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Direct In Vitro Effects of Androgens and Estrogens on *ob* Gene Expression and Leptin Secretion in Human Adipose Tissue

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In the present study, we have explored, in vitro, the possibility that short exposure to androgens and estrogens for 24 h may directly influence leptin expression (ARNm and secretion) in sc adipose tissue from men and women. In men, only dihydrotestosterone at high concentration (100 n*M*) induced a reduction in leptin secretion and ob mRNA level. In women, 17β-estradiol (10–100 n*M*) increased *ob* mRNA expression (+180 to +500%) and leptin release (+75%). Moreover, in adipose tissue of women, the estrogen precursors testosterone (100 n*M*) and dehydroepiandrosterone (1 µ*M*) also induced an increase in leptin secretion (+84 and +96%, respectively), an effect that was prevented by the aromatase inhibitor letrozole. Finally, the stimulatory effect of 17β-estradiol observed in women was antagonized by the antiestrogen ICI_{182780}. Altogether, these results suggest that the sexual dimorphism of leptinemia in humans is mainly owing to the estrogen receptor–dependent stimulation of leptin expression in adipose tissue by estrogens and estrogen precursors in women.

Key Words: *ob* mRNA; leptin secretion; sex hormones; human adipose tissue.

Introduction

Leptin, the obese (*ob*) gene product *(1)*, is a hormone mainly produced in adipose tissue *(2,3)* and secreted by adipocytes. Leptin is considered *ob* a homeostatic signal that contributes to regulation of body weight through modulation of feeding behavior and energy expenditure *(4,5)*.

It is well established that the net amount of body fat is the main determinant of serum leptin concentration *(6)*. However, a wide variability in leptin values for individuals with similar amounts of fat mass suggests that factors other than the number and size of adipocytes could modulate the secretion of leptin *(7)*. Among these factors, the sex steroid hormones could be particularly implicated for the following reasons: First, circulating leptin levels are higher in women than in men even after correction for body fat *(8,9)*. Second, before puberty, plasma leptin levels are similar in boys and girls, and during puberty plasma leptin levels increase in parallel with estrogens in girls *(10,11)*. Third, leptin is involved in the regulation of gonadal and placental functions and gestation *(12–14)*. Fourth, estrogen treatment increases leptinemia of male to female transsexuals or of women who undergo bilateral ovariectomy *(15–17)*, and testosterone treatment normalizes the abnormally elevated plasma leptin levels of hypogonadal men *(18,19)* or of female to male transsexuals *(15)*. However, the precise role of sex hormones on human adipose tissue leptin expression and secretion still remains unclear.

To determine this role, we have investigated in vitro the influence of a short-term exposure to androgens and estrogens on the *ob* mRNA and leptin secretion in human adipose tissue. This study has been realized with adipose tissue from the sc area of both genders because *ob* gene expression is higher in this localization than in other deeper fat deposits in humans *(20,21)*, and the sc fat mass is the predominant fat depot in normal weight women and in men *(22)*.

Results

Levels of *ob* mRNA were investigated on isolated adipocytes rather than in adipose tissue fragments because *ob* gene is mainly expressed and produced in these cells *(3)*. However, we have chosen to investigate the effects of sex steroids on leptin secretion in culture media of adipose fragments and not of isolated adipocytes because the amounts of leptin secreted in culture media of adipocytes are generally far below the detection range of the leptin radioimmunoassay (RIA). Moreover, tissue culture presents two advantages: enzymatic damages to cellular structures are avoided, and small adipose cells are not lost, as is usually the case when adipose cells are isolated after enzymatic dispersion from fragments *(23)*.

A 24-h exposure was chosen because preliminary kinetic experiments revealed that this time gave optimal results compared with those obtained after 6 or 48 h (data not shown). The steroids were tested at dosages higher than those generally

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used in dispersed cells in order to compensate for the reduced accessibility of the molecules to the inner cells of the cultured tissue *(24)* and also because an important part of the added steroids binds to the bovine serum albumin (BSA) (1.5%) present in the culture medium, thus reducing the concentration of the free and active hormone fraction. Finally, the validity of our experimental conditions was warranted by the positive response of *ob* gene expression to glucocorticoids *(25,26)*.

Effects of Sex Steroids in Men

As can be seen in Fig. 1A, no change in *ob* mRNA expression could be observed after exposure to dihydrotestosterone (DHT) (10 n*M*), testosterone (100 n*M*), dehydroepiandrosterone (DHEA) (1 μ*M*), or 17β-estradiol (100 n*M*). However, a significant decrease in *ob* mRNA expression was observed in the presence of a supraphysiologic concentration of DHT (100 n*M*). Furthermore, under the same conditions, exposure to dexamethasone (10 n*M*) resulted in a 147% increase in *ob* mRNA level.

The amount of leptin secreted into the culture media from adipose tissue was unchanged by DHT (10 n*M*), DHEA (1 μ*M*), or 17β-estradiol (100 n*M*) but half reduced by DHT (100 n*M*) (Fig. 1B). Moreover, the reduction in leptin secretion and *ob* mRNA expression caused by DHT (100 n*M*) was completely abolished when adipose tissue or isolated adipocytes were exposed simultaneously to DHT (100 n*M*) and the potent androgen receptor (AR) antagonist cyproterone acetate (1 μ*M*), which had no effect *per se* (Fig. 1A,B).

Effects of Sex Steroids in Women

As shown in Fig. 2A, 24-h exposure to 10 n*M* 17β-estradiol resulted in a 180% increase in *ob* mRNA expression. This upregulation was further enhanced (+502%) when 100 n*M* 17β-estradiol was tested. Moreover, exposure to 10 n*M* dexamethasone also induced a clear stimulation of *ob* mRNA expression (+407%) in adipose tissue from female donors (Fig. 2A). However, exposure to 100 n*M* DHT had no effect on *ob* mRNA level.

Leptin secretion was also significantly enhanced by 100 n*M* 17β-estradiol (+75%), 1 μ*M* DHEA (+96%), and 100 n*M* testosterone (+84%) but not by 100 n*M* DHT. Interestingly, the stimulatory effect of testosterone was abolished when adipose tissue was simultaneously exposed to 10 μ*M* letrozole, an inhibitor of aromatase and testosterone (Fig. 2B).

Moreover, the upregulatory effects of 17β-estradiol on both *ob* mRNA expression and leptin secretion were totally prevented when adipose tissue fat cells were exposed to 1 μ*M* ICI_{182780}, a selective estrogen receptor (ER) antagonist having no influence *per se* on leptin secretion (Fig. 2B).

Discussion

In the present study, 100 n*M* 17β-estradiol and the different androgens (10 n*M* DHT, 1 μ*M* DHEA, and 100 n*M*

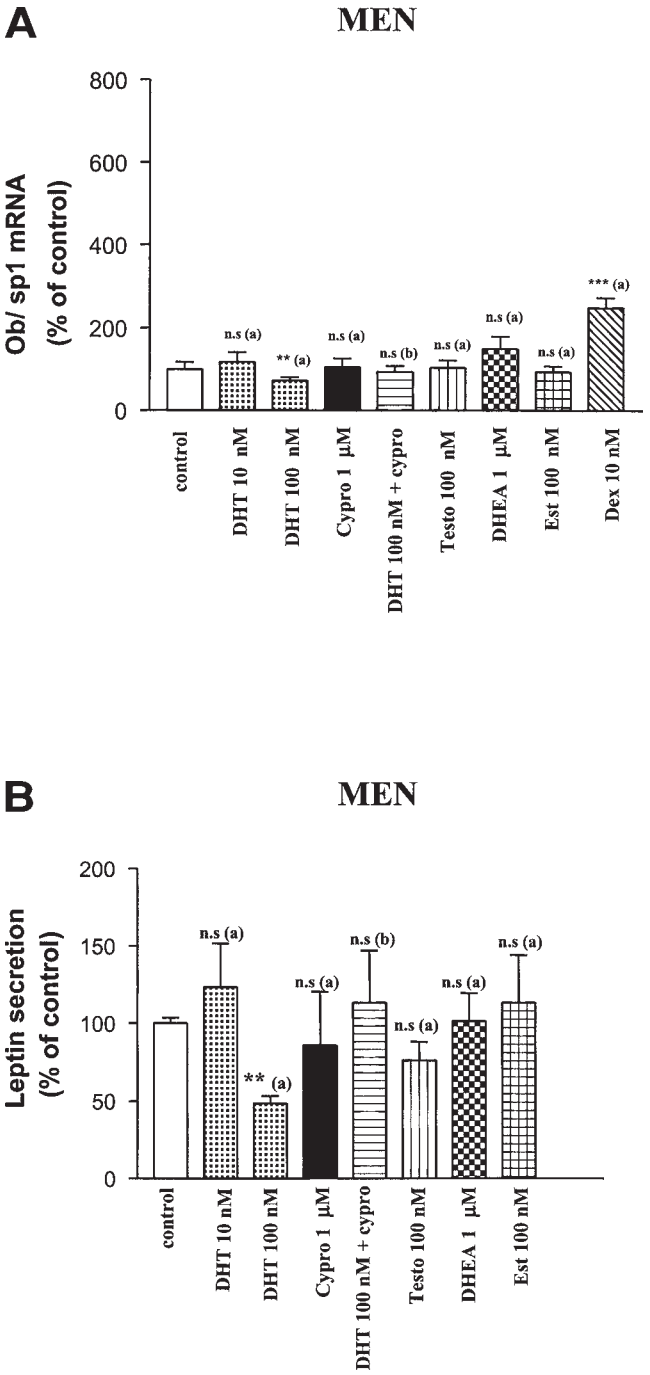

Fig. 1. In vitro effects of androgens and estrogens on *ob* mRNA and leptin secretion in men. Isolated adipocytes (**A**) or fragments (**B**) from adipose tissue of men donors were incubated for 24 h in the presence of DHT alone or combined with cyproterone acetate (Cypro) or testosterone (Testo) or DHEA or 17β-estradiol (Est) or dexamethasone (Dex) or without steroids (control). (A) Total RNA was extracted and subjected to reverse transcriptase polymerase chain reaction (RT-PCR) to determine *ob* mRNA levels using Sp1 as an internal standard. The band densities were quantified using an image analyzer. (B) At the end of the incubation, samples of the culture medium were collected and assayed for leptin by RIA. Results are means ± SEM of four to seven separate experiments and are normalized as percentage of the control value (without steroids) expressed as arbitrary units: for men = 50 ± 1.7 ng/(g of lipid·24 h). ***$p < 0.001$; **$p < 0.01$; ns, nonsignificant. (a) vs control; (b) DHT + Cypro vs Cypro.

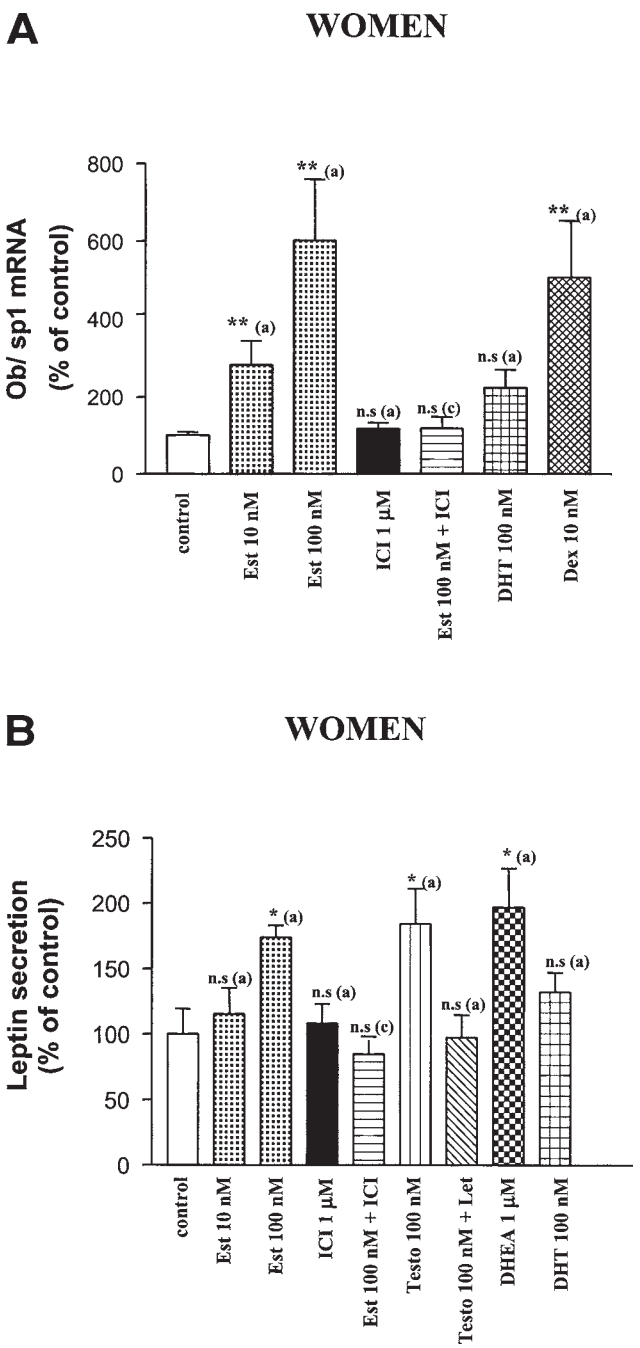

Fig. 2. In vitro effects of androgens and estrogens on *ob* mRNA and leptin secretion in women. Isolated adipocytes (**A**) or fragments (**B**) from adipose tissue of women donors were incubated for 24 h in the presence of 17β-estradiol (Est) alone or combined with ICI_{182780} (1 µ*M*), or in the presence of DHT or dexamethasone (Dex), or without steroids (control). Moreover, fragments (B) were incubated for 24 h in the presence of testosterone (Testo) alone or combined with letrozole (Let) or DHEA. (A) Total RNA was extracted and subjected to RT-PCR to determine *ob* mRNA levels using Sp1 as an internal standard. The band densities were quantified using an image analyzer. (B) At the end of the incubation, samples of the culture medium were collected and assayed for leptin by RIA. Results are means ± SEM of four to seven separate experiments and are normalized as percentages of the control values (without steroids) expressed as arbitrary units: for women = 56 ± 10.6 ng/(g of lipid.24 h). $^{**}p < 0.01$; $^{*}p < 0.05$; ns, nonsignificant. (a) vs control; (c) E2 + ICI vs ICI.

testosterone) tested failed to alter *ob* mRNA levels in human adipocytes from male donors. However, at a 10-fold higher concentration (100 n*M*) DHT, a nonaromatizable testosterone metabolite, induced a significant decrease in *ob* mRNA level and leptin secretion. These effects of DHT seem to be mediated through the ARs of human adipose tissue *(27,28)* because cyproterone acetate, a potent antagonist of these receptors, prevented the negative influences of high DHT concentrations on *ob* mRNA expression and leptin secretion. Our results are in accordance with other in vitro studies also reporting negative effects of androgens on leptin production in adipose tissue of obese adolescents *(24)* or in rat adipocytes *(29)*. Contrasting with the present data, inhibition of leptin secretion in vitro by DHT, androstenedione, and DHEA sulfate was recently reported in human female but not in male adipose tissue *(30)*. One possible explanation for these discrepant results could be the differences in the anatomic origin of the adipose samples studied: omental in ref. *(30)* vs sc in the present study. The present observation that leptin production from male sc adipose tissue is directly inhibited by supraphysiologic DHT concentrations thus warrants further study to establish the involved mechanisms.

We next examined the effects of estrogens, particularly 17β-estradiol, in adipose tissue from female donors. For the first time, we have observed a clear increase in both *ob* mRNA level and leptin secretion after 24-h exposure to 17β-estradiol. This effect seems to be mediated through the ERs because the antiestrogen ICI_{182780} prevented the positive effect of 17β-estradiol. These results are in accordance with those of another in vitro study that reported a positive effect of estradiol on leptin secretion in omental adipose tissue from women but not from men *(31)*. In addition, the present experiments also demonstrate a positive effect of testosterone on leptin secretion in human adipose tissue from female donors. The latter effect seems to require the conversion of testosterone into estradiol, which is dependent on aromatase, since the aromatase inhibitor letrozole abolished the testosterone stimulation of leptin secretion. In the same way, DHEA, the precursor of both testosterone and 17β-estradiol, also induced an increase in leptin release. However, in the presence of testosterone or DHEA, *ob* mRNA level was not modified in adipocytes from female donors (data not shown), which strongly suggests that this negative finding is linked to the very low aromatase expression observed in human adipocytes as compared to human preadipocytes *(32,33)*. Thus, the positive effects of testosterone and DHEA on leptin secretion in female adipose tissue appear to be essentially related to estradiol production by the preadipocytes. However, this conclusion does not explain why in male adipose tissue physiologic androgen concentrations have no effect on *ob* mRNA level and leptin secretion.

It is well established that sc adipose tissue from women is much more responsive to estrogens than adipose tissue from men *(31)*. The same sexual dimorphism was observed here for *ob* mRNA expression in response to dexamethasone,

confirming a previous report *(31)*. Although unestablished, the molecular basis of this sex specificity could involve different pattern of expression for the α and β ER subtypes (unpublished data) but not for ARs *(28)* and also for nuclear receptor cofactors in adipose tissue from males and females. In fact, these nuclear receptors regulate the transcriptional activity of specific genes by recruiting an array of coactivator proteins, including steroid receptor coactivator 1, whose expression was reported to be sex specific *(34)*.

As already demonstrated by us in rat adipocytes *(29)*, the increase in *ob* mRNA observed in the presence of 17β-estradiol could reflect a direct transcriptional effect of the estrogen on *ob* gene expression. Adding further weight to this suggestion are (i) our observation of a nonclassic estrogen-response element in the distal region of the cloned 3-kb human leptin promoter sequence (unpublished data), as was recently reported for the vascular endothelial growth factor gene *(35)*, and (ii) evidence demonstrating the presence of several Sp1-responsive elements in the human *ob* gene promoter region *(36)*. Thus, an ER-Sp1 association could represent an estrogen-mediated transactivation pathway that would not require direct ER binding to DNA, as recently described for various other genes *(37–39)*. Moreover, a recent study *(40)* has reported activation of the leptin gene promoter (3 kb) by estrogens through ERα in transiently transfected JEG-3 choriocarcinoma cells.

Finally, the positive effect of 17β-estradiol and its androgenic precursors DHEA and testosterone on in vitro leptin expression and/or secretion in female adipose tissue allows us to suggest that estrogens play a prominent role in the gender-specific differences in leptinemia observed in several in vivo studies.

In conclusion, the present in vitro study indicates that androgens have essentially no effect on leptin expression in adipose tissue of men. Conversely, estrogens positively modulate *ob* gene expression and leptin secretion in adipose tissue of women. Investigations using transfection systems are currently in progress in our laboratory to establish further the molecular mechanism of the *ob* gene positive regulation by estrogens in human adipocytes.

Materials and Methods

Materials

Superscript II RNase H-RT was obtained from Gibco-BRL (Grand Island, NY). Phenol red–free Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (50:50 mix), penicillin, streptomycin, vitamin E, phenylmethylsulfonyl fluoride (PMSF), BSA, DHT, DHEA, 17β-estradiol, testosterone, cyproterone acetate, and dexamethasone were purchased from Sigma (St. Louis, MO). ICI_{182780} was from Tocris (Bristol, UK). Letrozole was obtained from Novartis (Rueil-Malmaison, France). *Taq* Polymerase and RNA guard were provided by Pharmacia (Uppsala, Sweden). An RIA kit specific for human leptin was purchased from Linco (St. Charles, MO).

Subjects

Adipose tissue samples (40–60 g) were obtained from sc fat depots of women (mean age: 46.8 ± 7 yr) or men (mean age: 59.5 ± 4 yr). The tissue donor group was composed of six women (body mass index [BMI]: 23.9 ± 1.6 kg/m^2) and six men (BMI: 26.2 ± 1.2 kg/m^2). None of these patients suffered from malignant endocrine or chronic inflammatory diseases. All patients were undergoing elective surgery in accordance with the local ethical committee.

Tissue Preparation and Culture Conditions

The adipose tissue samples were collected in saline (150 m*M* NaCl) and immediately transferred to the laboratory. After removing blood vessels and connective tissue, adipose tissue was rinsed in saline containing antibiotics (100 U/mL of penicillin and 0.1 mg/mL of streptomycin) and cut into small pieces.

To study the effect of sex steroids on leptin secretion, tissue fragments were placed in 12-well dishes (300 mg of adipose tissue/well) containing 3 mL of DMEM/F12 supplemented with antibiotics, BSA (1.5%), PMSF (58 μ*M*), leupeptin (4 μ*M*), vitamin E (4 mg/mL), and the hormones or drugs tested for 24 h at 37ºC under 5% CO_2 and 95% air atmosphere as described elsewhere *(28)*. Twenty-four hours later, aliquots were taken from culture media and kept at –20ºC until they were used for leptin assay. When added to the medium, steroid hormones and antagonists of ARs and ERs were dissolved in ethanol (final ethanol concentration never exceeding 0.01%).

The effects of sex steroids on ob mRNA expression were investigated in mature adipocytes isolated as previously described *(35)* and then exposed for 24 h to the hormones and drugs tested.

RNA Extraction

Total RNA was isolated from the isolated adipocytes using the guanidium isothiocyanate procedure described by Chomczynski and Sacchi *(41)*. RNA recovery and quality were checked by measuring the 260/280 nm optical density ratio.

Reverse Transcriptase Polymerase Chain Reaction (42)

Total RNA was denatured for 10 min at 72ºC and reverse transcribed to cDNA as follows: Total RNA (0.5 μg) was incubated with 20 μL of reverse transcription mixture containing 50 m*M* Tris-HCl, 75 m*M* KCl, 3 m*M* $MgCl_2$, 20 m*M* dithiothreitol, 0.5 m*M* each dNTP, 16 mU of RNA guard, 50 ng of random hexamers, 200 U of Superscript II RT in a 20-μL final volume. Incubation was performed at 42ºC for 60 min, heated to 95ºC for 5 min and then quickly chilled on ice.

In this semiquantitative RT-PCR method, two different primer sets were used. One primer set used for the human *ob* gene had the following sequences: sense: 5'-GAC ACC AAA ACC CTC ATC AAG-3'; antisense: 5'-ATG TCC TGC

AGA GAG CCC TG-3'. With this primer set, the PCR generated a 383-bp fragment of the *ob* gene. To be sure that amplification of the *ob* gene was within the exponential range, different numbers of PCR cycles (20–30) were run. Finally, 24 cycles of PCR amplification were used to detect the *ob* mRNA.

The second primer set was specific for the human Sp1 gene, an ubiquitously housekeeping gene that is thus used as internal standard. Human Sp1-specific primers had the following sequences: sense: 5'-GAG AGT GGC TCA CAG CCT GTC-3'; antisense: 5'-GTT CAG AGC ATC AGA CCC CTG-3'. With this primer set, the PCR generated a 231-bp fragment of the Sp1 gene. In the same way, different numbers of PCR cycles (25–40) were run. Finally, 34 cycles of PCR amplification were found to be optimal for detection of the Sp1 mRNA. PCRs were performed with a thermocycler Gene Amp PCR 2400 (Perkin Elmer).

PCR products were analyzed on a 2% agarose gel in 90 m*M* Tris-borate, 2 m*M* EDTA buffer (TBE) (pH 8.0) and visualized by staining with ethidium bromide and ultraviolet transillumination. Quantification was realized with the Bio-gene software. Controls without RT were systematically performed in order to detect eventual genomic DNA contaminations.

Leptin Assay

Leptin levels in the incubation media were determined with a commercially available RIA kit specific for human leptin *(43,44)* using a human leptin antibody produced in rabbit. Leptin concentrations were within the detection range of the kit (0.5–100 ng of leptin/mL).

Statistical Analyses

All values were expressed as means ± SEM of four to seven different experiments, and statistical analyses were performed using analyses of variance with Bonferroni *p*.

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