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Leptin Antagonizes the Insulin-Like Growth Factor-I Augmentation of Steroidogenesis in Granulosa and Theca Cells of the Human Ovary*

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ABSTRACT

There is increasing evidence that leptin is a physiological link between obesity and infertility. Although leptin receptors have been demonstrated in human ovaries, there is no information regarding the effects of leptin on cells from developing ovarian follicles. To test the direct effects of leptin on human ovarian cells, granulosa cells (GC) and theca cells were isolated from the ovaries of regularly cycling women. Serum was obtained at the time of surgery, and follicular fluid was aspirated from the follicles before isolation of the ovarian cells. Leptin concentrations were similar in follicular fluid and serum. RT-PCR analysis demonstrated that the long, signaling form of the leptin receptor was expressed in both theca and GC. In cultured GC, leptin had no effect on estradiol production, alone or in the presence of FSH, but caused a concentration-related inhibition of the insulin-like growth factor I (IGF-I) augmentation of FSH-stimulated estradiol production. The effect of leptin was specific, because there was no effect on progesterone production. In cultured theca cells, leptin did not alter androstenedione production, alone or in the presence of LH. Leptin caused a concentration-related inhibition of the IGF-I augmentation of LH-stimulated androstenedione production. These data demonstrate that leptin can directly inhibit IGF-I action in ovarian theca and GC at concentrations commonly present in obese women. (*J Clin Endocrinol Metab* **84:** 1072–1076, 1999)

THE ELUSIVE link between obesity and infertility has recently been clarified with the cloning of leptin, a cytokine coded by the obese gene and secreted by adipocytes (1). Several studies have shown that serum concentrations of leptin increase proportionally with increasing body fat (2). In rodents, mutations causing leptin deficiency (*ob*) (3) or leptin receptor defects (*fatty, diabetic*) (4–6) cause obesity and infertility associated with hypogonadotropic anovulation (7). In humans, functional leptin receptor mutations have not been demonstrated (8–11); and leptin deficiency is extremely rare (12–16), but it causes severe childhood obesity when present (17). Hence, the vast majority of cases of human obesity are associated with high (rather than low) circulating leptin concentrations.

The leptin receptor is a member of the type I cytokine receptor family, with significant homology to gp130 (18), but gp130 does not seem to be involved in leptin signaling (19). In addition to a long isoform (thought to be the signaling form), the human leptin receptor is expressed in at least two other truncated isoforms, differing at their five prime ends (20). The short isoforms lack varying segments of the intracellular portion of the full-length receptor that contains motifs necessary for interaction with Janus kinases. Both long and short forms of the leptin receptor have been demonstrated in the human ovary, and leptin was shown to inhibit LH-stimulated estradiol production in granulosa luteal cells obtained from *in vitro* fertilization procedures (21). It is not known, however, whether leptin is capable of regulating GC and/or theca cell (TC) functions in developing follicles.

Because leptin deficiency is exceedingly rare in humans, the obese mouse model is not relevant to obesity-associated infertility in women. In contrast to the obese mouse, obese women have increased levels of leptin, compared with lean individuals (2). Animal studies in rat (22), porcine (23), and bovine (24) ovarian cells have demonstrated that leptin can exert direct inhibitory effects on steroidogenesis in GC and TC obtained from developing follicles. Leptin has no effect alone or on gonadotropin-stimulated steroidogenesis, but it is a potent inhibitor of the insulin-like growth factor I (IGF-I) augmentation of gonadotropin action in rat and bovine cells (22, 23). The role of leptin in regulating human ovarian steroidogenesis in developing follicles remains unknown. The objective of these studies was to test the hypothesis that leptin has direct inhibitory effects on steroidogenesis by human GC and TC.

Materials and Methods

Reagents

Human LH (NIDDK-hLH-B1) and human FSH (NIDDK-hFSH-B1) were a gift from the National Hormone and Pituitary Program of the NIDDK, NICHD, and USDA (Rockville, MD). Recombinant human IGF-I was obtained from R&D Systems, Minneapolis, MN. Recombinant

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human leptin was cloned and expressed in our laboratory by a previously published method (7). Leptin produced by this method has been shown to be active in *ob/ob* mice (7). Briefly, complementary DNA (cDNA) corresponding to the mature secreted form of human leptin was cloned from human adipose issue, using RT-PCR, and ligated in frame into the pQE30 expression vector (Qiagen, Valencia, CA). The construct was sequenced to confirm the identity of the clone with the Genbank sequence (U18915). The human leptin protein was expressed in *Escherichia coli* with a hexahistidine tag at the amino terminus and was purified by metal chelate affinity chromatography using nickel nitrilotriacetate resin under denaturing conditions. The eluted protein was refolded by slow dialysis of the denaturing agent, with final dialysis in three changes of PBS buffer. The protein concentration was measured by the method of Bradford using BSA as a standard. Analysis of the purified protein, by electrophoresis on a 12% SDS-polyacrylamide gel, demonstrated a single band. Measurement of the leptin content of a solution of the purified protein, by RIA, agreed with the total protein content, within experimental error.

Subjects and tissue isolations

All human tissue samples were obtained during the follicular phase of the menstrual cycle, from 19 healthy adult women with regular menstrual cycles, undergoing oophorectomy as part of their surgery for benign, nonendocrinological conditions unrelated to this study. The subjects were 41.7 ± 0.7 yr of age, with a BMI of 26.3 ± 1.2 kg/m^2, and were not taking medications known to affect reproduction, for a minimum of 3 months before surgery. Samples from 24 follicles, 13.0 ± 0.7 mm in diameter (range: 9.2–18.5 mm), were studied. Serum samples for determination of circulating leptin concentrations were collected from each subject before surgery. Cedars-Sinai Medical Center Institutional Review Board approval was obtained for this study.

Immediately after oophorectomy, the ovaries were transported to the laboratory in sterile, ice-cold saline. Follicular fluid (FF) was carefully and completely aspirated from the ovarian follicles, using a 25-gauge needle in an avascular region, to avoid blood contamination. The FF was centrifuged to remove the granulosa cells (GC). After centrifugation, the FF was frozen (−80 C) until assayed for leptin by RIA (Linco Research, Inc., St. Louis, MO). The aspirated follicles were hemisected, and the mural GC were collected by gently scraping with a platinum loop and were pooled with the GC collected from the FF. The GC were isolated from the blood cells and other cellular debris, using Percoll gradient centrifugation (25), and were resuspended in serum-free McCoy's 5a medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. The GC viability and concentration were determined by trypan blue exclusion.

After removal of the GC, the theca interna was microdissected from the follicular walls and incubated (37 C) in collagenase and deoxyribonuclease solution (26) until dispersed into a cell suspension. The TC were washed and resuspended in serum-free McCoy's 5a medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. The TC viability and concentration were determined by trypan blue exclusion.

Cell cultures

GC (5 × 10^4 viable cells/well) were cultured with 10^{-6} mol/L androstenedione in the presence and absence of FSH (50 ng/mL), IGF-I (50 ng/mL), and leptin (0–100 ng/mL), in a total vol of 200 μL, in 96-well plates, at 37 C, for 48 h. Estradiol and progesterone concentrations in the medium were determined by RIA (Diagnostic Products, Los Angeles, CA).

The isolated TC (5 × 10^4 viable cells/well) were cultured in the presence of LH (0–100 ng/mL), IGF-I (50 ng/mL), and leptin (0–100 ng/mL), as indicated, in a total vol of 200 μL, in 96-well plates, at 37 C, for 48 h. Androstenedione concentrations in the medium were determined by RIA (Diagnostic Products).

Identification of leptin receptor in GC and TC

The receptor splice variants for three isoforms of the leptin receptor were detected by RT-PCR. RNA was extracted from freshly isolated TC and GC. The messenger RNA (mRNA) was reverse transcribed into cDNA, then amplified by PCR using specific primer pairs for the long (13.2) and two short forms (12.1 and 6.4) of the human leptin receptor (27). Each primer pair shared a common 5′ primer (5′-ATCCCCATTGAGAAGTACCAG-3′) with specific 3′ primers for each isoform (13.2: 5′-GGCCTCATAGGTTACCTCAG-3′; 12.1: 5′-GCAGGGTCATAGGACA-ATAG-3′; 6.4: 5′-ACTGTTGGGAAGTTGGCACA-3′). The amplified bands (13.2: 535 bp; 12.1: 450 bp; 6.4: 350 bp) were separated by agarose gel electrophoresis and detected by ethidium bromide staining.

Statistical analyses

Treatments were performed in triplicate, and the experiments were repeated three times. ED_{50} values were calculated using the four-parameter logistic curve fitting method (28). Multiple comparisons were performed using one-way ANOVA, with *post hoc* comparisons employing Tukey's test. The paired *t* test was used to compare serum and FF leptin concentrations. Statistical significance was determined as $P \leq 0.05$.

Results

It is known that mature Graafian follicles are capable of concentrating steroids and gonadotropins in the FF. Certain molecules present in serum are excluded from FF, leading to the conclusion that the FF is a semiprotected environment. To determine whether there are significant differences in leptin between serum and FF, the concentration of leptin in FF was compared with the levels in serum collected at the time of surgery (Fig. 1). The mean concentrations of leptin in serum and FF were not significantly different (Fig. 1A). When the data are presented as paired values (Fig. 1B), it is apparent that leptin in FF varies up or down from serum values up to approximately 2-fold. In light of the pulsatile nature of leptin secretion, it is reasonable to infer that leptin is neither excluded from nor sequestered in FF.

We next examined the expression of the long (13.2) and two short (12.1 and 6.4) isoforms of the leptin receptor in the TC and GC of healthy developing ovarian follicles. RNA was isolated from the TC and GC dissected from dominant ovarian follicles with FF androstenedione:estradiol ratios less than 4. As shown in Fig. 2, the mRNA for the long form of the leptin receptor was expressed in both TC and GC, indicating that both cell types may be capable of responding to leptin in developing follicles. The mRNAs for the two short isoforms were also expressed in both TC and GC.

Because mRNA expression for the signaling isoform of leptin receptor was identified in both GC and TC, a series of cell culture experiments were performed to investigate the direct effects of leptin on steroidogenesis by these cell types. As shown in Fig. 3, leptin (50 ng/mL) had no effect on basal estradiol production by GC. As expected, FSH (50 ng/mL) stimulated estradiol production, and the addition of IGF-I (50 ng/mL) further augmented FSH-stimulated estradiol production. Leptin did not alter the FSH stimulation of estradiol production, but it completely abolished the IGF-I augmentation of FSH-stimulated estradiol production. The effect of leptin was dose-related (ED_{50} = 1.3 ± 1.8 ng/mL), with complete reversal of the IGF-I augmentation of FSH-stimulated estradiol production at physiological leptin concentrations present in obese women (Fig. 4). The effect of leptin was specific for estradiol production, having no effect on IGF-I augmentation of FSH-stimulated progesterone production by GC (Fig. 5).

We next examined the direct effects of leptin on TC an-

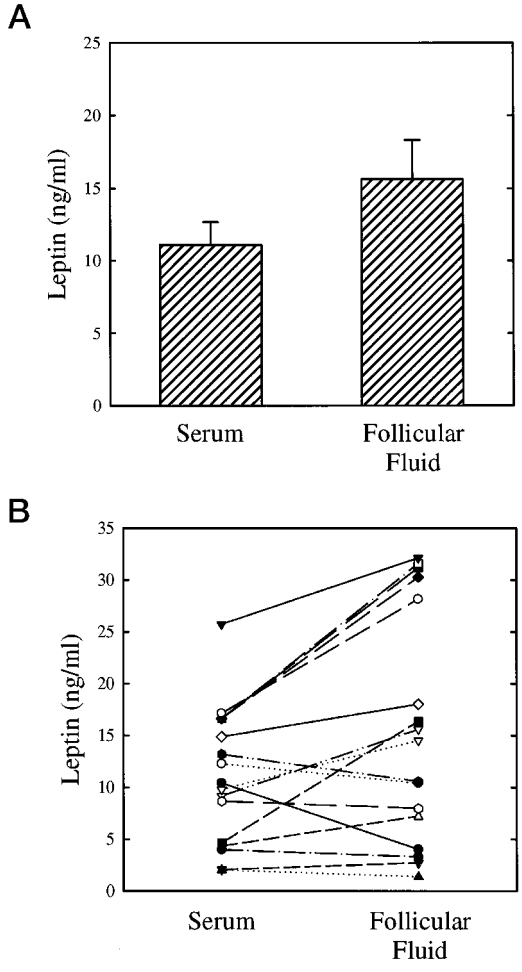

FIG. 1. Comparison of serum and FF leptin concentrations. Serum samples were obtained just before oophorectomy. Leptin concentrations in the serum and FF samples were measured by RIA. A, The data are the mean ± SEM of 17 women; B, serum and FF values are presented for individual women.

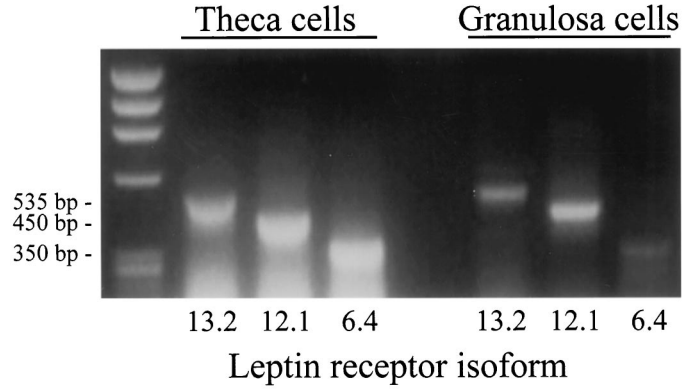

FIG. 2. Expression of leptin receptor mRNAs by human TC and GC. RNA was extracted from isolated TC and GC from healthy, dominant follicles. cDNA for the long form of the human leptin receptor (13.2) and two short isoforms (12.1 and 6.4) were amplified by RT-PCR. The amplified bands were detected on an ethidium bromide-stained agarose gel. The molecular weight markers are *Hae*III digested ϕX174 DNA.

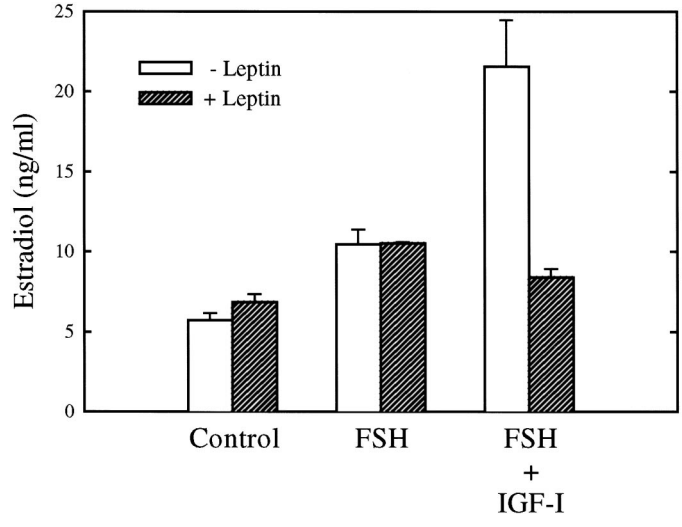

FIG. 3. Effect of leptin on GC estradiol production. GC were isolated from healthy dominant follicles and cultured (5×10^4 viable cells/well) in serum-free McCoy's 5a medium containing 10^{-6} mol/L androstenedione, for 2 days, in the presence and absence of FSH (50 ng/mL) and IGF-I (50 ng/mL) with and without leptin (50 ng/mL). Estradiol in the medium was measured by RIA. The data are the mean ± SEM of three experiments with triplicate cultures.

drogen production. As shown in Fig. 6, LH stimulated a dose-related (ED_{50} = 1.78 ± 2.1 ng/mL) increase in TC androstenedione production. Addition of IGF-I (50 ng/mL) increased androstenedione production, alone and at each concentration of LH, without altering the ED_{50} for LH stimulation. Concomitant treatment with leptin (50 ng/mL) completely abolished the stimulatory effect of IGF-I (Fig. 6). The inhibitory effect of leptin was dose-related (ED_{50} = 6.7 ± 5.5 ng/mL), with significant inhibition occurring at physiological concentrations typical of lean women and complete reversal of the IGF-I effect at physiological concentrations typical of obese women (Fig. 7).

Discussion

Small antral follicles seem to exclude certain molecules, such as FSH, from the FF (29). When a follicle is selected to become dominant, at approximately 7 mm in diameter, FSH bioactivity in the FF begins to increase (29); and by the time a follicle reaches the preovulatory stage of development it contains high concentrations of FSH, relative to the circulation (30). Our data demonstrate that the concentration of leptin in the FF of developing follicles is similar to the concentration measured in serum, indicating that leptin is neither excluded nor concentrated in the FF. There is variability within approximately a 2-fold range around the serum concentration. Because circulating leptin is pulsatile, with an amplitude of 120%, and exhibits diurnal variations on the order of 3-fold, it seems that there may be a diffusion gradient for leptin between the circulation and FF. It does not seem, however, that there is a physiologically important difference between circulating leptin levels and FF leptin concentrations. These data are consistent with the observation that leptin concentrations in the serum were similar to the levels in fluid obtained from follicle aspirations during *in vitro* fertilization oocyte retrievals (21).

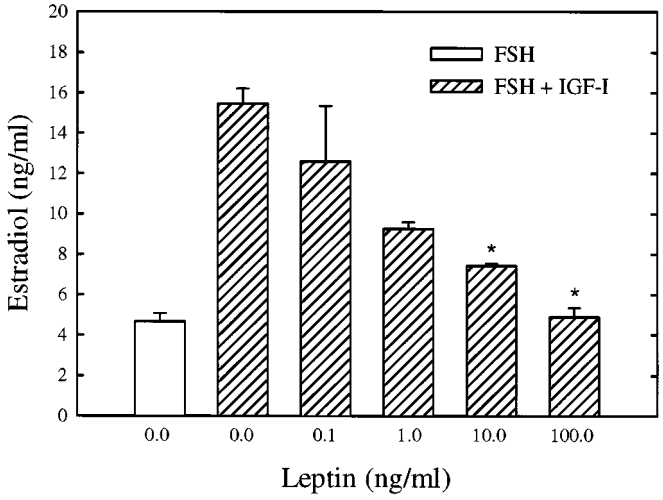

FIG. 4. Effect of leptin on the IGF-I augmentation of FSH-stimulated estradiol production by human GC. GC (8.8×10^4 viable cells/well) were cultured in serum-free McCoy's 5a medium containing 10^{-6} mol/L androstenedione and 50 ng/mL FSH, for 2 days, with and without IGF-I (50 ng/mL) and increasing concentrations of leptin (0–100 ng/mL). Estradiol in the medium was measured by RIA. The data are the mean ± SEM of three experiments with triplicate cultures.

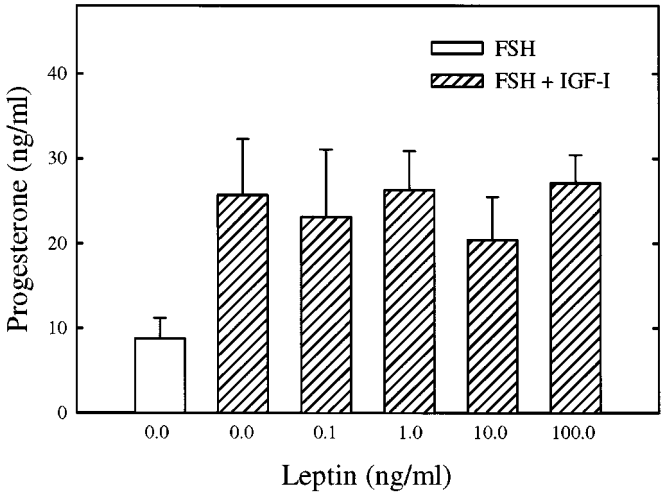

FIG. 5. Effect of leptin on IGF-I augmentation of FSH-stimulated progesterone production by human GC. GC (8.8×10^4 viable cells/well) were cultured in serum-free McCoy's 5a medium containing 10^{-6} mol/L androstenedione and 50 ng/mL FSH, for 2 days, with and without IGF-I (50 ng/mL) and increasing concentrations of leptin (0–100 ng/mL). Progesterone in the medium was measured by RIA. The data are the mean ± SEM of three experiments with triplicate cultures.

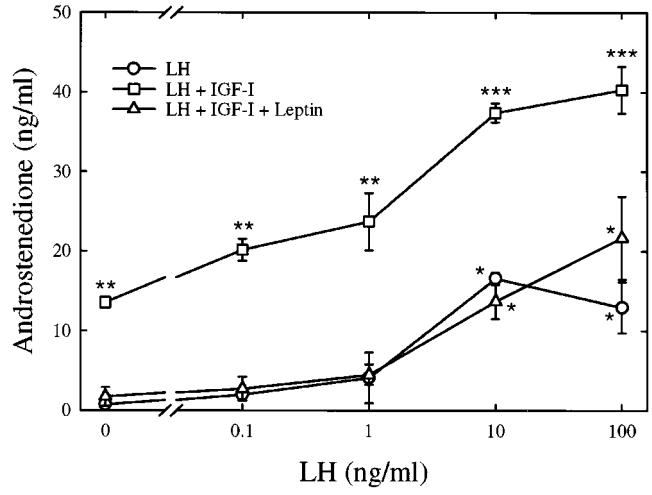

FIG. 6. Effect of leptin on androstenedione production by human TC. Isolated TC (6.6×10^4 viable cells/well) were cultured in serum-free McCoy's 5a medium, for 2 days, with increasing concentrations of LH (0–100 ng/mL) with and without IGF-I (50 ng/mL) and leptin (50 ng/mL). Androstenedione in the medium was measured by RIA. The data are the mean ± SEM of three experiments with triplicate cultures.

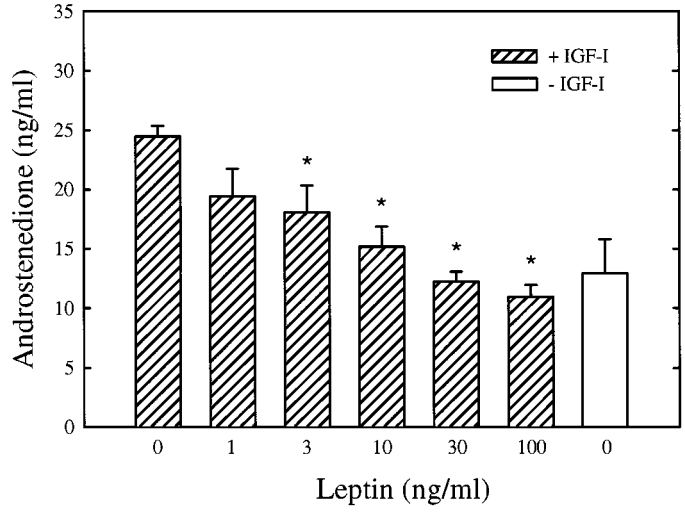

FIG. 7. Effect of leptin on IGF-I augmentation of LH-stimulated androstenedione production by human TC. Isolated TC (6.6×10^4 viable cells/well) were cultured in serum-free McCoy's 5a medium containing LH (50 ng/mL), for 2 days, with increasing concentrations of leptin (0–100 ng/mL) with and without IGF-I (50 ng/mL). Androstenedione in the medium was measured by RIA. The data are the mean ± SEM of three experiments with triplicate cultures.

Leptin receptor mRNA has been shown to be expressed in the human ovary (31). The long form mRNA was recently shown to be expressed in the TC and GC of the ovary (21). Our data confirm expression of the long form and demonstrate expression of two alternatively spliced short forms of the leptin receptor mRNA in both TC and GC.

We have previously shown that leptin has direct effects on the IGF-I augmentation of FSH-stimulated estradiol production by rat GC (22). These studies demonstrate very similar direct effects in human GC obtained from unstimulated developing follicles. In addition, we demonstrated direct inhibitory effects of leptin on the IGF-I augmentation of LH-stimulated androgen production by human TC. Interestingly, the concentrations of leptin that are commonly found in obese women completely suppressed the stimulatory effects of IGF-I *in vitro*. Taken together, these results indicate that high physiological concentrations of leptin could significantly interfere with the ability of the dominant follicle to produce estradiol, both by inhibiting the production of androgen substrate and by decreasing the aromatizing capacity of the GC. The inhibitory effect of leptin on the GC was specific to estradiol production and did not alter the effects of IGF-I on progesterone production. This observation

demonstrates that leptin does not block IGF-I signaling in the GC and suggests that leptin may specifically interfere with aromatase enzyme expression or activity.

The effects of leptin on insulin action in the ovary remain to be determined, but the potential exists for leptin to have a similar negative impact on insulin action in the ovary as it does on IGF-I action. If this were to be the case, elevated leptin concentrations in obese women might exacerbate the deleterious effects of insulin resistance in the polycystic ovary syndrome (PCOS). Initial evidence suggested that elevations of circulating leptin might be associated with a certain segment of women with PCOS (32); however, several subsequent papers were unable to demonstrate differences in leptin concentrations between women with PCOS and regularly cycling control women (33–36). Thus, the evidence does not support a causative role for leptin in PCOS; however, our data support the concept that the higher concentrations of leptin found in obese women can make them more susceptible to infertility.

The physiological significance of leptin, with respect to ovarian function, is likely to be as a modulator of ovarian responses to trophic stimuli. In obese women, high concentrations of leptin may counteract the sensitizing effects of locally produced growth factors. It is thought that production of sensitizing molecules, such as IGF-I by dominant follicles, augments the stimulatory effects of FSH, thereby giving dominant follicles a growth and developmental advantage, with respect to cohort follicles (37). Because the FSH concentration declines from the mid- to the late-follicular phase of the menstrual cycle, the dominant follicle is able to continue to grow and develop, whereas the cohort follicles have inadequate trophic stimulus and undergo atresia. The potential exists that high concentrations of leptin may interfere with development of the dominant follicle and suppress its estradiol production. Such an effect could result in an inadequate stimulus for the LH surge and may also lead to an immature preovulatory follicle or, in extreme cases, no preovulatory follicle at all. This type of mechanism is consistent with clinical observations that obese women have a higher incidence of infertility and that weight loss often causes significant clinical improvement (38).

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