Gonadotropin releasing hormone antagonists suppress aromatase and anti-Müllerian hormone expression in human granulosa cells

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Objective: To investigate the effects of a gonadotropin-releasing hormone antagonist (GnRH-ANT) on the expression of anti-Müllerian Hormone (AMH) and aromatase (via the exon CYP19IIa promoter), in cultured human granulosa cells (hGCs) and the human granulosa cell line (HGL5).

Design: Primary cell cultures of hGCs and culture of HGL5 cells.

Setting: Academic center.

Patient(s): Women undergoing IVF because of male factor, tubal infertility, or donor eggs.

Intervention(s): hGCs and HGL5 cells were treated with a GnRH-ANT (1 nM and 1 μM) alone or in combination with cAMP (1 mM). Media was collected and stored at −80°C until assayed.

Main Outcome Measure(s): mRNA levels of CYP19 IIa, AMH, steroidogenic factor 1 (SF-1) and liver receptor homologue-1 (LRH-1) were determined by quantitative polymerase chain reaction. ELISA was used to determine estradiol (E2) levels in the culture media. Pooled results from triplicate experiments were analyzed using one-way analysis of variance with Student–Newman–Keuls multiple-comparison methods.

Results: The GnRH-ANT decreased the expressions of CYP19 IIa, AMH, SF-1, and LRH-1. cAMP induced aromatase and AMH expression. Cotreatment with cAMP and GnRH-ANT caused a dose-dependent suppression of AMH and CYP19 IIa mRNA. A GnRH agonist (GnRH-A) increased the mRNA expressions of CYP 19 IIa and AMH. The GnRH-ANT decreased E2 production in cultured hGCs.

Conclusion(s): GnRH-ANTs, in addition to their central suppressive effects on the pituitary, may have a direct effect on ovarian granulosa cells with inhibition of aromatase and AMH expression. Furthermore, the inhibitory effect could be mediated via suppression of SF-1 and LRH-1, and may play a role in estrogen-mediated ovarian folliculogenesis. (Fertil Steril 2010;94:1832–9. ©2010 by American Society for Reproductive Medicine.)

Key Words: AMH, MIS, aromatase, CYP 19 IIa, GnRH antagonist, GnRH agonist, GnRH analogues

To date, gonadotropin-releasing hormone (GnRH) analogues are widely used in assisted reproduction to suppress the surge of the endogenous luteinizing hormone (LH) (1, 2). GnRH agonists (GnRH-A) and antagonists (GnRH-ANT) have been well characterized to have different mechanisms of action. Within the pituitary, GnRH-A stimulate the GnRH receptor (GnRHR), causing an initial flare of gonadotropin secretion followed by suppression because of desensitization of the GnRHR (3–5). In contrast, GnRH-ANT act as competitive inhibitors of the GnRHR, causing an immediate suppression of the gonadotropin secretion (6). In recent years there has been increased interest in the use of GnRH-ANT in patients undergoing controlled ovarian hyperstimulation (COH) (7–11). The use of GnRH-ANT has been associated with a shorter stimulation protocol and a lower incidence of ovarian hyperstimulation syndrome (12). Moreover, an additional clinical advantage of the GnRH-ANT over GnRH-A is the absence of an initial gonadotropin surge. However, an area of concern with the use of GnRH-ANT is the reported lower clinical embryo implantation rates compared with GnRH-A (1, 11, 13–17).

In addition to its central role in the pituitary, GnRH has also been demonstrated, via autocrine–paracrine mechanisms, to regulate extra-pituitary tissues including the ovary, uterus, placenta, and immune cells (18–26). The expression of GnRH and its receptor in these tissues are involved in the regulation of steroidogenesis, cell proliferation, and embryo implantation (18–26). Although the role of GnRH and its analogs at the pituitary level are well known, its mode of action in extra-pituitary tissues is still not completely understood.

Normal ovarian growth and function is mediated by proper ovarian folliculogenesis and steroidogenesis. Anti-Müllerian hormone (AMH) is a dimeric glycoprotein belonging to the transforming growth factor-β (TGF-β) superfamily. The AMH is produced in the ovary by the granulosa cells surrounding preantral and small antral follicles, and plays a pivotal role in folliculogenesis (27). In addition to its critical role in regulating folliculogenesis, AMH was found to be a sensitive marker of ovarian reserve. Serum basal levels of AMH were found to decrease significantly over time in young normal ovulatory women, and were found to be significantly lower in patients with premature ovarian failure (28, 29). Recent
studies have demonstrated a possible correlation between AMH and aromatase, a key enzyme in ovarian steroidogenesis, although their exact interaction is still not well understood (30–32).

Steroidogenesis in the ovary, specifically the production of estrogen, is essential for follicular growth and selection. The production of estrogen in the ovary is under the regulation of Cytochrome P450 aromatase (P450arom), which catalyzes the biosynthesis of estrogens from androgens. The human CYP19 (aromatase P450) gene is expressed in a number of tissues, including the placental syncytiotrophoblast, ovarian granulosa, and luteal cells and in the brain. Expression of aromatase in each of these tissues is controlled by unique tissue-specific promoters, with CYP19 IIa being the ovarian promoter (33–35).

The purpose of the present study was to investigate the effects of a GnRH-ANT on granulosa cell expression of P450arom and AMH. We also studied the effects of GnRH-ANT and GnRH-A on steroidogenic factor 1 (SF-1) and liver receptor homologue-1 (LRH-1), which are transcriptional regulators of steroidogenesis and folliculogenesis (35–39). We have hypothesized that GnRH-ANT directly influences AMH and CYP19 II a mRNA expression, and that these effects may be mediated through transcriptional factors SF-1 and LRH-1.

## MATERIAL AND METHODS

Experiments were performed on primary human granulosa-lutein cells (hGCs) in culture as well as on a well characterized human granulosa cell line (HGL5). The research protocol was approved by the University of Texas Southwestern Medical Center institutional review board.

### Cell culture

hGCs were obtained at the time of oocyte retrieval from women aged between 28 and 35 years, following COH for in vitro fertilization (IVF) because of male factor infertility, tubal pathology, or for egg donation. After harvesting the cumulus–oocyte complex for IVF, granulosa cells were isolated from follicular fluid samples through repeated centrifugation and washing with phosphate-buffered saline or culture medium (40). hGCs were washed twice with DMEM/F-12 medium (Invitrogen, Carlsbad, CA), and were incubated for 30 minutes at 37°C in DMEM/F-12 medium containing 0.1% hyaluronidase to disperse the granulosa cells. The dispersed cells were resuspended in 20 mL medium and transferred to 50 mL tubes containing 3.5 mL 50% percoll solution (Sigma Chemical Co., St. Louis, MO). hGCs were separated from red blood cells by centrifugation at 600 g for 30 minutes. hGCs formed a thin layer between the percoll and the medium. hGCs were removed and washed three times using DMEM/F-12 medium containing 10% FBS, 1% ITS Plus, and antibiotics. An overnight incubation (37°C, humidified atmosphere, 5% CO2) was undertaken to allow the attachment of the cells to the bottom of the plates and the recovery from any effects of the in vivo exposure to GnRH-A and gonadotropins during COH. Media were changed every 48 hours until 60% confluence was achieved.

In addition, HGL5 cells and control COS-7 cells were plated into six-well culture dishes at a density of 300,000 cells per well in a DMEM/F-12 medium containing 10% fetal bovine serum (FBS), 1% ITS Plus (insulin, human transferrin, selenous acid; Collaborative Research, Waltham, MA); and antibiotics. Cells were counted for viability by trypsin blue staining and plated into six-well culture dishes at a density of 300,000 cells per well in a DMEM/F-12 medium containing 10% fetal bovine serum (FBS), 1% ITS Plus, and antibiotics. An overnight incubation (37°C, humidified atmosphere, 5% CO2) was undertaken to allow the attachment of the cells to the bottom of the plates and the recovery from any effects of the drug (i.e., antagonist) or with sterile distilled water (vehicle).

### Experiments

All experiments were performed in triplicate dishes and repeated three times. The cultures derived from each patient were divided into control and treatment plates. Control cells were cultured with DMEM+F-12 + 2% FBS, 1% ITS+, and antibiotics and treated with sterile distilled water (vehicle). The treatment plates were exposed to GnRH-ANT cetrorelix acetate for 24 hours.

The experiments were performed by exposing the cells to GnRH-ANT cetrorelix (1 nM and 1 μM, EMD Serono Inc., Rockland, MA) for 24 hours with or without dibutyryl cAMP (B2cAMP; 1 mM, Sigma Chemical Co.). Additional cells were treated with GnRH-A, leuprolide acetate (1 nM and 1 μM; Sicor Pharmaceuticals, Irvine, CA), and incubated for 24 hours. After this period the culture media were collected and stored at −80°C and the cells were scraped into Trizol reagent (Invitrogen, Carlsbad, CA) for RNA extraction.

### Table 1: Real-time PCR primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH Forward</td>
<td>5’-AGGTACCAAGGGATTTGATGG-3’</td>
</tr>
<tr>
<td>AMH Reverse</td>
<td>5’-CTCTACGTGTCCTTCTCC-3’</td>
</tr>
<tr>
<td>GnRHR Forward</td>
<td>5’-ACCGCTCCGTGGCTATAC-3’</td>
</tr>
<tr>
<td>GnRHR Reverse</td>
<td>5’-ACTGTTCCGACTTTGCTTTGC-3’</td>
</tr>
<tr>
<td>LRH- 1 Forward</td>
<td>5’-TGGGAAAGGAGGAGGACATCT-3’</td>
</tr>
<tr>
<td>LRH- 1 Reverse</td>
<td>5’-CGAGACTCAGGGAGTGTGTGAA-3’</td>
</tr>
<tr>
<td>GYP 19 Ila Forward</td>
<td>5’-CAGAGGCTATAGTGACACCTTGGG-3’</td>
</tr>
<tr>
<td>GYP 19 Ila Reverse</td>
<td>5’-CTG ACA GGA GGT CCC TGG C-3’</td>
</tr>
<tr>
<td>GYP 19 l. 4 Forward</td>
<td>5’-CGG GTT CAG CAT TTC CAA AA-3’</td>
</tr>
<tr>
<td>GYP 19l.4 Reverse</td>
<td>5’-CAC TCT ACC CAC TCA AGG GCA-3’</td>
</tr>
<tr>
<td>GYP 19 l. 3 Forward</td>
<td>5’-TTG GCT TGA ATT GCA GCA TTT-3’</td>
</tr>
<tr>
<td>GYP 19 l. 3 Reverse</td>
<td>5’-GCCCTGAAACAGCAGAAAG-3’</td>
</tr>
<tr>
<td>SF-1 Forward</td>
<td>5’-GCCCTGTCTCACGCTTGAA-3’</td>
</tr>
<tr>
<td>SF-1 Reverse</td>
<td>5’-TGCATCATACCCCATTTCTATCA-3’</td>
</tr>
<tr>
<td>h3 6B4 Forward</td>
<td>5’-AAGGTGTAATCGGCTCCACAGAAG-3’</td>
</tr>
</tbody>
</table>

RNA Extraction, cDNA Synthesis, and Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA from tissue samples was extracted by the one-step method of Chomczynski and Sacchi (41) using Trizol reagent (Invitrogen). The isolated RNA was quantified by measuring the optical density of the samples at a wavelength of 260 nm. The quality of RNA was ascertained by the presence of ratios between 1.6 and 2.0 at 260/280 nm. RNA was treated with deoxyribonuclease (Invitrogen) to remove any contaminating DNA, and then was reverse-transcribed using random primers and Superscript II Reverse Transcriptase (Invitrogen). The relative abundance of each mRNA product in the tissue samples was determined by quantitative real-time (qRT)-PCR using a modification of previously published methods (42).

Primer sets directed against human CYP19 exons Ila (ovary), CYP19 exons I.3 (breast cancer), CYP19 exons I.4 (adipose tissue), AMH, SF-1, LRH-1, GnRH type I receptor, and h36B4 (ribosomal RNA for normalization) mRNA transcripts were designed using Primer Express software (PE Applied Biosystems, Foster City, CA) based on published sequences for these mRNAs (Table 1). For the quantitative analysis of mRNA expression, the ABI Prism 7700 Detection System (Applied Biosystems) was employed using the DNA binding dye SYBR Green (PE Applied Biosystems) for detection of PCR products. Thermocycling was done in a final volume of 12 μL containing 2 μL cDNA sample, 0.6 μL primer, 3.4 μL sterile water, and 6 μL SYBR Green I. The cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The cycle threshold was set at a level where the exponential increase in PCR amplification was approximately parallel among all samples. All primer sets produced amplifications of the expected size and sequence. We calculated the relative fold changes using the comparative cycle times (Ct) method with human ribosomal protein (h36B4) mRNA as the reference guide (42).

Hormone Assays

Estradiol levels in the media from hGC cultures were assayed by a commercially automated, chemiluminescent immunoassay (DPC Immulite 2000, Siemens Medical Solutions Diagnostics, Berkeley, CA). The intraassay coefficients of variation was 9.4%. Three replicates per sample were assayed.

Data Analysis and Statistical Methods

Pooled results from triplicate experiments were analyzed using one-way analysis of variance with Student–Newman–Keuls multiple-comparison methods, using SigmaStat version 3.0 (SPSS Inc., Chicago, IL). Treatments were considered significantly different when the value was P < .05. The results are presented as mean ± standard error of the mean (SEM).

RESULTS

The GnRHR and CYP 19 Ila is expressed in both HGL5 and hGCs

We used qRT-PCR to quantify the presence of GnRHR in the HGL5 cells and in hGCs. A monkey kidney cell line (COS-7 source) was used as a negative control. The GnRHR was highly expressed in both the HGL5 cells and hGCs, but was not detected in COS-7 cells (data not shown). Expression of the GnRHR was found to be higher in hGCs compared with the HGL5 cells.

We also used qRT-PCR to quantify expression of specific ovarian tissue promoters of aromatase (CYP 19 Ila) as well as of the breast (CYP 19 I.4) and adipose (CYP 19 I.3) tissue specific promoters, respectively, in both the HGL5 cells (Fig. 1A) and in hGCs (Fig. 1B). Both hGCs and the HGL5 cells selectively expressed the tissue ovarian specific promoter CYP 19 Ila (Fig. 1A and B).

The GnRH-ANT Suppresses CYP19Ila and AMH mRNA Expression in hGCs and in HGL5 Cells

hGCs were incubated for 24 hours in the presence of GnRH-ANT (1 nM and 1 μM). Incubation of hGCs with GnRH-ANT caused a dose-dependent suppression of both CYP19Ila and AMH mRNA compared with controls (Fig. 2A and B; P < .05). To assess if GnRH-ANT could antagonize cAMP-stimulated expression of aromatase (CYP 19 11a) or AMH, HGL5 cells were incubated for 24 hours in the absence (basal) or presence of Bt2cAMP (cAMP, 1 mM), the GnRH-ANT (1 nM and 1 μM), or the two agents in combination: cAMP (1 mM) + GnRH-ANT (1 nM and 1 μM).

As expected, cAMP markedly induced CYP 19 Ila mRNA expression in HGL5 cells (Fig. 2C; P < .05). Moreover, incubation of HGL5 cells with GnRH-ANT alone or in combination with cAMP, caused a dose-dependent suppression of aromatase (CYP 19 11a) (Fig. 2C; P < .05), cAMP also induced AMH mRNA, whereas the GnRH-ANT, alone or in combination with cAMP, decreased AMH expression (Fig. 2D; P < .05). When HGL5 cells were incubated for 24 hours in the presence of GnRH-ANT (1 nM and 1 μM), there was...
a dose-dependent decrease in $E_2$ production ($P<.05$, data not shown).

**The GnRH-Ant Suppresses SF-1 and LRH-1 mRNA Expression**

hGCs and HGL5 cells were incubated for 24 hours in the absence (basal) or presence of the GnRH-ANT (1 nM and 1 μM). Quantitative real-time PCR was used to quantify SF-1 and LRH-1 transcript levels. Incubation of hGCs with GnRH-ANT (1 nM and 1 μM) suppressed SF-1 mRNA expression in either the HGL5 cells or hGCs (Fig. 3A and B, respectively; $P<.05$). In addition, GnRH-ANT, in a dose-dependent manner, also decreased LRH-1 expression in both the HGL5 cells and hGCs as well (Fig. 3C and D, respectively).

**GnRH-A Stimulation Both CYP19 IIa and AMH mRNA Expression**

HGL5 cells were incubated for 24 hours in the presence of GnRH-A (1 nM and 1 μM) with or without Bt2cAMP (cAMP, 1 mM) or the
two agents in combination: cAMP 1 mM + GnRH-A (1 nM and 1 μM). CYP19 IIa mRNA levels were analyzed by qRT-PCR. Bt2cAMP markedly induced both CYP19 IIa and AMH mRNA expression in HGL5 cells (Fig. 4A and B). In addition, the treatment of HGL5 cells with GnRH-A alone caused a dose-dependent stimulation of aromatase mRNA expression via exon IIa (Fig. 4A; P < .05) and AMH mRNA (Fig. 4B; P < .05). Moreover, the combination of Bt2cAMP with GnRH-A caused further stimulation of both CYP19 IIa and AMH mRNA compared with GnRH-A alone or cAMP alone (Fig. 4A and B, P < .05).

DISCUSSION

Most vertebrate species possess at least two and usually three forms of GnRH (43–45). In humans, in addition to GnRH-I, GnRH-II has been identified (45–49). The most prominent difference in the tissue distribution of GnRH-I and GnRH-II in humans is that the latter is expressed at the highest level outside the brain while GnRH-I is expressed mainly in the brain (44). The GnRHR belongs to a member of the rhodopsin-like G protein-coupled receptor superfamily, which responds both to GnRH-I and GnRH-II (50).

In the ovary, expression of GnRH-I and GnRH-II mRNAs has been demonstrated in human corpus luteum, luteinized granulosa cells, epithelial ovarian carcinoma, and a number of ovarian cancer cell lines (51).

In our study we investigated the effects of GnRH-ANT on granulosa cells using two cell culture models: [1] primary hGCs obtained from patients undergoing COH during IVF cycles and [2] the HGL5 cell line. The HGL5 cells belong to an immortalized cell line that originated from transformation of luteinized HGCs using HPV 16. Compared with hGCs, the expression of CYP19 is lower in the HGL5 cells. The HGL5 cells can produce progesterone and E2 when stimulated with Forskolin or Bt2cAMP, but they are...
high doses of gonadotropins during COH treatment. Among the disadvantages of using primary hGCs is their heterogeneous response to treatments because of the underlying patient variability, the limited survival time in culture (7–8 days), and their high level of baseline aromatase expression because of exposure to high doses of gonadotropins during COH treatment.

In our study, we have demonstrated that GnRH-ANT alone as well as in combination with Bt2cAMP leads to a dose-dependent suppression of aromatase mRNA expression both in hGCs as well as in HGL5 cells with a 2.5- to 5-fold decrease in aromatase mRNA expression, respectively (Fig. 2 A and C). We also demonstrated in hGCs that the treatment with GnRH-ANT decreased E2 concentration in the culture media by twofold (data not shown). Our results support previous studies investigating the in vivo and in vitro effects of GnRH analogues on steroidogenesis. Issueh et al. (18, 19) reported that GnRH-ANT blocked the effects of FSH on induction of aromatase and 20α-hydroxysteroid dehydrogenase in immature hypophysectomized rats. Moreover, Minaretzis et al. (24) showed that granulosa-lutein cells obtained from antagonist-treated women showed significantly lower aromatase activity the first 6 hours after retrieval compared with granulosa cells of women treated with agonists. Our findings correlate well with the clinical observation that the use of GnRH-ANT is associated with lower E2 levels on the day of hCG administration. Whereas the lower E2 levels can be explained solely by central inhibitory effects of the GnRH-ANT, it is possible that the GnRH-ANT exerts a suppressive effect peripherally at the ovarian level through suppression of aromatase. Previous studies looking at the effect of GnRH-A on ovarian steroidogenesis in vitro and in vivo lead to conflicting results (24, 53–58). Our data on the effect of GnRH-A on hGCs demonstrated that the GnRH-A alone as well as in combination with Bt2cAMP caused a dose-dependent stimulation of aromatase and AMH mRNA with up to 50-fold increase in aromatase expression (Fig. 4A) and up to sevenfold increase in AMH expression (Fig. 4).

To elucidate the underlying mechanisms of action of the GnRH-ANT on ovarian steroidogenesis, we studied the effects of the GnRH-ANT on the expression of SF-1 and LRH-1. Orphan nuclear receptors SF-1 and LRH-1 are emerging as important factors in regulating female reproduction. Within the ovary, SF-1 (NR5A1) is expressed only in the somatic cells, such as granulosa, theca, luteal, and interstitial cells, whereas LRH-1 (NR5A2) is expressed only in the granulosa cells of the follicles and luteal cells within the ovary (33, 34). Transcriptional regulation of ovarian CYP19 IIA is mediated through a cAMP response element (CRE)-like sequence which binds the CRE-binding protein (CREB) transcription factor and two additional receptor response elements that bind SF-1 and LRH-1 (33, 34). Previous studies have shown that SF-1 and LRH-1 synergizes with cAMP to stimulate the aromatase promoter in the ovary as well as in the endometrium (59–61). SF-1, in addition to its role in regulating aromatase expression, is a transcriptional regulator of the AMH gene and of the GnRHR (62).

In the present study we showed that GnRH-ANT caused a dose-dependent inhibitory effect on SF-1 and LRH-1 in the HGL5 cells as well as in hGCs with a 1.5- and 1.8-fold decrease in mRNA expression, respectively (Fig. 3A–D). To the best of our knowledge, there are no previous studies that have looked at the effect of GnRH-ANT on these transcriptional regulators. Our findings suggest that the GnRH-ANT may exert its inhibitory actions on CYP19 IIA and AMH expression via suppression of these transcription factors.

Anti-Müllerian hormone is a member of the TGF-β family. In the mouse ovary, AMH has been found to inhibit recruitment of primordial follicles into the pool of growing follicles and decreases the sensitivity of small preantral follicles to FSH (63–69). In our study, we investigated the effect of GnRH-ANT on the expression of AMH mRNA in hGCs and HGL5 cells. We found that AMH mRNA was expressed in both the HGL5 cells as well as in the hGCs, with higher expression found in the HGL5 cells. Furthermore, we found that Bt2cAMP increased AMH mRNA expression by twofold.
(Fig. 2D). Treatment with the GnRH-ANT caused a dose-dependent suppression of AMH expression with a 2.7- and 3.5-fold decrease in mRNA expression in hGCs and HGL5 cells, respectively (Fig. 2B) suppression of AMH expression with a 2.7- and 3.5-fold decrease in mRNA expression in hGCs and HGL5 cells, respectively (Fig. 2B). However, the use of GnRH-A caused a stimulatory effect with a sevenfold increase of AMH mRNA expression (Fig. 4B). Treatment with the GnRH-ANT caused a dose-dependent suppression of AMH expression with a 2.7- and 3.5-fold decrease in mRNA expression, whereas GnRH-A showed opposite results in hGCs. Our finding suggest that GnRH-ANT may exert a direct ovarian effect not only by modulating ovarian steroidogenesis but also by modifying local expression of AMH and thus influencing follicular development.

We conclude that GnRH-ANT may play a role in decreasing aromatase and AMH expression in hGCs. These inhibitory effects may be mediated via suppression of SF-1 and LRH-1, two key transcriptional regulators of both enzymes. Our finding suggests that the lower E2 levels found in patients undergoing COH with the use of GnRH-ANT could be the results of a combined suppressive effects of the GnRH-ANT at both the pituitary and the granulosa cell level. GnRH-ANT may also influence ovarian function via the suppression of AMH expression. Additional studies are needed to understand the underlying mechanism of action of the GnRH-ANT on granulosa cells and the intimate relationship between the key ovarian steroidogenic enzymes and ovarian folliculogenesis.

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