Leptin directly stimulates aromatase activity in human luteinized granulosa cells

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Leptin, the obese (ob) gene product, is secreted by adipocytes and regulates appetite through interaction with hypothalamic leptin receptors. Leptin may also have a stimulatory effect on reproductive function. Furthermore, leptin receptor mRNA is expressed in the ovary, suggesting a direct effect on its function. The present study examines the direct role of leptin on the oestrogen-producing activity in human luteinized granulosa cells. The cells were obtained from in-vitro fertilization pre-ovulatory follicles, precultured for 24 h in the presence of 5% charcoal-treated serum, and incubated for 48–96 h in a serum-free medium containing recombinant human leptin, follicle stimulating hormone (FSH), and/or insulin-like growth factor-I (IGF-I). A single addition of leptin (0.5–10 ng/ml) stimulated aromatase activity with the incubation time of up to 96 h. The addition of leptin (1 ng/ml) further augmented the stimulation by a single addition of FSH (100 ng/ml) or IGF-I (100 ng/ml), or a combination of both. A single addition of leptin (1 ng/ml) or a combination of leptin (1 ng/ml), FSH (100 ng/ml), and IGF-I (100 ng/ml) gave rise to an increase in each parameter of oestrogen-producing activity measured, i.e. P450arom mRNA level, P450arom protein level, aromatase specific activity, and the oestradiol concentration in the culture supernatant. However, the production of progesterone did not change. These results indicate that leptin stimulates oestrogen production by increasing P450arom mRNA and P450arom protein expression and, consequently, aromatase activity by its direct action on the human luteinized granulosa cells.

Key words: aromatase/cytochrome P450/leptin/luteinized granulosa cells/oestrogen

Introduction

Leptin, the obese (ob) product, is synthesized and secreted by adipocytes (Zhang et al., 1994) and regulates appetite through interaction with hypothalamic leptin receptors (Tartaglia et al., 1995). Leptin binds to its receptors on the cell membrane and is involved in the activation of STAT3, a member of the signal transducer and activator of transcription family of proteins (Vaisse et al., 1996). Injecting leptin into ob/ob mice that are infertile with low gonadotrophin values increases the weight of the uterus and ovaries and the number of follicles (Barash et al., 1996), resulting in restoration of fertility (Chehab et al., 1996). Administering leptin treatment to normal female mice accelerates puberty (Ahima et al., 1997) and higher leptin levels have been shown to relate to the earlier onset of menarche in humans (Matkovic et al., 1997). These findings indicate that leptin is not only involved in energy metabolism but also may have a stimulatory effect on the reproductive function. Leptin is expressed in pre-ovulatory granulosa and cumulus cells in the human ovary (Cioffi et al., 1997), and leptin and STAT3 are expressed in polarized domains in human oocytes (Antczak and Van Blerkom, 1997, 1999). Furthermore, leptin receptor isoforms are expressed not only in the hypothalamus but also in luteinized granulosa cells (Cioffi et al., 1997; Karlsson et al., 1997) and cumulus cells (Cioffi et al., 1997) in the human ovary, suggesting that leptin may act directly on the ovary and modulate its function.

Adequately-regulated production of oestrogens by the ovary is essential in follicular development, ovulation and luteal function. Oestrogens are biosynthesized from androgens by aromatase, the major component of which is aromatase cytochrome P450 (P450arom) which is predominantly localized in granulosa cells (Tamura et al., 1992). The aromatase activity in granulosa cells is stimulated mainly by follicle stimulating hormone (FSH) in co-operation with insulin-like growth factor-I (IGF-I) (Erickson et al., 1989). In the culture system of rat (Zachow and Magoffin, 1997) and bovine (Spicer and Francisco, 1997) pre-ovulatory granulosa cells, leptin had no effect on basal oestrogen production; however, leptin inhibited IGF-I- (Zachow and Magoffin, 1997) or insulin-stimulated (Spicer and Francisco, 1997) oestrogen production, suggesting that leptin has a direct negative effect on ovarian function. However, the role of leptin on the steroidogenesis in the human reproductive tissues has not yet been investigated. The present study determines the direct role of leptin on oestrogen-producing activity in human luteinized granulosa cells.

Materials and methods

Chemicals

Recombinant human leptin was purchased from Immugenex (Los Angeles, CA, USA), human FSH from Sigma (St Louis, MO,
USA), and human recombinant IGF-I from Toyobo (Osaka, Japan). Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (1:1) with 15 mmol/l HEPES buffer without Phenol Red (DMEM/F-12) was purchased from Gibco-BRL (Grand Island, NY, USA).

Patients
Ovarian follicular aspirates were obtained from 33 women with a mean body mass index of 21.0 ± 2.4 (mean ± SD) undergoing oocyte retrieval as part of the in-vitro fertilization/embryo transfer programme at the Kyoto Prefectural University of Medicine. Informed consent was obtained from each patient. Patients were given intranasally 900 µg/day buserelin acetate (Suprecuro®; Hoechst Marion Roussel, Tokyo, Japan) starting from day 21 of the preceding cycle or from day 1 of the present cycle. Patients were given i.m. injections of human menopausal gonadotrophin (HMG, Humegon®; Organon, Oss, The Netherlands) or purified FSH (Fertinom P®; Serono, Geneva, Switzerland) at a daily dose of 150–300 IU until the dominant follicle reached 18 mm in diameter measured by transvaginal ultrasound sonography. Patients were injected i.m. with 10 000 IU human chorionic gonadotrophin (HCG) (Mochida, Tokyo, Japan) and after 36 h, oocyte retrieval was performed. There was no difference in the oestriadiol concentrations in the follicular fluid and the clinical outcome between the patients given injections of Humegon and Fertinom P.

Cell culture
After removing the oocytes, the aspirates were pooled and centrifuged at 300 g for 5 min. The cell pellets were resuspended in 0.25% collagenase (type 1; Sigma) in 10 ml of DMEM/F-12 medium, and dispersed by incubation at 37°C for 60 min. The suspension was centrifuged at 300 g for 5 min and the pellet was resuspended in 1.0 ml of DMEM/F-12 medium. The cell suspension was layered on to a 5 ml 50% Percoll® (Pharmacia Biotech, Uppsala, Sweden) solution and centrifuged at 400 g for 30 min to pellet the blood components. The granulosa cell layer collected at the middle part of the tube was collected by aspiration, and the cells were washed twice with DMEM/F-12 medium supplemented with 5% fetal bovine serum (FBS) (Gibco-BRL), penicillin (100 IU/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml) (antibiotics mixture), and then resuspended in a small volume of the same medium for cell counting. Cell viability was determined by Trypan Blue dye exclusion test and was found to be consistently >90%. The FBS was treated twice with charcoal (6.25 mg/ml) and Dextran T-70 (0.625 mg/ml), and then incubated at 56°C for 30 min to remove endogenous cytokines and steroids.

Each preparation of granulosa cells obtained from a single patient was inoculated into several 35 mm culture dishes (Becton Dickinson Labware, Lincoln Park, NJ, USA) at a density of 1 × 10^5 viable cells/2 ml/dish. The number of cells used in each individual experiment remained constant. The cells were precultured at 37°C in a humidified atmosphere of 5% CO_2-95% air for 24 h in the same medium containing 5% charcoal-treated FBS to let the cells attach to the dishes. The medium was discarded and the dishes were washed twice gently with the same medium but without serum. Then the cells were incubated in the serum-free medium containing the compound to be tested. To assay the oestradiol in the culture supernatant, 1 µmol/l of Δ_4-androstenedione (Sigma) was added together at this moment.

**Assay of aromatase activity**
Aromatase activity was determined by the tritiated water method (Bellino and Osawa, 1977) as previously described (Kitawaki et al., 1992) with modifications. The granulosa cells in 35 mm culture dishes were gently rinsed with DMEM/F-12 medium and incubated for 20 min at 37°C in a humidified atmosphere of 5% CO_2-95% air with 0.5 ml of medium containing the antibiotic mixture and [1β-^3H]-androstenedione (Dupont-New England Nuclear, Boston, MA, USA; 6.0 × 10^6 dpm, 150 pmol). The medium was transferred to a test tube, 0.2 ml of 20% trichloroacetic acid and 1.0 ml of 5% charcoal were added, and the mixture was incubated at 37°C for 30 min. The mixture was centrifuged at 800 g for 10 min and the supernatant was filtered through a cotton-plugged disposable pipette. The amount of ^3H-water in the eluate derived from the substrate was assessed using the 1β-elimination mechanism (75% release into water) (Bellino and Osawa, 1977). The tritiated water method was validated by the product isolation method as previously described (Kitawaki et al., 1992), and the data showed good agreement. The radioactivity of a dish containing no cells was subtracted from each count; however, it was usually negligible. Protein concentrations were determined by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as the standard.

**DNA isolation and reverse transcription–polymerase chain reaction (RT–PCR)**
Total RNA was extracted as previously described (Kitawaki et al., 1997) using Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA). The first-strand cDNA synthesis from total RNA was catalysed by Superscript II RT (Gibco-BRL) using oligo(dT)12–18 as previously described (Kitawaki et al., 1997). The resulting first-strand cDNA was used for PCR amplification with the following primers: 5'-CAAGGTATTTTGTATGCAATG-3' (forward, nucleotides 776–796) and 5'-TCTAAGGCTTTGGCCATGAC-3' (reverse, nucleotides 1361–1341) for human P450arom (Harada et al., 1992), and human G3PDH amplifier set for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Clontech, Palo Alto, CA, USA). The PCR mixture comprised 1 µl first-strand cDNA, 1 µmol/l each of the primers mentioned above, 0.2 mmol/l dNTP, and 2.5 IU KOD Dash (Toyobo, Osaka, Japan), in a total volume of 100 µl of PCR buffer provided by the manufacturer. The PCR conditions were 94°C for 3 min to denature the RNA/cDNA hybrid, then cycles of 94°C for 30 s, 55°C (for P450arom) or 60°C (for G3PDH) for 2 s, and 74°C for 30 s.

**Measurement of relative change in P450arom mRNA**
Duplicate aliquots of first-strand cDNA synthesized from control luteinized granulosa cells were subjected to PCR amplification for 16–32 cycles with two cycle intervals. The PCR products for P450arom and G3PDH were electrophoresed in 3% agarose gel and stained with ethidium bromide. Gels were photographed and scanned into a computer. The stained intensity of each band was measured by image analysing software, NIH Image 1.61. The intensity increased exponentially with PCR cycles up to 22 cycles until it eventually reached a plateau. Since the regression lines, which were determined by the linear portion of the curves, were parallel, we therefore set the PCR amplification at 22 cycles. To estimate the initial ratio of the P450arom mRNA amount among specimens, relative ratios of P450arom/G3PDH were compared.

**Determination of P450arom value**
The tissue value of P450arom was determined by sandwich enzyme-linked immunosorbent assay, as described previously (Kitawaki et al., 1989) with modifications. Briefly, after measuring the aromatase activity, the cells were homogenized in 10 mmol/l potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, 0.2% Emulgen 913 (donated by Kao Co, Tokyo, Japan), and 0.5 µmol/l Δ_4-androstenedione (buffer A), and centrifuged at 12 000 g for 10 min. Microtitre wells that were precoated with mouse monoclonal
antibody against human placental P450arom (MAb3-2C2) (Washida
et al., 1996) were incubated for 4 h at room temperature with
solubilized samples to be examined which had been serially diluted
with buffer A containing 1% BSA. Serially diluted purified P450arom
(Yoshida and Osawa, 1991) was used for the standard. The wells
were washed and incubated for 4 h at room temperature with rabbit
anti-human placental P450arom antiserum (PAb R-8–2) at a 1:3 000
dilution. The wells were washed and incubated for 4 h at room
temperature with donkey anti-rabbit immunoglobulin G (IgG) anti-
body horseradish peroxidase conjugate at a 1:20 000 dilution. After
the wells were washed, the wells were coloured by 3,3’,5,5’-
tetramethylbenzidine peroxidase substrate kit (Bio-Rad). The absorb-
ency was measured by the difference between those at 450 nm and
655 nm on a microplate reader Model 450 (Bio-Rad).

**Assay of oestradiol and progesterone**

The concentrations of oestradiol and progesterone in the culture
supernatant were measured by the corresponding enzyme immuno-
assay kits (Assay Designs, Ann Arbor, MI, USA) according to the
manufacturer’s protocol.

**Statistical analysis**

The figures were generated based on the experimental results from
33 separate culture series of the cells obtained from 33 patients. Data
are expressed as the mean ± SEM of a minimum of four replicate
cultures with duplicate determinations per culture. Statistical analyses
were performed by paired t-test and multiple comparisons are per-
formed using Bonferroni/Dunn’s procedure. *P < 0.05 was considered
to be significant.

**Results**

Luteinized granulosa cells were precultured for 24 h in the
presence of 5% charcoal-treated serum, incubated for various
periods of time in the serum-free medium containing leptin,
and then aromatase activity was measured. The activity
increased with the incubation time up to 96 h at a leptin
concentration of 1 ng/ml (*P < 0.01) and 10 ng/ml (*P < 0.05).
The extent of stimulation by 1 ng/ml leptin was greater than
that by 10 ng/ml (*P < 0.05) (Figure 1). In contrast, aromatase
activity was not changed by the addition of either concentration
of leptin under the condition without preculture with serum.
The cells were then exposed to varying doses of leptin for
72 h before the aromatase assay. A significant rise in the
activity was observed at leptin concentrations between 0.5
and 10 ng/ml, with the highest of 1.9 times that of the control at
1 ng/ml (*P < 0.001) (Figure 2). We therefore set the optimal
incubation condition for leptin at 72 h and 1 ng/ml with
preculture in the presence of serum. Data obtained from such
clinical materials fluctuated to a greater extent. However, under
our experimental conditions using the cells obtained from 21
patients, we observed consistently stimulatory, but not any
inhibitory, effect of leptin on the aromatase activity (Figure 3).

Single addition of FSH (100 ng/ml, *P < 0.01) or IGF-I
(100 ng/ml, *P < 0.0001) stimulated the aromatase activity
(Table I). Addition of FSH with IGF-I resulted in a greater
rise in the activity than that of each alone (*P < 0.001 and
*P < 0.05 respectively). When leptin was added in combination
with FSH, the aromatase activity was greater than that for
leptin or FSH alone (*P < 0.05). Similarly, the activity was
greater for combination of leptin and IGF-I than each alone
(*P < 0.01 and *P < 0.05 respectively). The stimulation by
combining FSH and IGF-I was augmented by the further
addition of leptin (*P < 0.001) (Table I).
Leptin stimulates aromatase in granulosa cells

Discussion

The present study indicates that leptin stimulates oestrogen-producing activity by increasing P450arom mRNA, P450arom protein expression, and aromatase activity by its direct action on the human luteinized granulosa cells. Leptin further augments oestrogen production stimulated by FSH and/or IGF-I, which have been known to be predominant stimulators of aromatase (Erickson et al., 1989). This may suggest that in a physiological hormonal environment, leptin is still stimulatory.

In animal studies using granulosa cells collected from pre-ovulatory antral follicles, a single addition of leptin had no effect on the oestrogen production of rat (Zachow and Magoffin, 1997) and bovine (Spicer and Francisco, 1997) cells. Leptin inhibited oestrogen production stimulated by IGF-I in rat (Zachow and Magoffin, 1997) and by insulin in bovine (Spicer and Francisco, 1997) granulosa cells. In contrast, the present study employed luteinized granulosa cells collected after HCG stimulus. One possible explanation for the different effects of leptin on animal versus human granulosa cells is that the former were non-luteinized whereas the latter were luteinized cells. Under our experimental conditions, repeated over 30 times using the cells obtained from >30 patients, we observed consistently stimulatory effects of leptin on the oestrogen-producing parameters. Although the data obtained from such clinical materials fluctuated to a greater extent, no inhibitory effect was observed throughout the experiments. We showed that preculture of cells with charcoal-treated serum was necessary for stimulation by leptin. Instead, the extent of stimulation by FSH and/or IGF-I was weakened compared with that in previous studies (Erickson et al., 1989). By contrast, the leptin action was negligible when cells were incubated with leptin in serum-free conditions from the beginning as shown in the present study and the animal studies (Spicer and Francisco, 1997; Zachow and Magoffin, 1997). It should be noted that the maximum effective concentration of leptin was 1 ng/ml both when leptin inhibited oestrogen production in animal non-luteinized granulosa cells and when leptin stimulated it in human luteinized cells. In contrast to stimulation of aromatase activity, the present study showed that leptin had no effect on progesterone production. However, since we did not determine 3β-hydroxysteroid dehydrogenase activity or mRNA, further studies are needed.

Systemic administration of leptin into ob/ob mice results in an increased uterine and ovarian weight and number of follicles (Barash et al., 1996), and restoration of fertility (Chehab et al., 1996). Administering leptin treatment to normal female mice accelerates puberty (Ahima et al., 1997). In humans, the higher leptin values are related to the earlier onset of menarche (Matkovic et al., 1997). These stimulatory actions of leptin on the reproductive functions have been explained to be secondary effects after its primary action on the neuroendocrine system via its hypothalamic receptors. In addition to the systemic actions, leptin has its receptors in various peripheral tissues including luteinized granulosa cells (Cioffi et al., 1997; Karlsson et al., 1997) and cumulus cells (Cioffi et al., 1997) in the human ovary. The present results provide evidence that leptin acts directly on the ovary and modulates its function.

Table I. Effect of leptin on follicle stimulating hormone (FSH) and/or insulin-like growth factor-I (IGF-I) stimulation of aromatase activity

<table>
<thead>
<tr>
<th></th>
<th>Aromatase activity (pmol/h/mg protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>12.0 ± 0.7</td>
</tr>
<tr>
<td>Leptin</td>
<td>16.7 ± 0.7</td>
</tr>
<tr>
<td>FSH</td>
<td>16.7 ± 0.7</td>
</tr>
<tr>
<td>FSH + leptin</td>
<td>20.0 ± 0.7</td>
</tr>
<tr>
<td>IGF-I</td>
<td>21.4 ± 0.6</td>
</tr>
<tr>
<td>FSH + IGF-I</td>
<td>21.6 ± 0.6</td>
</tr>
<tr>
<td>FSH + IGF-I + leptin</td>
<td>26.5 ± 1.5</td>
</tr>
</tbody>
</table>

Cells were precultured in the presence of 5% charcoal-treated serum, incubated in the serum-free medium for 72 h with single or concomitant addition of leptin (1 ng/ml), FSH (100 ng/ml), and/or IGF-I (100 ng/ml), and then aromatase activity was determined. Each assay pair of a single patient is linked. (A) Actual aromatase activity. **P < 0.001 versus control. (B) Replotted as percentage change versus controls.

Figure 3. Stimulation of aromatase activity by leptin in human luteinized granulosa cells. Cells obtained from 21 patients were individually cultured with or without leptin (1 ng/ml) for 72 h, and aromatase activity was determined (see text). Each assay pair of a single patient is linked. (A) Actual aromatase activity. **P < 0.001 versus control. (B) Replotted as percentage change versus controls.

We then measured various parameters related to oestrogen-producing activities. Incubation with 1 ng/ml leptin for 72 h resulted in a significant rise to a similar degree in each parameter of P450arom mRNA level, P450arom protein level (P < 0.0001), aromatase activity (P < 0.01), and the oestradiol concentration (P < 0.001) in the culture supernatant. However, the progesterone concentration in the culture supernatant was not increased significantly (Figure 4A). Actual control data of P450arom protein, aromatase activity, oestradiol, and progesterone concentrations were (23.0 ± 3.1)×10⁻⁴%, 12.0 ± 1.9 pmol/h/mg protein, 19.3 ± 2.4 nmol/l and 1.8 ± 0.3 μmol/l respectively. Similarly, each parameter except progesterone was increased to a similar degree after the cells were incubated with a combination of leptin (1 ng/ml), FSH (100 ng/ml) and/or IGF-I (100 ng/ml) (Figure 4A, B).

Table II. Effect of leptin on oestradiol (E2) and progesterone (P4) production

<table>
<thead>
<tr>
<th></th>
<th>Oestradiol (pmol/l)</th>
<th>Progesterone (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Leptin</td>
<td>21.4</td>
<td>0.9</td>
</tr>
<tr>
<td>FSH</td>
<td>26.5</td>
<td>1.1</td>
</tr>
<tr>
<td>FSH + leptin</td>
<td>21.4</td>
<td>0.9</td>
</tr>
<tr>
<td>IGF-I</td>
<td>21.6</td>
<td>1.1</td>
</tr>
<tr>
<td>FSH + IGF-I</td>
<td>21.6</td>
<td>1.1</td>
</tr>
<tr>
<td>FSH + IGF-I + leptin</td>
<td>26.5 ± 1.5</td>
<td></td>
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Each letter within a letter pair is also different (a,b,c,d,e,f,g,h,i,j,k,l,m,n). *P < 0.05; †P < 0.01; ‡P < 0.001; ‡‡P < 0.0001.
The serum leptin concentrations are correlated simply with body fat mass (Maffei et al., 1995; Considine et al., 1996) and do not fluctuate during the menstrual cycle (Mills et al., 1998), while several studies (Cioffi et al., 1997; Shimizu et al., 1997) reported that the serum leptin concentrations are higher in the luteal phase than in the follicular phase. In most reproductive-age women, the serum leptin values are 2–20 ng/ml (Laughlin and Yen, 1997; Shimizu et al., 1997), and in women with ovarian hyperstimulation, the serum values are compatible with those of the follicular fluid (Cioffi et al., 1997). Under the present experimental conditions, the optimal leptin concentration was 1 ng/ml, which is lower than the physiological range. The extent of stimulation was weakly decreased when the leptin concentration was higher. It is suggested that in lean women whose serum leptin concentrations are low, leptin gives weak stimulation on oestrogen production, and that in obese women whose serum leptin concentrations are high, leptin has little effect. The greater the body weight, the less leptin stimulates oestrogen production. The present data may suggest that within the physiological variation of the serum leptin values, the single action of leptin accounts for the change of aromatase stimulation to a lesser extent. However, the local sensitivity to leptin, i.e., the extent of leptin receptor expression in the granulosa cells, may vary during follicular and luteal phases. Further studies are needed to estimate the extent of the direct biological role for leptin on follicular and luteal development.

In conclusion, the present results indicate that leptin stimulates aromatase and thus oestrogen production by its direct action on the human luteinized granulosa cells. Leptin further augments the FSH- and/or IGF-I- stimulation of oestrogen production.

References
Leptin stimulates aromatase in granulosa cells


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