Compartmentalization of human chorionic gonadotrophin sensitivity and luteinizing hormone receptor mRNA in different subtypes of the human corpus luteum

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Introduction
Corpus luteum (CL) insufficiency has been characterized, from the hormonal point of view, by monitoring serum, urinary or salivary progesterone, serum oestrogen and tissue proglandins, as well as prolactin, luteinizing hormone (LH), follicle stimulating hormone (FSH) and inhibin (Bopp and Shoupe, 1993; Dawood, 1994; Himney et al., 1996). Based on Noyes’ classical work (Noyes et al., 1950), other studies have been directed towards the function of target organs (e.g. uterine endometrium), mainly by histological examination. The duration of luteal periods has also been taken into consideration for the diagnosis of luteal phase insufficiency (Finn et al., 1989; Soules et al., 1989; Nakajima and Gibson, 1991). Ultrasoundography was introduced to gynaecologists to observe growing preovulatory follicles and the formation of a CL (Hackløer et al., 1978). This tool has, together with other diagnostic methods, contributed much to the assessment of different causes of infertility. The smaller preovulatory size or decreased growing rate of a developing follicle and a thin or echogenic, types b and d were centrally echogenic, types a and b had thin surrounding ‘walls’ (<3 mm) and types c and d had thick walls (>3 mm). After luteectomy, the theca externa capsule was removed and tissue from directly beneath the surface (‘peripheral region’) and the layer immediately beneath (‘inner region’) minced into 4-6 mg pieces. Following preincubation, pieces were incubated for 3 h at 37°C in HEPES–minimal essential medium buffer with or without human chorionic gonadotrophin (HCG; 10 IU/ml), and subsequently progesterone accumulation in the medium was determined by radioimmunoassay. The highest progesterone production was consistently seen in the peripheral region. Type a had a significantly (P > 0.01) lower progesterone production (3.2 ± 1.5 nmol/g tissue wet weight, mean ± SEM, n = 4) than that of types b, c and d (17.7 ± 3.5 nmol/g tissue wet weight, n = 9). All types responded to HCG with an almost two-fold increase in progesterone production. However, the maximal progesterone produced following stimulation by HCG in the type a corpus luteum was <50% of the basal (unstimulated) progesterone synthesis of any other type of corpus luteum. Using in-situ hybridization, with a primate RNA probe complementary to the region coding the extracellular part of the luteinizing hormone (LH) receptor, a highly localized expression of LH receptor mRNA to the peripheral region was found. Negligible or low levels of expression were found in the theca externa capsule and the inner region. No obvious correlations between the different subtypes of corpora lutea and LH receptor mRNA expression were seen. Thus, the ultrasonographic detection of a thin-walled and centrally hypoechoic corpus luteum correlates well with reduced progesterone secretion. The underlying cellular mechanism does not appear to involve a diminished sensitivity to the gonadotrophic stimulation by LH or HCG.

Key words: corpus luteum/HCG/LH receptor mRNA/progesterone/ultrasonography
Materials and methods

Twenty-one women were recruited to the study. All had given informed consent and the study was approved by the ethical committee of Umeå University. In the first part of the study 13 corpora lutea were obtained from women undergoing hysterectomy for uterine fibroma at the Department of Obstetrics and Gynecology, Umeå University Hospital. The patients had not received any hormonal therapy during the preceding 6 months and were otherwise healthy. The average age of the patients was 43.5 years (range 34–48 years) (Table I). All patients had proven fertility, with an average parity of 2.2 (range 1–4 children) and had a history of regular menstrual cycles ranging between 24 and 30 days. The luteal age was determined according to last menstrual onset and verified by histological examination of endometrial specimens taken during hysterecnectomy using Noyes’ classification (Noyes et al., 1950). According to these two parameters all patients were in the mid-luteal phase of the menstrual cycle (between 5 and 9 days after ovulation). The mean luteal age was 7.6 ± 0.5 days (mean ± SEM, n = 13). On the day of surgery all patients were examined by ultrasonography by the same investigator (M.N.) and the corpora lutea were classified into four types, a–d, according to the combination of echogenicity of the central region (types a and c with hypoechoic central region; types b and d with echogenic central region) and the maximum wall thickness (types a and b with <3 mm maximum wall thickness; types c and d with ≥3 mm) (Nakata et al., 1992). After each ultrasound examination a blood sample was taken. Blood samples were, except for the last patient, also taken on the day before surgery. The serum was extracted with ether and progesterone concentrations determined by radioimmunoassay (Farnmos Diagnostica, Turku, Finland).

In-situ hybridization

In a subsequent experimental series of eight mid-luteal corpora lutea, the luteal age was determined by menstrual history and urinary detection of an ovulatory LH surge (Clearplan One Step, Unipath Ltd, Bedford, UK). Day 1 was defined as the first day after a positive LH test. The age of the corpora lutea ranged between 6 and 10 days and the mean ± SEM luteal age was 7.6 ± 0.5 (n = 8). During surgery the CL was extirpated and immediately cut into pieces and placed in liquid nitrogen. To localize the expression of LH receptor mRNA a 410 bp LH receptor cRNA probe was synthesized. A monkey LH receptor cDNA fragment was obtained by reverse transcriptase-polymerase chain reaction (RT–PCR) amplification using total RNA from monkey ovaries as template and with oligonucleotide primers complementary to the human LH receptor cDNA sequence (nucleotides 438–460 and 827–847). The obtained PCR product was cloned into a pGem-3Z plasmid and sequenced using an automated DNA sequencer (ALFexpress, Pharmacia Ltd.
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Uppsala, Sweden). Computer-based sequence analysis revealed that the monkey LH receptor cDNA fragment had a 99.56% homology with the known human cDNA sequence. The in-situ hybridization was performed essentially as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993). Briefly, 10 mm cryostat sections were collected on SuperFrost® Plus slides (Menzel-Glazer, Brann Scheig, Germany) and fixed in 4% paraformaldehyde (in PBS) for 10 min. Prehybridization was performed in a solution of 50% formamide, 5× SSC (sodium chloride, sodium citrate; Sigma), 5× Denhardt’s solution (Sigma), 250 mg/ml tRNA and 500 mg/ml herring sperm DNA (Sigma) at room temperature overnight. Hybridization was performed in the same solution containing ~1 µg/ml digoxigenin–UTP-labelled LH receptor antisense (Boehringer GmbH, Mannheim, Germany) RNA probe at 72°C overnight. After hybridization the slides were washed in 0.2× SSC at 72°C for 1 h. The slides were then incubated with blocking buffer (10% heat-inactivated normal goat serum, 0.1 M Tris, pH 7.5, 0.15 M NaCl) for 1 h at room temperature before addition of the alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer, 1:5000 dilution in 0.1 M Tris, pH 7.5, 0.15 M NaCl, 1% heat-inactivated goat serum). After incubation with the antibody at 4°C overnight, the slides were washed three times in Tris buffer (0.1 M Tris, pH 7.5, 0.15 M NaCl) before being equilibrated in alkaline phosphatase buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM MgCl2). Then substrate for alkaline phosphatase was added (450 mg/ml 4-nitroblue tetrazolium chloride (Boehringer) and 175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Boehringer) in alkaline phosphatase buffer). After 3 days in the dark, the colour reaction was terminated with 10 mM Tris, 1 mM EDTA (pH 8.0).

Figure 1. Time-dependent accumulation in vitro of progesterone from pieces of human corpora lutea incubated in HEPES–MEM medium. Two mid-luteal human corpus lutea (type d) were extirpated during hysterectomy and the peripheral region was minced into 4–6 mg pieces. Progesterone was measured in the incubation media by radioimmunoassay and the mean ± SEM values of eight pieces calculated.

Statistical analysis
Values are given as mean ± SEM or individual values. Differences between groups were tested by the Mann–Whitney U-test and differences within groups by Wilcoxon signed rank test. Spearman’s rank correlation (r) was used for correlation calculations. P < 0.05 was regarded as significant.

Results
Accumulation of progesterone in the peripheral region of the mid-luteal CL was found to increase almost linearly with incubation time, suggesting a continuous de-novo synthesis of progesterone (Figure 1). Therefore, in the following experiments an incubation time of 180 min was chosen to study progesterone production. The mean value of serum progesterone for these patients, calculated as the average value from the samples obtained on the preoperative and operation day for each patient (Table I), was 32.2 ± 5.6 nmol/l (mean ± SEM, n = 13). There was a significant correlation between serum progesterone concentrations on the day of operation and progesterone produced in vitro by the peripheral region of the CL (r = 0.69, P < 0.01). Basal progesterone accumulations following a 3 h incubation period for the different types of corpora lutea are shown in Table II and Figure 2. Notably, a CL produced significantly lower basal amounts of progesterone (P < 0.01) when compared to the other classes of corpora lutea (types b–d). This effect was more pronounced in the peripheral region than in the inner region (Figure 2). Basal synthesis of progesterone in the peripheral region of the human CL was consistently higher than in the inner region (Figure 2). The progesterone produced in the outermost layer (theca externa) was also measured in several experiments (data not shown) and found to be negligibly low (1.9 ± 0.8 nmol/g wet weight/3 h). When a high dose of HCG (10 IU/ml) was added to the incubation medium, progesterone production in the peripheral region was significantly (P < 0.05) stimulated (62% increase) when all types of corpora lutea were pooled (Table II). Notably, such a stimulation was not seen in the inner region (6.0 ± 2.4 versus 6.4 ± 2.0 nmol/g wet weight/3 h) for control and HCG-stimulated values, respectively (P > 0.05). Repetitive in-situ hybridization experiments revealed

Figure 2. In-vitro progesterone accumulation from peripheral and inner parts of human corpus luteum (CL) related to the pre-operative ultrasonographic classification (see Materials and methods). For both the peripheral and inner regions, type a (n = 4), characterized by a cystic CL with a thin ‘wall’, was compared to the pooled mean values of types b, c and d (n = 9). *P < 0.01 versus type a by Mann–Whitney U-test. †P < 0.01 versus peripheral region in corresponding group by Wilcoxon signed rank test.
that the expression of LH receptor mRNA is predominantly located to the peripheral region of the CL (Figure 3). Consistent throughout the series of different corpora lutea investigated \((n = 8)\), very low concentrations of LH receptor mRNA expression were noted in the outermost and inner layers. Furthermore, in a few corpora lutea sampled, a low but clearly discernible LH receptor message was localized to the ovarian surface epithelium lining the exterior region of the CL (Figure 3). No obvious differences in localization pattern or concentrations of expression of LH receptor mRNA were noted between the different classes of CL.

**Discussion**

The present study confirms our previous demonstration of a spontaneously occurring cystic CL with a thin wall concomitant with decreased systemic progesterone concentrations during the mid-luteal phase. Importantly, our results indicate that this type of CL has a low capacity for producing progesterone. Lenz and Lindenberg (1990) reported a higher incidence of corpora lutea with follicle-like structures in induced cycles than in normal cycles. They suggested that a sonographic cystic structure might indicate an abnormal CL, since their subjects did not exhibit normal serum concentrations of oestradiol or progesterone at the same luteal phase. Distinct from this is the luteinized unruptured follicle (LUF) syndrome, in which cystic ovarian structure(s) can be seen by ultrasonography in the mid-luteal phase. Low serum progesterone concentrations have been found in LUF patients (Couiss, 1985; Eissa et al., 1987). Hamilton et al. (1990) showed that luteal cyst formation occurred in both the supposed ruptured and unruptured follicle cycles in an unexplained infertility population. They concluded that the cyst formation was indicative of an infertile cycle, since the supposed ruptured follicle was followed by a somewhat lower plasma progesterone concentration than normal in the early to mid-luteal phase. Finn et al. (1989) showed that LUF is not the major cause of low progesterone concentrations during the luteal period in unexplained-infertile women by demonstrating a low frequency of LUF in this population. Based on these findings and our previous studies (Nakata et al., 1992; Bäckström et al., 1994), we conclude that, during ovulatory menstrual cycles, cystic corpora lutea occur spontaneously. These corpora lutea may cause fertility problems because of their reduced ability to support the endometrium, but this remains to be verified by further studies. The incidence of a cystic CL with a thin wall (type a) in this study was 23.8% (5/21). As the patients were older, they may have a higher rate of CL insufficiency, as has been discussed by Soules (1987). Nevertheless, this high incidence of a cystic CL with a thin wall should be taken into consideration during functional experiments using human corpora lutea.

Under the incubation conditions used in the present study, progesterone production showed an approximately linear relationship with time, similar to a previous study utilizing different incubation media (Hahlin et al., 1986). The dissected luteal tissue still actively secreted progesterone 300 min after the onset of incubation. Our finding that addition of HCG stimulated progesterone production to approximately double after 180 min incubation is also consistent with other in-vitro studies in the mid-luteal phase (Swanson et al., 1977; Hunter and Baker, 1981; Vijayakumar and Walters, 1983; Vega et al., 1987; Yamato et al., 1988; Retnales et al., 1994). In the present study it was confirmed that there was a large difference in progesterone production among the different layers of the CL and that the highest production was found in the peripheral region. Previous studies using incubated rabbit CL tissue (Dharmarajan et al., 1994) and luteinized human granulosa cells (Quirk et al., 1995) have indicated that luteal cells may undergo apoptosis when cultured in vitro. Although not directly studied herein, the differential response to HCG and the almost linear increase in progesterone output may suggest that the peripheral HCG-sensitive cell layer did not markedly undergo cell death during the incubation procedure.

A hitherto less recognized finding is that the majority of LH-receptors are located in the peripheral region, as
demonstrated by in-situ hybridization, which adds further support to the finding that regulated progesterone production is predominantly localized in this layer. A recently published study has localized LH receptor mRNA expression to the steroidogenic cells of the CL (Duncan et al., 1996) and, furthermore, demonstrated that the expression of LH receptors does not seem to be down-regulated following increased HCG concentrations administered to simulate maternal recognition of pregnancy. When the in-vitro progesterone synthesis by individual corpora lutea was compared, the difference in ultrasonographic characteristics between type a and the other types of corpora lutea corresponded well to their capacity for progesterone production per unit wet weight of luteal tissues. From these results it can be inferred that the decreased serum progesterone concentrations in patients exhibiting type a CL are not only caused by the smaller number of luteal cells in the thinner walls of these corpora lutea, but also by the attenuated function of the luteal tissue. The underlying cellular mechanism for the loss of function is still unclear, but it could be hypothesized that corpora lutea possessing thinner walls had a decreased blood vessel supply to the granulosa–luteal cell layers than corpora lutea with thicker walls. Jones et al. (1970) performed a histological study on luteal phase defect in clomiphene-induced ovulatory cycles, and demonstrated that CL with sparse blood vessels in the granulosa–lutein cell layers had lower 3β-hydroxysteroid dehydrogenase activity. Furthermore, the low serum progesterone values of patients with type a CL is not likely to be caused by HCG insensitivity. This is mainly based on the finding of equal responsiveness to HCG but also upon the lack of obvious differences in LH receptor mRNA expression between the various types of corpora lutea. However, it is notable that the maximal progesterone produced following stimulation by HCG in type a CL was <50% of the basal (unstimulated) progesterone synthesis of any other type of CL. Two populations of luteal cells of different size exist in many mammalian species including the human (Ohtani et al., 1987). In the human CL the large luteal cells produce more progesterone while the small luteal cells respond better to HCG (Retmales et al., 1994). Considering these cell types, it is possible that the condition of luteal insufficiency could be due to changes in the proportions of these cell types. Whether the condition of type a CL described here represents such changes is not clear at present.

In summary, we have demonstrated that the ultrasonographic detection of a thin-walled, centrally hypoechoic corpus luteum is indicative of a sub-functional state as judged by luteal progesterone production. The underlying mechanism(s) does not appear to involve structural tissue rearrangements, since in all CL types the bulk of progesterone was produced in the peripheral layer where the functional sensitivity to HCG and the expression of LH receptor mRNA was also found to be higher than in the outermost and inner layers of the CL.

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References

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