RAPID COMMUNICATION

Absence of leptin expression and secretion by human luteinized granulosa cells

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Abstract

Whether leptin is secreted by the human ovary is not known. The available data on leptin gene (ob gene) expression by human granulosa cells are conflicting. The aim of the present study was first to re-examine the expression of leptin messenger RNA (mRNA) by human granulosa cells and second to investigate if these cells have the ability to secrete leptin in cultures. Human luteinized granulosa cells were obtained from normal women undergoing *in vitro* fertilisation treatment after ovarian stimulation and follicle aspiration. The expression of ob gene was studied by Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) both in primary granulosa cells treated immediately after oocyte recovery and in cells cultured up to 24 h under baseline and hormonally stimulated conditions (FSH: 100 ng/ml, LH: 100 ng/ml). ob mRNA transcripts were not detected in luteinized granulosa cells, while they were present in adipose tissue cDNA. Actin gene expression was detected in all studied samples. Using a sensitive radioimmunoassay (lower limit of detection 0·05 ng/ml), leptin was undetectable in the culture media at all points during the 72 h cultures, while at the same time significant amounts of oestradiol and progesterone were produced particularly after the addition of androstendione (1 μM) to the incubation media. These results demonstrate for the first time that leptin is not secreted by human luteinized granulosa cells in cultures. From a physiological point of view, this may contribute to the development of the optimal follicular environment for oocyte maturation during the preovulatory period.

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Introduction

Leptin is a 167-amino acid protein, coded by the ob gene, which is located in the long arm of chromosome 7 (Zhang *et al.* 1994). Its receptors (both long and short isoforms) have been detected in the hypothalamus and the pituitary (Huang *et al.* 1996, Guan *et al.* 1997) as well as in granulosa and theca cells of various species including humans (Cioffi *et al.* 1997, Karlsson *et al.* 1997, Agarwal *et al.* 1999).

Although leptin is present in human follicular fluid (Cioffi *et al.* 1997), it is still unknown whether this hormone is produced by the human ovary. Clinical studies addressing the role of the ovaries in leptin homeostasis have not shown convincing data. For example, in women superovulated with FSH an increase in serum leptin concentrations was found during the treatment that was not in proportion to the degree of ovarian hyperstimulation (Messinis *et al.* 1998, Butzow *et al.* 1999). Also, following ovariectomy in

premenopausal women, serum leptin values declined significantly, but this could be prevented by treating women with oestradiol plus progesterone (Messinis *et al.* 1999, 2000). On the other hand, the existing data concerning leptin's messenger RNA (mRNA) expression in the ovary are conflicting. In particular, using reverse transcriptase polymerase chain reaction (RT-PCR), Karlsson *et al.* (1997) demonstrated that ob gene expression is not detected in human granulosa and theca cells obtained from normal women undergoing laparotomy, while Cioffi *et al.* (1997) and Löeffler *et al.* (2001) showed expression of leptin mRNA by human luteinized granulosa and cumulus cells of preovulatory follicles aspirated for *in vitro* fertilisation (IVF). It is interesting to note that Löeffler *et al.* (2001) have also investigated the localization of leptin by immunochemistry in growing and regressing follicles in intact ovaries, where leptin seemed absent in the granulosa layer of antral follicles, a finding that is in controversy to their RT-PCR results. Further to these discrepancies, no data exist concerning the potential of human granulosa cells to secrete leptin *in vivo* or *in vitro*.

The aim of the present study was to re-examine the expression of leptin mRNA by human luteinized granulosa cells and to investigate for the first time if these cells have the ability to secrete leptin in cultures.

Materials and methods

Subjects and tissue isolation

Human luteinized granulosa cells were obtained from follicle aspirates of women undergoing oocyte retrieval in stimulated cycles in the context of IVF treatment. Informed consent was obtained from all women and the study was approved by the local ethical committee. The treatment protocol in all cases involved pituitary and ovarian suppression by a GnRH agonist, followed by follicle stimulation with recombinant FSH. Final follicle and oocyte maturation was induced by human chorionic gonadotrophin (hCG). Oocyte retrieval was performed 36 h later. After the removal of the oocyte, the aspirated granulosa cells were transported on ice to the laboratory for purification (Yong *et al.* 1992). Granulosa cells aspirates were washed several times with phosphate-buffered saline (PBS, Biochrom AG, Germany) containing 0·1% bovine serum albumine (BSA, Sigma) until cleared from blood cells contamination and were centrifuged at 500 ***g*** for 5 min (Yong *et al.* 1992). Cell pellet was resuspended in serum free media 199 Earle's salts and Hepes (Gibco, Life Technologies, BRL, Scotland) containing 500 IU/ml sodium G penicillin and 500 μg/ml streptomycin (Biochrom AG), 0·1% BSA and 5% L-glutamine (Biochrom AG). Cell viability was determined by trypan blue exclusion. Granulosa cells were divided into two groups, cells that were used for RNA extraction (Chomczynski & Sacchi 1987) and cells that were seeded in cultures (Yong *et al.* 1992).

Cell cultures

Granulosa cells from each patient were adjusted at 5×10^6 cells/ 0·5 ml of serum free media, transferred into 24-well plate (Greiner Cellstar, Germany) and treated with different concentrations of FSH (0, 10 and 100 ng/ml) and LH (0, 10 and 100 ng/ml) with or without the addition of androstendione (1 μM) (A_4) (NIDDK's National Hormone and Peptide Program, Harbor-UCLA Medical Center) as a substrate for the aromatase in the culture media. Plates were incubated for 72 h at 37 °C in humidified atmosphere air containing 5% CO_2. Culture media was replaced every 24 h.

Leptin assay

For leptin measurement in culture media, a human leptin radioimmunnoassay was used (Sensitive Human Leptin RIA Kit, Linco Research, Ann Arbor, USA). This assay has been developed to increase sensitivity approximately 5x as compared with the regular human leptin assay, used for measuring leptin in blood samples. The lower limit of detection of the assay was 0·05 ng/ml. The antibody used was a polyclonal antibody raised in guinea pig against highly purified recombinant human leptin. Intra- and inter-assay coefficients of variation were 5·6% and 5·7% respectively. All measurements were performed in duplicate.

Steroid assay

Steroids, 17β-oestradiol and progesterone, in culture media were measured by commercially available enzyme immunoassays (Assay Designs,

Inc., Ann Arbor, USA). The lower limits of detection for 17β-oestradiol and progesterone were 37 pmol/L and 0·6 nmol/L, respectively. The intra- and inter-assay coefficients of variation for 17β-oestradiol and progesterone were 6·6% and 6·2% and 5·4% and 8·3%, respectively.

RNA extraction and cDNA preparation

Total cellular RNA from lutein granulosa cells was isolated by the guanidinium isothiocyanate method (Chomczynski & Sacchi 1987) either immediately after oocyte retrieval or following a 24 h pre-incubation period in serum free media with or without FSH (100 ng/ml) or LH (100 ng/ml).

In vitro reverse transcription was performed using Moloney Murine Leukaemia Virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA), random hexamers as primers (Gibco-BRL, Gaithersburg, MD, USA) and adipose tissue total cellular RNA substrate. After an initial denaturation at 65 °C for 5 min cDNA synthesis was performed at 37 °C for 60 min.

As a control for the presence of amplifiable RNA, 5 μl of the reverse transcription cDNA product was amplified by PCR. The following primers specific for β-actin gene were used; A1: 5′-GTGACGGCCCAGAGCAAGAG-3′ and A2: 5′-AGGGGCCGGACTCATCGTACT-3′. Amplification consisted of an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C/min, annealing at 57 °C/min and extension at 72 °C/min with a final extension step of 10 min at 72 °C.

Both in RT and the ensuing amplification reactions, recommended measures to prevent cross-contamination of samples were followed (Kwok & Higuchi 1989). In addition, a negative control reaction without template was included in all experiments to check for the presence of contaminants.

PCR amplification of ob gene cDNA sequence

5 μl of the reverse transcription reaction were amplified by PCR in a reaction using oligonucleotides specific for exons 2 (ob1: 5′-GCATT GGGGAACCCTGTG-3′, nucleotides 3–20) and 3 of ob gene (ob2: 5′-AGACCCAGGGCTGAGGT-3′, nucleotides 502–485) (Karlsson *et al.* 1997). PCR reaction was carried out in a final volume of 50μl with 25 pmol of each oligonucleotide primer, 200 μM each of dNTP and 2,5 U Taq polymerase (Gibco-BRL, Gaithersburg, MD, USA) in PCR buffer (50 mM KCL, 10 mM Tris–HCl pH 8·0, 1·5 mM MgCl2, 0·01% gelatin). Amplification consisted of an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C/min, annealing at 58 °C/min and extension at 72 °C/min with a final extension step of 10 min at 72 °C. In each experiment, cDNA from adipose tissue (Clontech, USA) was amplified and used as a positive control for the ob gene expression. The PCR products were separated on 2% agarose gel containing ethidium bromide and visualized by UV light. Determination of the specific PCR products was done by direct sequencing. For the sequencing analysis, PCR specific bands were isolated and purified from 2% low melting point agarose gel and sequenced by the dideoxy-chain termination method (Sanger *et al.* 1977) using a modified version of T7 DNA polymerase (Sequenase 2·0, US Biochemicals, OH).

Statistical analysis

All experiments were performed in triplicate. Data are presented as mean ± S.E.M. Statistical significance of combined data was determined by Student's *t*-test for paired samples. Statistical Package for Social Sciences (SPSS) 10·0 software was used.

Results and Discussion

In the present study, we have shown that leptin production was undetectable in the media of both unstimulated and hormonally stimulated human luteinized granulosa cell cultures (Table 1). Although it is known that leptin is present in human follicular fluid at concentrations similar to those in serum (Cioffi *et al.* 1997, Karlsson *et al.* 1997), our study demonstrates for the first time that human luteinized granulosa cells from antral follicles are not a place of leptin synthesis at least *in vitro*.

Even when the granulosa cells were stimulated in cultures by FSH or LH with or without the presence of androstendione as a substrate of aromatase in the media and although a sensitive radioimmunoassay was used for the measurement

Table 1

	Control	FSH (100 ng/ml)	LH (100 ng/ml)	A_4 (1 μM)	FSH (100 ng/ml)+ A_4 (1 μM)	LH (100 ng/ml)+ A_4 (1 μM)
Leptin (ng/ml)	nd*	nd*	nd*	nd*	nd*	nd*
Oestradiol (pmol/L)	15620±6002	28575±10042†	11613±1999	291462±34838‡	258272±44068	438346±65106
Progesterone (nmol/L)	599±129	1574±343†	1381±249	856±118†	923±327	1431±391

*nd: not detectable.
†$p<0.05$ (difference from control).
‡$p<0.01$ (difference from control).

of leptin, this protein was undetectable in all samples. One would argue that the cells lost their functional integrity under the *in vitro* conditions. This possibility, however, is rather unlikely due to the fact that these cells produced steroids *in vitro* under the same environmental conditions.

Maximum secretory capacity of the cells was seen at 24 h declining thereafter. The production of progesterone ($P<0{\cdot}05$) and particularly that of oestradiol ($P<0{\cdot}01$) was significantly enhanced by the addition of androstendione to the incubation media at 24 h (Table 1). However, neither FSH nor LH was able to enhance further the androstendione-supplemented steroidogenesis (Table 1), showing that the granulosa cells were somewhat refractory to the effects of the gonadotrophins. The present findings in terms of the steroidogenic capacity of granulosa cells *in vitro* are in agreement with earlier reports (Wickings *et al.* 1986, Erickson *et al.* 1989, Föeldesi *et al.* 1998).

The possibility that leptin is produced by luteinized granulosa cells in small amounts undetectable by our assay, as well as the possibility that these cells may produce leptin only *in vivo* are rather unlikely as leptin mRNA was not detected in primary cell samples. In particular, by the qualitative RT-PCR protocol, ob cDNA sequences were not detected in any sample from human granulosa cells grown under baseline or hormonally stimulated conditions (Fig. 1A, lanes 1–5), while ob mRNA transcripts were present in cDNA from human adipose cells (Fig. 1A, lane 6). Previous studies investigating this issue have given conflicting results.

Using RT-PCR, Karlsson *et al.* (1997) reported absence of ob gene expression in human granulosa cells obtained from women at laparotomy; in contrast, Cioffi *et al.* (1997) and Löeffler *et al.* (2001) have reported that ob gene is expressed in luteinized granulosa cells of preovulatory follicles aspirated for IVF. In our study, ob mRNA transcripts were not detected in luteinized granulosa cells either in those treated immediately after retrieval or in cells stimulated for 24 h with FSH or LH (Fig. 1A). The similar PCR signal of the reporter gene prior to or following short-term growth of the cells under serum-free conditions (Fig. 1B, lanes 1 & 2) indicates that by analogy to the apparent lack of inhibitory influence on steroid secretion (Table 1), the serum-free growth conditions used did not affect the gene expressional properties of the cellular system studied. The coordinated absence of leptin-specific and presence of actin-specific PCR product in the various cDNA preparations of human luteinized granulosa cells, along with the presence of both leptin- and actin-specific PCR product in human adipose cell cDNA (Fig. 1), strongly suggest that the ob gene is not expressed by the former cellular system.

The discrepancies between the present study and the studies by Cioffi *et al.* (1997) and Löeffler *et al.* (2001) are difficult to explain. The only apparent difference is that in the previous two studies experiments were performed in cells obtained from preovulatory follicles, while in the present study a mixture of granulosa cells from antral follicles of various sizes was used. It is important to note that Löeffler *et al.* (2001) were unable to confirm their findings with immunolocalisation of leptin in antral follicles. Importantly, in our study leptin mRNA and protein data seem to be consistent, indicating that granulosa lutein cells neither produce nor express leptin *in vitro*.

Certainly, the present data do not precisely reflect the capacity of an individual antral follicle as a unit to produce leptin, since first the granulosa cells used had already been exposed *in vivo* to hCG and second that leptin may be produced in other parts of the follicle, such as the theca cells (Löeffler *et al.* 2001). We cannot, therefore, exclude the possibility that granulosa cells can produce leptin before they luteinize, a process that may be attenuated or even inhibited under the influence of the midcycle LH surge and/or the suppressive effect on leptin synthesis from intra-ovarian sources. Previous studies have shown that leptin may act as a paracrine factor within the ovary affecting oocyte maturation and steroidogenesis with low levels in follicular fluid having beneficial effect (Antczak & Van Blerkom 1997, Agarwal *et al.* 1999, Mantzoros *et al.* 2000, Brannian *et al.* 2001, Ghizzoni *et al.* 2001). It is possible that the lack of leptin secretion by luteinized granulosa cells may contribute to the development of the optimal environmental conditions for the maturation of the oocyte within the follicle.

In conclusion, the present results demonstrate for the first time that human luteinized granulosa cells do not produce leptin *in vitro*. In terms of physiology, this may be important for leptin homeostasis within the follicle that provides support

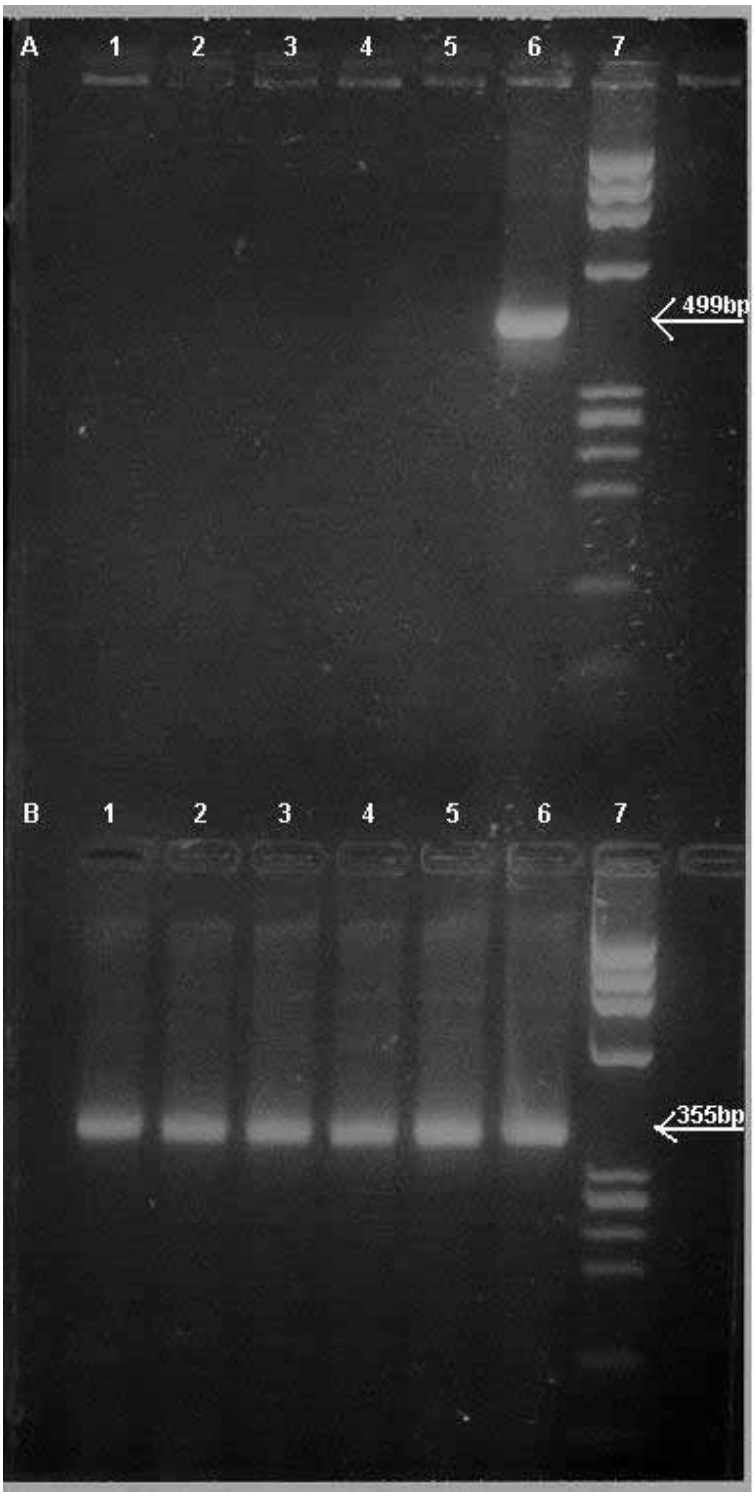

Figure 1 RT-PCR analysis for ob-gene (A) and β-actin gene (B) in human luteinized granulosa cells. Total mRNA isolated from human luteinized granulosa cells prior to (lane 1) or following culture for 24 h in the absence (lane 2), or presence of FSH (100 ng/ml) (lane 3), LH (100 ng/ml) (lane 4), androstendione (1 μM) (lane 5). PCR product of reporter β-actin cDNA was detected in all examined samples (Fig.1B). cDNA from human adipose cells was used as positive control for ob mRNA transcripts (lane 6). ϕX174/HaeIII was used as molecular weight marker (lane 7).

to the trophic and mitotic demands of the oocyte and early embryo.

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