EXPERIMENTAL STUDY

Actions of gonadotropin-releasing hormone antagonists on steroidogenesis in human granulosa lutein cells

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Abstract

Objective: GnRH antagonists have recently been introduced for the prevention of premature LH surges during controlled ovarian hyperstimulation (COH). We have here investigated whether the GnRH antagonists cetrorelix and ganirelix exert effects on ovarian steroidogenesis. Since there is some controversy about the action of GnRH agonists in the human ovary we also tested the effect of triptorelin on steroid production in cultured human granulosa lutein cells.

Methods: Cells were obtained from patients treated with different protocols of COH. In addition to gonadotropins they received triptorelin, cetrorelix, ganirelix or no GnRH analogue.

Results: Such *in vivo* treatment did not result in significant effects of triptorelin or the two GnRH antagonists on spontaneous or human chorionic gonadotropin (hCG)-stimulated steroidogenesis. To exclude the possibility that the *in vivo* treatment might not affect *in vitro* steroid production because of low or absent peptide activity, we performed *in vitro* treatments with triptorelin, cetrorelix and ganirelix for up to 96 h. However, these treatment paradigms did not influence basal or hCG-stimulated steroid production.

Conclusions: We conclude that GnRH antagonists do not exert any significant effects on ovarian steroidogenesis *in vitro* and therefore their introduction into protocols of COH is unlikely to impair ovarian function.

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Introduction

Gonadotropin-releasing hormone (GnRH) agonists and the GnRH antagonists cetrorelix and ganirelix are currently used in different protocols of controlled ovarian hyperstimulation (COH) for assisted reproduction techniques to prevent premature luteinizing hormone (LH) surges and thus reduce cancellation rates of COH cycles (1–6). GnRH antagonists have demonstrated their ability to prevent premature LH surges with a comparable clinical performance to GnRH agonists (2, 4, 5, 7–9). In contrast to GnRH agonists, which induce an initial rise of gonadotropin secretion (flare-up) before suppression is achieved through desensitization of gonadotrophs, administration of the antagonists leads to a rapid inhibition of LH release, which results from competitive binding of the antagonists to pituitary GnRH receptors. These compounds are therefore more suitable for the blockage of premature LH surges than the GnRH agonists.

Apart from its pituitary actions GnRH has been shown to bind to specific receptors in a number of extrapituitary tissues (10–15). The recent cloning of the GnRH receptor led to the demonstration of GnRH

receptor gene expression in the human ovary (16–18). However, controversial data exist on GnRH receptors in the human ovary (19–21). Recent observations by Brus *et al.* (22) indicated that high-affinity ovarian GnRH receptors are present predominantly in ovarian tissue after the LH surge. Unlike in the rat, it is still controversial whether these receptors are functional (11, 13). Studies on the actions of GnRH analogues on steroidogenesis in granulosa lutein cells *in vitro* revealed contradictory effects (17, 23–27). Minimal information is available on the actions of GnRH antagonists in the human ovary (3). Since GnRH antagonists will be more widely used in protocols of COH in the near future it is important to characterize their ovarian effects, which might be advantageous or detrimental for the treatment goal. Furthermore, such knowledge could enhance our understanding of the physiological role of GnRH in the human ovary.

In the present paper we describe the actions of the two new GnRH antagonists ganirelix and cetrorelix and the GnRH agonist triptorelin on steroidogenesis of human granulosa lutein cells. Since there is controversy about the presence of GnRH receptors in the human ovary we determined the GnRH receptor mRNA

in granulosa lutein cells that were used for these studies.

Materials and methods

Protocols of COH

Human granulosa lutein cells were obtained from patients undergoing *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI). Four different stimulation protocols were used for COH. Patients in group A ($n = 9$) were treated with 2 ampoules human menopausal gonadotropin (hMG; Ferring, Kiel, Germany) from day 2 of the cycle. On day 6 the dose of hMG was increased to 3 ampoules and thereafter the amount of hMG was adjusted according to the ovarian response. In group B, patients ($n = 20$) received 3.75 mg D-6-tryptophane-GnRH (triptorelin; Ferring) subcutaneously 14 days before hMG application. The protocol of hMG treatment was the same as in group A. In group C ($n = 20$) patients were treated with 2 ampoules hMG from day 2 of the cycle. From day 7 the GnRH antagonist cetrorelix (ASTA Medica, Frankfurt, Germany) was co-administered subcutaneously with hMG at a dose of 0.25 mg/day as described previously (5). The dose of hMG was adapted to the ovarian response in the same way as in groups A and B. In group D ($n = 14$) patients were treated as in group C except that 2 ampoules recombinant follicle-stimulating hormone (FSH; Serono, Freiburg, Germany) and the GnRH antagonist ganirelix (N.V. Organon, Oss, The Netherlands) at a dose of 0.25 mg/day was used instead of cetrorelix. When at least three follicles reached a diameter of 20 mm each, ovulation was induced by administration of 10 000 IU human chorionic gonadotropin (hCG). Transvaginal ovarian puncture was performed 36 h later. The studies were approved by the ethical committee of the Medical University Lübeck.

Granulosa lutein cell preparation and culture conditions

Follicular aspirates were gained from patients treated with various protocols of COH (see above). After collection of oocytes, aspirates were centrifuged at 200 *g* for 10 min. The pellet was washed twice in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} . The cell pellet was resuspended in PBS, overlaid on Ficoll (Pharmacia, Uppsala, Sweden) and centrifuged at 300 *g* for 20 min. Granulosa lutein cells were aspirated from the interphase and resuspended in Ham's F10 medium containing 10% fetal calf serum, 100 IU/ml penicillin, 100 ng/ml streptomycin and 5 μM testosterone. Cells were cultured on multiwell dishes (100 000 cells/well) in 5% CO_2 , 95% air and saturated humidity at 37 °C. The viability of the cells was always >90% as demonstrated by trypan blue exclusion. (For experimental protocol see also reference 44.)

Effects of *in vivo* treatments with GnRH analogues

Cells obtained from patients who were treated with triptorelin, cetrorelix, ganirelix or no GnRH analogue were cultured for different periods to examine the possible actions of GnRH analogues on steroidogenesis. To test the possibility that the effect of the *in vivo* treatment with these GnRH analogues is transient, estradiol and progesterone production was determined during the initial 1 h of the culture period. Thereafter media were collected at 24-h intervals for up to 72 h for estradiol and progesterone measurements. To investigate whether the *in vivo* GnRH analogue treatment had an effect on agonist-induced steroid production, cells were stimulated for 6 h with 1 IU/ml hCG at 24-h intervals for up to 96 h.

Effects of *in vitro* treatments with GnRH analogues

To exclude the possibility that the *in vivo* treatment with GnRH analogues does not result in changes of steroidogenesis of cultured granulosa lutein cells because of the absence of peptide activity during the culture period, we employed different *in vitro* treatment protocols using GnRH agonists and GnRH antagonists. Steroid production was determined in cells that were obtained from patients who had been treated with triptorelin or no GnRH analogue. Such cells were exposed to 1 nmol/l triptorelin, cetrorelix or ganirelix for up to 96 h. We used such a long culture period because the COH treatment of the patients includes high doses of exogenous gonadotropins which might down-regulate GnRH receptor expression (17). The prolonged culture period might be sufficient to allow the recovery of GnRH receptors. Estradiol and progesterone release were examined at 24-h intervals. Stimulations with hCG (1 IU/ml) were performed every 24 h for 6 h. Controls were present in each culture and each plate.

Steroids were determined by enzyme linked immunosorbent assay (Serono). The sensitivity of the assay was 0.2 ng/ml and 5 pg/ml for progesterone and estradiol respectively. Inter- and intra-assay variations were below 10%.

RT-PCR for GnRH receptor

Total RNA from human granulosa lutein cells that were obtained from different groups of patients was purified by Trizol purification (Gibco-BRL, Eggenheim, Germany). In a one-tube assay, total RNA was reverse transcribed (RT; Superscript II RNase H-Reverse Transcriptase; Gibco-BRL) and amplified using recombinant *Thermus aquaticus* YT1 (Taq) polymerase (Superscript One Step RT-PCR System; Gibco-BRL) to synthesize cDNA. RNA (0.5 μg) was dissolved in 50 μl reaction

mix containing 2.4 mM MgSO₄, 0.4 mM dNTP mixture, 0.4 µM sense and antisense primers each and 2 units polymerase. For PCR amplification, a sense primer (nucleotides 601 to 621; 5'-TCTAGCAGA CAGCTCTGGACA-3') and antisense primer (nucleotides 832 to 851; 5'-GAGTCTTCAGCCGTGCTCTT-3') taken from the published GnRH receptor cDNA (16) were used. The amplicons are derived from exons 2 and 3 after RT. Human β-actin was used as control. PCR conditions were: 50 °C for 30 min for RT followed by 94 °C for 2 min, and 34 cycles at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 90 s followed by a final extension at 72 °C for 10 min (Perkin-Elmer Thermocycler Gene Amp 9600, Norwalk, CT, USA). After PCR, an aliquot of the reaction was resolved in a 1.3% agarose gel stained with ethidium bromide.

Data presentation and statistical analysis

Each experiment was carried out with granulosa lutein cells from one patient and was performed in triplicate.

Data from 9 to 20 experiments were combined and presented as absolute values for progesterone or expressed in per cent of basal release (100%). Treatment groups were analyzed for statistically significant differences by Mann–Whitney U test or by a Kruskal–Wallis test if more than two treatments were tested.

Results

GnRH receptor and mRNA

The granulosa lutein cells obtained from patients undergoing different protocols of COH were analyzed by RT-PCR for the expression of GnRH receptor mRNA. A pair of primers was designed based on the human pituitary GnRH receptor cDNA sequence (16) located on different exons (exons 2 and 3). PCR products with the predicted size from pituitary and granulosa lutein cells, but not from skeletal muscle, placental and testicular tissue cDNA were detected (Fig. 1). To exclude the possibility of RNA degradation, an RT-PCR for β-actin was run in parallel showing the expected 353 bp fragments (data not shown).

Effects of triptorelin on steroid secretion

Since data on direct actions of GnRH agonists on ovarian function are controversial, we tested the possibility that triptorelin exerts effects on steroidogenesis in cultured granulosa lutein cells. Cells obtained from patients who received triptorelin during COH secreted slightly lower amounts of progesterone than control cells (no GnRH analogue *in vivo*) (Fig. 2a). However, this inhibitory action was not statistically significant. There were no significant differences in the estradiol or progesterone response to hCG between treatment groups (Fig. 2b). To exclude the possibility

Figure 1 Detection of GnRH receptor transcript by RT-PCR in human granulosa lutein cells (3–5), pituitary (1), placental tissue (2), musculus rectus (6) and testicular tissue (7). C, control (H₂O); M, λ DNA marker.

that the *in vivo* action of triptorelin cannot be demonstrated after prolonged culture periods we measured estradiol and progesterone release during the initial 1 h after plating the cells. Even under those conditions, there were no differences between the control, cetrorelix- and triptorelin-treated group (data not shown). There were no differences in estradiol production after all treatment paradigms (data not shown).

Effects of cetrorelix and ganirelix on steroid secretion

We investigated the effect of cetrorelix and ganirelix treatment on steroidogenesis of granulosa lutein cells and compared them with control or triptorelin treatment. When steroid accumulation was measured in granulosa lutein cells a time-dependent increase was observed. Spontaneous estradiol and progesterone production was not significantly influenced by the GnRH antagonists. This was the case when GnRH antagonist treatment was compared with control or triptorelin treatments (Fig. 2). As described above, we determined estradiol and progesterone release during the first hour of the culture period after the three different treatment paradigms without any significant effects (data not shown). hCG-stimulated steroid secretion was not influenced by cetrorelix (Fig. 2). Since we intended to exclude the possibility that the actions of cetrorelix might not be present after *in vivo* treatments, we exposed cells that were obtained from patients who received no GnRH analogue or triptorelin to cetrorelix *in vitro*. Steroid accumulation was measured up to 96 h with time-dependent increases of estradiol and progesterone production. There was no significant effect of cetrorelix compared with control or triptorelin treatment (Figs. 3 and 4). Also, hCG-stimulated steroid release was not influenced by cetrorelix (Figs. 3 and 4).

In a second series of experiments we used cells from patients that were treated with a COH protocol that included the application of ganirelix. We did not

Figure 2 Effects of *in vivo* treatment with triptorelin and cetrorelix on spontaneous and hCG-stimulated progesterone (P) release from cultured granulosa lutein cells. Cells were obtained from patients treated with triptorelin, cetrorelix or no GnRH analogue and cultured for 72 h. (a) Progesterone accumulation was determined at 24-h intervals. (b) hCG stimulation was performed every 24 h for 6-h periods. Representative data (means $\pm$ S.E.M.) from 9 to 20 experiments at selected time-points are depicted.

observe any significant differences in steroidogenesis when these cells were kept in culture for up to 72 h when compared with triptorelin treatment *in vivo*. Furthermore ganirelix did not influence hCG-stimulated estradiol and progesterone production (Fig. 5). In addition, we performed *in vitro* treatments with ganirelix and observed no significant differences in basal or hCG-induced steroid production by control, triptorelin- or ganirelix-treated cells (Figs. 6 and 7). Data on estradiol production are not shown, since there were no differences between the treatment groups.

Figure 3 Effects of *in vitro* treatment with cetrorelix on spontaneous and hCG-stimulated progesterone (P) release from cultured granulosa lutein cells. Cells were obtained from patients treated with triptorelin and cultured for 72 h. (a) Progesterone accumulation was determined at 24-h intervals. (b) hCG stimulation was performed every 24 h for 6-h periods. Representative data (means $\pm$ S.E.M.) from 20 experiments at selected time-points are depicted.

Discussion

In the present study we have investigated the effects of GnRH analogues on steroidogenesis in human granulosa lutein cells. The specific aim was to characterize the actions of the two new GnRH antagonists cetrorelix and ganirelix, the use of which has already been approved for COH in some countries. The prerequisite for effects of GnRH analogues on steroidogenesis is the presence of GnRH receptors in human ovarian cells. There is consensus that the rodent ovary contains

Figure 4 Effects of *in vitro* treatment with cetrorelix on spontaneous and hCG-stimulated progesterone (P) release from cultured granulosa lutein cells. Cells were obtained from patients treated without any GnRH analogue and cultured for 72 h. (a) Progesterone accumulation was determined at 24-h intervals. (b) hCG stimulation was performed every 24 h for 6-h periods. Representative data (means $\pm$ S.E.M.) from 9 experiments at selected time-points are depicted.

GnRH receptors. Conflicting data exist on their expression in the human ovary (19, 21). In a careful study by Brus *et al.* (22), GnRH receptors have been demonstrated in a large proportion of follicular aspirates containing granulosa lutein cells but not in preovulatory follicles, suggesting that GnRH receptors are present in human ovarian tissue predominantly after the LH surge. The expression of the GnRH receptor gene in the human ovary has been shown by several groups (3, 16, 17, 28). GnRH receptor gene expression is up-regulated by GnRH, but down-regulated by hCG (17). The presence of GnRH receptors in human granulosa lutein cells suggests that they mediate the

actions of GnRH. However, concentrations of hypothalamic GnRH in the peripheral circulation are much too low to activate these receptors. In contrast, the application of GnRH analogues to patients who undergo COH results in serum concentrations which are sufficient to interact with the receptors.

Since there was no consensus on the effects of agonistic GnRH analogues on steroidogenesis in the human ovary we compared ganirelix and cetrorelix treatments with triptorelin and control. We found that the administration of the GnRH agonist triptorelin to patients who underwent COH did not lead to changes in estradiol and progesterone production by granulosa lutein cells obtained from these patients. This was the case when estradiol and progesterone levels were measured in the initial hour or after prolonged culture periods. Since it could be possible that the *in vivo* exposure of the ovary to GnRH analogues was ineffective because of the absence of the peptide during the culture period we applied the GnRH agonist to the culture medium. Even under these conditions we did not detect any changes in steroid production in the presence of triptorelin either on basal or on hCG-stimulated hormone release. These observations confirm those of other authors who were also unable to demonstrate GnRH agonist actions on ovarian steroidogenesis (24, 29–31). On the other hand, there have been numerous reports that have shown inhibitory and stimulatory actions of GnRH agonists (17, 23, 26, 32–35). Such controversy might result from differences in experimental conditions, type of GnRH agonist, cells and experimental designs. A few studies which investigated the effect of GnRH agonist treatments *in vivo* on *in vitro* steroidogenesis by granulosa lutein cells have demonstrated impaired progesterone production (36–39). In granulosa cells from early to late follicular phase, GnRH or GnRH agonists were without inhibitory action on progesterone secretion (23, 24, 26, 35). The majority of these studies have shown inhibitory action which is astonishing since follicular-phase granulosa cells did not express GnRH receptors in the recent investigation by Brus *et al.* (22). Interestingly, it seems that certain GnRH agonists exert inhibitory actions while others are inactive (27). In our series of experiments, the GnRH agonist triptorelin showed no effects on steroidogenesis under different experimental conditions.

Limited information is available on the actions of GnRH antagonists in the human ovary. Recently Minaretzis *et al.* (3) have reported on the effect of the GnRH antagonist Nal-Glu compared with GnRH agonist leuprolide acetate treatment of patients undergoing COH. The progesterone accumulation in granulosa lutein cell cultures was not different between the two groups. Aromatase activity was reduced in the GnRH antagonist-treated group during the first 6 h of culture. Lin *et al.* (40) reported on the possibility that the luteal function in GnRH antagonist (cetrorelix)

Figure 5 Effects of *in vivo* treatment with triptorelin and ganirelix on spontaneous and hCG-stimulated progesterone (P) release from cultured granulosa lutein cells. Cells were obtained from patients treated with triptorelin or ganirelix and cultured for 72 h. (a) Progesterone accumulation was determined at 24-h intervals. (b) hCG stimulation was performed every 24 h for 6-h periods. Representative data (means $\pm$ S.E.M.) from 14 experiments at selected time-points are depicted.

treatment is less impaired compared with control (no GnRH analogue) and GnRH agonists. Both GnRH antagonists had no effect on basal or hCG-induced estradiol or progesterone production by granulosa lutein cells, independent of whether the cells were exposed to the compounds *in vitro* or *in vivo*. A recent study by Lin *et al.* (41) suggested that the luteal function in GnRH antagonist-treated patients may be less impaired. They compared cetrorelix with GnRH

Figure 6 Effects of *in vitro* treatment with ganirelix on spontaneous and hCG-stimulated progesterone (P) release from cultured granulosa lutein cells. Cells were obtained from patients treated with triptorelin and cultured for 72 h. (a) Progesterone accumulation was determined at 24-h intervals. (b) hCG stimulation was performed every 24 h for 6-h periods. Representative data (means $\pm$ S.E.M.) from 20 experiments at selected time-points are depicted.

agonist buserelin and found that cells from patients treated with cetrorelix respond earlier to *in vitro* hormone stimulation. In the latest study by Dor *et al.* (42) FSH-induced estradiol production by granulosa lutein cells was significantly lower in patients treated with GnRH agonists compared with patients treated exclusively with hMG for assisted reproduction techniques (ART). However, they could not detect that effect in cells that were not treated with FSH. Nathwani *et al.* (43) demonstrated, in human granulosa luteal cells,

Figure 7 Effects of *in vitro* treatment with ganirelix on spontaneous and hCG-stimulated progesterone (P) release from cultured granulosa lutein cells. Cells were obtained from patients treated without any GnRH analogue and cultured for 72 h. (a) Progesterone accumulation was determined at 24-h intervals. (b) hCG stimulation was performed every 24 h for 6-h periods. Representative data (means $\pm$ S.E.M.) from 9 experiments at selected time-points are depicted.

that estradiol (24 h) decreases GnRH mRNA and regulates its receptor gene expression in a biphasic way, such as short-term estradiol treatment (6 h) enhances and long-term treatment (48 h) decreases GnRH receptor mRNA levels. If an inhibitory autocrine/paracrine system based on GnRH is present in the human ovary one would assume that steroid production in cultured granulosa lutein cells from women treated with GnRH antagonists would be increased. Obviously data from our experiments do not exclude this possibility because the COH treatment of the patients includes high doses of exogenous gonadotropins which might down-regulate GnRH receptor expression (17). On the other hand, GnRH antagonists did not affect steroidogenesis after a culture period of 2 days, which might be sufficient to allow recovery of

GnRH receptors. Furthermore, there are reports that have demonstrated GnRH receptors in granulosa lutein cells from IVF patients that have not been cultured but analyzed by receptor assays directly after retrieval by follicle aspiration (22). These receptors bind the GnRH antagonist D-Tyr-antide and presumably the GnRH antagonists used in the present study (22). Given the fact that granulosa lutein cells express GnRH receptors they do not necessarily mediate GnRH or GnRH antagonist actions on steroidogenesis. For example, in another of our own investigations, we have demonstrated that ganirelix did not exert any effect on cAMP accumulation in human granulosa lutein cells (44). In ovarian and endometrial carcinoma cells, activation of GnRH receptors leads to antiproliferative effects of GnRH agonists and GnRH antagonists (45). Also, in these cells GnRH receptor activation does not lead to hydrolysis of phosphoinositides by phospholipase C as in pituitary gonadotrophs but inhibits mitogenic activity and signalling of epidermal growth factor (46). Therefore, the lack of effects of GnRH antagonists on steroidogenesis does not exclude ovarian actions of these compounds in general.

In conclusion, we have demonstrated that the GnRH antagonists cetrorelix and ganirelix do not affect steroidogenesis of human granulosa lutein cells. The absence of such activity, together with the data from recent clinical studies, show that these compounds are unlikely to have detrimental effects on ovarian function of patients who receive cetrorelix or ganirelix in protocols of COH.

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