Leptin and Soluble Leptin Receptor in Follicular Fluid

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Purpose: Previous studies suggest that follicular fluid leptin levels predict successful assisted reproduction. The relationship between intrafollicular leptin and the soluble leptin receptor, ovarian hormones, and oocyte quality was examined to determine potential factors contributing to this finding.

Methods: Follicular fluid leptin, soluble leptin receptor, hormones, and oocyte quality were examined in 84 individual follicles from 30 women undergoing *in vitro* fertilization.

Results: Follicular fluid leptin and soluble leptin receptor levels correlated inversely with each other ($r = -0.354$; $p = 0.001$). Follicular fluid leptin levels correlated with intrafollicular estradiol ($r = 0.42$; $p < 0.001$), progesterone ($r = 0.48$; $p < 0.001$), and androstenedione ($r = 0.49$; $p < 0.001$), whereas soluble leptin receptor levels correlated with activin ($r = 0.38$; $p < 0.001$) and follistatin ($r = 0.35$; $p < 0.002$). There was no relationship between follicular fluid leptin or soluble leptin receptor levels and pretreatment serum hormone levels, stimulated serum estradiol, follicle number, oocyte quality, fertilization, or embryo grade.

Conclusion: The data demonstrate that leptin and the soluble leptin receptor are highly interrelated with each other and with other intrafollicular hormones, but not with markers of oocyte quality, fertilization, or embryo grade.

KEY WORDS: Activin; estradiol; follistatin; leptin; oocyte.

INTRODUCTION

Leptin, the protein product of the *ob* gene, is produced by adipose tissue and is secreted into the circulation in proportion to the amount of energy stored in adipose tissue (1,2). Leptin acts at its hypothalamic receptors to suppress food intake and stimulate energy expenditure (3–5). Alternative splicing of the leptin receptor results in the formation of the long form of the leptin receptor, found in the hypothalamus, and several short forms which have a wide tissue distribution (6).

Recently, a soluble form of the leptin receptor has been identified (soluble leptin receptor; sOB-R). The sOB-R consists only of the extracellular domain of the leptin receptor, binds leptin with an affinity similar to membrane bound receptors and represents the most abundant leptin binding protein in serum (7–9). The sOB-R appears to modulate leptin actions. It delays leptin clearance and in excess, may compete for leptin binding to membrane bound receptors (10). Thus, sOB-R may have direct physiological implications for potential leptin actions.

Leptin is found in the ovary and has been demonstrated to have direct ovarian actions *in vitro*. The long form of the leptin receptor has been identified on granulosa and theca cells in the human ovary. Leptin is present in follicular fluid (11) and may be produced by granulosa cells, oocytes, or both in the ovary (11,12). Leptin inhibits gonadotropin stimulated steroidogenesis either directly (11) or by antagonizing IGF-I or insulin (13,14), demonstrating direct leptin action at

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the ovary. However sOB-R, a potential modulator of leptin action, has not previously been measured in the ovary. Further, the relationship of sOB-R to leptin and other ovarian steroid and protein factors has not been examined.

In addition to its potential action on ovarian steroidogenesis, there is evidence that serum and follicular fluid leptin levels may predict treatment success in assisted reproduction. Follicular fluid leptin levels were lower in patients who became pregnant during assisted reproductive technology treatment compared to those who did not, independent of body mass index (BMI) (15). Similarly, higher serum (16,17) and follicular fluid leptin levels (18) were associated with adiposity and a poorer ovarian response to FSH. Taken together with evidence for leptin action at the ovary, leptin may exert direct influence on ovarian estradiol production, the follicle or oocyte, affecting the potential for pregnancy. Further, serum sOB-R and free leptin levels vary during gonadotropin induction (19). Therefore, sOB-R may also modify leptin's action in follicular fluid.

Several markers can be obtained from the oocyte and follicular fluid during assisted reproduction that may be related to cycle outcome, such as oocyte stage, oocyte grade, fertilization, embryo grade, steroid and protein levels. To test the hypothesis that leptin, the sOB-R, or both are related to these follicular measurements of cycle outcome, leptin and sOB-R were measured in follicular fluid from women undergoing *in vitro* fertilization, and compared to other follicular fluid hormone levels and oocyte quality, fertilization, and embryo grade within the same follicle. In addition, by examining leptin and sOB-R in the follicular fluid microenvironment, this study was able to investigate hormonal relationships with leptin and sOB-R. The findings provide evidence that leptin and sOB-R levels are highly interrelated with each other and with other intrafollicular hormones, but not with markers of oocyte quality, fertilization, and embryo grade.

METHODS

Patient Selection

The study was approved by the Institutional Review Board of the Massachusetts General Hospital and all subjects gave their written informed consent.

Follicular fluid samples from 84 individual follicles were collected from 30 consecutively enrolled women undergoing *in vitro* fertilization (IVF). Subjects were 24–45 years of age, with the following

diagnoses leading to IVF: tubal factor ($n = 10$), endometriosis ($n = 7$), anovulation ($n = 1$), male factor ($n = 6$), idiopathic ($n = 5$), and diethylstilbestrol (DES; $n = 1$). After pituitary desensitization with leuprolide acetate (Lupron; TAP Pharmaceuticals, Chicago, IL, 0.5 mg/day, subcutaneously), subjects were treated with human menopausal gonadotropins (Perganol; Serono, Randolph, MA) and/or human follicle stimulating hormone (FSH; Metrodin, Serono). Blood was sampled for estradiol and human chorionic gonadotropin (hCG; Profasi, Serono) was administered when the lead follicle was 16 mm or greater. Follicles were aspirated 36 h later. Oocytes were isolated and evaluated and follicular fluid was centrifuged to remove debris, after which it was aliquoted and stored at -20°C until assayed. Embryos were assessed daily from day 2 until the time of embryo transfer.

Oocyte and Embryo Classification

Oocytes were graded into maturational stages 1–3 (20) or were described as degenerating (deg). Oocytes were also classified into stage: GV if a germinal vesicle was visible, M1 if neither the germinal vesicle nor first polar body were visible, and M2 if the first polar body was visible. Oocytes were labeled postmature (PM) if they were fractured at retrieval. After incubation with sperm, oocytes were classified as normal fertilization (two pronuclei), failed fertilization (no evidence of sperm penetration), or abnormal fertilization (polyspermy and oocytes with only one pronucleus).

Embryos were classified into 3 grades. Grade 1 embryos had blastomeres of equal size and fragmentation less than 20%. Grade 2 embryos had unequal blastomeres and fragmentation between 20 and 50%. Embryos were classified as grade 3 if any of the following characteristics were noted: fragmentation more than 50%, unequal blastomeres more than 50%, extensive cytoplasmic granulation, blastomeres incompletely separated or zona pellucidae of abnormal thickness. Grade 1 and 2 embryos were considered highest quality and were preferentially transferred, unless an insufficient number were available, at which time grade 3 embryos were also transferred.

Hormone Assays

Estradiol, progesterone, and androstenedione were measured by radioimmunoassay (RIA) as previously described (21). Inhibin A, inhibin B, and activin A were measured using enzyme-linked immunosorbent

assays (ELISA; Serotec, Oxford, England), as previously described (22). Total immunoreactive inhibin was measured using the Monash RIA as previously described for follicular fluid samples (21). Free follistatin (288 and 315) was determined using a two-site solid phase chemiluminescent assay (23). Total leptin was measured using a RIA (Linco Research, St. Louis, MO). sOB-R was measured using an ELISA (BioVendor Laboratory Medicine, Brno, Czech Republic), as previously described (24). Results are reported in U/mL with 1 unit equivalent to 2 ng sOB-R. The sOB-R ELISA sensitivity was 0.8 ng/mL and intraassay CV 3.9–5.6%. In addition, the sOB-R ELISAs from Biovendor and DSL (Webster, TX) were used to measure sOB-R in 84 separate samples of follicular fluid. The two methods correlated significantly with each other ($r = 0.80$, $r^2 = 0.64$ and $p < 0.0001$).

Data Analysis

Follicular fluid hormone concentrations were corrected for dilution by the flush used to aspirate the follicles. The free leptin index (FLI) was calculated as leptin/sOB-R. Spearman correlations were used to examine associations between average leptin, FLI, and sOB-R levels in individual subjects and baseline subject characteristics and cycle characteristics. Spearman correlations were used to examine associations between hormone levels. An ANOVA on ranks was used to investigate leptin and sOB-R levels in relation to indexed data including subject, oocyte grade, oocyte stage, fertilization, and embryo grade. A p value of <0.05 was considered significant for all analyses except correlations in which a Bonferroni correction was used to account for multiple comparisons, and a p value <0.005 was considered significant. The mean and standard error are reported, unless otherwise indicated.

RESULTS

Leptin (11.2 ± 1.0 ng/mL, mean $\pm$ SE; 1.3–49.6 ng/mL, range) and sOB-R (6.1 ± 0.4 U/mL, mean $\pm$ SE; 2.2–20.1 U/mL, range) were detectable in follicular fluid. Follicular fluid leptin and sOB-R levels were compared between individual subjects and were significantly different ($p < 0.05$). The average coefficients of variation (CV) for leptin (30%) and sOB-R (28%) in the follicles of individual subjects were higher than expected from the assay CVs (max-

imum 8 and 6%, respectively), suggesting that leptin and sOB-R levels are specific to individual follicles and are not determined by overall serum levels in subjects. Leptin and sOB-R were inversely correlated (-0.354 ; $p = 0.001$) and exhibited a curvilinear relationship (Fig. 1).

The average follicular fluid leptin levels in subjects correlated with weight and BMI at baseline (Table I; Fig. 2). In contrast, the average sOB-R in subjects correlated inversely with weight and BMI and exhibited a curvilinear relationship (Fig. 2). There was no relationship between leptin or sOB-R and other cycle parameters (Table I). There was also no relationship between average leptin or sOB-R and patient diagnosis ($p = \text{NS}$).

Intrafollicular leptin in individual follicles correlated with intrafollicular estradiol, progesterone, and androstenedione (Table II). Intrafollicular sOB-R in subjects correlated with activin A and follistatin. As previously described (22) androstenedione, progesterone, total inhibin, inhibin A, activin A, and follistatin correlated with each other. Estradiol also correlated with these hormone levels, although it did not reach significance in the larger sample studied previously (22).

There was no relationship between follicular fluid leptin or sOB-R in individual follicles and oocyte parameters including oocyte grade, stage, fertilization, and embryo grade (Table III).

DISCUSSION

The follicular fluid is a unique microenvironment that compartmentalizes steroid and protein factors secreted by the granulosa cells under endocrine and autocrine/paracrine control. This study demonstrates that sOB-R is present in follicular fluid, extending a previous report of leptin binding activity (18). The difference in leptin and sOB-R levels between individual follicles in each subject suggests that levels are regulated within the microenvironment of the follicle. Although cause and effect relationships cannot be determined with the current data, the relationship between leptin and sOB-R suggests a role for sOB-R in leptin control within this compartment. Further, the data suggests that leptin may be influenced by steroidogenic factors while sOB-R is related to autocrine/paracrine protein factors.

The relationship between leptin and estradiol in this study is consistent with previous data suggesting that estradiol stimulates leptin production. *In vivo*,

Fig. 1. Soluble leptin receptor (sOB-R) as a function of leptin levels in individual follicles. Note the inverse curvilinear relationship between sOB-R and leptin.

estradiol replacement in postmenopausal women increased leptin levels in the absence of changes in fat mass (25). Further, animal and human studies suggest that the estradiol stimulated leptin increase is related to increased *Ob* gene expression (26–28) and leptin release from adipose tissue (28,29). The positive correlation between leptin and estradiol stands in contrast to previous experimental evidence in which leptin inhibits gonadotropin stimulated estradiol secretion

(11,13), perhaps related to the absence of sOB-R and other follicular fluid factors in these *in vitro* models. Testosterone and dehydroepiandrosterone also increase leptin expression and secretion via aromatization to estradiol (28), perhaps accounting for the relationship between androstenedione and leptin in this study. Finally, experimental data demonstrate contrasting results for progesterone, with some studies stimulating (30,31) and others eliciting no effect (25). Thus, the positive correlation between leptin and sex steroids provides support for a role of estradiol in follicular fluid leptin regulation.

In contrast to leptin, sOB-R is inversely related to BMI and leptin and correlated to follicular fluid proteins. Previous studies in serum also demonstrate an inverse relationship between sOB-R and leptin and adiposity (24). Experimental data further demonstrate that leptin directly suppresses sOB-R and prevents its fasting induced increase (24). Thus, leptin appears to regulate its own binding protein. In contrast to previous studies, however, there is no relationship between sOB-R and estradiol or androgens in the current study (24). Rather, sOB-R correlates with activin and follistatin. Activin is an autocrine/paracrine factor known to promote granulosa cell and follicle differentiation, prevent premature luteinization, and

Table I. Relationship Between Average Follicular Fluid Levels of Leptin and sOB-R and Subject/Cycle Characteristics

	Leptin	sOB-R	FLI
Age	−0.032	0.024	−0.068
Height	−0.078	−0.290	0.057
Weight	0.577	−0.749	0.700
BMI	0.713	−0.705	0.783
Day 3 FSH	−0.156	0.104	−0.081
Day 3 E2	−0.074	0.108	−0.001
Serum E2 ^a	−0.009	0.362	−0.163
Follicle number	−0.056	0.291	−0.155
Total hMG/FSH ^b	0.047	−0.176	0.137
Leptin		−0.572	0.943
sOB-R			−0.770

Note. Values in bold are significant ($p < 0.005$).

^a Serum estradiol level at the time of hCG administration.

^b Total number of ampules of hMG or FSH administered in the cycle.

Fig. 2. Leptin (A) and sOB-R (B) as a function of BMI. Note the linear relationship between leptin and BMI and the inverse curvilinear relationship between sOB-R and BMI.

enhance oocyte maturity, while follistatin is an activin binding protein important for limiting activin's action (32). It is therefore possible that sOB-R plays a similar role in autocrine/paracrine regulation of follicular development, either directly or indirectly through leptin.

This study examined individual follicles so that oocyte quality, fertilization outcome, and hormone levels from each follicle could be evaluated and related to leptin and sOB-R levels. Leptin and

sOB-R did not predict oocyte stage, oocyte grade, fertilization, or embryo grade. Further, although other studies have demonstrated a relationship between higher follicular fluid inhibin B (33) and pregnancy outcome, leptin and sOB-R demonstrated no relationship to inhibin B. In contrast, follicular fluid progesterone was lower in follicles responsible for pregnancy (34) and leptin correlated with progesterone, although experimental data has not been definitive when evaluating leptin regulation by

Table II. Relationship Between Follicular Fluid Levels of Leptin and sOB-R and Other Hormones

	Progesterone	Androstenedione	Total Inhibin	Inhibin A	Inhibin B	Activin	Follistatin	Leptin	sOB-R	FLI
Estradiol	0.533	0.649	0.432	0.471	-0.111	0.281	0.326	0.422	0.009	0.295
Progesterone		0.666	0.466	0.607	-0.225	0.617	0.623	0.476	0.268	0.220
Androstenedione			0.520	0.507	-0.044	0.416	0.473	0.493	0.183	0.270
Total inhibin				0.501	-0.073	0.494	0.482	0.174	0.293	0.022
Inhibin A					-0.029	0.768	0.531	0.261	0.253	0.095
Inhibin B						-0.044	0.040	0.186	-0.080	0.020
Activin							0.719	0.156	0.377	-0.035
Follistatin								0.174	0.353	-0.015
Leptin									-0.354	0.892
sOB-R										-0.708

Note. Values in bold are significant ($p < 0.005$).

Table III. Leptin and sOB-R Levels in Relation to Oocyte Stage, Oocyte Grade, Fertilization and Embryo Grade

	Leptin (ng/mL) ^a	sOB-R (U/mL) ^a
Oocyte grade ^b		
1 (n = 2)	16.6 (11.0–22.2)	4.7 (3.8–5.5)
2 (n = 58)	8.2 (4.3–17.3)	5.9 (4.1–8.2)
3 (n = 17)	7.7 (4.0–14.0)	4.3 (3.0–6.5)
Deg (n = 7)	4.0 (3.1–19.5)	4.4 (3.0–6.1)
Oocyte stage ^b		
GV (n = 19)	5.1 (2.8–13.6)	4.2 (2.8–5.9)
MI (n = 29)	7.7 (4.6–14.1)	5.7 (3.7–8.3)
MII (n = 33)	8.1 (4.4–18.4)	5.9 (4.2–8.2)
PM (n = 3)	24.6 (8.6–24.9)	6.1 (3.5–7.4)
Fertilization ^b		
Normal (n = 37)	8.9 (5.1–19.0)	5.6 (4.1–8.2)
Abnormal (n = 11)	10.1 (5.3–14.6)	5.7 (3.2–7.5)
Failed (n = 18)	4.9 (3.7–12.1)	5.1 (3.2–7.4)
Embryo grade ^b		
1 (n = 7)	5.7 (4.4–8.3)	5.9 (5.0–6.6)
2 (n = 14)	14.2 (8.6–22.2)	5.0 (3.8–7.9)
3 (n = 10)	11.3 (7.0–23.2)	4.8 (3.1–6.3)

^a Median (25–75% confidence limits).^b See methods for description.

progesterone (25,30,31). Intrafollicular leptin does not vary as a function of the baseline subject or cycle characteristics that typically predict outcome, as demonstrated previously (12,15), with the exception of BMI (15). Similarly, sOB-R varies only with BMI. Serum leptin levels have also been demonstrated to predict response to gonadotropins (16,17), but were not evaluated in the current study. Therefore, whereas our previous studies and others suggested that lower intrafollicular leptin levels predicted better cycle outcome (15,18), a potential mechanism for these findings was not elucidated.

In summary, the current data demonstrate the presence of sOB-R in follicular fluid and suggest that leptin regulates its own binding protein within this compartment. They further suggest that intrafollicular estradiol may play a role in local leptin regulation, while activin and follistatin are more strongly associated with sOB-R. Finally, leptin and sOB-R do not appear to directly effect oocyte quality, fertilization, or embryo grade. Thus, the intrafollicular compartment demonstrates relationships between leptin, sOB-R, and other intrafollicular hormone levels, however, the role of leptin and sOB-R in follicular function will require further studies.

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