CLINICAL STUDY

Actions of GnRH antagonists on IGF-II, IGF-binding protein-2 and pregnancy-associated plasma protein-A in human granulosa-lutein cells

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Abstract

Objective: Recently, GnRH antagonists (GnRHants) have been introduced for the prevention of premature LH surges during controlled ovarian hyperstimulation (COH). Here we investigated whether the GnRHants cetrorelix and ganirelix exert effects on the human ovarian IGF system. Since controversy exists on the action of GnRH agonists in the human ovary, we also tested the effect of triptorelin on IGF-II, IGF-binding protein-2 (IGFBP-2) and pregnancy-associated plasma protein-A (PAPP-A) in cultured human granulosa-lutein cells.

Design: *In vitro* cell culture study in a research laboratory of a university hospital.

Patients: Cells were obtained from patients treated with different protocols of COH. In addition to gonadotropins they received triptorelin or cetrorelix. Cells were treated with triptorelin, cetrorelix or ganirelix, 1 nmol/l each, for 48 h. IGF-II, IGFBP-2 and PAPP-A were measured by RIA and enzyme immunoassay respectively.

Results: GnRHants and triptorelin did not affect IGF-II, IGFBP-2 or PAPP-A.

Conclusions: We conclude that GnRHants do not exert any significant effects on the IGF system of granulosa-lutein cells and therefore their introduction into protocols of COH is unlikely to impair ovarian function.

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Introduction

Gonadotropin-releasing hormone (GnRH) agonists (GnRHags) and more recently the GnRH antagonists (GnRHants) cetrorelix and ganirelix have been employed for several clinical indications like sex hormone-dependent diseases and infertility to suppress luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion by gonadotropes (1). Before GnRHags suppress gonadotropins they induce an initial rise of gonadotropin secretion (flare-up), whereas GnRHants lead to a prompt inhibition of LH release by competitive binding to pituitary GnRH receptors. Apart from its pituitary actions, GnRH binds to specific receptors in numerous extrapituitary tissues (2–7). After cloning of the GnRH receptor its gene expression has been demonstrated in the human ovary (8–10). Brus *et al.* (11) indicated that high-affinity ovarian GnRH receptors are present predominantly in ovarian tissue after the LH surge. Minimal information is available on the actions of GnRHants in the human ovary (12). Recently we demonstrated that GnRHants exert no effects on ovarian steroidogenesis (13). Furthermore

GnRHants did not affect cAMP production in human granulosa-lutein cells (14).

GnRH analogs are currently used in different protocols of controlled ovarian hyperstimulation (COH) for assisted reproduction techniques to prevent premature LH surges, which are detrimental for COH cycles (12, 15–19). GnRHants have been demonstrated to have a comparable clinical performance to GnRHags (16–18, 20). Recently, however, three large prospective randomized trials have compared GnRHants with GnRHags. Patients treated with GnRHants showed lower pregnancy rates. This difference was not statistically significant. But it was consistent in all three studies no matter whether cetrorelix was used in a single- (21) or multiple-dose (22) protocol or ganirelix (23) was used (24). Since GnRHants will be more widely used in protocols of COH in the near future it is important to further 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. The lower pregnancy rates in GnRHants-treated patients with lower estradiol

concentrations and lower implantation rates and the finding of GnRH receptors in human ovarian tissue prompted us to investigate whether GnRHants exert direct actions on human granulosa-lutein cells.

A debate arose whether GnRHants may affect growth factors responsible for folliculogenesis and mitosis, which itself is a prerequisite for human implantation. Growth factors, especially insulin-like growth factors (IGFs) are responsible for adequate folliculogenesis and embryonal development. An impairment of IGFs by GnRHants could explain a disturbed implantation (25). The predominant IGF in the human ovary is IGF-II (26, 27). The synthesis of IGF-II in the estrogen-dominant follicle increases dramatically at the time of follicle selection (28). It binds to IGF-binding proteins IGFBP-2 and IGFBP-4 (29). Recent studies noted that in dominant and luteinized follicles levels of IGFBP-4 were low. In contrast, high levels of pregnancy-associated plasma protein-A (PAPP-A), identified as a protease of IGFBP-4, were found (30). PAPP-A cooperates with IGF-II, whereas its binding proteins counteract its actions.

In the present paper we describe the actions of the two new GnRHants, ganirelix and cetrorelix, and the GnRHag triptorelin on IGF-II, its binding proteins IGFBP-2 and -4 and the protease of IGFBP-4, PAPP-A, as pivotal factors of the IGF system in human granulosa-lutein cells.

Materials and methods

Protocols of COH

Human granulosa-lutein cells were obtained from patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection. Two different stimulation protocols were used for COH; these are the long protocol with a GnRHag and the multiple-dose GnRHant protocol. In the long protocol, patients ($n = 25$) received 3.75 mg D-6-tryptophan-GnRH (triptorelin; Ferring, Kiel, Germany) s.c. 14 days before recombinant FSH application. Patients were treated with 2 ampoules (amps) of FSH (75IE) from day 2 of the cycle. On day 6 the dose of FSH was increased to 3 amps and thereafter the amount of FSH was adjusted according to the ovarian response. In the GnRHants group patients ($n = 25$) were treated with two amps of FSH from day 2 of the cycle. From day 7 the GnRHant cetrorelix (ASTA Medica, Frankfurt, Germany) was co-administered s.c. with FSH at a dose of 0.25 mg per day as described previously (17). The dose of FSH was adapted to the ovarian response in the same way. When at least three follicles reached a diameter of 18–20 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 been approved by the ethical committee of the Medical University of Lübeck.

Granulosa-lutein cell preparation and culture conditions

Follicular aspirates were obtained from patients treated with two different protocols of COH (see above). After collection of oocytes, aspirates were centrifuged at 175 *g* for 10 min. The pellet was washed twice in PBS without Ca^{2+} and Mg^{2+} . The cell pellet was resuspended in PBS, overlayed on Ficoll (Pharmacia, Uppsala, Sweden) and centrifuged at 300 *g* for 20 min. Granulosa-lutein cells were aspirated from the interphase. After filtration to remove red blood cells, granulosa cells were resuspended in Ham's F10 medium containing 10% fetal calf serum, 100 IU/ml penicillin and 100 ng/ml streptomycin. Cells were cultured on multiwell dishes (200 000 cells/well) in 5% carbon dioxide, 95% air and saturated humidity at 37°C. Viability of the cells was always >90% as demonstrated by trypan blue exclusion.

Effects of *in vivo* treatments with GnRH analogs

To test the hypothesis that long-term *in vivo* treatment with cetrorelix or triptorelin may affect the IGF system, granulosa-lutein cells from patients treated with the long agonistic protocol were compared with cells derived from patients to whom the antagonistic multiple-dose regimen was administered. Those cells experienced no *in vitro* treatment. Media were collected after 24 h.

Effects of *in vitro* treatments with GnRH analogs

To exclude the possibility that fetal calf serum, which contain growth factors, may have an influence on the results of the experiments, incubation medium was replaced by medium which is free of serum and growth factors (Serum Replacement 2; Sigma, Steinheim, Germany). We employed different *in vitro* treatment protocols using GnRHags and GnRHants. Cells were exposed to 1 nmol/l triptorelin, cetrorelix or ganirelix for 48 h. We used such a long culture period because the COH treatment of the patients includes high doses of exogenous gonadotropins, which might downregulate GnRH receptor expression (10). The prolonged culture period might be sufficient to allow recovery of GnRH receptors. We used the same concentration of 1 nmol/l for all GnRH analogs as we did in our previous studies (13), since this concentration was derived from studies on pharmacokinetics of GnRHants (31).

IGF-II, IGFBP-2 and PAPP-A measurements

IGF-II, IGFBP-2 and PAPP-A were measured in culture media. IGF-II was determined by RIA (Mediagnost,

Reutlingen, Germany). Extraction of IGFBPs was performed by acid extraction. This method delivered consistent results. The sensitivity was 0.1 ng/ml. Inter- and intra-assay variations were <10%. To test the consistency of the IGF-II assay we performed all experiments in triplicate. We obtained similar results in all sets, demonstrating that enough IGFBPs have been extracted by acidification. Therefore the binding proteins could not bind to IGF-II, which would interfere with results.

For IGFBP-2 measurement an RIA kit was used (DSL, Sinsheim, Germany). The sensitivity was 0.5 ng/ml. Inter- and intra-assay variations were <8.5%.

No commercial RIA kit for IGFBP-4 was available. We designed one as follows: recombinant human IGFBP-4 and IGFBP-4 polyclonal anti-human antibody were obtained by R&D systems (Wiesbaden, Germany). IGFBP-4 was iodinated. The second antibody used was anti-goat mouse serum. BSA at 0.25% in PBS buffer was used for the RIA.

Since no IGFBP-4 was detected by RIA we performed Western blot analysis using the same antibodies and recombinant human IGFBP-4.

In accord with recent studies by Conover *et al.* (30, 32) we were not able to detect IGFBP-4 in human granulosa-lutein cells, no matter which method was used.

Therefore we investigated PAPP-A, which acts as specific protease of IGFBP-4. Concentrations of the protein were determined by using an enzyme immunoassay (Biocat, Heidelberg, Germany). The intra- and interassay variations were below 5%.

Controls

To test the sensitivity of all assays we tested different compounds serving as positive or negative controls as described in the literature. This would exclude the possibility that no differences between treatment groups is due to a lack of sensitivity of the assays. For IGF-II assay, cells were additionally treated with 70 ng/ml hCG as a positive control. For IGFBP-2 assay cells were treated with IGF-II (2 nmol/ml) as negative and insulin (1 µmol/ml) as positive control.

Data presentation and statistical analysis

Each experiment was carried out with granulosa-lutein cells from one patient and was performed in triplicate. Data from 9–20 experiments were combined and presented as percent of basal release (100%). Treatment groups were analyzed for statistically significant differences by the Mann–Whitney U-test or ANOVA followed by a Newman–Keuls test for comparison of individual groups, if more than two treatments were tested.

Results

Effects of triptorelin, cetrorelix and ganirelix on IGF-II

To test the sensitivity of IGF-II measurements we stimulated IGF-II release by hCG as a positive control; 70 ng/ml hCG could significantly enhance IGF-II secretion by 460% (data not shown). That is in the range described before (33). It showed that our assay is sensitive enough to detect even small stimulatory or inhibitory effects of the GnRH analogs on IGF-II. No differences in IGF-II levels could be demonstrated whether cells were from patients treated with cetrorelix compared with triptorelin. Cells received no *in vitro* treatment (Fig. 1).

Neither triptorelin, cetrorelix nor ganirelix *in vitro* treatment for 48 h was able to affect IGF-II secretion from granulosa-lutein cells. There were no differences between treatment groups (Fig. 2).

Figure 1 Granulosa-lutein cells from patients treated *in vivo* with triptorelin or cetrorelix were compared, 25 patients/group. Cells received no *in vitro* treatment. IGF-II was measured by RIA. Values are expressed as means $\pm$ S.E.M.

Figure 2 Granulosa-lutein cells were treated *in vitro* for 48 h with cetrorelix, ganirelix or triptorelin, 1 nmol/l each. IGF-II was measured by RIA. Untreated controls were set to 100%. IGF values of different patients were in the range 0.47–1.1 ng/ml. 5–10 replicates were performed per group. Values are expressed as means $\pm$ S.E.M.

Effects of triptorelin, cetrorelix and ganirelix on IGFBP-2

We again tested the function of the assay used before experimental measurements. IGF-II as a negative control attenuated IGFBP-2 release to 90.3%. On the contrary, insulin as positive control enhanced IGFBP-2 to 112% (data not shown). IGFBP-2 concentrations did not differ in cells from patients treated with triptorelin or cetrorelix (Fig. 3).

No differences in IGFBP-2 secretion were found whether cells were treated *in vitro* for 48 h with 1 nmol/l triptorelin, cetrorelix or ganirelix (Fig. 4).

IGFBP-2 was not affected by GnRH analogs.

Effects of triptorelin, cetrorelix and ganirelix on PAPP-A

Since the detection of IGFBP-4 by different methods failed, we included the measurement of PAPP-A, the

Figure 3 Granulosa-lutein cells from patients treated *in vivo* with triptorelin or cetrorelix were compared, 25 patients/group. Cells received no *in vitro* treatment. IGFBP-1 was measured by RIA. Values are expressed as means $\pm$ S.E.M.

Figure 4 Granulosa-lutein cells were treated *in vitro* for 48 h with cetrorelix, ganirelix or triptorelin, 1 nmol/l each. IGFBP-2 was measured by RIA. Untreated controls were set to 100%. IGFBP-2 values of different patients were in the range 10.2–38.9 ng/ml. 5–10 replicates were performed per group. Values are expressed as means $\pm$ S.E.M.

Figure 5 Granulosa-lutein cells were treated *in vitro* for 48 h with cetrorelix, ganirelix or triptorelin, 1 nmol/l each. PAPP-A was measured by enzyme immunoassay. Untreated controls were set to 100%. PAPP-A values of different patients were in the range 0.2–0.4 mIU/ml. 5–10 replicates were performed per group. Values are expressed as means $\pm$ S.E.M.

protease of IGFBP-4. This is in agreement with recent studies showing high levels of PAPP-A and consequently no detected IGFBP-4 in dominant follicles or granulosa-lutein cells (30, 32).

Triptorelin, ganirelix and cetrorelix did not affect PAPP-A release of granulosa-lutein cells. No differences between treatment groups could be observed (Fig. 5).

Discussion

In the present study we investigated the effects of GnRH analogues on IGF-II, IGFBP-2 and PAPP-A, the most important factors of the human ovarian IGF system, in human granulosa-lutein cells. The specific aim was to identify the actions of the two new GnRHants cetrorelix and ganirelix, which have both been approved for COH in some countries. Cetrorelix, ganirelix and triptorelin did not exert any effects on IGF-II, IGFBP-2 and PAPP-A.

The requirement for effects of pharmacological doses of GnRH analogues on the function of the human ovary is the presence of active and functional GnRH receptors. Emerging evidence exists for their expression in the human ovary. Binding studies with human luteal tissue or cells have revealed no, or low- or high-affinity binding sites for GnRH (34). High-affinity GnRH receptors have also been detected by quantitative autoradiography in human granulosa cells at a late stage of follicular maturation (35). Brus *et al.* (11) have demonstrated GnRH receptors 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 luteinizing hormone surge. The expression of GnRH receptor gene in the human ovary has been shown by several groups (8–10, 13, 36). GnRH receptor gene expression is upregulated by GnRH, but downregulated by hCG (10). The presence of GnRH receptors in human granulosa-lutein cells points to the possibility that they mediate actions of

GnRH. However, concentrations of hypothalamic GnRH in the peripheral circulation are presumably much too low to activate extrapituitary GnRH receptors. In contrast, the application of GnRH analogs to patients in protocols of COH results in serum concentrations which might be sufficient to interact with these receptors (37). The GnRH analog concentration of 1 nmol/l we used for all *in vitro* experiments should be sufficient to detect any differences, since compared with our recently published pharmacokinetic data on cetrorelix the levels in plasma and follicular fluid were lower (32).

The actions of GnRHants in the human ovary are not understood. Minaretzis *et al.* (12) reported that treatment of patients undergoing COH with the GnRHant Nal-Glu has no effect on the progesterone production of granulosa-lutein cells obtained from these patients. Aromatase activity, however, was reduced in the GnRHant group during the first 6 h of culture. Two studies suggested that the luteal function in GnRHant-treated patients may be less impaired compared with GnRHags (38, 39). They compared cetrorelix with GnRHag buserelin and found that cells from patients treated with cetrorelix respond earlier to *in vitro* hormone stimulation. Our previous studies, however, did not find any ovarian effects of GnRHants. Neither did they influence cAMP production (14), nor steroidogenesis in human granulosa-lutein cells (13).

Given the fact that granulosa-lutein cells express GnRH receptors they do not necessarily mediate GnRH or GnRHants actions. For example in ovarian and endometrial carcinoma cells, activation of GnRH receptors leads to antiproliferative effects of GnRHags and GnRHants (40). Also, in these cells GnRH receptor activation does not lead to hydrolysis of phosphoinositides by phospholipase C, as in pituitary gonadotropes, but inhibits mitogenic activity and signaling of epidermal growth factor (41). Therefore the lack of GnRHants effects on steroidogenesis and cAMP production in human ovary does not exclude any ovarian actions of these compounds in general.

The lower pregnancy rates in GnRHant-treated patients remain a hurdle to a wide acceptance of these compounds. The contributing factors are still a matter of debate. Some blame lower estradiol levels before oocyte pick-up (42), others discuss an impaired endometrial receptiveness caused by GnRHants (25). The proper function of the ovarian IGF system is another prerequisite for normal follicular development and finally implantation (25).

The IGF family is composed of IGF-I and IGF-II peptides, six structurally homologous high-affinity IGFBPs (IGFBP-1 to -6), IGF-specific proteases and two types of IGF receptors. IGF-I and IGF-II are small, mitogenic and antiapoptotic peptides that promote differentiation and also have insulin-like metabolic effects mediated upon binding to specific membrane receptors (28, 43, 44).

In the human ovary, the granulosa cell is a site of IGF-II rather than IGF-I gene expression (26, 27, 45). IGF-I mRNA is hardly detectable in the human ovary and in granulosa cells at no time of follicle maturation, whereas IGF-II synthesis has been found in granulosa-lutein cells and in preovulatory granulosa cells (26, 46). IGF-II stimulates, as the primary IGF in the human ovary, steroidogenesis along with FSH in granulosa cells. At the time of follicle selection, in the estrogen-dominant follicle, granulosa IGF-II synthesis increases dramatically (28). In human ovaries IGFBP-2 and -4 are known to inhibit the action of IGF-II, which they predominantly bind. IGFBP-2 and -4 can be localized in granulosa and theca cells of atretic follicles, but are negligible in primordial, preantral and antral follicles (29). The follicular fluid of atretic/androgen-dominant follicles contains high levels of IGFBP-2 and -4, subsequently low levels and activity of IGF-II (47–49). In contrast an increase of lower molecular mass forms of IGFBP-2 and -4 in dominant follicles suggests an increased activity of proteases of IGFBPs (50), resulting in a decreased affinity of IGFBP-2 and -4 for IGF-II. Moreover, protease activity is also observed in follicular fluid of developing but not atretic follicles (51, 52).

The effects of IGF-II are inhibited by IGFBP-4 and are enhanced by cleavage of IGFBP-4 through a specific protease decreasing its affinity for IGFs. The protease of IGFBP-4 was found to be PAPP-A (32). PAPP-A is present in follicular fluids of estrogen-dominant follicles and luteinized granulosa cells, but could not be detected in androgen-dominant follicles (30, 51, 53). Consequently highest activity of IGF-II and low levels of IGFBP-4 in estrogen-dominant follicles and luteinized granulosa cells were noted. These observations are in agreement with our failure to detect IGFBP-4 in human granulosa-lutein cells. In contrast, the levels of PAPP-A, degrading IGFBP-4, were consistently high, which is in accord with previous data as well (54).

This is substantial evidence that IGFs and IGFBPs are involved in folliculogenesis and steroidogenesis in the ovary and therefore an imbalance of the intraovarian IGF system due to the application of GnRHants would disturb the physiological differentiation of the follicle growth and maturation, but also of the endometrium and the implantation of the embryo.

In conclusion, we have shown for the first time that the GnRHants cetrorelix and ganirelix do not affect the IGF system of human granulosa-lutein cells. It is therefore unlikely that the slightly diminished pregnancy rates in GnRHants cycles are due to ovarian effects of GnRHants.

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