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Specific gonadotrophin-releasing hormone analogue binding predominantly in human luteinized follicular aspirates and not in human pre-ovulatory follicles

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In an attempt to resolve the apparent controversy in the observed effects of gonadotrophin-releasing hormone (GnRH) analogues on the ovary, conventional binding studies were conducted with a GnRH agonist and an antagonist in various ovarian tissues to demonstrate possible GnRH receptor binding. In human luteinized granulosa cells derived from unstimulated in-vitro fertilization cycles, high affinity receptor binding was present in 17 out of 24 patients, while binding was not observed in any of the six pre-ovulatory follicles removed during abdominal surgery. Apparently contradictory observations on the direct ovarian effects of GnRH analogues may be the result of the intermittent presence of high affinity GnRH receptors. Our observations indicate that in the human, high affinity ovarian GnRH receptors are present predominantly in ovarian tissue after the luteinizing hormone surge. We also propose the possibility of regulation and activation of a human follicular GnRH receptor in the ovary as a physiological process which may be influenced pharmacologically.

Key words: GnRH receptor/gonadorelin agonist/gonadorelin antagonist/granulosa cells/human ovary

Introduction

In the past decade substantial progress has been made in the development of gonadotrophin-releasing hormone (GnRH) analogues as both diagnostic and therapeutic tools. GnRH agonists are widely used in in-vitro fertilization (IVF) procedures for the prevention of premature luteinization.

A meta-analysis suggested that the use of GnRH agonists in IVF procedures results in more and better quality oocytes (Hughes *et al.*, 1992). In the near future, GnRH antagonists may also become available for this purpose (Diedrich *et al.*, 1994), with perhaps even better results (Minaretzis *et al.*, 1995a). With this further application, more insight is required into the direct ovarian effects of GnRH analogues. A number of studies in primates and non-primates have already focused on this. In the rat, direct effects of GnRH agonists and antagonists on granulosa cells could easily be explained by the presence of high affinity GnRH receptors (Clayton *et al.*, 1979; Seguin *et al.*, 1982). In the human and non-human primate, however, effects were rather contradictory (Hsueh and Jones, 1981; Clayton and Huhtaniemi, 1982; Casper *et al.*, 1982, 1984; Parinaud *et al.*, 1988; Wickings *et al.*, 1990). There are still conflicting data on the presence of GnRH receptors in the human ovary (Bramley *et al.*, 1987; Clayton, 1989; Latouche *et al.*, 1989). Recently, several studies have demonstrated independently expression of the GnRH receptor gene in human ovarian tissue (Kakar *et al.*, 1992; Peng *et al.*, 1994; Minaretzis *et al.*, 1995b; Fraser *et al.*, 1996). Based on these results, we postulated that any direct ovarian effects of GnRH analogues may not only on affinity type, but also on the timing of the appearance of GnRH receptors. In an attempt to resolve the apparent controversy in observed direct effects, we conducted conventional binding studies in human pre-ovulatory non-luteinized follicles and in luteinized follicular aspirates with a GnRH agonist and antagonist so as to estimate affinity type and the timing of the appearance of possible GnRH receptor binding.

Materials and methods

The study was approved by the local ethical committee of the Free University Hospital of Amsterdam (The Netherlands), where the study took place. Written informed consent was given by all participants.

Collection of human pre-ovulatory follicles

Human pre-ovulatory follicles were obtained at the time of surgery from six women aged 32–43 years (mean 37.3), having regular menstrual cycles of ~28 days; five of the women were undergoing hysterectomy for various benign gynaecological diseases [uterine myomas (n = 2), pelvic pain (n = 3)], and one a Burch operation with sterilization. Surgery was planned such that all the women were in late follicular phase. The stage of the menstrual cycle was determined by the patients' history and confirmed by pre-operative vaginal ultrasound examinations. None of the patients used any medication. Examination of the ovaries did not reveal any abnormalities. Under general anaesthesia, the pre-ovulatory follicles were sharply dissected and immediately snap frozen in liquid nitrogen until further analysis.

Collection of human luteinized granulosa cells

Follicular aspirates were obtained from 24 women aged 31–40 years (mean 37.0) with tubal occlusions, who were undergoing oocyte retrieval for IVF in a spontaneous unstimulated cycle. When the follicle was 20–22 mm in diameter, human chorionic gonadotrophin (HCG; 10 000 IU; Profasi; Serono, Aubonne, Switzerland) was administered i.m., 36 h before oocyte retrieval. Follicle aspiration

was carried out under local anaesthesia. Follicular aspirates were collected in Erb's medium and stored at –20°C until further processing.

Collection of rat tissue

Pituitaries and ovaries from rats on the day of pro-oestrus were employed to test the binding ability of the iodinated GnRH analogues used for the human pre-ovulatory follicle homogenates. Adult female rats (200–240 g) from the Wistar-derived colony kept in our laboratory were allowed free access to food and water in an animal room illuminated from 0700 to 1900 h and maintained at a constant temperature of 22°C. Vaginal smears were taken daily from regular 4 day cyclic rats. The rats were killed by decapitation, and pituitaries and ovaries were dissected and stored at –20°C until analysed.

Granulosa cells from 28 day old rats were used as a positive control for the human luteinized follicular aspirates. These rats were kept under the same conditions as the adult female rats, but were treated with 100 µl (5 mg/ml saline) diethylstilbestrol (Organon, Oss, The Netherlands) for 3 days and killed by an overdose of ether inhalation. Ovaries were dissected and the follicles were punctured; the cells collected were stored at –20°C until use.

Peptides

The GnRH antagonist antide and D-Tyr-antide for iodination were kindly supplied by Ares Serono (Geneva, Switzerland). The GnRH agonist buserelin was generously donated by Hoechst Holland NV (Amsterdam, The Netherlands). Radio-iodinated NaI was purchased from Amersham (Amersham, UK).

Radio-iodination of GnRH analogues

Buserelin was radio-iodinated by the hypochloride method (Redshaw and Lynch, 1974) and purified using a QAE Sephadex column (Hazum, 1981). D-Tyr-antide was radio-iodinated by the lactoperoxidase method (Copeland *et al.*, 1979; Li *et al.*, 1994) and purified using a Sephadex G25 column.

Preparation of human pre-ovulatory follicles and rat pituitaries and ovaries

After thawing, the pre-ovulatory follicles were homogenized in incubation buffer [0.0025 M Tris–HCl, 0.01 M $MgCl_2$ and 0.1% bovine serum albumin (BSA), pH 7.4] using 1 ml incubation buffer per half a human follicle. In parallel, the rat pituitaries and rat ovaries were thawed and homogenized in the same buffer using 1 ml per pituitary and 1 ml per two ovaries.

The material was centrifuged for 5 min at 225 *g*. The supernatant of the first spin was centrifuged for 15 min at 12 000 *g*. The pellet of the second spin was resuspended with the buffer; 250 µl of the suspension were used containing the equivalent of one quarter of a human follicle, one quarter of a rat pituitary, or one half of a rat ovary. The mean amounts of DNA were 1150, 1430 and 1210 µg DNA/ml respectively.

Preparation of human luteinized granulosa cells and rat granulosa cells

After thawing, 2 ml of the follicular aspirates were added to 1 ml of the buffer and centrifuged for 15 min at 225 *g*. Subsequently, the supernatant was centrifuged for 30 min at 12 000 *g*, after which the pellet was resuspended in the assay buffer and 250 µl (810 mg DNA/ml) were used for the binding assay.

Binding assay procedure

For total binding, iodinated 5.0×10^{-11} M D-Tyr-antide (10 000 c.p.m.) or 2.5×10^{-11} M iodinated buserelin (10 000 c.p.m.) were added to all the tubes. To determine non-specific binding, 2 µg/100 µl GnRH analogue of either antide or buserelin were added to half of the tubes in a total volume of 500 µl. In each assay, D-Tyr-antide and buserelin were tested in duplicate.

Incubation was terminated after 90 min at 4°C for buserelin and after 24 h at 4°C for D-Tyr-antide by centrifugation for 15 min at 12 000 *g*. Subsequently radioactivity in the pellets was counted. The specific binding was calculated (i.e. total binding – non-specific binding) and expressed as a percentage of the total counts added. The specific activity, as determined by self-displacement (Clayton, 1983) in human granulosa cells, was 606 µCi/mg for D-Tyr-antide and 891 µCi/mg for buserelin. The counting efficiency of the gamma spectrometer was 75%. Maximal binding ability was established by adding excess rat pituitary or ovarian homogenate or pooled granulosa cells to 3000 c.p.m. of the iodinated peptides. Maximal binding was 20–40% for D-Tyr-antide and 15–35% for buserelin. The K_a value was calculated using a computerized Scatchard analysis of saturation assays (Munson and Rodbard, 1979). Because of the limited availability of human granulosa cells, the K_a values were estimated in pooled material.

Table I. The number of cases with specific binding of the gonadotrophin-releasing hormone (GnRH) agonist buserelin and the GnRH antagonist D-Tyr-antide to luteinized granulosa cells from human follicular aspirate obtained from spontaneous in-vitro fertilization cycles

	Specific binding		
	Yes	No[a]	Total
D-Tyr-antide	17 (2.5–7.0)[b]	7	24
Buserelin	17 (3.0–3.5)	7	24

Values in parentheses are percentages. Fisher's exact test: 95% confidence interval 48.91–87.28%.
[a]All cases negative for D-Tyr-antide binding were also negative for buserelin.
[b]Range of specific binding expressed in percentage of total of counts added.

Results

Human pre-ovulatory follicles

No binding was found in any of the six human pre-ovulatory follicles with either D-Tyr-antide or buserelin (Fisher's exact test: 95% confidence interval 54.07–100.00%).

Human follicular aspirate

Specific binding in the follicular aspirate was found in a significant number of patients (17/24) (Table I) with both D-Tyr-antide (3–7%) and buserelin (3–4%). In all other cases no binding of either D-Tyr-antide or buserelin was detectable.

Rat tissue

As expected, specific binding of rat pituitaries, ovaries and granulosa cells could be detected in all samples with values of 24, 20 and 13% respectively with D-Tyr-antide and of 10, 5 and 6% respectively with buserelin (Table II).

Binding characteristics

The binding characteristics of the GnRH analogues with the various homogenates are given in Table III. Specific high affinity binding sites were demonstrated in rat pituitaries, rat ovaries, pooled rat granulosa cells and pooled human granulosa

Table II. Mean and range of total, non-specific and specific binding expressed as a percentage of total counts added of the gonadotrophin-releasing hormone (GnRH) agonist buserelin and the GnRH antagonist D-Tyr-antide in human granulosa cells and in tissue from adult female rats

	Total	Non-specific	Specific
Buserelin			
Rat pituitary (n = 10)	15 (8–22)	5 (3–7)	10 (5–15)
Rat ovary (n = 20)	10 (5–15)	5 (2–8)	5 (3–7)
Rat granulosa (n = 20)	12 (6–18)	6 (3–9)	6 (3–8)
Human granulosa (n = 17)	9 (5–12)	5 (2–9)	3.25 (3.0–3.5)
D-Tyr-antide			
Rat pituitary (n = 10)	57 (37–75)	32 (18–44)	24 (19–31)
Rat ovary (n = 20)	48 (35–61)	28 (20–36)	20 (15–25)
Rat granulosa (n = 20)	36 (25–47)	23 (15–31)	13 (10–16)
Human granulosa (n = 17)	17 (11–19)	12 (9–12)	4 (3–7)

Table III. K_a values (10^9 M^{-1}) of human follicular aspirate obtained in spontaneous in-vitro fertilization cycle and rat control tissue

	D-Tyr-antide	Buserelin
Rat pituitary	5.7	4.5
Rat ovary	6.5	4.1
Rat granulosa	4.7	3.0
Human granulosa	3.6	2.7

cells. The binding capacities (receptor concentrations) for human granulosa cells were 74 fmol/mg DNA for D-Tyr-antide and 61 fmol/mg DNA for buserelin.

Displacement curves of iodinated D-Tyr-antide and iodinated buserelin are given in Figure 1.

Binding and IVF and embryo transfer outcome

The absence or presence of binding was not significantly related to any of the IVF and embryo transfer outcome parameters such as fertilization rate (Fisher's exact test: P = 0.6225) or pregnancy rate (P = 0.5165). In all seven cases with no binding oocytes were obtained, whereas no oocytes could be obtained in six out of the 17 subjects in whom specific binding was demonstrated (P = 0.13).

Discussion

Our study shows that specific GnRH agonistic and antagonistic binding is present in human luteinized granulosa cells. The K_a values indicate high affinity binding sites, comparable with the binding that we measured in rat pituitary, ovary and granulosa cells. In contrast, no binding was measured in homogenates of pre-ovulatory follicles.

Controversies exist with respect to the direct ovarian effects of GnRH agonists and antagonists *in vitro*. The many differences in observation with respect to the production of major ovarian hormones indicate that a variety of factors may be involved, such as type and dose of the analogue (Bussenot *et al.*, 1993), ovarian cell types and the hormonal pretreatment of these cells (Casper *et al.*, 1982; Tureck *et al.*, 1982; Birnbaumer *et al.*, 1985). The most important determinant, however, is probably the presence or absence of a GnRH

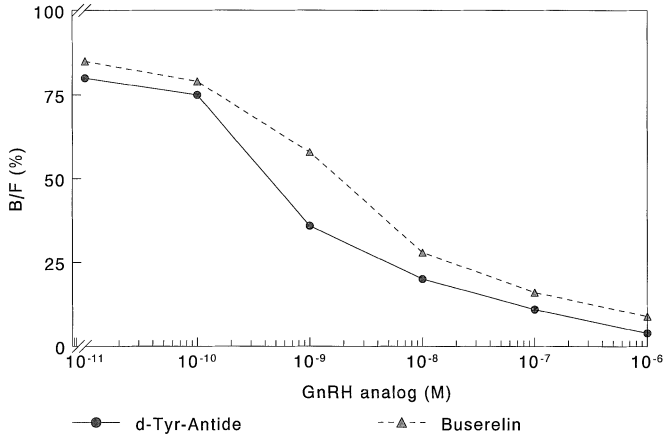

Figure 1. Displacement of labelled D-Tyr-antide or labelled buserelin from gonadotrophin-releasing hormone receptor sites in human granulosa cells by increasing concentrations of unlabelled homologous peptide. B/F = bound/free ratio.

receptor. This seems to be highly species dependent. High affinity GnRH receptors have been demonstrated in rat ovary (Clayton *et al.*, 1979; Seguin *et al.*, 1982) and explain the opposite effects established by GnRH antagonists and GnRH agonists (Wickings *et al.*, 1990).

Indeed, conflicting data exist on the presence of GnRH receptors in primate ovarian tissue. Latouche *et al.* (1989) showed GnRH agonist binding sites with high affinity in the granulosa of a single human pre-ovulatory follicle. Bramley *et al.* (1985) demonstrated low affinity binding in mid-luteal corpora lutea. However, no GnRH receptor binding was demonstrated in corpora lutea by Clayton and Huhtaniemi (1982), while another study showed the presence of specific binding sites for GnRH and its analogues in human luteal homogenates (Popkin *et al.*, 1983). Fraser *et al.* (1996) demonstrated low levels of expression of GnRH receptor mRNA with in-situ hybridization in human corpora lutea. In addition, Kakar *et al.* (1992) demonstrated, by the polymerase chain reaction amplification technique, the presence of cDNA coding for the human GnRH receptor in some samples of human ovarian tissue. Even more recently, GnRH receptor mRNA has been demonstrated both in human luteinized granulosa cells (Peng *et al.*, 1994) and in human ovarian homogenates across different functional stages of the menstrual cycle, implying that multiple ovarian compartments may express GnRH receptors (Minaretzis *et al.*, 1995b).

Our data indicate, however, the temporary presence of functional GnRH binding in human ovarian tissue, predominantly around ovulation. This temporary presence would largely explain the wide variety of results of experiments testing the direct ovarian effects of GnRH analogues.

All pre-ovulatory follicles were collected under general anaesthesia, and follicular aspiration was carried out under local anaesthesia. Therefore the theoretical possibility of anaesthetic conditions influencing GnRH receptor binding cannot be excluded. There are, however, no literature data to support this. Therefore the question as to what regulates expression of the GnRH receptor remains.

GnRH itself increased the amount of mRNA in precultured

human granulosa luteal cells (Peng *et al.*, 1994). The same report showed that in cultured human granulosa luteal cells GnRH receptor gene expression is down-regulated by prolonged exposure to HCG. Previous studies in the rat ovary have shown that the injection of HCG caused a 60% loss of GnRH receptor (Harwood *et al.*, 1980). Whitelaw *et al.* (1995) observed that the administration of follicle stimulating hormone or human menopausal gonadotrophin to hypophysectomized female rats enhanced the expression of GnRH receptor mRNA. So these data do not support the notion that luteinizing hormone (LH) itself is the primary cause of a rise in the number of GnRH receptors in the ovary. However, the most important endocrine changes in the ovary following the peri-ovulatory LH surge are progesterone production and the generation of cAMP (Yong *et al.*, 1992, 1994). Importantly, Fan *et al.* (1995) have shown that the GnRH receptor gene contains cAMP and progesterone responsive sites. This may indicate that ovarian GnRH receptor expression following the LH surge is mediated by these ovarian products.

Expression of the GnRH receptor gene in the human ovary provides evidence that the ovary could be a target of extra-pituitary GnRH action. The concentration of hypothalamic GnRH seems to be too low to interact with the receptor in peripheral tissue. However, GnRH, capable of binding to the GnRH receptor, is present in human ovary and may act in an autocrine or paracrine fashion (Aten *et al.*, 1986; Peng *et al.*, 1994). Peng *et al.* (1994) showed that when GnRH receptor mRNA is up-regulated, GnRH inhibits progesterone production; however, when GnRH receptor mRNA is unaffected, GnRH has no effect on progesterone accumulation. The direct modulatory effect of GnRH on steroidogenesis, together with the finding that both GnRH and GnRH receptor genes are expressed in human granulosa luteal cells, support further the notion that GnRH plays an autocrine and/or paracrine role in the human ovary. In addition, a potential source from which extra-hypothalamic GnRH originates is the placenta (Lin *et al.*, 1995). Thus, hypothetically, GnRH receptors in the human ovary may mediate an endocrine signal resulting from pregnancy. What these putative effects are requires further evaluation.

It is possible that the GnRH receptors are expressed only in specific cells and only at or near the time of ovulation to enhance meiotic maturation and indirectly the fertilization rate. It has been reported that ovarian GnRH receptor mRNA varies during the rat ovulatory cycle, with amounts increasing 2-fold between met-oestrus and pro-oestrus (Bauer Dantoin and Jameson, 1995). In bovine oocytes, GnRH and GnRH agonists increase cleavage rates in IVF (Funston and Seidel, 1995). GnRH antagonists diminished this effect of GnRH. This study has demonstrated gene expression of the GnRH receptor in cumulus–oocyte complexes. These findings provide evidence for a mechanism to increase cleavage rates in IVF procedures, possibly through the binding of GnRH to its receptor.

In conclusion, specific high affinity binding in human follicular aspirates and not in pre-ovulatory follicular homogenates may indicate that the presence of GnRH receptors varies dramatically during the course of the ovulatory cycle. Apparently, its presence and absence is controlled by a carefully regulated physiological mechanism that may be influenced pharmacologically. The effects of stimulation or inhibition of the human ovarian GnRH receptor need to be elucidated further. In view of the likelihood of regulation by GnRH itself, more prudence should be applied to the use of GnRH agonist, and in the near future the GnRH antagonist, immediately prior to or during the time of ovulation.

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