GnRH receptor mRNA expression by in-situ hybridization in the primate pituitary and ovary

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Gonadotrophin-releasing hormone (GnRH) receptors are present on the ovary as well as in the anterior pituitary gland. GnRH analogues may exert their actions in part via these ovarian receptors. However, in the primate ovary, GnRH receptors are of low affinity and their significance is questionable. The aim of the present study was to compare pituitary and ovarian expression of the GnRH receptor mRNA by in-situ hybridization to gain further information on the possible significance of the ovarian receptor. Pituitaries and ovaries were obtained from two stump-tailed macaque monkeys and three marmoset monkeys at the mid-luteal phase of the ovariative cycle. Human corpora lutea were obtained during the early and mid-luteal phase and after 'rescue' by human chorionic gonadotrophin (HCG) and a whole ovary obtained during the late luteal phase (n = 1 per group). Frozen tissue sections were incubated with a 3²P-labelled probe to the human GnRH receptor and exposed for 4 weeks. All pituitary glands exhibited intense silver grains in the anterior pituitary gland. In the ovaries, grains were present at low levels in the granulosa cells of antral follicles, just above tissue background in corpora lutea and indistinguishable from tissue background in the remaining ovarian compartments. These results demonstrate that the GnRH receptor mRNA in the primate pituitary is present in sufficient quantities to be clearly detectable in the anterior pituitary gland by in-situ hybridization. In contrast, in the human and monkey, ovary levels of mRNA appear to be very low.

Key words: corpus luteum/follicle/GnRH mRNA/in-situ hybridization

Introduction

Gonadotrophin-releasing hormone (GnRH) receptors have been described in the ovary of the rat and human as well as on the anterior pituitary gland (Popkin et al., 1983a; Bramley et al., 1985; Fraser et al., 1986). In the rat, receptor affinity is similar to that of the pituitary GnRH receptor and GnRH and its analogues have been shown to exert direct effects upon the ovary both in vivo and in vitro (Hsueh and Jones 1981; Popkin et al., 1983b; Billig et al., 1994). However, in the human, GnRH binding affinity is low and it may be more appropriate to term these 'receptors' as binding sites (Fraser et al., 1986). Also, the majority of studies which have examined effects of GnRH analogues on granulosa or luteal cell function have shown either no or minimal effects (Casper et al., 1982, 1984; Richardson et al., 1984; Wickings et al., 1990). This has led us to question the physiological and clinical significance of GnRH receptors in the human ovary (Fraser and Eidne 1989).

The cloning of the GnRH receptor complementary DNA has allowed the exploration of the expression of the mRNA in the pituitary gland, the ovary and other extra-pituitary tissues. In the rat, in-situ hybridization has revealed that the GnRH receptor mRNA is highly expressed in the granulosa cells of both healthy and atretic follicles and in the corpus luteum (Whitelaw et al., 1995). Comparisons of the rat GnRH receptor nucleotide sequence found in the anterior pituitary (Eidne et al., 1992) with that found in the ovary (Whitelaw et al., 1995) indicate that these sequences are identical. Similarly, the nucleotide sequences of human GnRH receptors in ovarian tumours are identical with that found in the pituitary (Kakar et al., 1994). The GnRH receptor mRNA in the human ovary has been investigated by Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) and does not appear to be detectable without PCR amplification (Kakar et al., 1992; Peng et al., 1994; Minaretzis et al., 1995). Using this approach, the mRNA has also been found in a number of tissues both within and outwith the reproductive system (Kakar et al., 1992). The observation of the GnRH receptor mRNA in the ovary has led to further speculation that a physiological system exists involving the production of GnRH-like peptides to modulate function through a specific receptor (Oikawa et al., 1990; Peng et al., 1994; Irmer et al., 1995; Minaretzis et al., 1995). It is also possible that this system may be susceptible to interference from the GnRH agonist and antagonist analogues used clinically.

The aim of the present study was to localize the GnRH receptor mRNA using in-situ hybridization to determine the ovarian compartments which expressed the mRNA and to obtain a perspective on the level of expression of the GnRH receptor in the primate ovary compared with that in the anterior pituitary gland. Should this approach demonstrate the presence of mRNA in the primate ovary further studies could determine its physiological control. Since most of the information obtained on the human ovarian GnRH receptor is based upon luteal binding sites, the current study also focused upon luteal phase ovaries.
Figure 1. Dark field photomicrographs showing expression of gonadotrophin-releasing hormone (GnRH) receptor mRNA in the marmoset pituitary and ovary by in-situ hybridization. (a) Whole pituitary gland expressing high levels of mRNA in the anterior pituitary (AP) with absence of expression in the posterior pituitary (PP). (b) Higher magnification of a second marmoset pituitary over the anterior-posterior pituitary junction showing high expression in the anterior pituitary with uniform low level of grains in the posterior pituitary which is just above the acellular background (bg). (c) Portion of a marmoset corpus luteum (CL) showing low grain density. (d) Portion of a marmoset ovary showing low levels of expression in the granulosa cells (g) of an antral follicle (AF) with absence of expression in the theca (t). Bar = 200 μm.

Materials and methods

Tissue collection

Pituitaries and ovaries were obtained from marmoset monkeys (Callithrix jacchus), a New World primate (n = 3) and the stump-tailed macaque (Macaca arctoides), an Old World primate (n = 2), during the mid-luteal phase as reported previously (Fraser et al., 1993, 1995). Three human corpora lutea were obtained from women undergoing hysterectomy during the early luteal phase, the mid-luteal phase and after ‘rescue’ by human chorionic gonadotrophin (HCG) (see Rodger et al., 1995). For luteal rescue, HCG (Profasi; Serono Laboratories, Welwyn Garden City, UK) was administered by i.m. injection starting on day 7 of the luteal phase at a dose of 125 IU then in doubling doses for 5 consecutive days. In addition a whole ovary obtained during the late luteal phase (n = 1 per group) was studied. Ethical approval for this study was granted by the Reproductive Medicine Subcommittee of Lothian Research Ethics Committee. Tissue was snap-frozen in liquid nitrogen, transported to the laboratory and stored at -70°C. Pituitary and ovary frozen sections were cut on a cryostat and thaw-mounted on poly-l-lysine-coated RNase-free microscope slides. Slides were stored in air-tight boxes at -70°C prior to hybridization.

Riboprobe production

A human pituitary gland 5'-stretch cDNA library in λgt10 (Clontech, Palo Alto, CA, USA) was screened with the rat GnRH receptor cDNA (Eidne et al., 1992) according to standard methods. A positive clone, containing a 1800 base pair (bp) insert, was isolated. DNA sequencing was carried out using an Applied Biosystems 373A automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). This confirmed that the isolated clone corresponded to bases 190 to 1947 of the published human GnRH receptor sequence (Chi et al., 1993). The clone was amplified by PCR using λgt10 insert screening amplimers (Clontech, Palo Alto, CA, USA) and inserted into the polylinker of the vector pCR1 (Invitrogen, San Diego, CA, USA).

Linearized recombinant plasmid containing the insert was transcribed in vitro, using an RNA transcription kit (Promega, Madison, WI, USA), with either T7 or SP6 polymerases (Boehringer Mannheim UK, East Sussex, UK) in the presence of nucleotides and [α-32P]UTP (>1000 Ci/mmol, Amersham International, Aylesbury, UK) to obtain labelled sense and antisense riboprobes using a RNA transcription kit (Promega, Madison, WI, USA). After transcription, the DNA template was degraded with DNase. The riboprobe was then subjected to hydrolysis at 60°C for 18 min to produce fragments of ~400 bp.

Marmoset pituitary RNA was prepared and subjected to RT-PCR amplification. Subsequent DNA sequencing of the PCR product revealed that the marmoset GnRH receptor sequence was ~96% homologous with the human GnRH receptor sequence (results not shown). As it is reasonable to assume an even greater homology.
Figure 2. Dark field photomicrographs showing expression of gonadotrophin-releasing hormone (GnRH) receptor mRNA in the macaque pituitary and macaque and human ovary by in-situ hybridization. (a) Portion of macaque pituitary showing high expression of mRNA in the anterior pituitary (AP) with absence of expression in the posterior pituitary (PP). (b) Portion of a macaque ovary showing low levels of expression in the granulosa cells (g) of an antral follicle with absence of expression in the theca (t) and a portion of the corpus luteum (CL) showing low grain density. (c) Higher magnification of the anterior pituitary gland of the second macaque showing high expression for direct comparison with (d) expression in the granulosa cells of an antral follicle in the human ovary. Bar = 200 µm.

between the macaque and human sequence it was considered valid to employ the human probe for hybridization on all three species.

In situ hybridization

The in-situ hybridization procedure was similar to that described previously (Zabavnik et al., 1993; Fraser et al., 1993). Sense and antisense probes were diluted in prehybridization buffer containing 10% dextran sulphate to give a specific concentration of 1X10^6 counts per min/slide. Duplicate tissue sections were hybridized with sense or antisense probes in hybridization buffer at a volume of 60 µl. Slides were covered with Gelbond film (Flowgen FMC, Sittingbourne, Kent, UK) and placed overnight in a humidified oven at 42°C.

Following the overnight incubation, the slides were treated as described previously and after dipping in photographic emulsion were exposed for 4 weeks. After developing, slides were stained with haematoxylin and mounted with coverslips.

Results

The sections were assessed by two investigators experienced in quantitative image analysis for in-situ hybridization (see Chowen-Breed et al., 1989; Fraser et al., 1993, 1995). Pituitaries from the two macaques and three marmoset monkeys exhibited high expression of the GnRH receptor mRNA as reflected by high grain density over cells in the anterior pituitary gland (Figure 1a, b and Figure 2a, c). Grains were clustered over individual cells scattered throughout the anterior pituitary. The distribution of these cells suggested regional variation, cells with grain clusters being most dense in the latero-posterior region and lowest in the anterior region. Quantitative image analysis of grain density was not performed because of extensive grain coalescence as a result of high signal. This was due to the fact that the sections had been exposed for 4 weeks to allow maximal signal to develop in the ovarian tissue with subsequent overexposure in the anterior pituitary. In the posterior pituitary, expression of the mRNA was either the same as or slightly above acellular background, without any evidence of clustering. Sense probes showed grain concentrations which were indistinguishable from or just above acellular background.

In the six marmoset ovaries and two macaque ovaries, low signal was observed in granulosa cells of antral follicles and in the corpora lutea (n = 8 in marmoset, n = 2 in macaque) (Figures 1 and 2). Signal was not above tissue background in primary and secondary follicles. A good example of this pattern of grain density is shown in Figure 2b where the compartments can be graded as follows: granulosa cells > corpus luteum > theca cells = tissue background > acellular background (antrum of follicle). In both species, this low level
signal was absent from antral follicles which were grossly atretic but some of the follicles in which it was present were likely to be undergoing early stages of atresia. The four human corpora lutea showed weak grain counts which were above acellular background levels. Because some of the sense sections were also above acellular background the significance of this level of grain density is difficult to assess. In the whole human ovary, low grains were present in the granulosa cells of 3 of 6 antral follicles (Figure 2d).

Discussion
The issue of the significance of GnRH receptor binding sites on the primate ovary has remained unresolved for several years. The availability of a probe for the GnRH receptor mRNA permitted this attempt to localize the sites of production within the ovary using a sensitive (13P) in-situ hybridization technique and a long (4 week) exposure time. The use of pituitary glands and ovaries obtained from the same monkey allowed direct comparison of expression between these tissues. The results show that while anterior pituitary glands of the marmoset and macaque demonstrate high levels of signal for the GnRH receptor mRNA, the ovaries of these animals and the human corpus luteum expressed only low levels of mRNA.

In general, mRNAs for G-protein coupled receptors are expressed at comparatively low levels but the use of this sensitive technique questions a physiological role for the GnRH receptor mRNA in the primate ovary. Previous results have shown the presence of the GnRH receptor mRNA in luteinized granulosa cells of the human ovary, whole ovarian tissue and in post-menopausal ovary (Peng et al., 1994; Minaretzis et al., 1995). However, levels of mRNA are low and RT-PCR has been required to locate and quantify possible changes in mRNA. The use of in-situ hybridization has the advantage that differences in expression of the GnRH receptor mRNA may be detectable in specific ovarian compartments and different stages of follicular or luteal development. The present results demonstrate only low expression of the GnRH receptor mRNA in the marmoset, macaque and human ovary, expression being localized to granulosa cells of antral follicles and to the corpus luteum. This low level of expression contrasted with studies on the rat in our Centre using the same approach as in the current study: high expression was observed in granulosa cells of healthy follicles, in follicles in the early stages of atresia and in corpora lutea (Whitelaw et al., 1995). Although we cannot exclude the possibility that the sequence of the primate ovarian GnRH binding site is different from that encoding the pituitary receptor this seems unlikely (Kakar et al., 1994). Our results concur with those of Minaretzis et al. (1995) using RT-PCR who estimated that the level of GnRH receptor mRNA was ~200-fold lower in the human ovary than in the pituitary. Comparison of the clear expression of the GnRH receptor mRNA in the rat ovary with the low expression in the primate ovary correlates with the contrasting results of receptor binding studies and direct effects of GnRH analogues between the species.

This is also the first report showing localization of the GnRH receptor in the pituitary gland of the primate. The mRNA is clearly detectable in the marmoset and macaque anterior pituitary by in-situ hybridization using a riboprobe based upon the sequence of the human GnRH receptor. Studies on changes in the GnRH receptor in the primate pituitary have been severely limited because they have required membrane preparation and binding to labelled GnRH analogues (Adams et al., 1981). The use of in-situ hybridization on tissue sections should provide the basis for detailed quantitative analysis during different reproductive states.

In conclusion, our results show that in contrast to the anterior pituitary gland and the rat ovary the primate ovary is not a site of high GnRH receptor mRNA production. The low levels of message found in granulosa cells of antral follicles and in the corpus luteum mean that a function cannot be excluded. It is possible that more detailed studies of ovaries at other stages of the cycle may reveal increased hybridization at specific stages of follicular growth or luteal development or that quantitative RT-PCR of mRNA of individual follicles or corpora lutea may prove a more sensitive technique to investigate the physiological control of the GnRH receptor mRNA. The issue of whether a GnRH ligand-receptor system operates within the primate ovary and whether it may be a site of action of GnRH analogues used clinically remains to be established.

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