Effect of In Vivo GnRH Agonist and GnRH Antagonist on hCG and Insulin-Stimulated Progesterone Production by Human Granulosa-Lutein Cells In Vitro¹

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Purpose: To investigate hCG and insulin-stimulated progesterone (P) production by human granulosa-lutein cells (hGLC) in vitro.

Methods: hGLCs were isolated from patients undergoing IVF-ET cycles in which GnRH agonist or GnRH antagonist was used to prevent a midcycle gonadotropin surge. The cells were cultured for 3 days, and then treated with hCG 0.5, 1, and 10 IU/l, and insulin 0.01, 0.1, and 1 μ M in serum free conditions. In vitro P production was measured by enzyme immunoassay.

Results: hCG stimulated P production by hGLCs from cycles in which GnRH antagonist was used, but a blunted response was seen in GnRH-agonist treated cycles. Insulin-stimulated P production was similar in cells from cycles in which GnRH-agonist or GnRH-antagonist treatment was used.

Conclusions: Because insulin and hCG may share common pathways beyond the level of receptor activation, we hypothesize that GnRH agonist, but not GnRH antagonist, may affect the expression and/or activation of LH receptors in the hGLCs.

KEY WORDS: GnRH agonist; GnRH antagonist; granulosa cells; progesterone.

INTRODUCTION

During controlled ovarian hyperstimulation (COH) for assisted reproduction, suppression of the endogenous LH and FSH surge is necessary to avoid cancellation of the treatment cycles. Suppression of gonadotropin secretion can be achieved with either GnRH agonists or GnRH antagonists. GnRH

agonists induce pituitary desensitization of the gonadotropes by reducing the number of GnRH receptors on the cell membrane, a mechanism called down-regulation. On the other hand, GnRH antagonists achieve pituitary suppression of gonadotropin secretion by the competitive blockade of the GnRH receptors (1–6).

Pituitary suppression by GnRH agonists is known to increase gonadotropins required for an adequate response during COH and result in the need for luteal phase support by administration of exogenous progesterone (P) and/or hCG. These effects are believed to be due to suppression of endogenous pituitary gonadotropin concentrations that accompany GnRH-agonist administration and/or a possible direct effect of GnRH agonists on the ovary.

GnRH receptors have been found in the human granulosa cells, and a possible direct effect of GnRH on ovarian steroidogenesis has been suggested (7,8).

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However, there is debate concerning the effect of GnRH agonists on granulosa cell function because studies on the actions of GnRH analogues on steroidogenesis in granulosa lutein-cells in vitro revealed contradictory effects (7,9–13). Moreover, little is known about any direct action of GnRH antagonists on ovarian steroidogenesis (3).

Although the concentrations of the hypothalamic GnRH in the peripheral circulation are too low to activate the peripheral GnRH receptors in the ovaries, the application of GnRH analogues, agonists, and antagonists to patients undergoing controlled ovarian hyperstimulation results in serum concentrations which are high enough to interact with the receptors.

Since GnRH agonists and antagonists are widely applied in protocols of controlled ovarian hyperstimulation, it is important to study their ovarian effects, which might be advantageous or detrimental for the treatment outcome. Moreover, such knowledge would improve our understanding of the physiological role of GnRH in the human ovary.

The aim of this study was to evaluate the in vitro P production by human granulosa-lutein cells (hGLCs) isolated from women undergoing IVF, who had COH with FSH injections and pituitary suppression with the GnRH agonist leuprolide acetate, or the GnRH antagonist ganirelix.

PATIENTS AND METHODS

Approval by the local Research Ethics Board was obtained to use hGLC from women undergoing IVF-ET for in vitro studies. Women undergoing IVF had pituitary suppression by either the long GnRH agonist leuprolide acetate (Lupron; TAP Pharmaceuticals, Deerfield, IL) protocol or the GnRH antagonist ganirelix (Antagon; Organon, Oss, The Netherlands). The patients were randomized to receive GnRH agonist ($n = 3$) or GnRH antagonist ($n = 3$) as part of a clinical trial in IVF. The patients' ages, peak estradiol levels, number of preovulatory follicles on the day of hCG, day of hCG, and number of oocytes collected are outlined in Table I. The patients had infertility due to tubal obstruction or male factor. All patients were younger than 38 years old and none of the patients had endocrine abnormalities.

Women who had GnRH agonist for pituitary down-regulation received leuprolide acetate 0.5 mg s.c. daily starting on Day 19–21 of the previous cycle and continued till the day of hCG administration. Women who had GnRH antagonist to prevent an endogenous gonadotropin surge received ganirelix

Table I. Patients' Characteristics

	GnRH agonist ($n = 3$)	GnRH antagonist ($n = 3$)
Age	32 $\pm$ 2.5	33 $\pm$ 2.5
Peak estradiol level (pmol/L)	7061 $\pm$ 658	7122 $\pm$ 647
No. of preovulatory follicles	6.7 $\pm$ 0.94	7 $\pm$ 1.6
Day of hCG administration	12 $\pm$ 0.8	12 $\pm$ 0
No. of oocytes collected	7.3 $\pm$ 0.5	7.3 $\pm$ 1.25

Note. Data presented as Mean $\pm$ SD.

0.25 mg s.c. daily from Day 6 of FSH injection till the day of hCG administration. Highly purified FSH (Fertinorm®; Serono, Oakville, Ontario, Canada) or recombinant FSH (Gonal-F®; Serono, Oakville, Ontario, Canada, or Puregon®; Organon, Scarborough, Ontario, Canada) was given at a dose of 150–225 IU/day. In both groups of women, hCG (Profasi®; Serono or Pregnyl, Organon) 10,000 IU s.c. was administered when at least three leading follicle(s) reached a diameter of 2 cm and ovum retrieval was performed 34–36 h later using a transvaginal ultrasound-guided procedure.

Isolation and Culture of Granulosa Cells (GC)

After recovering the oocytes from the follicular fluid, the hGLC clumps were collected manually under the microscope from the follicular fluid using Pasteur pipettes. The cell clumps were then washed repeatedly in prewarmed medium 199 (Gibco, Grand Island, NY) to remove the red blood cells. Finely drawn Pasteur pipettes were used to disperse the washed cell clumps. We did not use any enzymatic digestion for the cell clumps. Also, passing the cells through the finely drawn Pasteur pipettes was done gently to avoid any disruption of the cell membrane structures including the hormonal receptors. Cell number was counted using a hemocytometer and cell viability was determined by 0.5% Trypan blue (Sigma, St. Louis, MO) exclusion. Cell viability was checked before starting the culture and the end of the culture after collecting the media for hormonal assay. Cell viability ranged from 80 to 90% in both cases without any significant difference between the different culture wells. Culture media M199 (Gibco, Grand Island, NY) that contained 1% of L-glutamine 200 mM (Gibco, Grand Island, NY), 1% penicillin 10,000 IU/mL, and streptomycin 10 mg/mL (Gibco, Grand Island, NY) was used. Cells were cultured in 24-well culture dishes.

Each well contained 50,000 cells in a volume of 0.5 mL of 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) containing culture media. Cells were cultured in 5% CO₂, 95% air and saturated humidity at 37°C. On the second day, similar volume of the FBS-containing culture media was added to the individual wells (0.5 mL per well). On the third day, culture dishes were washed using prewarmed phosphate buffered saline (Gibco, Grand Island, NY) followed by shift into serum free culture medium that contained 1% human albumin (Sigma St. Louis, MO) substituting for FBS. On the fourth day, cells were treated in three replicates for each treatment condition. Serum free media containing 1% human albumin and different hCG or insulin concentrations were used in equal volumes of 1 mL for each replicate. The experiment was repeated several times and the results of three perfect experiments were included. The cells were treated with concentrations of hCG (Profasi®; Serono, Oakville, Ontario, Canada) 0.5, 1, and 10 IU/L, or insulin (Humulin®; Lilly, Toronto, Ontario, Canada) 0.01, 0.1, and 1 µM in serum free conditions for 18 h. The conditioned media was then collected for P measurement by enzyme immunoassay. The cells in the control condition received no treatment.

STATISTICS

The results are expressed as the mean ± SEM of three representative experiments expressed as percentage from control. Statistical difference between P production under different treatments and the basal (no treatment) was calculated using ANOVA and Bonferroni *t* test and a *P*-value <0.05 was considered significant. The statistical tests were performed with SigmaStat for Windows Version 1.0 software (SigmaStat Software HighEdit Professional Copyright® 1993, MicroHelp Inc and HeilerSoftware GmbH, San Rafael, CA).

RESULTS

The mean ± SEM of basal P accumulation after 18 h in serum free medium (control condition) was 32.33 ± 1.9 and 67.32 ± 2.4 pmol/µg protein cells in the GnRH-agonist and GnRH-antagonist groups respectively (*P* < 0.0001). In the hGLC from GnRH agonist-treated women, hCG was not effective in significantly stimulating P production until the 10 mIU concentration (135% from control ± 14%; *P* < 0.05). In contrast, hCG stimulated a robust increase in

Fig. 1. Effect of GnRH-agonist and GnRH-antagonist pituitary suppression on in vitro hCG-stimulated P production by hGLCs. hCG-stimulated significantly (*P* < 0.001) higher levels of P production by granulosa-lutein cells from cycles in which GnRH antagonist was used.

P production by hGLC from GnRH antagonist-treated women (2.4–3.3 times higher than basal secretion). The pattern of hCG-stimulated P production was significantly different between the GnRH-agonist and GnRH-antagonist groups (*P* < 0.001) (Fig. 1).

Insulin stimulated a similar increase in P production in both GnRH agonist- and antagonist-treated groups (1.1–1.5 times higher than basal secretion in cells from GnRH-agonist cycles and 1.1–1.6 times higher than basal secretion in cells from GnRH-antagonist cycles) (Fig. 2).

DISCUSSION

Although there are many studies that investigated the possible direct effect of various GnRH agonists

Fig. 2. Effect of GnRH-agonist and GnRH-antagonist pituitary suppression on in vitro insulin-stimulated P production by hGLCs. The pattern of insulin-stimulated granulosa-lutein cell P production was similar in cycles in which GnRH-antagonist or GnRH-agonist treatment was used.

on ovarian steroidogenesis, there is no consensus of the effects on steroidogenesis in the human ovary. Also, there is little information concerning the direct effect of GnRH antagonists on ovarian steroidogenesis (14). Moreover, fewer studies have investigated the effect of GnRH-agonist and GnRH-antagonist treatments in vivo on in vitro steroidogenesis by hGLC (15,16). While some authors were unable to demonstrate GnRH-agonist actions on ovarian steroidogenesis (10,17,18), others have shown inhibitory and stimulatory actions of GnRH agonists (7,9,12,19–22). Such controversy might have resulted from differences in experimental design and the type of GnRH agonist. A few studies that looked at the effect of GnRH-agonist treatments in vivo on in vitro steroidogenesis by hGLC found an impairment of P production (14,23–25). However, when non-luteinized granulosa cells were studied, GnRH or GnRH agonists were found to have no effects on P production (9,12,22). This could be explained by the fact that these cells do not express GnRH receptors (26).

There is not much information on the actions of GnRH antagonists in the human ovary. 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 controlled ovarian hyperstimulation. The P production in hGLC cultures was not different between the two groups. Lin *et al.* (16,27) reported on the possibility that the luteal function in GnRH-antagonist (cetrorelix) treatment was found to be less impaired. Both GnRH antagonists had no effect on basal or hCG-induced estradiol or P production by granulosa-lutein cells, independent of whether the cells were exposed to the compounds in vitro or in vivo.

Our results support the hypothesis that GnRH-antagonist treatment in vivo does not affect ovarian P production in vitro by hGLC compared to the effect of in vivo GnRH-agonist treatment. However, an in vitro study has demonstrated an inhibitory effect of GnRH antagonist on gonadal steroid secretion (28).

Our findings support previous findings by Pellicer and Miro who found that GnRH-agonist exposure in vivo may affect hGLC function in vitro. They compared the P accumulation in hGLC cultures between patients treated with clomiphene citrate/gonadotropin and GnRH agonist/gonadotropin. Granulosa cells obtained from patients treated with the GnRH agonist had lower P production than cells isolated from women treated with clomiphene citrate/gonadotropin (15). However, Minaretzis *et al.* compared

the hGLC steroidogenesis in vitro from Nal-Glu and leuprolide acetate treated women respectively. They reported that basal and gonadotropin-stimulated P secretion was similar in the two treatment groups (3).

Lin *et al.* (16) showed that granulosa cells from women treated with GnRH antagonist (cetrorelix) responded earlier to the in vitro hormone stimulation in terms of P accumulation than women treated with the GnRH agonist (buserelin). The authors explained the difference in P accumulation by the difference in time of in vivo exposure to the analogue (16). More recently, Weiss *et al.* found that in vivo treatment with triptorelin or the two GnRH antagonists, cetrorelix and ganirelix, did not have an effect on spontaneous or hCG-stimulated steroidogenesis. The authors also performed in vitro treatments with triptorelin, cetrorelix, and ganirelix for up to 96 h and did not find any effect of these treatments on basal or hCG-stimulated steroid production (14). These findings are generally similar to ours.

In all prior reports, which studied the effect of GnRH-agonist treatment on ovarian steroidogenesis, only the effect of hCG was investigated. In the present study, we examined the effect of two known physiologic stimuli of ovarian steroidogenesis, hCG and insulin. Our results show that GnRH-agonist treatment affected both the basal and hCG-stimulated P production by hGLC but the pattern of insulin-stimulated P secretion was not affected. Because insulin and hCG may share common pathways beyond but not at the level of receptor activation, we hypothesize that in vivo GnRH agonist might affect the expression and/or activation of LH receptors.

In our study, we found that hGLC from patients treated with GnRH antagonist responded to hCG in a fashion generally consistent with previously reported hCG-stimulated hGLC P production (14,16). In addition, the basal P production and absolute levels of insulin-stimulated P production were both significantly higher than observed following GnRH-agonist treatment. In contrast, hGLC previously exposed in vivo to GnRH agonist had a blunted P response to hCG. Our results, therefore, support the hypothesis that GnRH agonists may have a direct negative effect on human ovarian steroidogenesis and suggest that luteal function may be less affected during IVF-ET cycles when GnRH antagonist is used. In a recent study, which supports this hypothesis, the authors concluded that GnRH antagonists can be safely administered in gonadotropin-stimulated intrauterine insemination cycles without luteal phase

supplementation because no deleterious effects of GnRH-antagonist administration were noted on luteal P concentration or on the duration of the luteal phase (29). However, in a more recent study, GnRH-antagonist therapy in women undergoing ovarian stimulation was found to be associated with a significant effect on ovarian follicular steroidogenesis. The authors found the mean follicular fluid estradiol concentration significantly lower in patients treated with GnRH antagonist than in those treated with GnRH agonist. However, no significant differences were found between groups in follicular fluid P concentrations (30). However, further studies are required to investigate the effect of GnRH agonist and antagonist on gonadotropin receptors and different enzymes involved in ovarian steroidogenesis.

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