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Expression of Functional Leptin Receptors in the Human Ovary*

CECILIA KARLSSON, KAJSA LINDELL, EVA SVENSSON, CHRISTINA BERGH, PETER LIND, HÅKAN BILLIG, LENA M. S. CARLSSON, AND BJÖRN CARLSSON

Research Centre for Endocrinology and Metabolism, Departments of Internal Medicine (C.K., K.L., L.M.S.C., B.C.) and Department of Obstetrics and Gynecology (E.S., C.B., H.B.), Sahlgrenska University Hospital; and the Department of Physiology and Pharmacology (E.S., H.B.), Goteborg University, Goteborg; and the Department of Molecular Biology, Pharmacia and Upjohn (P.L.), Stockholm, Sweden

ABSTRACT

The size of body fat stores is known to influence fertility, indicating a link between adipose tissue and the reproductive system. Studies in mice have identified the adipocyte-derived hormone, leptin (Ob protein), as a possible mediator of this effect. The aim of this study was to investigate the possibility that leptin may have direct effects on the human ovary. To probe this hypothesis we first analyzed the expression of leptin receptors in the human ovary. Transcripts encoding both the long and short isoforms of the leptin receptor were present in human granulosa cells and thecal cells; however, the short isoforms were expressed at much higher levels. Immunoreactive leptin was present in follicular fluid at levels similar to those found in serum. *ob* gene expression, however, was undetectable in the ovary, as determined by reverse transcription-PCR, whereas it was easily detected in adipose tissue. To determine whether leptin could induce a biological response in ovarian cells, we examined the effect of leptin on estradiol production in cultured granulosa cells. Leptin (100 ng/mL) inhibited LH (0.1 ng/mL)-stimulated estradiol production. In contrast, leptin had no effect on estradiol production in the absence of LH.

In conclusion, this study has demonstrated that the leptin receptor is expressed in the human ovary, that leptin is present in follicular fluid, and that leptin can induce a biological response in ovarian cells. These results suggest that leptin may have a direct effect on the human ovary. (*J Clin Endocrinol Metab* **82:** 4144–4148, 1997)

SEVERAL observations suggest a link between body fat mass and fertility (1–3). Both too low as well as too high percentages of body fat are known causes of female infertility (4). The former can be seen in vigorously exercising women; the latter in women with polycystic ovary syndrome (PCOS). The amount of body fat is also a determinant of menarche (5). Furthermore, weight loss in anovulatory obese women results in significantly increased pregnancy and ovulation rates (6).

Leptin is an adipocyte-derived protein (7) that acts in an endocrine fashion by reporting the size of the adipose tissue mass to hypothalamic leptin receptors, and serum leptin levels correlate with the amount of body fat (8, 9). Administration of recombinant leptin to mice reduces their body weight by increasing energy consumption and decreasing food intake (10–12). Leptin is also a candidate for mediation of the signal between fat stores and the reproductive system (13–18). Genetically obese, *ob/ob* mice, are infertile (19). Thinning of *ob/ob* female mice by diet restriction failed to correct their sterility; however, administration of recombinant leptin restored their fertility (14). The mechanism by which leptin modulates fertility is not clear. In rodents, there are indications that leptin modulates the reproductive endocrine system in the hypothalamus, pituitary, and ovary (20–23).

Leptin exerts its effects via the leptin receptor, which is a member of the cytokine receptor superfamily (24). Several isoforms of the leptin receptor are produced by alternative splicing, resulting in receptors with different intracellular domains (24–26). Only the isoform with a long intracellular domain is capable of activating the JAK-STAT signaling pathway (27, 28), whereas the function of the short isoforms is unknown. The leptin receptor is expressed in the human ovary (25); however, it is unclear which of the isoforms of the receptor is present in the ovary.

The aim of our study was to determine whether there are prerequisites for a direct action of leptin on the human ovary.

Subjects and Methods

Subjects

Ovarian and adipose tissue were obtained from women with regular menstrual cycles undergoing laparotomy for reasons unrelated to ovarian pathology. Serum, follicular fluid, and granulosa cells were obtained from four women undergoing oocyte retrieval in stimulated cycles in the course of *in vitro* fertilization (IVF)/embryo transfer. The patients, numbered 1–4 (Fig. 4) had body mass indexes (BMI) of 27.4, 23.1, 21.6, and 29.1 kg/m^2, respectively. Informed consent was obtained from all women, and the study was approved by the local ethical committee.

Ribonucleic acid (RNA) isolation and complementary DNA (cDNA) synthesis

Total RNA was isolated as described by Chomczynski and Sacchi (29). First strand cDNA was generated as described previously (30).

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Address all correspondence and requests for reprints to: Dr. Björn Carlsson, Research Center for Endocrinology and Metabolism, Department of Internal Medicine, Gröna Stråket 8, Sahlgrenska University Hospital, S-413 45 Goteborg, Sweden. E-mail: bjorn.carlsson@ss.gu.se.

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Ribonuclease (RNase) protection assay

A leptin receptor probe that specifically allows analysis of expression of the long and short isoforms of the receptor was generated by reverse transcription-PCR (RT-PCR; Fig. 1A). The primers, 5′-CCAGTTCAGTCTTTACCC-3′ (nucleotides 2421–2438) and 5′-CTCAGCCTCAGA GAAGTT-3′ (nucleotides 2904–2887), were based on the human leptin receptor cDNA sequence (24). cDNA was amplified using a stepdown procedure, with annealing temperatures of 60, 57, 54, and 51 C (3 cycles each) and finally 57 C (30 cycles). The amplified 483-bp fragment was subcloned into pBluescript SK (Stratagene, La Jolla, CA), generating pCK6:95. Plasmids were purified (Qiagen, Chatsworth, CA), and the subcloned fragment was sequenced using a Terminator Double-Stranded DNA Sequencing Kit (Applied Biosystems Division, Perkin-Elmer, Foster City, CA).

The vector (pCK 6:95) was linearized with *Hind*III and used as template (0.25 μg) for the probe synthesis (31) in 1 $\times$ transcription buffer (MAXIscript, Ambion, Austin, TX), 10 mmol/L dithiothreitol, 0.5 mmol/L ATP, 0.5 mmol/L CTP, 0.5 mmol/L GTP, 5 μmol/L UTP (Ambion), 10 U RNase inhibitor (Promega, Madison, WI), 2.75 μmol/L [α-^{33}P]UTP (DuPont, Dreieich, Germany), and 10 U T3 polymerase (Promega). After synthesis for 15 min at room temperature, 10 U RNase-free deoxyribonuclease were added (37 C for 15 min). Finally, the probe was purified using NICK columns, Sephadex G-50 DNA grade (Pharmacia Biotech, Uppsala, Sweden). Hybridization was performed overnight at 45 C in 1 $\times$ hybridization buffer (RPA II Kit, Ambion) using 100 μg RNA. Subsequently, the samples were treated with 25 U RNase A and 1000 U RNase T1 for 30 min at 37 C. Protected fragments were precipitated and analyzed on a denaturing polyacrylamide gel. The gel was exposed on a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for 10 days and then analyzed.

RT-PCR

Leptin receptor cDNA. Amplification of leptin receptor cDNA was performed in 1 $\times$ *Taq* extender reaction buffer (Stratagene) containing cDNA, 0.8 mmol/L deoxy-NTP (Boehringer-Mannheim, Mannheim, Germany), and 1 μmol/L each of sense and antisense primers, 5′-TAGAATTCCCTCGAATGTTAA-3′ (nucleotides 2358–2378) and 5′-CGTGATTTTCTTCAGGAA-3′ (nucleotides 3217–3199; Fig. 1A). The forward primer was based on the mouse leptin receptor cDNA sequence, and the reverse primer was based on the rat leptin receptor cDNA sequence. After denaturation (4 min at 94 C), 5 U each of *Taq* DNA polymerase (B/M) and *Taq* extender PCR additive (Stratagene) were added, resulting in a final volume of 50 μL. cDNA was amplified using a stepdown procedure, with annealing temperatures of 56, 53, and 50 C (3 cycles each) and finally 48 C (30 cycles). The identity of the PCR product (Fig. 3A) was verified by DNA sequencing using the Prism Dye Primer Cycle Sequencing Kit (Applied Biosystems Division, Perkin-Elmer) and an ABI 373A automatic sequencer (Applied Biosystems).

Ob cDNA. Amplification of Ob cDNA was performed with 1 μmol/L each of sense and antisense primers, 5′-M13-forward-GCATTGGGGAACCCTGTG-3′ (nucleotides 3–20) and 5′-biotin-AGCACCCAGGGCTGAGGT-3′ (nucleotides 502–485), as previously described (30). The primers were based on the human *ob*-gene sequence (32) and were designed to span one intron (Fig. 1B).

Cyclophilin cDNA. Amplification of cyclophilin cDNA using the same cDNA templates as those described above was included as a positive control and was performed with 1 μmol/L each of sense and antisense primers, 5′-biotin-GCAGACAAGGTCCCAAAGA-3′ (nucleotides 76–94) and 5′-GCAGCGAGAGCACAAAGA-3′ (nucleotides 231–209; Fig. 1C) and an annealing temperature of 57 C (30 cycles). The primers were based on the human cyclophilin gene sequence (33) and were designed to span 3 introns (Fig. 1C).

The PCR products were separated on a 0.8% agarose gel containing ethidium bromide and visualized by UV light (Fig. 3, A–C). Controls for possible PCR contamination were included in all experiments and were always negative (Fig. 3, A–C, lane 6).

Leptin RIA

Serum and follicular fluid leptin concentrations were determined in duplicate by a human leptin RIA (Linco Research, St. Charles, MO). The limit of sensitivity for the assay was 0.5 μg/L. The intraassay coefficient of variation was 6.3% at a leptin concentration of 15.6 μg/L. All samples were analyzed in the same assay.

Leptin

Recombinant human leptin was produced essentially as described by Halaas *et al.* (11). In brief, human leptin was expressed as a hexahistidine tag leptin fusion protein and purified with Q-Sepharose (Pharmacia Biotech). The hexahistidine tag was removed by thrombin cleavage, and leptin was further purified by gel filtration (Superdex 75, Pharmacia Biotech) to near homogeneity (>99%). The endotoxin concentration, determined by *Limulus* amoebocyte endotoxin assay, was 0.00065 U/μg leptin.

Leptin stimulation of primary isolated human granulosa cells

Human granulosa cells were obtained in connection with IVF through follicle aspiration via transvaginal ultrasound-guided puncture. The patients were treated with a GnRH agonist for 3 weeks (Suprefact, Hoechst, Germany) before inducing follicle development with recombinant FSH (Gonal-F, Serono, Italy). The follicle aspirations were performed 36–38 h after the administration of hCG (Profasi, Serono, Italy). The granulosa cells were washed in medium 199 with Earle's salt (Life Technologies, Paisley, UK), 25 mmol/L $NaHCO_3$, 50 mg/L gentamicin (Life Technologies), 1% FBS (Flow Laboratories, Labkemi, Goteborg, Sweden), and 0.1 mg/mL testosterone (Sigma Chemical Co., St. Louis, MO). The cells from 4 subjects were pooled and cultured in 24-well plates (Falcon 3047, Becton Dickinson Labware, Lincoln, NJ; 96,000 cells/well) in medium 199 with the additives described above. The cells were precultured for 2–4 days and then cultured for 2 days with different combinations of leptin (100 ng/mL) and LH (0.1 ng/mL). Before adding hormones, the medium was changed. The amount of estradiol was measured in spent culture medium by Delfia assay (Wallac Oy, Finland). The experiment was performed three times, with four observations in each experiment.

Statistical analysis

Differences between groups were analyzed by Student-Newman-Keuls multiple range test. $P < 0.05$ was considered significant.

Results

Analysis of leptin receptor expression in ovarian cells

The expression of leptin receptors in granulosa cells and thecal cells was analyzed by a RNase protection assay that differentiates between long and short receptor isoforms. The probe was designed to protect a 483-bp fragment for the long isoform and a 252-bp fragment for the short isoforms (Fig. 1A). Only a 252-bp fragment was detected (Fig. 2), indicating that the short isoforms of the leptin receptor are the most abundant and that the long isoform is expressed at levels below the sensitivity of our assay.

As only the long isoform of the leptin receptor appears to have signaling capacity (27, 28), we specifically assayed the expression of this receptor isoform by RT-PCR. The primers were designed to generate an 859-bp fragment corresponding to part of the intracellular domain of the leptin receptor extending over regions corresponding to boxes 1 and 2 of the cytokine receptor superfamily (34). Leptin receptor transcripts of the expected size were detected in granulosa cells, thecal cells, and interstitial cells (Fig. 3A, lanes 1–3). DNA sequencing verified that the PCR product was identical to the cDNA sequence of the long isoform of the leptin receptor (GenBank accession no. U43168). Genomic DNA was included as a negative control.

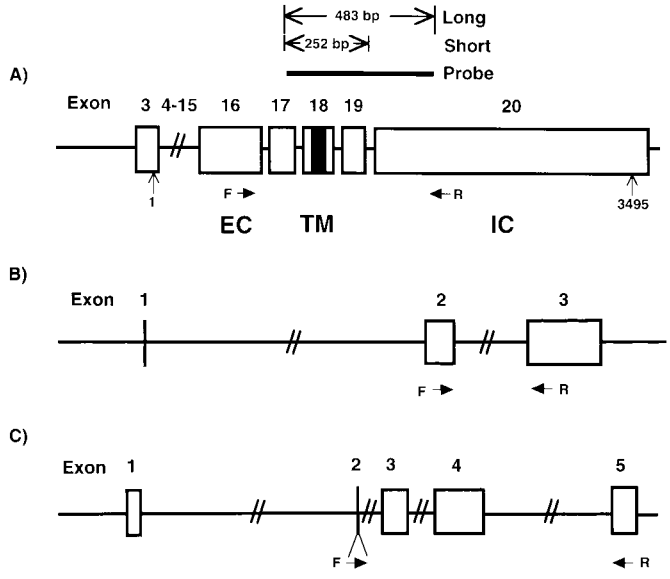

FIG. 1. Schematic illustration of the genes encoding the leptin receptor (A), leptin (B), and cyclophilin (C) and the locations of the primers (*arrows*; F, forward; R, reverse) used for RT-PCR. *Boxes* represent exons. Nucleotides are numbered, with position 1 referring to the first nucleotide of the start codon. The position of the leptin receptor probe used for the RNase protection assay is indicated by a line, and the expected lengths (base pairs) of the protected fragments are given. EC, Extracellular; TM, transmembrane; IC, intracellular.

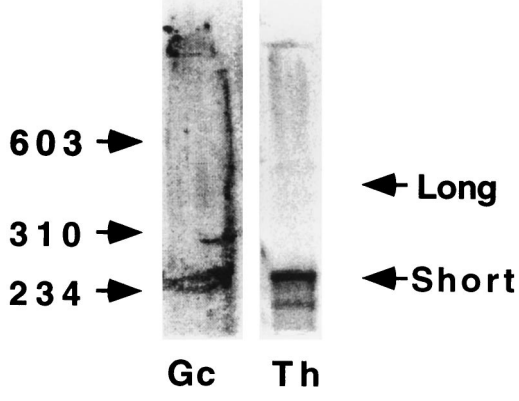

FIG. 2. Expression of the short and long isoforms of the leptin receptor in human granulosa and thecal cells analyzed by an RNase protection assay. The positions of the molecular markers are indicated on the *left*. The positions where protected fragments corresponding to the long and short isoforms of the leptin receptor migrate are indicated on the *right*. Gc, Granulosa cells; Th, thecal cells.

Analysis of immunoreactive leptin in follicular fluid

Recent studies suggest that leptin circulates in both bound and free forms in human blood (35, 36). A binding protein may interfere with the diffusion of leptin from blood vessels to the avascular compartment within the follicle. We, therefore, measured immunoreactive leptin in serum and follicular fluid obtained from four women undergoing IVF. Serum and follicular fluid leptin concentrations ranged from 18.3–29.7 μg/L. In all four patients, leptin concentrations in follicular fluid were similar to those in serum (Fig. 4).

To examine whether leptin is produced in the ovary, we analyzed the expression of the *ob* gene in human ovarian cells

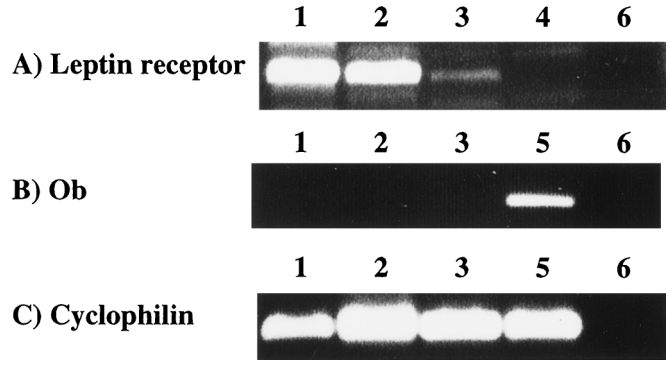

FIG. 3. Tissue distribution of mRNA encoding the long isoform of the leptin receptor (A), *ob* mRNA (B), and cyclophilin mRNA (C) analyzed by RT-PCR. Numbers indicate 1) granulosa cells, 2) thecal cells, 3) interstitial cells, 4) genomic DNA, 5) adipose tissue, and 6) negative control.

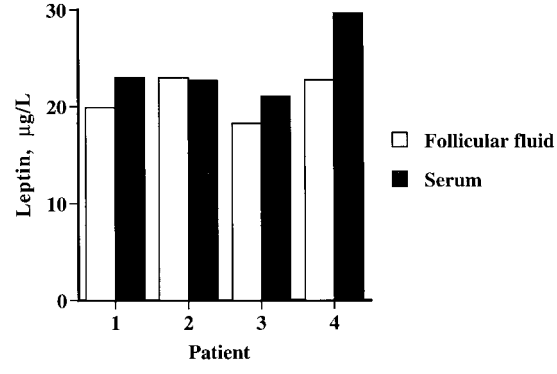

FIG. 4. Concentrations of leptin in serum and follicular fluid from four women undergoing IVF, determined by RIA.

by RT-PCR. *ob* gene expression was detected in adipose tissue, but not in granulosa cells, thecal cells, or interstitial cells (Fig. 3B). To verify the quality of the RNA and the cDNA, cyclophilin gene expression was analyzed (Fig. 3C).

Effects of leptin on human granulosa cells

The effect of leptin (100 ng/mL) on basal and LH-stimulated (0.1 ng/mL) steroid production was investigated in granulosa cells obtained in connection with IVF. Leptin had no effect on basal estradiol production (Fig. 5). However, LH-induced estradiol production in primary cultures of human granulosa cells was suppressed by leptin (Fig. 5).

Discussion

In this study we have shown that the leptin receptor is expressed in human ovarian cells, that immunoreactive leptin is present in human follicular fluid (although not produced in the ovary) and that leptin can exert biological effects on granulosa cells. These findings are consistent with an endocrine action of leptin on the ovary.

Leptin has recently been shown to be of critical importance for normal function of the female reproductive system in rodents (14–18). It is not known whether the effects of leptin on the reproductive system are exerted at the ovarian or pituitary/hypothalamic level or at both levels. Several leptin receptor isoforms exist that are generated by alternative

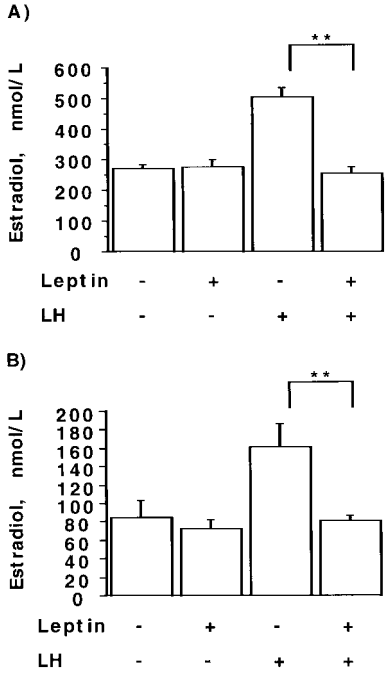

FIG. 5. Effects of LH (0.1 ng/mL) and leptin (100 ng/mL) on estradiol production in primary cultures of human granulosa cells during culture days 2–4 (A) and 4–6 (B). The granulosa cells were obtained in connection with IVF. Data are presented as the mean ± SEM. **, $P < 0.01$.

splicing, and the isoforms differ in the intracellular domain. Transfection experiments have shown that only the long isoform of the leptin receptor has signaling capacity (27, 28). This is in line with studies of several other members of the cytokine receptor superfamily that have shown that two semiconserved regions, boxes 1 and 2, in the intracellular region of the receptors are required for efficient activation of protein tyrosine kinases of the JAK family (37). Leptin receptor expression was recently demonstrated in the human ovary (25); however, the analysis used detected all isoforms of the leptin receptor, including those without signaling capacity. In this study we show that the long isoform of the leptin receptor is expressed in several human ovarian cell types, although transcripts encoding the short isoforms were much more abundant. It has been proposed that the short isoform may act as a dominant negative receptor (38) as has been shown for receptors of the tyrosine kinase family (39). The biological response to leptin in granulosa cells was, therefore, unexpected because of the high ratio of the short/long receptor isoforms of the leptin receptor. However, transfection experiments have shown that the long isoform of the leptin receptor is relatively resistant to dominant negative repression by truncated receptor isoforms (40), and the effects of leptin may, therefore, be exerted through the low abundant long leptin receptor. As the long isoform of the leptin receptor was only detected by RT-PCR, which is a very sensitive method for messenger RNA (mRNA) detection, it is possible that the long receptor isoform is expressed at levels that are biologically insignificant. An alternative explanation is, therefore, that the short leptin receptor has some signaling capacity. This idea is supported by a recent study in which leptin inhibited the effects of insulin on cultured hepatocytes that only express the short isoform of the receptor (41). However, a potential problem when using recombinant proteins produced in bacteria is contamination with endotoxins that may exert biological effects. As leptin had no effect on basal estradiol production, and the endotoxin levels were low in our leptin preparation, the suppression of LH-induced estradiol production is likely to be caused by leptin itself.

In rodents, *ob* gene expression appears to be restricted to adipocytes (7). In contrast, in man, *ob* gene expression has also been detected in placenta and heart (42). However, it remains to be established which cell type within these tissues expresses the *ob* gene. The presence of leptin in follicular fluid and the absence of *ob* gene expression in the ovary indicate that leptin acts in an endocrine fashion on the ovary.

Leptin was identified based on its importance in the regulation of adipose tissue mass in an animal model of obesity (*ob/ob* mice) (7). Leptin seems to have similar effects in humans, as defects in the *ob* gene cause obesity in humans (43). However, *ob* gene defects are rare in human obesity (30, 43–45), and to date, there are no reports of mutations in the leptin receptor gene in man (46). Obese subjects, as a group, have elevated levels of leptin in the blood (8, 9), and it has been proposed that obese subjects are leptin resistant (9). It is not known whether the elevated levels of leptin in serum contribute to diseases secondary to obesity. It has been speculated that leptin may be of importance in obesity-associated dysfunction of the reproductive system (13, 23). One study has shown that a substantial portion of women with PCOS have leptin levels higher than expected for their BMI (47). In contrast, three independent laboratories recently provided evidence against the concept of elevated leptin levels in PCOS patients compared to those in age- and weight-matched control subjects (48–51). However, as there may be subgroups of women with PCOS, it is possible that a subgroup of PCOS women exists that has higher leptin levels (48). Interestingly, leptin inhibits the production of estradiol, but not that of progesterone, in cultured rat granulosa cells (23), suggesting that it may promote a steroid microenvironment in the follicle similar to that present in PCOS (52). Based on the tissue distribution of leptin receptors (Refs. 24–26 and this study), it is possible that leptin acts on the reproductive system both at the hypothalamic-pituitary level (GnRH-FSH/LH) (20–22) and directly on the ovary (Ref. 23 and this study). The relative importance of leptin interactions at these levels is not known and could also vary during physiological and pathophysiological situations. It is possible that the major site of action of leptin may differ depending on the concentration of leptin in the blood. The direct action of leptin on the ovary may be of importance under certain conditions with elevated concentrations of leptin in the blood, such as obesity. It was recently shown that the cerebrospinal fluid/plasma leptin ratio is lower in obese compared to lean subjects, and it was suggested that this was due to a reduced efficiency of the transport of leptin from plasma to cerebrospinal fluid (53, 54). This creates a situation where peripheral tissues may be exposed to very high leptin concentrations while the central nervous system is exposed to only moderately increased levels of leptin. Conversely, leptin action at the hypothalamic level may be of relatively greater importance during conditions with low concentra-

tions of leptin in the blood, as in women with low BMI and in *ob/ob* mice (20, 21, 55–57).

We conclude that both the short and long isoforms of the leptin receptor are expressed in human ovarian cells and that immunoreactive leptin is present in human follicular fluid. In addition, leptin significantly suppressed LH-induced estradiol production. These findings are consistent with an endocrine action of leptin on the human ovary, with possible implications for female reproduction in health and disease.

Acknowledgments

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