The mechanism responsible for the supraphysiologic gonadotropin surge in females treated with gonadotropin-releasing hormone (GnRH) agonist and primed with GnRH antagonist

Joelle E. Taylor, M.D.,[a,b] *Bradley T. Miller, M.D.,*[a,c] *Karen D. Gray, Ph.D.,*[d] *Richard T. Scott, Jr., M.D.,*[e] *William H. Catherino, M.D., Ph.D.,*[c] *and James H. Segars, M.D.*[a,c]

[a] Reproductive Biology and Medicine Branch, National Institutes of Health, Bethesda, Maryland; [b] Department of Obstetrics and Gynecology, Wake Forest University School of Medicine, Winston-Salem, North Carolina; [c] Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Bethesda, Maryland; [d] National Cancer Institute, National Institutes of Health, Frederick, Maryland; and [e] Reproductive Medicine Associates of New Jersey, Morristown, New Jersey

Objective: To elucidate the physiologic mechanism responsible for the supraphysiologic gonadotropin release from the pituitary induced by gonadotropin-releasing hormone (GnRH) agonist in female rats primed with GnRH antagonist.
Design: Controlled experimental intervention.
Setting: Government research facility.
Animal(s): Forty 8-week-old Sprague-Dawley rats.
Intervention(s): Forty oophorectomized rats were randomized into four treatment groups of 10: group A, control vehicles; group B, GnRH agonist (leuprolide acetate; 1.7 μg/kg twice a day) on day 4; group C, GnRH antagonist (Nal-Lys; 3 mg/kg each day) days 1 to 4; or group D, GnRH antagonist (Nal-Lys; 3 mg/kg each day) days 1 to 4 plus GnRH agonist (1.7 μg/kg twice a day) on day 4.
Main Outcome Measure(s): Immunohistochemical methods, Northern and in situ hybridization to quantitate pituitary follicle-stimulating hormone beta (FSH-β), luteinizing hormone beta (LH-β), and GnRH receptor (GnRH-R) messenger RNA (mRNA), and receptor protein levels in all treatment groups.
Result(s): Treatment with GnRH antagonist was associated with increased storage of gonadotropin in the pituitary for FSH-β and LH-β, but mRNA levels were unchanged. The GnRH-R mRNA decreased after GnRH-agonist treatment but remained stable in the GnRH-antagonist treatment groups. Levels of GnRH-R were decreased after GnRH-antagonist treatment.
Conclusion(s): These data indicate that the in vivo mechanism responsible for the exaggerated release of gonadotropins in rats primed with GnRH antagonist and treated with GnRH agonist was an increase in releasable gonadotropin pools coupled with a reduction in GnRH-R, but receptor function was preserved. (Fertil Steril® 2010;93:1668–75. ©2010 by American Society for Reproductive Medicine.)

Key Words: LH-RH, GnRH receptor, ovulation induction, LH surge, OHSS, ovarian hyperstimulation syndrome, pituitary

Gonadotropin-releasing hormone (GnRH) analogues play an important role as treatment options for many reproductive diseases (1). Commonly, GnRH agonists are used to induce a transient menopausal state for the treatment of estrogen-dependent diseases such as endometriosis and uterine leiomyomata, and in assisted reproductive technologies (ART) during ovulation stimulation (1–3). In the clinical setting of ART where ovarian hyperstimulation syndrome (OHSS) is a concern, GnRH agonists have been used to trigger a luteinizing hormone (LH) surge in women pretreated with a GnRH antagonist (4–8). This strategy was appealing because endogenously produced LH has a shorter half-life than human chorionic gonadotropin (hCG) (7) and thus may reduce the risk of developing OHSS (9–13). Quantitatively, this surge of LH is supraphysiologic compared with surges induced by GnRH agonists in the non–GnRH-antagonist primed female.

There are more than 24 reports of this strategy in the literature (14), and four randomized, controlled studies have examined clinical outcomes after a surge induced by a GnRH agonist in women pretreated with a GnRH antagonist (7, 10, 11, 13). In all four studies, no statistically significant differences were noted in the number of oocytes retrieved, proportion of metaphase II oocytes, fertilization

Received October 7, 2008; revised November 20, 2008; accepted December 10, 2008; published online February 6, 2009.
J.E.T. has nothing to disclose. B.T.M. has nothing to disclose. K.D.G. has nothing to disclose. R.T.S. has received research grants from EMD Serono, Organon-Schering Plough, and Ferring. W.H.C. has nothing to disclose. J.H.S. has nothing to disclose.
Supported in part by the Intramural Research Program of the Reproductive Biology and Medicine Branch, NICHD, NIH.
Reprint requests: James H. Segars, M.D., NICHD, Program of Reproductive and Adult Endocrinology, Building CRC, Room 1E3140, 10 Center Drive, Bethesda, MD 20814 (FAX: 301-402-0884; E-mail: segarsj@mail.nih.gov).

0015-0282/10/$36.00
doi:10.1016/j.fertnstert.2008.12.047

rate, or embryo quality score in women treated with GnRH agonists compared with those treated with hCG (7, 10, 11, 13). Fauser et al. (7) and Engmann et al. (13) reported a comparable clinical pregnancy rate for GnRH agonists compared with hCG treatment. In contrast, studies by Humaidan et al. (10) and Kolibianakis et al. (11) were terminated before completion because of the lower pregnancy rate observed in the GnRH-agonist treatment arm compared with hCG. Of note, only the trials by Fauser et al. (7) and Engmann et al. (13) used luteal supplementation with intramuscular progesterone, a very critical detail for women treated with GnRH analogues (15). Humaidan et al. (10) stopped luteal phase support immediately after a positive pregnancy test was obtained, and they documented a high early pregnancy loss. Despite the two trials suggesting a reduction in pregnancy (10, 11), if the luteal phase was supported, the pregnancy rates were comparable (7, 13), and the strategy may be effective for patients at risk for OHSS (13, 14).

Although both GnRH agonists and GnRH antagonists ultimately reduce gonadotropin levels, the mechanisms of pituitary desensitization differ. Administration of a GnRH agonist induces a transient rise in gonadotropins, known as a flare, as GnRH receptors (GnRH-R) are initially bound and activated. This is followed by a state of pituitary desensitization, resulting in a decrease in GnRH receptors and a diminished response to GnRH stimulation (1, 16–19). In contrast, GnRH antagonists reduce gonadotropin levels without producing an initial flare, a characteristic of benefit in many therapeutic applications (20, 21).

The rat has often been used as a model to dissect in vivo responses to GnRH agonists and GnRH antagonists at the level of the pituitary (19, 22–24). Some studies have reported that GnRH antagonists competitively inhibit GnRH-R without reducing the GnRH message (24–26). More recent studies have shown marked decreases in GnRH-R messenger RNA (mRNA) and GnRH-R after treatment with a GnRH antagonist (19, 22, 27). In vivo studies clearly show that the antagonistic effects of GnRH-antagonist may be overcome by a GnRH agonist, leading to a supraphysiologic surge of gonadotropins (28, 29), but the mechanism responsible for the supraphysiologic LH surge remains unclear.

Our study explored the mechanism responsible for the LH surge induced by a GnRH agonist in ovariectomized (OVX) female rats pretreated with a GnRH antagonist. The results suggest that releasable pools of gonadotropin accumulate in the pituitaries of rats treated with a GnRH antagonist and that GnRH-R levels are reduced, but the remaining receptors are sufficient to cause the LH surge on GnRH-agonist treatment.

MATERIALS AND METHODS

Animal Treatment and Collection of Tissue

All studies were conducted in accordance with U.S. federal guidelines, and the study protocol was reviewed and approved by the National Institutes of Health (NIH) Animal Care and Use Committee. Ten days after ovariectomy of 8-week-old Sprague-Dawley rats, the animals were randomized and placed into four treatment groups consisting of 10 animals each. The experimental design consisted of 4 consecutive days of treatment; the rats were killed on the morning of day 5 (Fig. 1). Treatment groups were as follows. The control group (group A) was treated with both vehicles and received a daily morning subcutaneous injection of corn oil on days 1 to 4 (Sigma Chemical Company, St. Louis, MO) and two subcutaneous injections of saline given 12 hours apart on day 4. Group B, the GnRH-agonist group, received two doses of leuprolide acetate (1.7 μg/kg) diluted in saline (TAP Pharmaceuticals, Lake Forest, IL) 12 hours apart on day 4. Group C, the GnRH-antagonist group, received a daily morning subcutaneous injection of Nal-Lys Antide (3 mg/kg) dissolved in corn oil (Organon USA, Roseland, NJ) days 1 to 4. Group group D, the combined treatment group, received four consecutive daily morning injections of GnRH antagonist (Antide) and two doses of GnRH agonist (leuprolide acetate) given 12 hours apart on day 4 at the same doses as in groups B and C. All animals were killed by CO_2 asphyxiation on day 5, 12 hours after the last GnRH-agonist or saline treatment.

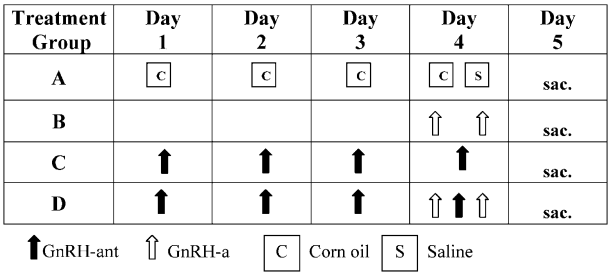
The pituitaries of four to five animals from each group were fixed in 10% buffered formalin for 24 hours, paraffin embedded, sectioned to a thickness of 7 μm, and placed on silanized slides for immunohistochemical studies of LH and follicle-stimulating hormone (FSH) protein expression or for in situ hybridization of GnRH-R expression. The remaining pituitaries from each group were snap frozen in liquid nitrogen and stored at –70°C until processed either for RNA isolation to analyze steady-state levels of mRNA for LH, FSH, and GnRH-R by Northern hybridization or for cell membrane isolation for hormone-binding studies to determine free GnRH-R–binding sites.

RNA Isolation, Northern and in situ Hybridizations

Total RNA was isolated from pituitaries by homogenization of frozen tissue in Tri-Reagent (Sigma Chemical) using a Polytron (Brinkman Instruments, Westbury, NY) and following the manufacturer's specifications. The RNA samples were suspended in water treated with diethylpyrocarbonate (DEPC), and the RNA concentration was determined by absorbance at 260 nm. The RNA was fractionated by electrophoresis in a formaldehyde 1% agarose gel, stained with ethidium bromide, transferred to a nylon membrane, and baked under vacuum at 80°C for 1 hour. The blots were used to evaluate the steady-state levels of mRNA for the beta subunits of LH and FSH and GnRH-R. This was done by hybridization of the blots at 60°C overnight in 0.4 M sodium phosphate, pH 7.2, 7% SDS, 1% bovine serum albumin (BSA), and 0.02 M ethylenediaminetetraacetic acid (EDTA) with ^{32}P-labeled probes generated by random priming of purified complementary DNA (cDNA) fragments specific for the beta subunits of rat LH, 350-bp fragment in pGEM-2 (30) and FSH, 1.0-kb fragment in pGEM-2 (31), and the rat GnRH-R, nucleotides 1–360 (accession #X76635.Gb-Ro). A fragment of the rat GnRH-R cDNA was cloned in our laboratory by polymerase chain reaction techniques into the

FIGURE 1

Diagram of the experimental protocol. Eight-week-old ovariectomized Sprague-Dawley rats were randomized to four treatment groups. Control group A was treated with both vehicles and received a daily morning subcutaneous injection of corn oil on days 1 to 4 and two subcutaneous injections of saline given 12 hours apart on day 4. Group B received two doses of a GnRH agonist, leuprolide acetate (1.7 μg/kg), diluted in saline 12 hours apart on day 4. Group C received subcutaneous injections of 3 mg/kg of a GnRH-antagonist (Nal-Lys; Antide) dissolved in corn oil daily on days 1 to 4. Group D received daily morning injections of a GnRH antagonist on days 1 to 4 and two doses of GnRH agonist on day 4 given 12 hours apart. All animals were killed on the fifth day, 12 hours after the second dose of leuprolide acetate. Black up-arrows indicate the GnRH antagonist; white up-arrows indicate the GnRH agonist; and boxes represent the vehicles, corn oil (C) and saline (S).



Taylor. Supraphysiologic LH surge. Fertil Steril 2010.

BamHI and Xb1 sites of pGEM4Z (Promega, Madison, WI), based on the sequences provided by Kaiser et al. (32).

After hybridization, the blots were washed sequentially three times for 15 minutes each in 2XSSC-0.1% SDS then three times for 15 minutes each in 0.2XSSC-0.1% SDS at 60°C and exposed to x-ray film. The relative optical density of the products was measured using a PDI J densitometer and software (Huntington Station, NY) and normalized to the ribosomal RNAs.

For in situ hybridization, dewaxed slides were pretreated with 0.2 N HCL for 30 minutes at room temperature, digested with 10 μg/mL proteinase-K in 0.1 M Tris-HCL (pH 7.4) and 0.05 M EDTA for 15 minutes at 37°C, and then treated with 0.1 M triethanolamine 0.25% acetic anhydride for 5 minutes and 0.1 M Tris-glycine (pH 7.4) for 30 minutes at room temperature. The sections were prehybridized at 50°C for 1 hour in 2XSSC, 10 mMDTT, 5X Denhardt's solution, 100 μg/mL of both salmon sperm DNA and yeast tRNA, and 50% formamide. The slides were hybridized overnight at 50°C in the same medium with 10% dextran sulfate and digoxigenin-labeled sense or antisense riboprobes to rat GnRH-R prepared by transcription with SP6 or T7 RNA polymerases from the appropriate linearized GnRH cDNA plasmid (Genius 4 kit; Boehringer-Mannheim, Indianapolis, IN).

After hybridization, the slides were washed twice in 1XSSC for 10 minutes, digested with RNases (2.8 μg/mL RNase-A and 0.3 μg/mL RNase-T1 in 10 mM Tris-HCL, pH 7.4), washed twice more in 1XSSC at 55°C for 20 minutes, and then twice in 0.1XSSC at 55°C for 30 minutes. The sections were equilibrated in Tris-buffered saline (TBS) with Tween-20 (TBST: 0.01 M Tris-HCL, pH 7.6, 0.9% NaCL, 0.05% Tween-20) and incubated with antidigoxigenin alkaline-phosphatase antibody (Boehringer-Mannheim) according to the manufacturer's directions, washed with TBST, and visualized using BCIP/NBT as a substrate.

Immunohistochemistry and Quantitation

Detection of FSH and LH was performed with guinea pig antibodies (1:250–1:500 dilution) specific to their respective beta subunits obtained from the National Pituitary Hormones and Antisera Center (Torrance, CA). In addition, a commercially available rabbit antibody against FSH-β subunit was used at a 1:50 dilution (Biomeda, Foster City, CA). Unless otherwise stated, all incubations were performed at room temperature.

Before immunostaining, the deparaffinized tissue sections were subjected to antigen retrieval using a pressure cooker in a 0.01 M citrate buffer, pH 6.0, followed by incubation in 3% H_2O_2 in methanol for 10 minutes to block endogenous peroxidase activity. The slides were then washed thoroughly and equilibrated in TBS with Tween-20 (TBST: 0.01 M Tris-HCL, pH 7.6, 0.9% NaCL, 0.05% Tween-20). Nonspecific sites were blocked by incubation for 20 minutes with 1% normal serum from a Vectastain ABC elite kit for guinea pig primary antisera (Vector Laboratories, Burlingame, CA) diluted in TBST and were incubated overnight at 4°C with the primary antiserum (1:100 dilution). The slides were washed in TBST twice for 10 minutes each and then incubated with the appropriate biotinylated secondary antibody and peroxidase-conjugated streptavidin, as suggested by the manufacturer (Vectastain ABC Elite kit; Vector Laboratories). The FSH and LH were immunolocalized using 2,2-diaminobenzidine as a substrate.

To ensure consistency in immunohistochemical detection of the gonadotropins, the specimens were cut on the same day, placed on the same lot of silanized slides, and stained simultaneously in one experimental run. The entire staining procedure was repeated at least two times using the same or different antisera when available. Background staining was estimated by substituting nonimmune serum for the primary antisera where the staining intensity was found to be negligible (data not shown).

The relative intensity of the immunoreaction was estimated by measurement of the diaminobenzidine peroxidase product in pituitaries from the various treatment groups using light microscopic image analysis consisting of digital Pro-ImageJ software (Media Cybernetics, Silver Spring, MD), a color CD-camera, and an Olympus microscope (Opelco, Sterling

VA). The system was first set to measure linear optical density with the larger values corresponding to more intense staining followed by calibration so that immunoreactive cells in all samples would fall within the linear range. At least four random areas were selected from each pituitary (four to six pituitaries per group) using ×400 magnification. All immunostained gonadotropes within the field were manually traced to obtain the relative area and optical density values. Greater than 300 cells were traced and quantified for each treatment group. The mean and standard error were determined for the gonadotrope cell area and relative optical density of staining for either FSH or LH.

Statistical significance between two groups for parametric data was assessed by the Student's *t* test, and multiple group comparisons were analyzed using one-way analysis of variance, with Duncan's multiple range test applied to determine statistically significant differences between groups. Results are expressed as the mean ± standard deviation (SD), and $P<.05$ was considered statistically significant.

GnRH-Receptor–Binding Assays

Four to six frozen pituitaries from each treatment group were thawed and homogenized in assay buffer (0.025 M Tris-HCL, pH 7.4, 0.01 M $MgCl_2$, and 0.1% BSA) using 1 mL of buffer per pituitary. One hundred microliters of cell homogenate were used for each binding point according to the method of Nett et al. (33), in the presence of [^{125}I]-buserelin (Hoescht-Roussel Pharmaceuticals, Frankfurt, Germany). Nonspecific binding was assessed in the presence of 5 μg/tube of unlabeled GnRH analog. Measurements were repeated three times, and the coefficient of variance was calculated. Results are expressed as the mean ± SD, and $P<.05$ was considered statistically significant.

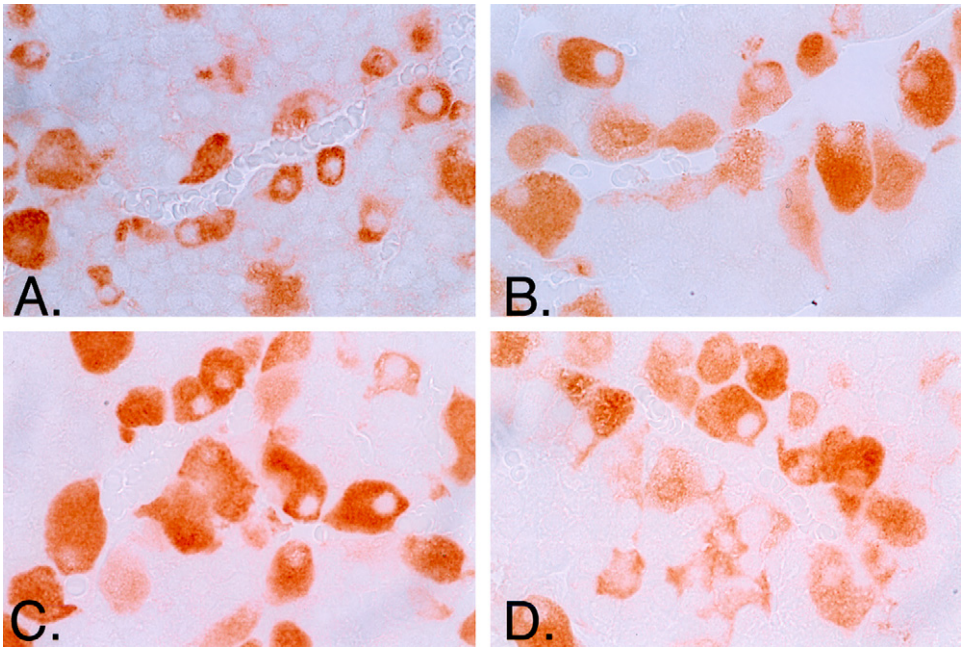
RESULTS

The response of pituitary gonadotropes to GnRH agonist and GnRH antagonist was examined in 40 sexually mature female rats 10 days after ovariectomy (see Fig. 1). The OVX rats were divided into four groups. Group D was intended to mimic the clinical use of GnRH agonist in women primed with GnRH antagonist. The control group (group A) only received vehicle injections of corn oil and saline. Group B was treated with two doses of GnRH agonist, leuprolide acetate (1.7 μg/kg) given 12 hours apart on day 4. Group C received 3 mg/kg of the GnRH antagonist (Nal-Lys; Antide) daily for 4 consecutive days. Group D was given 3 mg/kg of GnRH antagonist (Nal-Lys; Antide) daily for 4 days and two doses GnRH agonist, leuprolide acetate (1.7 μg/kg) on day 4. All animals were killed on the fifth day, 12 hours after the second dose of the GnRH agonist.

Immunohistochemical staining was performed on four to six pituitaries from each treatment group to analyze intracellular pools of gonadotropin. A representative staining of pituitary sections (Fig. 2) from the four different treatment groups

FIGURE 2

Immunohistochemical staining for gonadotropins. Pituitary sections stained for FSH-β from ovariectomized rats after (**A**) no treatment and treatment with (**B**) GnRH agonist alone, (**C**) GnRH antagonist alone, or (**D**) both. In all samples antibody–antigen interaction was visualized as brown staining. Staining for FSH-β was visibly increased after treatment with GnRH antagonist alone. (*A–D:* original magnification, ×400.)



Taylor. Supraphysiologic LH surge. Fertil Steril 2010.

demonstrated increased FSH-β staining in animals treated with a GnRH antagonist (group C and D). Pituitary sections stained for LH-β revealed similar findings in the GnRH-antagonist treatment animals (not shown). The intensity of the staining for LH-β and FSH-β was quantified using a computer-based density quantification software program (Image Pro Plus). Quantitative analysis showed that the animals treated with GnRH antagonist alone had a statistically significant increase in staining for both LH-β and FSH-β (Fig. 3).

To determine whether the increased pools of FSH and LH were associated with increased transcription, steady-state levels of RNA were harvested from the pituitaries of all treatment groups and probed with cDNA corresponding to either FSH-β or LH-β. We observed no change in the steady-state levels of transcripts encoding either FSH-β or LH-β in any of the treatment groups (Fig. 4, left panel). This finding, coupled with the immunohistochemical staining of the pituitaries, suggests that the increase in FSH and LH protein in the pituitary resulted from an increase in the storage of gonadotropin and not increased transcription.

Next, we examined GnRH-R mRNA in all treatment groups. Despite equal loading, GnRH-R transcripts were reduced in the animals treated with a GnRH agonist; however, no change was observed in the GnRH-antagonist-only or combined treatment groups (see Fig. 4, right panel). To corroborate these findings, in situ hybridization for GnRH-R was performed on pituitaries from all treatment groups (Fig. 5). Consistent with the Northern blot analysis, no change in GnRH-R mRNA was apparent in pituitaries harvested from the two GnRH-antagonist treatment groups (see Fig. 5C–D); however, a reduction in signal was noted in the GnRH-agonist treatment group (see Fig. 5B). As expected, staining was localized to gonadotropes and was most pronounced in the combination group (see Fig. 5D). As a control, sense riboprobe showed no staining (see Fig. 5E). These observations indicate that steady-state levels of mRNA encoding the GnRH-R were not reduced in rats treated with the GnRH antagonist, in contrast to rats treated with GnRH agonist alone.

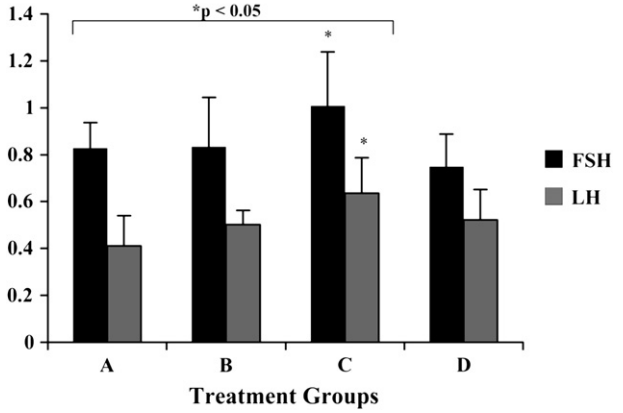
To determine whether there was a change in the level of GnRH-R protein in the pituitaries among the four treatment groups, we used a receptor-binding protein assay. Pituitary homogenates were prepared from each of the four treatment groups, and receptor levels were assessed by the binding of [I^{125}]-labeled buserelin (33). The level of GnRH-R (fentamole/gram) was increased after treatment with GnRH-agonist, but was reduced in pituitaries harvested from animals that received the GnRH antagonist (Fig. 6). In pituitaries harvested from rats treated with both GnRH analogs, receptor levels were decreased compared with the control although not to the extent of those treated with GnRH antagonist alone.

DISCUSSION

We were unable to identify previous reports in the literature that described the mechanism responsible for the

FIGURE 3

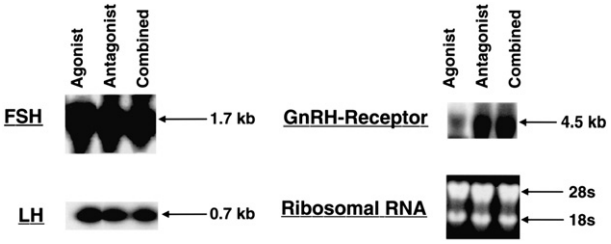
Quantification of immunohistochemical staining for FSH and LH using computer-based density quantification software. The relative intensity of the immunoreaction was estimated by measurement of the diaminobenzidine peroxidase product from pituitaries of all treatment groups using light microscopic image analysis. Groups demonstrated statistically significantly increased pituitary pools of FSH-β and LH-β in rats treated with GnRH antagonist alone compared with control ($P<.05$). Dark bars represent FSH. Light bars represent LH. Y-axis = optical density units. Error bars = standard deviation. $P<.05$ was considered statistically significant compared with the control group (all asterisked values).



Taylor. Supraphysiologic LH surge. Fertil Steril 2010.

FIGURE 4

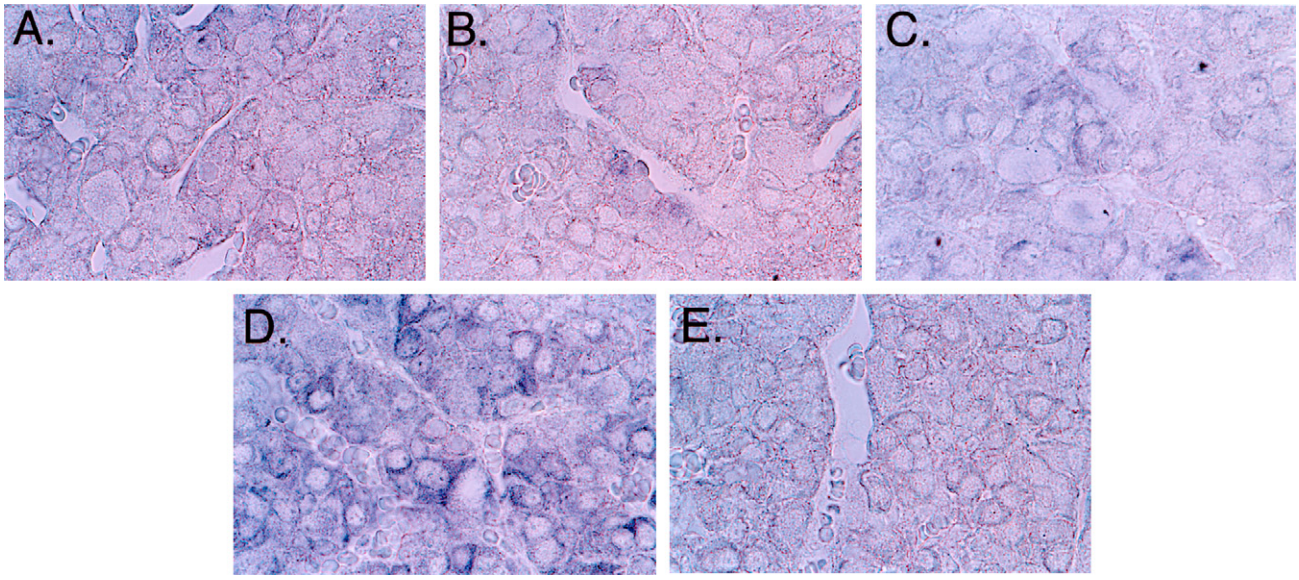
Northern blot analysis. RNA harvested from ovariectomized rats after treatment with GnRH agonist (group B), GnRH antagonist (group C), or both (group D) were probed with cDNA. Ribosomal RNA was used as a control for loading. Levels of steady-state FSH and LH transcripts remained constant regardless of treatment group. Steady-state level GnRH-R transcripts were reduced after GnRH-agonist treatment (group B).



Taylor. Supraphysiologic LH surge. Fertil Steril 2010.

FIGURE 5

In situ hybridization of pituitaries from treatment groups (A–D) using a riboprobe generated from a cDNA encoding GnRH-R. Compared with (**A**) the control group, GnRH-R transcript levels showed a reduction in staining in (**B**) the GnRH-agonist treatment group. No reduction was observed in (**C**) the GnRH-antagonist treatment group or (**D**) the combination group. (**E**) Sense riboprobe served as control.



Taylor. Supraphysiologic LH surge. Fertil Steril 2010.

supraphysiologic LH surge induced by GnRH agonists in females primed with a GnRH antagonist. We found increased staining for LH-β and FSH-β in pituitaries of rats during GnRH-antagonist treatment, accompanied by reduced but functional GnRH-R that could be activated to effect release of gonadotropin pools upon GnRH-agonist treatment. We confirmed depletion of gonadotropin pools in the pituitary (i.e., release) by immunohistochemistry (see Figs. 2 and 3).

Studies conducted in a primate model have demonstrated that GnRH agonists cause a supraphysiologic release of gonadotropins in animals pretreated with GnRH antagonist (34). Subsequently, this strategy has been used in controlled ovarian hyperstimulation (COH) with a single dose of GnRH agonist to trigger ovulation and induce final oocyte maturation (7, 10, 11, 13, 14, 34–36). This strategy is of clinical relevance because it may reduce the risk of OHSS (13, 14).

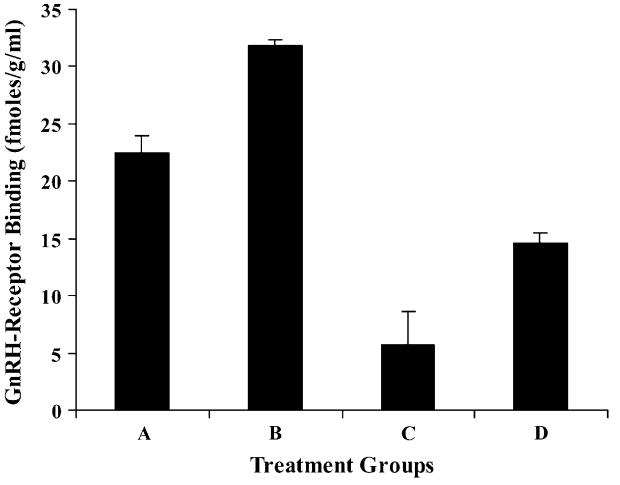
Previous studies have focused on the mechanism of action of individual gonadotrope analogues upon the pituitary. The results we obtained in the GnRH–antagonist-only treatment group (group C) were consistent with prior reports (23, 27). These reports also showed the level of GnRH-R protein decreased while GnRH-R mRNA remained stable after the GnRH antagonist (23, 27). Additional studies extended these findings and showed that a single injection of GnRH antagonist significantly reduced the number of membrane receptors for LH–RH (GnRH-R) in a time-dependent manner after 10 days of treatment (19) and 30 days after depot injection (37). Notably, an earlier study showed that the inhibitory effects of GnRH-antagonist resulted from reduced occupancy of the GnRH-R binding sites as well as sustained reduction in membrane receptors (38).

In contrast, animals treated exclusively with the GnRH agonist (group B) demonstrated a marked decrease in GnRH-R mRNA and an increase in receptor protein compared to the control 12 hours after treatment (see Fig. 6). The increased GnRH-R protein likely results from an initial flare response to the GnRH agonist because the pituitaries were collected within 12 hours of treatment. In support of our findings, Horvath et al. (27) showed a decrease in GnRH-R mRNA after 10 days of a microcapsule GnRH-agonist treatment and an increase in receptor protein after 10 days of daily injection. Murase et al. (39) reported similar results with two other GnRH agonists at multiple time points. Studies have reported that GnRH agonists cause a rapid decrease in GnRH-R mRNA in the rat pituitary as early as 2 hours after treatment and a decrease in LH mRNA after 48 hours and FSH mRNA after 3 hours (22, 39). In our current study, the two-dose regimen of GnRH-agonist (groups B and D) was sufficient to reduce the levels of GnRH-R mRNA, but not FSH and LH mRNA, a result likely explained by the termination of our study 12 hours after GnRH agonist administration.

Increased intracellular staining for LH and FSH after treatment with an GnRH antagonist, without appreciable alterations in mRNA concentration, suggests that GnRH antagonists stimulate intracellular storage of FSH and LH within pituitary gonadotropes. Given the short delay between

FIGURE 6

Levels of GnRH-R protein in pituitary homogenates from treatment groups A through D. The level of GnRH-R increased after treatment with GnRH agonist (group B) and decreased in both the GnRH-antagonist and combination groups (group C and D). Y-axis = GnRH-R binding in fentamolar/gram of pituitary lysate. Error bars = standard deviation. $P<.05$ was considered statistically significant for all treatment groups compared with the control.



Taylor. Supraphysiologic LH surge. Fertil Steril 2010.

agonist treatment and necropsy (12 hours), we interpret the finding of decreased intracellular pools of gonadotropins in the combined treatment group (group D; see Figs. 2 and 3) to indicate that gonadotropes have released storage pools of gonadotropin in response to the GnRH-agonist treatment. These results support the exaggerated release of gonadotropins caused by the GnRH agonist in females primed with a GnRH antagonist. This observation is consistent with preservation of intact signaling despite reduced GnRH receptors and indicates receptor function.

One limitation of the current study is that experiments were performed on castrated sexually mature female rats 10 days after ovariectomy to eliminate estrogen's effect upon control of gonadotropin in vivo, at the level of the pituitary. Pituitary secretion of gonadotropins has been shown to vary depending on the length of time after oophorectomy in the rat and other species (4, 40). Our results in OVX female rats may not be applicable to intact rats because there is a rise in follistatin and follistatin mRNA after gonadectomy (41–43). Despite limitations inherent in the model, our approach mirrors that of previous studies in which OVX rats were used for the expected higher levels of GnRH in the pituitary (19, 23).

These findings suggest the mechanism of supraphysiologic release of gonadotropin in GnRH-antagonist primed female is that some GnRH receptors, albeit reduced in amount, remain active and responsive to pharmacologic doses of GnRH agonist, which in turn stimulate the release of large storage of pools of gonadotropins that accumulate during GnRH-antagonist treatment.

Acknowledgments: The authors thank Dr. Terry Nett of Colorado State University for the GnRH-R analogs and Drs. Gharib and Chin for providing the cDNA clones for beta-subunits of rat FSH and LH. Drs. Prabir Chakraborty and Anitha Nair provided essential assistance and expertise. Maggie Cisar provided technical assistance and support and performed several of the experiments. The authors also thank Drs. Barbara Stegmann and Alan DeCherney for statistical support and guidance, respectively.

REFERENCES

1. Schally AV. Luteinizing hormone-releasing hormone analogs: their impact on the control of tumorigenesis. Peptides 1999;20:1247–62.
2. Filicori M. Gonadotropin-releasing hormone agonists. A guide to use and selection. Drugs 1994;48:41–58.
3. Olivennes F. The use of gonadotropin-releasing hormone antagonists in ovarian stimulation. Clin Obstet Gynecol 2006;49:12–22.
4. Illions EH, Scott RT, Carey KD, Navot D. Evaluation of the impact of concurrent gonadotropin-releasing hormone (GnRH) antagonist administration on GnRH agonist-induced gonadotrope desensitization. Fert Steril 1995;64:848–54.
5. Olivennes F, Fanchin R, Bouchard P, Taieb J, Frydman R. Triggering of ovulation by a gonadotropin-releasing hormone (GnRH) agonist in patients pretreated with a GnRH antagonist. Fertil Steril 1996;66:151–3.
6. Itskovitz-Eldor J, Kol S, Mannaerts B. Use of a single bolus of GnRH agonist triptorelin to trigger ovulation after GnRH antagonist ganirelix treatment in women undergoing ovarian stimulation for assisted reproduction, with special reference to the prevention of ovarian hyperstimulation syndrome: preliminary report: short communication. Hum Reprod 2000;15:1965–8.
7. Fauser BC, De Jong D, Olivennes F, Wramsby H, Tay C, Itskovitz-Eldor J, et al. Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for in vitro fertilization. J Clin Endocrinol Metab 2002;87:709–15.
8. Orvieto R, Zagatsky I, Yulzari-Roll V, La Marca A, Fisch B. Substituting human chorionic gonadotropin by gonadotropin-releasing hormone agonist to trigger final follicular maturation, during controlled ovarian hyperstimulation, results in less inflammation. Gynecol Endocrinol 2006;22:437–40.
9. Reissmann T, Felberbaum R, Diedrich K, Engel J, Comaru-Schally AM, Schally AV. Development and applications of luteinizing hormone-releasing hormone antagonists in the treatment of infertility: an overview. Hum Reprod 1995;10:1974–81.
10. Humaidan P, Bredkjaer HE, Bungum L, Bungum M, Grondahl ML, Westergaard L, et al. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: a prospective randomized study. Hum Reprod 2005;20:1213–20.
11. Kolibianakis EM, Schultz-Mosgau A, Schroer A, van Steirteghem A, Devroey P, Diedrich K, et al. A lower ongoing pregnancy rate can be expected when GnRH agonist is used for triggering final oocyte maturation instead of hCG in patients undergoing IVF with GnRH antagonists. Hum Reprod 2005;20:2887–92.
12. Griesinger G, von Otte S, Schroer A, Ludwig AK, Diedrich K, Al-Hasani S, et al. Elective cryopreservation of all pronuclear oocytes after GnRH agonist triggering of final oocyte maturation in patients at risk of developing OHSS: a prospective, observational proof-of-concept study. Hum Reprod 2007;22:1348–52.
13. Engmann L, DiLuigi A, Schmidt D, Nulsen J, Maier D, Benadiva C. The use of gonadotropin-releasing hormone (GnRH) agonist to induce oocyte maturation after cotreatment with GnRH antagonist in high-risk patients undergoing in vitro fertilization prevents the risk of ovarian

hyperstimulation syndrome: a prospective randomized controlled study. Fertil Steril 2008;89:84–91.
14. Griesinger G, Diedrich K, Devroey P, Kolibianakis EM. GnRH agonist for triggering final oocyte maturation in the GnRH antagonist ovarian hyperstimulation protocol: a systematic review and meta analysis. Hum Reprod 2006;12:159–68.
15. Beckers NG, Macklon NS, Eijkemans MJ, Ludwig M, Felberbaum RE, Diedrich K, et al. Nonsupplemented luteal phase characteristics after the administration of recombinant human chorionic gonadotropin, recombinant luteinizing hormone, or gonadotropin-releasing hormone (GnRH) agonist to induce final oocyte maturation in in vitro fertilization patients after ovarian stimulation with recombinant follicle-stimulating hormone and GnRH antagonist cotreatment. J Clin Endocrinol Metab 2003;88: 4186–92.
16. Conn PM, Crowley WF Jr. Gonadotropin-releasing hormone and its analogues. N Engl J Med 1991;325:93–103.
17. Mason DR, Arora KK, Mertz LM, Catt KJ. Homologous down-regulation of gonadotropin-releasing hormone receptor sites and messenger ribonucleic acid transcripts in alpha T3-1 cells. Endocrinology 1994;135: 1165–70.
18. Scheele F, Hompes PG, Lambalk CB, Schoute E, Broekmans FJ, Schoemaker J. The GnRH challenge test: a quantitative measure of pituitary desensitization during GnRH agonist administration. Clin Endocrinol 1996;44:581–6.
19. Halmos G, Schally A. Changes in subcellular distribution of pituitary receptors for luteinizing hormone-releasing hormone (LH-RH) after treatment with the LH-RH antagonist cetrorelix. Proc Natl Acad Sci USA 2002;99:961–5.
20. Huirne JA, Lambalk CB. Gonadotropin-releasing-hormone antagonists. Lancet 2001;358:1793–803.
21. Tarlatzis BC, Fauser BC, Kolibianakis EM, Diedrich K, Devroey P. GnRH antagonists in ovarian stimulation for IVF. Hum Reprod 2006;12:333–40.
22. Lerrant Y, Kottler ML, Bergametti F, Moumni M, Blumberg-Tick J, Counis R. Expression of gonadotropin-releasing hormone (GnRH) receptor gene is altered by GnRH agonist desensitization in a manner similar to that of gonadotropin beta-subunit genes in normal and castrated rat pituitary. Endocrinology 1995;136:2803–8.
23. Kovacs M, Schally AV. Comparison of mechanisms of action of luteinizing hormone-releasing (LHRH) antagonist cetrorelix and LHRH agonist triptorelin on the gene expression of pituitary LHRH receptors in rats. Proc Natl Acad Sci USA 2001;98:12197–202.
24. Roth C, Hegemann F, Hildebrandt J, Balzer I, Witt A, Wuttke W, et al. Pituitary and gonadal effects of GnRH (gonadotropin releasing hormone) analogs in two peripubertal female rat models. Pediatr Res 2004;55: 126–33.
25. Stojilkovic SS, Reinhart J, Catt KJ. Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. Endocr Rev 1994;15:462–99.
26. Kaiser UB, Conn PM, Chin WW. Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. Endocr Rev 1997;18:46–70.
27. Horvath JE, Bajo AM, Schally AV, Kovacs M, Herbert F, Groot K. Effects of long-term treatment with the luteinizing hormone-releasing hormone (LHRH) agonist Decapeptyl and the LHRH antagonist Cetrorelix on the levels of pituitary LHRH receptors and their mRNA expression in rats. Proc Natl Acad Sci USA 2002;99:15048–53.
28. Leal JA, Gordon K, Williams RF, Danforth DR, Roh SI, Hodgen GD. Probing studies on multiple dose effects of antide (NAL-LYS) GnRH antagonist in ovariectomized monkeys. Contraception 1989;40:623–33.
29. Scott RT, Illions EH, Carey KD, Navot D. Gonadotropin-releasing hormone antagonist administration enhances gonadotrope responsiveness at doses inadequate to suppress immunoassayable gonadotropin levels. Fertil Steril 1994;64:1069–71.
30. Chin WW, Godine JE, Klein DR, Chang AS, Tan LK, Habener JF. Nucleotide sequence of the cDNA encoding the precursor of the beta-subunit of rat lutropin. Proc Natl Acad Sci USA 1983;80:4649–53.
31. Gharib SD, Roy A, Wierman ME, Chin WW. Isolation and characterization of the gene encoding the b-subunit of rat follicle-stimulating hormone. DNA 1989;8:339–49.
32. Kaiser UB, Zhao D, Cardona GR, Chin WW. Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. Biochem Biophys Res Commun 1992;189:1645–52.
33. Nett TM, Crowder ME, Moss GE, Duello TM. GnRH-receptor interaction. V. Down-regulation of pituitary receptors for GnRH in ovariectomized ewes by infusion of homologous hormone. Biol Reprod 1981;24:1145–55.
34. Ron-El R, Raziel A, Schachter M, Strassburger D, Kasterstein E, Friedler S. Induction of ovulation after GnRH antagonists. Hum Reprod Update 2000;6:318–21.
35. Kol S, Itskovitz-Eldor J. Severe OHSS: yes, there is a strategy to prevent it!. Hum Reprod 2000;15:2266–7.
36. de Jong D, Van Hooren EG, Macklon NS, Mannaerts BM, Fauser BC. Pregnancy and birth after GnRH agonist for induction of final oocyte maturation in a woman undergoing ovarian stimulation for ICSI, using a GnRH antagonist (Orgalutran/Antagon) to prevent a premature LH surge: a case report. J Assist Reprod Genet 2001;18:30–3.
37. Horvath JE, Toller GL, Schally AV, Bajo AM, Groot K. Effects of long-term treatment with low doses of the LHRH antagonist Cetrorelix on pituitary receptors for LHRH and gonadal axis in male and female rats. Proc Natl Acad Sci USA 2004;101:4996–5001.
38. Halmos G, Schally AV, Pinski J, Vadillo-Buenfil M, Groot K. Down-regulation of pituitary receptors for luteinizing hormone-releasing hormone (LH-RH) in rats for LH-RH antagonist cetrorelix. Proc Natl Acad Sci USA 1996;93:2398–402.
39. Murase M, Uemura T, Gao M, Inada M, Funabash T, Hirahara F. GnRH antagonist-induced down-regulation of the mRNA expression of pituitary receptors: comparisons with GnRH agonist effects. Endocr J 2005;52:131–7.
40. Roth C, Schricker M, Lakomek M, Strege A, Heiden I, Luft H, et al. Autoregulation of the gonadotropin-releasing hormone (GnRH) system during puberty: effects of antagonistic versus agonistic GnRH analogs in a female rat model. J Endocrinol 2001;169:361–71.
41. Kaiser UB, Jakubowiak A, Steinberger A, Chin WW. Regulation of rat pituitary gonadotropin-releasing hormone receptor mRNA levels in vivo and in vitro. Endocrinology 1993;133:931–4.
42. Depaolo LV, Mercado M, Guo Y, Ling N. Increased follistatin (activin-binding protein) gene expression in rat anterior pituitary tissue after ovariectomy may be mediated by pituitary activin. Endocrinology 1993;132:2221–7.
43. Kirk SE, Dalkin AC, Yasin M, Haisenleder DJ, Marshall JC. Gonadotropin-releasing hormone pulse frequency regulates expression of pituitary follistatin messenger ribonucleic acid: a mechanism for differential gonadotrope function. Endocrinology 1994;135:876–80.