Evidence of leptin expression in normal and polycystic human ovaries

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Leptin, the ‘obese’ protein, is found in cultured granulosa cells derived from human pre-ovulatory follicles. However, the occurrence of leptin has not been studied in intact ovaries, either normal or polycystic, until now. Paraffin sections from 25 human ovaries of different cycle stages and 25 wedge resections of polycystic ovaries were investigated by means of immunochemistry. Additionally, three ovaries were available for reverse transcription–polymerase chain reaction analysis. Leptin-positive cells were located in the granulosa cells of pre-antral follicles, and distinctly in the thecal layer of intact and regressing antral follicles. In the corpus luteum (CL) in the developmental stage, the former epithelioid leptin-positive thecal cells became fibroblast-like in the septum. In the CL of the secretory stage, single leptin-positive cells were detected between luteal cells. In polycystic ovaries, leptin-positive cells were noted both in the hypertrophied thecal layer and in the luteinized granulosa layer. Our findings on leptin expression at the protein level were confirmed by a positive mRNA signal for leptin in granulosa cells and in the CL. Additionally, mRNA of the full-length leptin receptor OB-R and of the short isoforms B219.1–B219.3 was identified in granulosa cells and the CL, as well as in the cortex and medulla. We conclude that leptin is produced in the ovary and may act in autocrine and paracrine ways.

Key words: human/leptin/leptin receptor/ovary/polycystic ovary

Introduction

Leptin, the obesity protein that is produced by the adipose tissue (Zhang et al., 1994), regulates food intake and energy expenditure (Trayhurn et al., 1999) and also plays an influential role in reproduction, as indicated by leptin deficiency. Ob/ob mice from the C57BL/6J strain, which have a spontaneous mutation in the Ob gene and synthesize a truncated, inactive leptin molecule, develop profound obesity and become infertile (Coleman, 1973, 1978; Friedman, 1997). Fertility can be restored by treatment with human recombinant leptin (Chehab et al., 1996). Women undergoing IVF therapy who tend to obesity and show a reduced ovarian response display increased serum leptin concentrations, but unchanged leptin concentrations in follicular fluid (Bützow et al., 1999). For women with polycystic ovary syndrome (PCOS), whether or not high leptin levels participate in this disturbed gynaecological event is still a matter of debate (Mantzoros et al., 2000).

Leptin action in the ovary is conceivable, since the mRNAs for leptin and its receptors, both long and short isoforms (Glasow et al., 1998; Kutoh et al., 1998; Kitawaki et al., 2000), have been detected in granulosa and cumulus cells of pre-ovulatory follicles from women undergoing IVF (Cioffi et al., 1997). Furthermore, leptin immunofluorescence has been located in inner granulosa cells, and in non-fertilized and fertilized oocytes as well as in preimplantation stage embryos (Antczak and Van Blerkom, 1997). Leptin suppresses steroidogenesis in granulosa cell cultures when co-stimulated by FSH and dexamethasone (Barkan et al., 1999). Leptin also weakly inhibits gonadotrophin- and/or insulin-like growth factor (IGF)-I-induced steroidogenesis in bovine thecal and granulosa cells cultured for 2 days in serum-free medium (Spicer et al., 2000). However, other findings have demonstrated leptin’s stimulatory effect on aromatase activity and oestrogen production in luteinized granulosa cells from human pre-ovulatory follicles (Kitawaki et al., 1999). With regard to the intact ovary, leptin receptor mRNA has been found in porcine (Lin et al., 2000) and in human ovaries (Karlsson et al., 1997), although the long and short receptor isoforms were not distinguished. However, there is still little or no information on leptin localization in the intact human ovary. For this reason, the objective of this study was to analyse leptin and its receptor expression both at the protein and mRNA levels during various stages of follicle growth and in a corpora lutea (CL).
Table I. Design of the study

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th>Conservation/method</th>
<th>Number of samples from different ovarian cycle phases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Follicular phase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Development</td>
</tr>
<tr>
<td>Normal ovaries&lt;sup&gt;a&lt;/sup&gt; (21)</td>
<td>Fixed with 4% formaldehyde embedded in paraffin-wax/immunohistochemistry</td>
<td>7</td>
</tr>
<tr>
<td>Polycystic ovaries&lt;sup&gt;b&lt;/sup&gt; (25)</td>
<td>Fixed with 4% formaldehyde, embedded in paraffin-wax/immunohistochemistry</td>
<td>25</td>
</tr>
<tr>
<td>Normal ovaries&lt;sup&gt;c&lt;/sup&gt; (3)</td>
<td>Shock frozen in liquid nitrogen/simple RT-PCR</td>
<td>–</td>
</tr>
<tr>
<td>IVF-granulosa cells&lt;sup&gt;d&lt;/sup&gt; (5)</td>
<td>Shock frozen in liquid nitrogen/simple RT-PCR</td>
<td>5</td>
</tr>
</tbody>
</table>

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Materials and methods

**Tissues**

The design of the study and sample numbers are presented in Table I.

**Immunohistochemistry of normal ovaries as a retrospective study**

A total of 21 women of reproductive age (18–44 years) underwent oophorectomy for gynaecological diseases. The ovaries were fixed in 4% formaldehyde and embedded in paraffin wax at the Institute of Pathology, University of Leipzig. The timing of the patient’s menstrual cycle was determined by the pathohistological report on the endometrium. All ovaries were serially sectioned. Each section of 7 µm thickness was mounted on object slides coated with paper glue (Cementit<sup>e</sup>; Merz und Benteli AG, Niederwangen, Switzerland) for section adherence. The first section of each series was stained with haematoxylin and eosin for histological classification of the ovarian cycle: (i) follicular phase (n = 7) when large antral follicles were present and CL regression was advanced; (ii) luteal phases (n = 14) according to the criteria of Corner and Clement (Corner, 1956; Clement, 1987). In CL of the developmental stage (n = 4), the basal membrane had disappeared between luteinizing granulosa and thecal cells; the CL of the secretory stage (n = 5) consisted of large luteal cells and a well developed capillary bed; and the early regressing CL (n = 5) displayed luteal cells with vacuolated cytoplasm as a sign of degeneration and almost no capillaries.

**Immunohistochemistry of polycystic ovaries as a retrospective study**

Twenty-five small wedge resections of polycystic ovaries were reviewed at the Private Institute of Pathology, Ulm, Germany. The clinician had diagnosed PCOS and this was confirmed by the pathologist’s diagnosis. Since detailed clinical information was missing, we refer only to polycystic ovaries.

**Tissues for extraction of mRNA**

Two ovaries with CL at the secretory stage and one with CL at the developmental stage were obtained from women aged 36, 41 and 56 years, having undergone oophorectomy for non-malignant gynaecological diseases (Department of Gynaecology and Obstetrics, St Georg Hospital, Leipzig, Germany). Written consent from the patients had been obtained. Samples of ovarian cortex and medulla, CL and abdominal adipose tissue as positive controls were snap-frozen in liquid nitrogen and stored at −80°C until use. Furthermore, granulosa cells from mature follicles were obtained from five patients undergoing IVF therapy (Centre of Reproductive Medicine, Leipzig, Germany). The experimental protocol and the use of the cells were submitted to the patients’ written consent. Aspirates from different follicles of each patient were pooled and centrifuged in 45% isotonic Percoll<sup>f</sup> (Amersham Pharmacia Biotech, Freiburg, Germany) at 200 × g for 5 min. The upper band was removed. Cells were washed three times (300 × g for 3 min), and 1 × 10<sup>5</sup> cells/2 ml medium were seeded into a 6-well plate and cultured in complete Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 (GIBCO BRL, Grand Island, NY, USA) with 15 mmol/l HEPES, 22 mmol/l NAHCO<sub>3</sub> and 5% fetal calf serum for 3 days.

**Indirect immunohistochemistry**

We used the avidin-biotin-horseradish peroxidase (HRP) complex technique (Vectastain<sup>®</sup> Elite ABC kit; Vector Laboratories, Alexis, Grünberg, Germany) for immunohistochemistry. A polyclonal antibody against leptin 1:300, Ob (A-20) (Santa Cruz Biotechnology Inc., CA, USA) was used. For antibody dilution, phosphate-buffered saline (PBS), pH 7.4 with 0.25% bovine serum albumin (BSA; A-4378, Sigma, Deisenhofen, Germany) and for wash, buffer, 0.05 mol/l Tris-HCl-buffered saline (TBS, pH 7.6) were prepared. Staining was performed as described previously (Löffler et al., 2000). Briefly, deparaffinized sections were pre-treated for antigen retrieval with 0.05% pronase E (type XIV; Sigma) in 0.5 mol/l Tris-HCl, pH 7.6, at 37°C for 15 min. Endogenous peroxidase activity was quenched with 0.3% H<sub>2</sub>O<sub>2</sub> in absolute methanol for 30 min. For reduction of the background staining, the sections were treated with 1.5% normal goat serum (Vector Laboratories) for 20 min. The samples were incubated with the primary antibody in a humid chamber at 4°C overnight. Negative controls were incubated with normal rabbit immunoglobulin fraction (20 g/l, DAKO, Hamburg, Germany) or with leptin-antibody which had been blocked by a blocking peptide (Santa Cruz Biotechnology). Oocytes known to be leptin-positive (Antczak and Van Blerkom, 1997; Cioffi et al., 1997) were considered as positive controls.

The next day, sections were incubated at room temperature for 30 min with the second goat-anti-rabbit antibody (BA-1000, Vector Laboratories), diluted 1:200 in TBS with 1.5% normal goat serum. After incubating the sections with the avidin-biotin-HRP complex, the reaction product was histochemically detected with 0.02% 3’,3’-diaminobenzidine (DAB; Aldrich, Basel, Switzerland) in 0.05 mol/l Tris-HCl, pH 7.6, and with 0.03% H<sub>2</sub>O<sub>2</sub>. Sections were counter-stained with haematoxylin, dehydrated and mounted in Histokitt (Carl Roth GmbH, Karlsruhe, Germany).

By a similar method, leukocytes were located using a monoclonal antibody against the leukocyte common antigen (LCA; CD 45, DAKO) diluted 1:100. A goat-anti-mouse antibody (BA-9200, Vector Laboratories) diluted 1:200 was used as a secondary antibody,
Table II. Primer sequences, length of amplified templates, and cycle conditions for RT–PCR

<table>
<thead>
<tr>
<th>Gene product, Genbank accession number</th>
<th>Primer sequence 5′–3′</th>
<th>Product length (bp)</th>
<th>Number of cycles</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin, D63581</td>
<td>up cttgccccatcaaaaaagtcc</td>
<td>366</td>
<td>40</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>down cccccagctgtcaaggtt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OB-R, long form U43168</td>
<td>up gctatttgggaagagtt</td>
<td>501</td>
<td>40</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>down tgcctgggctctatctc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OB-R219.1, U52912</td>
<td>up tggaagcctctgatgaa</td>
<td>822</td>
<td>36</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>down acgagataaacaagtgaacaaag</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OB-R219.2, U52913</td>
<td>up tggaagcctctgatgaa</td>
<td>772</td>
<td>36</td>
<td>62</td>
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<tr>
<td></td>
<td>down aggctgcaagagtggaga</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OB-R219.3, U52914</td>
<td>up atccaatgggtgtcctgtt</td>
<td>573</td>
<td>36</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>down catggtggttcctgtgga</td>
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<td></td>
</tr>
</tbody>
</table>

Reverse transcription–polymerase chain reaction analysis

Total cellular RNA was isolated from different ovarian areas and granulosa cell cultures using the QIAGEN total RNA isolation kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol. Genomic DNA was digested with 0.02 IU DNase/μg RNA (Roche Molecular Biochemicals, Mannheim, Germany) at 25°C for 10 min. Total RNA of 5 μg was taken to synthesize cDNA using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) in a reaction volume of 15 μl.

Primer sequences and annealing temperatures for the simple reverse transcription–polymerase chain reaction (RT–PCR) technique are shown in Table II. The primer sequences for the long form of the OB-R have been published by Ciolfi et al. (1997), and those for leptin and the short forms of the OB-R by Glasow et al. (1997; Glasow et al., 1998). Each 25 μl amplification reaction contained 2.5 μl 10× concentrated PCR buffer (15 mmol/l MgCl₂), 0.3 IU Taq DNA polymerase (Roche Molecular Biochemicals), 100 μmol/l dNTPs (Perkin-Elmer, Weiterstadt, Germany), 0.1 μmol/l of each primer and 1 μl sample cDNA. Variations between the cDNA concentrations of different preparations were corrected by adjusting all cDNA samples to contain equal concentrations of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, as described previously for a semi-quantitative competitive RT–PCR (Aust et al., 1997). The GAPDH competitor (10⁻¹⁸ mol) was co-amplified with the sample cDNA in the same PCR tube and resolved by gel electrophoresis. The amount of cDNA used was that which gave an equal ratio between the sample cDNA signal and competitor signal intensity, thus equal amounts of cDNAs were used for each amplification. A PCR without sample cDNA served as a negative control, and mRNA from abdominal adipose tissue was used as a positive control.

Results

Leptin-positive cells in growing and regressing follicles and in the CL

Leptin-positivity occurred in the granulosa cells of pre-antral follicles (Figure 1A,B). An intact antral follicle displayed a strong leptin-positive response in the thecal layer, but not in the granulosa layer (Figure 1C). Leptin-positivity also occurred in the thecal layer of regressing antral follicles, which often showed epithelioid hypertrophy of the thecal layer and absence of the granulosa layer (Figure 3A,B). Oocytes exhibited a uniform staining all over the cytoplasm in pre-antral and regressing antral follicles. Yet the oocyte of the one intact antral follicle appeared to express leptin-positivity adjacent to the oolemma only (Figure 1C,D). The zona pellucida was judged to be non-responsive (Figure 1D,E). For CL, the structure, amount and distribution of leptin-positive cells depended on the stage of the cycle. In the developing CL, positively stained cells of fibroblast-like structure were distinctly noted in the former thecal layer which was transforming into septa (Figure 2A,B). In the subsequent stage of secretion, the number of leptin-positive cells was significantly lower. The positive cells were located between luteal cells (Figure 2C). By comparing similar regions in subsequent sections, LCA-positive leukocytes clearly differed in morphology compared with leptin-positive cells (Figure 2D). In the regressing CL, we observed single cells with a strong positive leptin response between degenerating luteal cells (not shown).

Leptin-positive cells in polycystic ovarian follicles

Intact antral follicles were absent in polycystic ovaries. Follicular cysts developed with and without hypertrophy of the thecal layer. In addition, we found cysts with a luteinized thecal and granulosa layer. These follicular cysts with luteinization appeared to be the largest in diameter and their granulosa cells were distinctly larger in size than the thecal cells. Most follicular cysts displayed a moderate to strong leptin response (Table III). It was nicely expressed in the thecal layer, but negligible in the decaying granulosa layer (Figure 4A). Follicular cysts with luteinization displayed a conspicuous leptin immunorespose both in the granulosa and thecal layers (Figure 4B).

Leptin and leptin receptor mRNA in different ovarian regions and IVF granulosa cells

Leptin mRNA was detected in IVF granulosa cells and in the developing CL in high quantities, whereas lower quantities were found in the secretory CL (Figure 5). The cortical region of the ovaries showed a very faint signal. No mRNA could be detected in the medulla. All samples expressed the mRNA of the full-length leptin receptor OB-R as well as the mRNA of the isoforms OB-R219.1–3. The signals were stronger in the case of OB-R219.1 and OB-R219.3 than in the case of OB-R and OB-R219.2 (Figure 5).

Discussion

This is the first report that shows leptin-positive cells which change in distribution and density in the intact human ovary.
Leptin was located in the granulosa layer of pre-antral follicles. Yet in antral follicles, leptin was strongly expressed in the thecal layer, but seemed absent in the granulosa layer. However, one should interpret this finding with caution because our RT-PCR analysis shows leptin mRNA in pre-ovulatory granulosa cells, and because others have detected leptin in pre-ovulatory granulosa cells and in cumulus cells (Cioffi et al., 1997). Regressing pre-antral and antral follicles contained oocytes with a leptin-positive response all over the cytoplasm. In one intact antral follicle, a strong leptin response seemed to be associated with the oolemma, i.e. with the cortical region of the oocyte. A similar observation has been published for mature follicles of stimulated mouse ovaries (Antczak and Van Blerkom, 1997). The cortically located leptin probably indicates an unknown oocyte–granulosa cell interaction in mature follicles which is missing in regressing follicles. To verify this suggestion, further studies are required.

Leptin-positive cells display an epithelioid appearance in the thecal layer of antral follicles. The cells, which change to fibroblast-like cells in the CL in the early developmental stage, differ in morphology compared with LCA-positive leukocytes. In the secretory CL, the small-sized, leptin-positive cells are
Leptin in human ovaries

Figure 2. Immunolocalization of leptin in corpora lutea. (A) Leptin-positive cells (asterisk) in the developing septum (i.e. in the former thecal layer) of an early CL. (B) The leptin-positive cells, as in A, appear fibroblast-like. (C) Small-sized leptin-positive cells (arrows) between luteal cells of a corpus luteum in the secretory stage. (D) LCA positive leukocytes (arrows) differ in morphology compared with leptin-positive cells in a comparable region of C. (A): scale bar = 320 µm, (B): scale bar = 25 µm, (C) and (D): scale bar = 160 µm.

Table III. The number of follicles and follicular cysts was evaluated in 25 wedge resections from polycystic ovaries and the immunohistological leptin response was graded semi-quantitatively

<table>
<thead>
<tr>
<th>Immunoresponse</th>
<th>Number</th>
<th>Antral follicles</th>
<th>Follicular cysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>intact</td>
<td>regressing</td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>Weak</td>
<td>44</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Moderate</td>
<td>46</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>Strong</td>
<td>26</td>
<td>–</td>
<td>1</td>
</tr>
</tbody>
</table>

Luteinization of granulosa cells.

distributed in the septa and between luteal cells, often close to capillaries. The high number of leptin-positive luteal cells in the early CL and their decrease in the secretory stage was confirmed by the strong and moderate leptin mRNA signals respectively in developing and secretory CL. An accurate statement on different mRNA levels has not been obtained because of the simple RT–PCR analysis used. The faint leptin mRNA signal observed in the cortex is likely to have been caused by oocytes in primordial and primary follicles, which may have been present in the PCR samples. No mRNA signal was found in the medulla, thus confirming the negative outcome for leptin staining by immunohistochemistry.

In PCOS, the initial recruitment and growth of follicles seems intact, yet the selection of the dominant follicle is disturbed and cysts develop (Erickson and Yen, 1993). Some authors have reported higher serum leptin levels in women with PCOS than in those without PCOS (El Orabi et al., 1999), but others contradict these findings for women at a given body mass index (Caro, 1997; Gennarelli et al., 1998). In this study, we have provided evidence of the conspicuous occurrence of leptin in the wall of polycystic follicles. This suggests that the local production of leptin is increased independently of the serum levels, which were probably high or normal in the 21 patients investigated. High local leptin concentrations may cause abnormalities in the FSH control system and in the synthesis of steroid hormones by granulosa cells, since hormonal dysregulations have been described for granulosa cells from polycystic follicles (Erickson et al., 1992).
Figure 3. Leptin positivity in regressing follicles. (A) Regressing antral follicles show leptin-positivity in the thecal layer of epithelioid appearance (asterisk). (B) The granulosa layer (arrowhead), as in A, is missing. (A): scale bar = 80 µm, (B): scale bar = 40 µm.

Figure 4. Leptin-positivity in follicles of polycystic ovaries. (A) In the hypertrophied thecal layer (asterisk), a moderate leptin response is seen. It is negligible in the decaying granulosa layer (arrowhead). (B) The conspicuous leptin response is stronger in the luteinized granulosa layer (arrowhead) than in the thecal layer (asterisk) of a follicular cyst with luteinization. (A, B): scale bar = 80 µm.

Figure 5. Leptin and leptin receptor mRNAs. RT–PCR analysis shows conspicuous detection of leptin mRNA in granulosa cells (GC) and in the developing CL (CL_dev); a weaker reaction in the secretory CL (CL_secr); and no reaction in the medulla. mRNA of the full-length receptor (OB-R) and the short isoforms (B219.1–219.3) in CL_dev, CL_secr, cortex, medulla and GC are expressed at different strengths. Abdominal adipose tissue (ad. tiss.) was used as positive control, and RT–PCR was carried out without sample cDNA as negative control (neg. control). GAPDH mRNA was determined by competitive RT–PCR with concentrations of GAPDH cDNA in the samples (upper band) and in the competitor (lower band).
Furthermore, a local excess of leptin production may interfere with the steroidogenic response induced by intra-ovarian factors e.g. IGF-I in the dominant follicle. A reduced oestrogen secretion signifies an inadequate signal for LH secretion and thus anovulation (Caprio et al., 2001).

This is the first report on the occurrence of transcripts for the long and short isoforms of the leptin receptor in various regions of the human ovary. The mRNA of both the long receptor and the short isoforms were found in granulosa cells and in the CL, cortex and medulla, where the receptors are expressed at varying intensities. However, we cannot exclude the possibility that the signal is caused by the presence of leukocytes, since both CD4 and CD8 T-lymphocytes express leptin receptors (Martin-Romero et al., 2000). Mouse macrophages are also known to have functional leptin receptors (Lee et al., 1999). The long form of leptin receptor mRNA has been detected in both thecal and granulosa cells isolated from ovaries of women with regular cycles (Agarwal et al., 1999). Cultured granulosa cells display an inhibitory effect on steroidogenesis under the influence of leptin (Karlsson et al., 1997; Barkan et al., 1999), when otherwise they have augmented oestrogen production (Kitawaki et al., 1999). It is thus likely that leptin is also involved in steroidogenesis in the intact ovary. The full-length leptin receptors in follicles, CL and the medulla may indicate a leptin-dependent autocrine and paracrine loop for steroid production and for any other as yet unknown peripheral leptin function.

Acknowledgements

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References


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