Gonadotrophin-releasing hormone and triptorelin inhibit the follicle stimulating hormone-induced response in human primary cultured granulosa-lutein cells

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Introduction

Gonadotrophin-releasing hormone (GnRH) and its agonists have been shown to exert direct effects on ovarian function. In animals, most of the reported effects are agonadotrophic. GnRH inhibits follicle stimulating hormone (FSH)-stimulated follicular development (Ying and Guillemin, 1979), granulosa cell steroidogenesis (Hsueh and Erickson, 1979) and cyclic AMP (c-AMP) accumulation (Knecht and Catt, 1981). In granulosa cells, GnRH binding to its receptor stimulates phospholipases C and D, with subsequent production of intracellular second messengers. Cyclic AMP (c-AMP)-dependent cell shape changes can be used to study the gonadotrophic response in cultured human granulosa-lutein cells. This approach has been developed here to determine the direct potential antigonadotrophic effect of gonadotrophin-releasing hormone (GnRH) and a GnRH agonist (triptorelin) on human granulosa-lutein cells. Treatment with triptorelin or GnRH alone for 1 h did not affect granulosa-lutein cell morphology. However, in the presence of stimulatory doses of follicle stimulating hormone (FSH), triptorelin (5×10⁻⁷-5×10⁻⁶ M) and GnRH (10⁻¹⁰-10⁻⁹ M) inhibited the FSH-induced c-AMP-dependent response. The antigonadotropic effect of triptorelin was prevented by two GnRH antagonists, indicating that triptorelin acts via specific GnRH binding sites. On the other hand, triptorelin failed to inhibit human chorionic gonadotrophin- and forskolin-mediated morphological changes. Our results suggest that the GnRH agonist interacts specifically with the FSH-induced c-AMP-dependent cascade of events, at a site located ahead of that of c-AMP generation. In conclusion, GnRH and triptorelin strongly inhibit FSH-mediated function in human granulosa-lutein cells in culture. This inhibition might play a role in the low follicular development rates observed in some patients treated with GnRH agonist + gonadotrophins for ovarian stimulation.

Key words: cell shape changes/FSH/GnRH/human granulosa-lutein cells/triptorelin

Materials and methods

Hormones and chemicals

Hormones and chemicals were obtained from the following sources: human FSH of the pituitary gland (50 IU/mg) from UCB-Bioproducts (Saint-Quentin-Fallavier, France); and HCG (12 000 IU/mg) was kindly provided by Organon (Serifontaine, France). Hormones and chemicals were obtained from the following sources: human FSH of the pituitary gland (50 IU/mg) from UCB-Bioproducts (Saint-Quentin-Fallavier, France); and HCG (12 000 IU/mg) was kindly provided by Organon (Serifontaine, France). Hormones and chemicals were obtained from the following sources: human FSH of the pituitary gland (50 IU/mg) from UCB-Bioproducts (Saint-Quentin-Fallavier, France); and HCG (12 000 IU/mg) was kindly provided by Organon (Serifontaine, France). Hormones and chemicals were obtained from the following sources: human FSH of the pituitary gland (50 IU/mg) from UCB-Bioproducts (Saint-Quentin-Fallavier, France); and HCG (12 000 IU/mg) was kindly provided by Organon (Serifontaine, France). Hormones and chemicals were obtained from the following sources: human FSH of the pituitary gland (50 IU/mg) from UCB-Bioproducts (Saint-Quentin-Fallavier, France); and HCG (12 000 IU/mg) was kindly provided by Organon (Serifontaine, France). Hormones and chemicals were obtained from the following sources: human FSH of the pituitary gland (50 IU/mg) from UCB-Bioproducts (Saint-Quentin-Fallavier, France); and HCG (12 000 IU/mg) was kindly provided by Organon (Serifontaine, France). Hormones and chemicals were obtained from the following sources: human FSH of the pituitary gland (50 IU/mg) from UCB-Bioproducts (Saint-Quentin-Fallavier, France); and HCG (12 000 IU/mg) was kindly provided by Organon (Serifontaine, France). Hormones and chemicals were obtained from the following sources: human FSH of the pituitary gland (50 IU/mg) from UCB-Bioproducts (Saint-Quentin-Fallavier, France); and HCG (12 000 IU/mg) was kindly provided by Organon (Serifontaine, France).
Figure 1. Phase-contrast microscopy showing the morphology of human granulosa-lutein cells after different treatments. Cells were cultured for 48 h and incubated for 1 h with (a) serum-free medium, (b) follicle stimulating hormone (FSH; 0.1 IU/ml), (c) FSH + triptorelin ($10^{-6}$ M) and (d) FSH + triptorelin + gonadotrophin-releasing hormone antagonist (G98) ($10^{-5}$ M). Note that cells become rounded after FSH treatment (b); this effect is inhibited by triptorelin (c), and G98 totally prevents the triptorelin effect (d). Bar = 25 μm.

C.Furger et al.

**Results**

**Direct effects of drugs and hormones on human granulosa-lutein cells**

Incubation of the cells for 1 h with human FSH (0.005–0.100 IU/ml; Figures 1b and 2), HCG (0.5 IU/ml) and forskolin ($5 \times 10^{-5}$ M) (Figure 3) substantially increased the number of rounded versus flattened cells. In contrast, GnRH ($10^{-14}$–$10^{-7}$ M) (Figure 4, left) and triptorelin ($5 \times 10^{-7}$–$5 \times 10^{-6}$ M) alone (Figure 2, left) failed to stimulate morphological changes.

**Effect of triptorelin on FSH-, HCG- and forskolin-induced cell response**

The effects of increasing concentrations of triptorelin on gonadotrophin and forskolin actions were determined. Triptorelin ($5 \times 10^{-7}$–$5 \times 10^{-6}$ M) totally inhibited the promotion of morphological changes induced by FSH (Figure 2). The effect of triptorelin was detectable after only 10 min of incubation (data not shown) and was maximal at 1 h of treatment (Figure 1c). In contrast, triptorelin failed to inhibit HCG-induced cell shape changes (Figure 3), suggesting that the triptorelin inhibitory effect is related specifically to the FSH action upon human granulosa-lutein cells. The same concentrations of triptorelin were also ineffective towards forskolin-induced morphological changes (Figure 3).

**Effect of GnRH on the FSH-induced c-AMP-mediated cell response**

As shown in Figure 4, the addition of GnRH ($10^{-14}$–$10^{-7}$ M) to FSH-treated cells resulted in a dose-dependent, biphasic response. At concentrations of $10^{-10}$–$10^{-9}$ M, GnRH signific-
FSH action in human granulosa cells

Figure 2. Dose-dependent inhibitory effect of triptorelin on follicle stimulating hormone (FSH)-induced cell response in human granulosa-lutein cells. After 48 h of culture, cells were incubated for 1 h with FSH (0.1 IU/ml) and with increasing doses of triptorelin. Values are means ± SE of the percentage determination of all microscopic fields observed in each treatment, and represent cumulative data from seven patients. (*) P < 0.01 when compared with human FSH alone.

Figure 3. Absence of the effect of increasing doses of triptorelin (5 × 10⁻⁹-5 × 10⁻⁶ M) on 5 × 10⁻⁵ M human chorionic gonadotrophin (HCG)- and 5 × 10⁻⁵ M forskolin (FK)-induced cell response. Values are means ± SE of the percentage determination of all microscopic fields observed in each treatment, and represent cumulative data from five patients.

Figure 4. Dose-dependent effect of gonadotrophin-releasing hormone (GnRH) on follicle stimulating hormone (FSH)-induced cell response in human granulosa-lutein cells. After 48 h of culture, cells were incubated for 1 h with FSH (0.05 IU/ml) and with increasing doses of GnRH. Values are means ± SE of the percentage determination of all microscopic fields observed in each treatment, and represent cumulative data from six patients. (*) P < 0.01 when compared with human FSH alone.

Evidence for the involvement of GnRH receptors in triptorelin action in human granulosa-lutein cells

To determine whether the triptorelin effect is mediated by specific GnRH binding sites, FSH- and triptorelin-treated cells were co-incubated with two specific GnRH antagonists: [Ac-3,4-dehydro-Pro¹, d-p-F-Phe², d-Trp³]-GnRH (G98) and [Ac-d-p-Cl-Phe¹,², d-Trp³, d-Arg⁶, d-Ala¹⁰]-GnRH (G90). In the presence of FSH (0.1 IU/ml) and triptorelin (10⁻⁶ M), G98 and G90 (5 × 10⁻⁶ M) totally prevented a triptorelin inhibitory effect on FSH-promoted cell shape changes (Figure 5), showing that the triptorelin acts via specific GnRH binding sites in human granulosa-lutein cells. Figure 1 illustrates the effects on cell morphology of FSH, triptorelin and a GnRH antagonist (G98).

Discussion

We clearly demonstrated, using biochemical and morphological approaches, that GnRH and its analogue triptorelin exert an inhibitory effect on the FSH-mediated response in human granulosa-lutein cells. Inhibition by triptorelin is total after 1 h of incubation with FSH (0.1 IU/ml), indicating an interaction between GnRH- and FSH-generated transduction pathways. Co-incubation of the cells with selective GnRH antagonists prevents the inhibitory effect of triptorelin, confirming that...
specific GnRH binding sites are present at the granulosa cell surface. GnRH (10^{-10}–10^{-9} \text{ M}) partially but significantly inhibited the FSH-induced response. At higher concentrations (10^{-8}–10^{-7} \text{ M}) GnRH failed to exert inhibitory effects, suggesting that the GnRH-mediated response may undergo a dose-dependent desensitization process.

It is now well recognized that FSH stimulates granulosa cell function by activating c-AMP production (Kolena and Channing, 1972). Here we have shown that the percentage of rounded versus flattened human granulosa-lutein cells in culture strongly increases after 1 h of incubation with FSH, HCG or forskolin. These effects on cell morphology are totally abolished when triptorelin is concomitantly added to FSH-incubated cells. Furthermore, this antigonadotropic effect is detected as early as 10 min after treatment with FSH. These data are unique in their demonstration of a short-term antigonadotropic effect of GnRH (or agonists) on human granulosa-lutein cells, and are consistent with an interaction between GnRH-dependent intracellular second messengers and a c-AMP-dependent cascade of events leading to morphological changes. Because triptorelin failed to inhibit the forskolin-induced response, the site of interaction between the two signalling pathways could be located upstream from adenyl cyclase activation, at a site where various receptor-coupled G proteins may interact. To date, the only studies reporting antigonadotropic effects of GnRH or agonists on human granulosa cells (Tureck et al., 1982; Parinaud et al., 1981) concerned long-term hormonal incubations (24–48 h). In that case, a direct effect of these decapeptides on gonadotrophin-dependent c-AMP pathway activation was not evident. In such long-term effect studies, an alternative pathway could involve the stimulation by GnRH or related peptides of gene expression, leading to the synthesis of proteins implicated in the generation, degradation or action of c-AMP.

In the rat, inhibition by GnRH agonists of FSH-induced granulosa cell shape changes was reported in earlier studies (Amsterdam et al., 1981; Knecht et al., 1981, 1982). A recent study was designed to ascertain the transduction pathway via which GnRH inhibits FSH-induced morphological changes in this species (Amsterdam et al., 1994). The antigonadotropic effect of GnRH is mimicked by phosphatidic acid and phorbol esters, two potent activators of protein kinase C activity, suggesting that the decapeptide exerts its inhibitory effects through phospholipases C and D activation.

Although the presence of high-affinity GnRH receptors has been observed in some animal species, their presence in the human ovary remains highly controversial. Early studies of GnRH binding in human corpus luteum failed to show the presence of high-affinity pituitary-like GnRH binding sites (Clayton and Huhtaniemi, 1982; Popkin et al., 1983). However, specific low-affinity binding sites, which may be part of the locally active ovarian GnRH-like system hypothesized by Aten et al. (1987) and Li et al. (1987), have been detected in the same model (Popkin et al., 1983; Bramley et al., 1986).

Here we have shown that triptorelin antigonadotropic effects are totally prevented when cells are co-incubated with two different GnRH antagonists. Both of them are well known to inhibit circulating gonadotrophin concentrations and ovarian maturation in humans (Nekola et al., 1982; Dahl et al., 1988). One of these antagonists, \{Ac-\text{A}^3\text{Pro}^1, \text{d}-\text{p}-\text{F}-\text{Phe}^2, \text{d}-\text{Trp}^3, \text{d}\}GnRH, is also able to prevent GnRH-induced inositol 1,4,5-triphosphate generation after 5 min in rat granulosa cell (Davis et al., 1984, 1986), thus indicating a probable direct interaction between GnRH and its antagonist at the level of the receptor. Considering these data, our results strongly support the fact that specific GnRH binding sites are present in human granulosa-lutein cells. Our observations, which arise from experiments performed with human primary cultured granulosa cells recovered at the time of ovulation, are in good agreement with the recent data reporting the detection of GnRH receptor mRNA in the same model (Peng et al., 1994) and the results of Latouche et al. (1989), who studied the binding of GnRH on sections of six different human ovaries using quantitative in-vitro autoradiography. In one of these sections, the presence of high-affinity binding sites was reported but only in a single dominant pre-ovulatory follicle and exclusively in the granulosa cell layer.

In patients who respond normally to ovulation induction, one obvious clinical advantage to complete exogenous gonadotrophin treatment by the addition of GnRH agonists lies in prevention of the endogenous luteinizing hormone surge and a higher number of recovered oocytes (Zorn et al., 1988). However, in some patients low responses are observed, despite high dosages of HMG or FSH. In most cases, the reason for this suboptimal response to ovarian stimulation remains elusive. One intuitive approach to the treatment of low-responder patients was to increase the dosage of administered gonadotrophins in an effort to maximize the recruitment of follicles from the gonadotrophin-sensitive pool. Two recent studies (Karande et al., 1990; Davis and Rosenwaks, 1993) have suggested that increasing doses of exogenous gonadotrophins do not improve ovarian response to stimulation. Another strategy in the management of low-responder patients was to reduce the dose of adjunctive GnRH agonist, at least in women with a history of idiopathic hyporesponsiveness. In a group of 17 women, the dose of GnRH agonist (leuprolide acetate) was halved in 21 subsequent treatment cycles. A significant increase in the mean peak 17β-oestradiol concentration (958 versus 586 pg/ml) and a significant enhancement of the clinical pregnancy rate per transfer (30.8 versus 14.3%) were observed (Davis and Rosenwaks, 1993), indicating that, at least in a subpopulation of low-responder patients, the IVF success rate could be inversely proportional to the circulating concentration of GnRH agonist.

Although a direct link has been established between the presence of the GnRH agonist and granulosa cell biological activity, we must bear in mind the fact that various effects are exerted by the different available GnRH agonists at the granulosa cell level. For example, diverse ovarian effects were reported during therapeutic trials with buserelin, triptorelin and leuprorelin (Parinaud et al., 1992). Differences were also observed \textit{in vitro} in a recent study using cultured human granulosa cells. Comparative effects of either GnRH or five different agonists upon steroidogenesis without gonadotrophin stimulation were evaluated (Bussenot et al., 1993). Gly^{10}-substituted analogues (buserelin, leuprorelin, H4065 and
H4055) were shown to increase 17β-oestradiol production significantly, whereas triptorelin (substituted only in Gly) and GnRH had no effect.

To our knowledge, no reports exist on the structure–function changes caused by the effects of various GnRH agonists upon granulosa cell activity after gonadotrophin stimulation. Our data show that triptorelin (5×10−7 M) inhibits FSH-dependent granulosa cell responses. Under the same experimental conditions, Parinaud et al. (1988) reported that buserelin (10−7 M) was able to inhibit luteinizing hormone-stimulated progesterone secretion. Other studies in non-human primates have confirmed that buserelin can exert a strong inhibitory effect on FSH-dependent oestradiol, progesterone and c-AMP production in cultured marmoset granulosa cells (Wickings et al., 1990) and on FSH-dependent follicular morphological changes in vivo (Gougeon et al., 1992).

Further studies are needed to evaluate the effects of other structurally different GnRH agonists on the gonadotrophin-dependent granulosa cell response. The finding of an analogue that does not exert a direct ovarian antigonadotrophic effect (or exerts a less potent effect) should represent a useful tool in the management of low-responder patients, one of the most vexing challenges in assisted reproductive technology.

Acknowledgements
This work was supported by IPSEN-Biotech (Paris, France) through an operating grant and a fellowship (to C.F.).

References


Received on January 25, 1995; accepted on December 20, 1995