in leptin values for any given proportion of body fat suggest
that other hormonal and nutritional factors may contribute to its regulation (Caro et al. 1996). In this sense, serum leptin concentrations have been shown to be lower in men than in women with similar amounts of body fat (Maffei et al. 1995, Caprio et al. 1996, Considine et al. 1996, Hassink et al. 1996, Havel et al. 1996, Ostlund et al. 1996, Rosenbaum et al. 1996), a fact that has already been described at birth, when no differences in the adipose tissue reserves are expected (Matsuda et al. 1997, Tome et al. 1997).

When considering the gender-based differences in leptin secretion, testosterone and other androgen steroids have been postulated as the putative mediators. Several indirect pieces of evidence obtained in vivo suggest that testosterone normalized the abnormally elevated leptin levels in hypogonadal men (Jockenhovel et al. 1997, Sih et al. 1997), while other studies failed to find any significant relationship between leptin concentrations and androgenic hormones (Haffner et al. 1997). In peripubertal boys, leptin rises in parallel with body fat until the time of the initial testosterone rise, when a sudden leptin reduction occurs (Garcia-Mayor et al. 1997). As the majority of these reports are based on indirect and inferential evidence, the exact role of androgens in leptin secretion is still an open question. The aims of the present work were twofold: to study the effect of androgens on leptin secretion by using an in vitro system of human omental adipose tissue organ culture; and to assess any gender-based difference in the action of androgens.

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Materials and Methods

Omental adipose tissue was obtained from 44 non-obese patients during elective abdominal surgery; malignancy was an exclusion criterion. The tissue donor group was composed of 21 women (age 56·8 ± 4·0 years, body mass index (BMI) 27·2 ± 1·09) and 23 men (57·0 ± 3·6 years, BMI 26·8 ± 0·76). Patients were taking no drugs or antibiotics. The study was approved by the hospital ethical committee, and each participating subject provided informed consent.

Excised adipose tissue was immediately transported to the laboratory in ice-cold Krebs–Ringer–Hepes buffer (NaCl, 125 mM; KCl, 5 mM; MgSO4, 1·2 mM; CaCl2, 2 mM; KH2PO4, 1·2 mM; glucose, 6 mM; Hepes, 25 mM; pH 7·4). After removing blood vessels and connective tissue, adipose tissue was washed with sterile Krebs–Ringer–Hepes and cut into small pieces with sharp scissors. Tissue fragments were placed in six-well dishes (300–400 mg adipose tissue/well) containing 2·5 ml Dulbecco’s modified Eagle’s medium plus 0·5% fetal calf serum, supplemented with penicillin (100 U/ml) and streptomycin sulphate (100 µg/ml), and incubated at 37 °C under a humidified atmosphere of 95% air–5% CO2. After a preincubation period of 1 h the media were discarded and 2·5 ml fresh medium (with or without stimuli) were dispensed into each well. Culture media were then collected completely every 24 h and replaced with fresh medium, again with or without stimuli. The drugs tested were testosterone, dihydrotestosterone (DHT), stanozolol, dehydroepiandrosterone sulphate (DHEA-S) and androstenedione, all at 1 µM, with the appropriate vehicle added to control samples; this dose was selected as the most appropriate for the organ culture system used (Casabiell et al. 1998). Unless specifically indicated, all drugs and reagents were from Sigma Chemical Co. (St Louis, MO, USA).

For each tested variable, either untreated or treated sample, the adipose tissue from a given subject was independently incubated in triplicate, and the medium was collected and analysed every 24 h to obtain the 24 h secretion, the cumulative secretion till 96 h, and the integrated secretion (area under the curve, AUC), in such a way that each subject acted as her/his own control. Samples were stored at −20 °C until assayed for leptin. Serum leptin levels were measured in duplicate by RIA using commercial kits (Human Leptin RIA, Linco Research Inc., St Charles, MO, USA). The limit of sensitivity was 0·5 µg/l, the intraassay coefficient of variation was 8·3%, and the interassay coefficient of variation was 6·2%.

Statistical analysis

The mean BMI, defined as the weight in kilograms divided by the square of the height in metres was calculated. Data are presented as the mean ± s.e.m. of the group. Leptin secretion was expressed as the total amount of leptin secreted into the well by a given sample (in nanograms) divided by the amount of fat tissue of the sample and the time considered, i.e. ng leptin/g tissue per 48 h (Barr et al. 1997, Casabiell et al. 1998). The integrated AUC was calculated by the trapezoidal method. Comparisons between groups were made by the paired and unpaired t-tests when appropriate. Values of P<0·05 were considered significant.

Results

Spontaneous (non-stimulated) leptin secretion into the incubation medium was well maintained in samples from both men and women, from 0 to 96 h (Fig. 1). No tissue damage was observed even in longer incubation periods (Casabiell et al. 1998). As there was a progressive decrease in the spontaneous leptin secretion after 96 h, no longer times were studied. The leptin secretion in periods of 24 h reached its maximum net increment at the 48 h point, afterwards the leptin secretion reached a plateau state; for this reason the integrated secretion was analysed in 48 h.

Analysis of leptin secretion over longer periods (96 h) did not qualitatively change the results obtained by the standard 48 h periods (data not shown). Spontaneous leptin secretion at both 24 and 48 h was significantly higher \( (P<0.01) \) in women than in men (Fig. 1, lower panel).

Analysed as the integrated secretion (AUC as ng leptin/g tissue per 48 h, Fig. 1, bars in upper panel), leptin secretion in female samples \( (n=21, 4149 \pm 301) \) was significantly higher \( (P<0.01) \) than that of male samples \( (n=23, 2456 \pm 428) \).

The addition of testosterone (1 µM) in the incubation medium throughout the 96 h of incubation, induced a moderate non-significant increase in the rate of leptin secretion into the medium of samples taken from both men and women (Fig. 2). This non-significant enhancement was quite consistent at any moment studied and when analysed as integrated secretion in 48 h (see later Fig. 7), the secretion of leptin in samples treated with testosterone was higher \( (P<0.05) \) in female \( (n=15, 4856 \pm 366) \) than in male samples \( (n=16, 3322 \pm 505) \), but not different from the respective control values.

On the contrary, when the adipose tissue was incubated with DHT (1 µM), a clear-cut decrease in leptin secretion was observed in female, but not in male samples (Fig. 3). The time-period at which maximal reduction in leptin occurred was in women at the 24 h and in men at the 72 h period. Examined as ng leptin/g tissue per 48 h (Fig. 7), the spontaneous leptin release from tissue fragments from female donors \( (n=16, 4253 \pm 320) \) was significantly decreased by DHT \( (3119 \pm 322, P<0.05) \), while it was not affected in samples from male donors \( (n=15, 2188 \pm 405 \text{ in controls vs } 2042 \pm 430 \text{ in samples treated with DHT}) \).

When the adipose tissue fragments were incubated throughout the 96 h period in the presence of the non-aromatizable androgen stanozolol (1 µM), the spontaneous leptin secretion in samples from male donors was unaffected, while a clear decrease was observed in female samples (Fig. 4). The inhibitory action of stanozolol on
female samples was evident at the 24 h period ($P<0.01$). As integrated secretion (Fig. 7), in samples taken from women ($n=10$), the spontaneous secretion ($3906 \pm 511$) was inhibited by stanozolol ($2809 \pm 383$, $P<0.05$), but was not affected in those taken from men ($n=7$, $1254 \pm 425$ control vs $1553 \pm 671$ stanozolol).

As Fig. 5 shows, DHEA-S induced a decrease in leptin release on tissue samples taken from women ($P<0.05$ at the 24 h period and $P<0.01$ at 48 h), while in samples from men there was a weak inhibition which was only statistically significant at the 72 h point ($P<0.05$). The spontaneous release of leptin in female samples ($4321 \pm 420$) (Fig. 7) was significantly ($P<0.01$) inhibited by DHEA-S ($n=10$, $2996 \pm 473$), leaving again unaffected the secretion in male samples ($n=10$, $1670 \pm 510$ in controls vs $1596 \pm 528$ in samples with DHEA-S).

Incubation of adipose tissue fragments with 1 µM androstenedione (Fig. 6) induced a clear-cut inhibition of leptin secretion in samples from females. In fact, when the 24 h incubation periods were considered, a significant difference vs control samples was observed for all periods ($P<0.01$ at 24 and 48 h, $P<0.05$ at 72 and 96 h) in adipose tissue from female donors, while no action was observed in male samples. The integrated leptin secretion after the administration of 1 µM androstenedione (Fig. 7) was significantly lower ($P<0.01$, $2231 \pm 264$) than secretion in the control incubations ($4321 \pm 420$) in women’s samples ($n=10$). In male samples androstenedione was devoid of action, the spontaneous secretion ($n=10$; $1670 \pm 510$) not being altered by the steroid ($1605 \pm 544$).

It appears that adrenal androgens, such as DHEA-S and androstenedione, were more potent inhibitors of leptin secretion than either DHT or stanozolol.

Discussion

It is undisputed that the net amount of body fat is the main determinant of leptin concentrations in serum (Caro.
et al., 1996, Ferron et al., 1997, Leal-Cerro et al., 1998). However, the wide variability in leptin values from individuals with similar amounts of fat mass, and the lack of stoichiometry between fat mass and leptin values, suggest that in addition to the number and size of adipocytes, other factors could operate by modulating the release of leptin. As it is now accepted that women have higher serum leptin levels than men, even after adjusting for adiposity (Hassink et al., 1996, Ostlund et al., 1996, Rosenbaum et al., 1996, Tome et al., 1997), sex steroids have been considered as potential modulators of leptin release. In fact, oestradiol is an in vitro releaser of leptin with a potency similar to that of glucocorticoids (Casabiell et al., 1998), and this may well be the background for the gender-based differences in serum leptin concentrations.

It is not known whether, besides oestrogens, other sex steroids may contribute to leptin regulation in humans; testosterone, for example, has been suggested to exert a negative regulation over leptin secretion based on indirect evidence (Haffner et al., 1997, Jockenhovel et al., 1997, Matsuda et al., 1997, Sih et al., 1997, Tome et al., 1997). However controversial, the main support for a negative role of testosterone on leptin comes from the studies in hypogonadal men who present inappropriately high leptin levels that are normalized after testosterone replacement therapy (Jockenhovel et al., 1997, Sih et al., 1997). Nevertheless, in most of these studies the steroid treatment induced changes in body composition that may per se explain the leptin changes. Interestingly, when shorter treatment schedules were used to prevent variations in body composition, testosterone was devoid of relevant action over circulating leptin levels in both hypogonadal and eugonadal men (V Popovic and F F Casanueva, unpublished observations).

To further clarify this topic, in the present work the action of different androgenic compounds has been systematically studied in an adipose tissue organ culture

**Figure 5** Mean ± s.e.m. leptin secretion into the incubation medium after treatment with 1 μM DHEA-S, expressed as the increment over the respective control values for cumulative secretion (lines) or for each 24 h incubation period (bars); women n=10, men n=10. **P<0.01 or *P<0.05 vs control secretion in the same period.

**Figure 6** Mean ± s.e.m. leptin secretion into the incubation medium after treatment with 1 μM androstenedione, expressed as the increment over the respective control values for cumulative secretion (lines) or for each 24 h incubation period (bars); women n=10, men n=10. **P<0.01 or *P<0.05 vs control secretion in the same period.
Although subcutaneous fat is the main contributor to circulating leptin levels, the participation of the abdominal omental adipose tissue to the amount of circulating leptin is qualitatively similar to that of the subcutaneous fat (Masuzaki et al. 1995, Hardie et al. 1996, Halleux et al. 1998), the former being easier to prepare and manipulate due to its relative lack of fibrous and non-adipose components. Furthermore, in vitro leptin secretion has been followed for more than 96 h with no morphological or biochemical signs of tissue damage, and well-preserved leptin secretion. The good response to glucocorticoids and oestrogens plus the inhibition by forskolin (Casabiell et al. 1998), and the here reported inhibition by androgenic compounds, further support the functionality of the assay.

On the other hand, no histological evidence of gender-based differences in adipocyte size or number between women and men’s samples was found (Casabiell et al. 1998), ruling out differences in secretion rate as an explanation for the findings observed. In this study, steroids were employed at doses slightly over their habitual use in dispersed cells in order to compensate for the reduced accessibility to the inner cells of organ culture (Wabitsch et al. 1997).

The first observation of the present work was the confirmation, with different samples from different patients, of previous work (Casabiell et al. 1998) reporting that in vivo female adipose tissue samples secreted more leptin than those from male donors, a finding that may be the biological basis of the gender differences reported in vivo for leptin secretion. Interestingly enough, testosterone was devoid of inhibitory action on leptin secretion both in female and in male samples, showing only non-significant mild stimulatory activity.

In female samples of human omental adipose tissue, both DHT, the active metabolite of testosterone, and the non-aromatizable androgen stanozolol were clear inhibitors of leptin secretion. It is possible that the tendency of testosterone to increase leptin levels may be the final result of two antagonistic actions, i.e. an inhibitory one mediated by its conversion to DHT, plus the stimulatory one exerted by its previous aromatization to oestradiol (Gray et al. 1979). In fact, oestradiol is as potent as glucocorticoids for stimulating leptin secretion in vivo and in vitro (Casabiell et al. 1998). It is worthy of note that the two adrenal androgens tested, i.e. androstenedione and DHEA-S, were the strongest inhibitors of leptin secretion in samples from female donors. In fact, while DHT and stanozolol have inhibitory actions only in the first 48 h after their addition to the culture medium, the inhibitory action of the adrenal androgens was evident at all periods of incubation analysed until the 96 h point. This suggests a long-lasting activity of these compounds, or the fact that no receptor desensitization to their activity occurs. There is no clear explanation for such differences in potency among compounds that are acting through the same cytoplasmic receptor.
By far the most surprising finding was the weak activity, or lack of activity, of the androgens studied on leptin secretion by male samples in vitro. These results are consistent with our previous report in which both oestradiol and dexamethasone, which act as potent leptin secretagogues in female adipose tissue, were devoid of action in male samples (Casabiell et al. 1998). This then results in a generalized absence of response to steroid hormones (glucocorticoids, oestrogens and androgens) in adipose tissue coming from male donors. Considering that leptin secretion in male samples is inhibited in vitro by forskolin (Casabiell et al. 1998), a general lack of modulation may be ruled out. As the existence of androgen receptors in human adipose tissue is well established, and no known sex-based differences in the amount of steroids receptors have been reported in peripheral tissues, the biological basis for the observed gender-based differences in leptin secretion in vitro has made future molecular analysis mandatory. Whether these differences in leptin release reflect gender-based different regulatory mechanisms leading to altered transcription or stabilization of the Ob gene mRNA in adipocytes merits further study. Alternatively, it cannot be excluded that the androgenic action on leptin release may be mediated by indirect mechanisms of action such as regulation of lipolysis. In fact, androgens do regulate lipolysis (Xu et al. 1990), and fatty acids seem to modulate the expression of the Ob gene (Rentsch & Chiesi 1996), although, to date, a sex-based difference in lipolysis regulation seems an unlikely possibility.

In any case, the results here reported make it unlikely that the reported gender-based differences in circulating leptin levels in humans can be due to any androgenic action. First of all, the inhibitory role of androgens, if any, is little in comparison with the clear-cut stimulatory action of oestrogens. Secondly, both compounds, androgens and oestrogens, act exclusively on women’s samples, being devoid of effect on male-derived adipose tissue. These considerations, together with the well-known fact that women have higher levels of oestrogens than androgens, make minimal any role of circulating androgens in the regulation of plasma leptin levels in humans.

In conclusion: (a) it is confirmed that spontaneous leptin secretion by human omental adipose tissue in vitro is larger in female than in male samples; (b) testosterone is devoid of action on leptin secretion; and (c) androgenic steroids are potent inhibitors of leptin secretion in the adipose tissue of female donors, while they are scarcely or not effective in samples from men. The lack of effect of steroid compounds on male adipose tissue, together with the stimulatory role previously reported for oestrogens on female adipocytes, strongly suggests that the gender-based differences in leptin levels observed in vivo are due to the hormonally modulated leptin secretion in women rather than to an inhibitory effect of androgens on men, as previously postulated.

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