Naturally Occurring Antihormones: Secretion of FSH Antagonists by Women Treated with a GnRH Analog

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Follicle-stimulating hormone (FSH) is a glycoprotein essential for gonadal development and steroidogenesis. Recent studies suggest that deglycosylation of FSH results in the formation of antagonistic proteins that are capable of binding to gonadal receptors but that are devoid of bioactivity. Treatment of hypogonadal women with an antagonist of gonadotropin-releasing hormone substantially decreased serum FSH bioactivity with minimal changes in immunoreactivity. Chromatofocusing and size fractionation of the serum samples indicated the secretion of immunoreactive FSH isoforms that are devoid of bioactivity but that are capable of blocking FSH action in ovarian granulosa cells. These findings provide the first demonstration of naturally occurring circulating antihormones. These FSH antagonists may play an important role in the physiology and pathophysiology of the gonads.

GONADOTROPIN-RELEASING HORMONE (GnRH) is secreted in a pulsatile manner by the hypothalamus to activate and maintain gonadotropin synthesis, glycosylation, and secretion (*1*). The gonadotropins, in turn, regulate gametogenesis and gonadal steroidogenesis (*2*). Gonadotropins [follicle-stimulating hormone, luteinizing hormone, and chorionic gonadotropin] and thyroid-stimulating hormone (TSH) are protein dimers, consisting of noncovalently linked alpha and beta subunits. All of these glycoproteins have the same alpha subunit in combination with a hormone-specific beta subunit (*3*). Studies have shown that the anterior pituitary contains a family of FSH isohormones with different molecular weights (M_r), isoelectric properties, bioactivities, and circulating half-lives (*4*); microheterogeneity of carbohydrate moieties is believed to be responsible for these isoforms. The lack of a sensitive FSH bioassay prevented the evaluation of the bioactivity of circulating FSH isoforms. However, deglycosylation of purified pituitary glycoprotein hormones by chemical or enzymatic means generates antagonists that bind to receptors but block the action of native hormones on steroidogenesis (*5*).

In vivo administration of GnRH antagonists suppresses gonadotropin levels and gonadal function (*6*) and can provide the basis for designing contraceptive compounds. We have developed a sensitive in vitro bioassay for FSH (*7*) based on the FSH stimulation of aromatase activity by cultured ovarian granulosa cells and have demonstrated that the ratio of bioactive FSH to immunoreactive FSH (the B/I ratio) dramatically decreases after GnRH antagonist administration to men and women in different physiological states (*8*). In the present study, we used chromatographic techniques to isolate FSH isoforms from these serum samples. Using the in vitro bioassay, we showed that treatment of hypogonadal women with a GnRH antagonist causes the secretion of FSH isoforms with antagonistic properties. The antagonistic effect of the FSH isoforms was blocked by antibodies specific for human FSH.

Samples were obtained from hypogonadal women before and 8 hours after infusion of a GnRH antagonist (Ac-Δ^3-Pro^1,*p*-F-D-Phe^2,D-Trp^{3,6}-GnRH; 30 μg/kg per hour). A pronounced decrease (62 ± 3%; $n = 4$) in FSH bioactivity was detected in serum of GnRH antagonist-treated patients as compared to pretreatment levels (*8*). In contrast, only a minimal (30 ± 3%) decrease in immunoreactivity was found, with a decrease in the B/I ratio from 1.8 to 1.0.

Because gonadotropin isoforms can be separated on the basis of their isoelectric properties, serum samples were chromatographed on a PBE-94 column with a *p*H gradient ranging from 7 to 4 (*9*) (Fig. 1, inset). Each fraction was analyzed both by FSH radioimmunoassay (RIA) (Fig. 1A) and the granulosa cell FSH bioassay (Fig. 1B). Four major isohormone peaks were distinguished with apparent *p*I > 7.0 (peak I), 6.6 to 6.2 (peak II), 6.0 to 5.2 (peak III), and 4.9 to 4.0 (peak IV). For serum samples obtained both before and after GnRH antagonist treatment, peak IV contained most of the FSH isoforms (70 to 80%) as judged from both assays. After GnRH antagonist treatment, a substantial increase in the percentage of the most basic peak (peak I) was apparent and was accompanied by decreases in the other peaks. The B/I ratios were 0.1, 8, 57, and 2 for peaks I, II, III, and IV, respectively, indicating that peak I was the least bioactive. The low B/I ratio in peak I suggests that these isoforms may be a mixture of FSH molecules with both agonist and antagonist activities. Studies of purified pituitary FSH preparations demonstrated similar FSH microheterogeneity profiles based on RIA analysis (*10*).

To estimate the M_r of the newly formed FSH isoforms at *p*H >7, we pooled peak I fractions (Fig. 1) from serum samples of GnRH antagonist-treated patients and added them to a gel filtration column (Ultrogel AcA 54). On this column, iodinated human pituitary FSH eluted as a single peak at an M_r of 38,000, in agreement with prior estimates (*11*). The applied sample displayed a wide range of M_r from 45,000 to 25,000 as assessed by RIA and the in vitro bioassay. However, the B/I ratio of each fraction varied. Fractions with M_r and B/I ratios lower than intact FSH were pooled and run on a chromatofocusing column (PBE-118) with a *p*H gradient from 11 to 7 (*12*). The resulting fractions were again analyzed by RIA and the bioassay (Fig. 2A). Fractions from *p*H 9.6 to 9.4 contained most of the FSH immunoreactivity, but these isoforms were not bioactive. Although the samples applied to this column contained some bioactivity, these activities were not found between *p*H 11 and 8.7 and could have

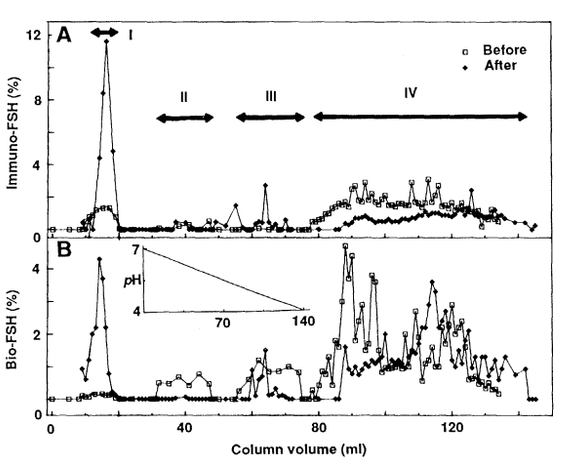

Fig. 1. Chromatofocusing analysis of serum samples from hypogonadal women before (open squares) and after (closed diamonds) GnRH antagonist infusion, with the use of a *p*H gradient of 7 to 4 (inset). Fractionated samples were assayed (**A**) by RIA, and (**B**) by a granulosa cell aromatase bioassay. Results are expressed as the percentage of total activity eluted from the column.

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eluted at lower *p*H values. The possible antagonistic effects of these FSH isoforms were investigated by adding human FSH (2 mIU/ml) along with the column fractions in the bioassay. Samples with a *p*H of 9.6 to 9.4 inhibited FSH-stimulated estrogen production (Fig. 2B, open squares), but fractions with higher or lower *p*H were ineffective. A dose-dependent inhibition of estrogen production by the *p*H 9.5 fraction, but not by the *p*H 10.1 fraction, was found (Fig. 2B, inset). Nonspecific inhibitory effects were ruled out by adding forskolin ($10^{-5}M$) along with the same fractions; forskolin bypasses the FSH receptor and directly activates adenyl cyclases to increase estrogen biosynthesis in the granulosa cells. There was no inhibition of forskolin-stimulated estrogen production by any fractions (Fig. 2B, crosses), an indication that the suppression of FSH action by these fractions probably occurs before adenyl cyclase activation.

To demonstrate further that the antagonistic effect of the samples with *p*H values between 9.6 and 9.4 is due to FSH-like molecules, we performed immunoneutralization experiments. Although treatment of cultured granulosa cells with antibodies directed against human FSH or with pooled *p*H 9.6 to 9.4 fractions (iso-FSH) alone did not increase estrogen biosynthesis, a near saturating dose (1 ng/ml) of ovine FSH did stimulate production (Fig. 3). Because the antibody to human FSH is species-specific, no significant decrease in estrogen biosynthesis was detected when the antibody was added along with ovine FSH. When iso-FSH was added with ovine FSH, there was an 86% inhibition of estrogen production. However, when the antibody to human FSH (1:1 or 1:5 dilutions) was added together with ovine FSH and the human iso-FSH, the antibody blocked the inhibitory effects of iso-FSH in a dose-dependent manner. Therefore, the FSH antagonist isoforms were preferentially bound by the antibodies, with the result that their receptor-specific inhibitory effects were blocked.

This is the first demonstration of a naturally occurring circulating antagonistic isohormone. In tissue extracts of a mouse tumor, a TSH antagonist immunologically related to TSH was demonstrated, but this isoform has not been found in the circulation (*13*). Although fractionated follicular fluid contains FSH-antagonist activities (*14*), these uncharacterized factors are probably not FSH isoforms, because no associated FSH immunoreactivity was detected.

Because of the lack of a sensitive FSH bioassay, earlier investigators used radioligand receptor assays to assess FSH bioactivity (*15*). The receptor assay, however, cannot differentiate between agonistic and antagonistic isoforms of FSH. To facilitate the separation of heterogeneous agonistic and antagonistic FSH isoforms, we used chromatofocusing techniques to separate molecules with different *p*I values and then estimated bioactivity by means of a sensitive in vitro FSH bioassay. It is clear that treatment with a GnRH antagonist causes the release of new FSH isoforms (*p*I values between 9.6 and 9.4) with potent FSH antagonistic properties. In addition, we have shown that the antagonist activities are due to immunoreactive FSH molecules, because the observed antagonistic effect is blocked by antibodies to human FSH.

Studies with gonadotropins from different species have shown variations in isohormone activities (*11, 15, 16*). Disparate bio- to immunoactivities of different acidic FSH isoforms are believed to be due to variations in sialic acid residues that would confer a longer biological half-life and higher in vivo biopotency (*3, 4, 17*). In addition, removal of carbohydrate residues (including sialic acid) from purified pituitary FSH preparations results in more basic FSH isoforms with antagonistic properties (*5*). Although we demonstrated an increase in more basic FSH isoforms and those with lower M_r after GnRH antagonist treatment, the FSH antagonists we observed may not be due only to the removal of sialic acids.

The demonstration of circulating FSH antagonist isoforms after GnRH antagonist administration provides clues to the mechanism of GnRH action. The GnRH antagonist could affect the pituitary action of GnRH on at least three levels—transcription of the alpha and FSH beta subunits, glycosylation of the FSH apoprotein cores, and secretion of FSH. The major short-term effects of the GnRH antagonist are probably not at the levels of transcription or of secretion of preformed FSH because minimal decreases in immuno-FSH were detected during the first few hours of GnRH antagonist administration (*8*). The initial suppression of FSH B/I ratios by the GnRH antagonist is probably due to changes in glycosylation, possibly by affecting the enzymes responsible for the addition of specific sugar moieties. Indeed, GnRH has been shown to stimulate the glycosylation of luteinizing hormone in rat pituitary glands (*18*).

In conclusion, these findings demonstrate a naturally occurring antihormone and provide the basis for elucidating the role of hormone antagonists in various physiological, pharmacological, and pathophysiological states. It is possible that certain patients with resistant ovary syndrome have circulating gonadotropin antagonist isoforms. Further characterization and eventual purification of these FSH isoforms may aid our understanding of the glycosylation and the action of FSH and would also provide clinical approaches for antihormone therapies. Both GnRH antagonists and the observed FSH antagonist isoforms may provide prototypes for the design of contraceptives.

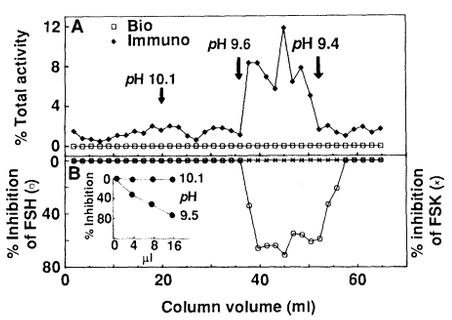

Fig. 2. Further chromatofocusing analysis of GnRH antagonist-treated patient samples with the use of a *p*H gradient of 11 to 8. FSH isoforms with M_r and B/I ratio lower than those of intact FSH from an Ultrogel AcA 54 column were applied to the chromatofocusing column. (**A**) Column fractions were assayed by RIA and by bioassay and are shown as percentages of total activity. (**B**) Antagonistic effects of FSH isoforms on FSH-stimulated (open squares) and forskolin-stimulated (crosses) estrogen production and dose-dependent inhibition of FSH-stimulated estrogen production by FSH isoforms (inset). Results were expressed as percent inhibition of estrogen production induced by FSH or forskolin (FSK).

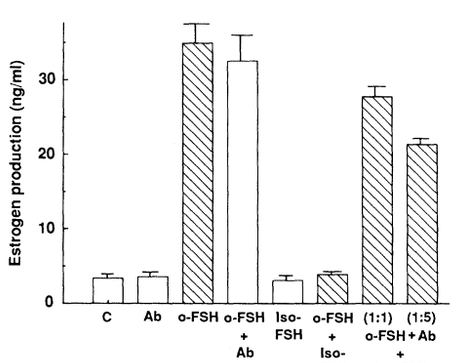

Fig. 3. Immunoneutralization of the antagonistic effects of FSH isoforms (*p*H 9.6 to 9.4) with antibodies against human FSH. Cultured granulosa cells were treated with ovine FSH (o-FSH), antibodies against human FSH (Ab) (immunoglobulin G at 750 μg/ml), and pooled fractions *p*H 9.6 to 9.4 from the chromatofocusing column (iso-FSH) alone or in combination. The last two columns indicate incubations with ovine FSH, iso-FSH, and two different dilutions of the antibody. Estrogen production by granulosa cells is shown (mean ± SE; $n = 3$). Similar results were obtained in three separate experiments.

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9. A column (40 cm by 1 cm) was packed with PBE-94 (Pharmacia) and equilibrated with 0.025*M* imidazole-HCl (*p*H 7.4). Serum to be chromatofocused was pretreated with 12% polyethylene glycol, and centrifuged (14,000*g*). The supernatant, which contained 99% of the total FSH activity, was applied to the column, and 5 ml of equilibration buffer was added. The column was eluted with a 1:8 dilution of Polybuffer 74 in distilled water, adjusted to *p*H 4.0 with HCl, with a flow rate of 30 ml/hour. Fractions (2 ml) were collected until the *p*H of the eluant reached the *p*H of the elution buffer. The chromatography experiments were performed on pooled serum samples from individual patients. These experiments were run at least three times with samples from different patients, and one representative set of results is shown.
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12. A column (40 cm by 1 cm) was packed with PBE-118. The column was equilibrated with 0.025*M* triethylamine HCl (*p*H 11). Serum to be chromatofocused was applied to the column, and 5 ml of equilibration buffer was added. The column was eluted with a 1:45 dilution of Pharmalyte in distilled water, adjusted to *p*H 8.0 with HCl, with a flow rate of 30 ml/hour. Fractions (2 ml) were collected until the *p*H of the eluant reached the *p*H of the elution buffer. With pooled samples from different individuals, the experiment has been repeated three times with similar results.
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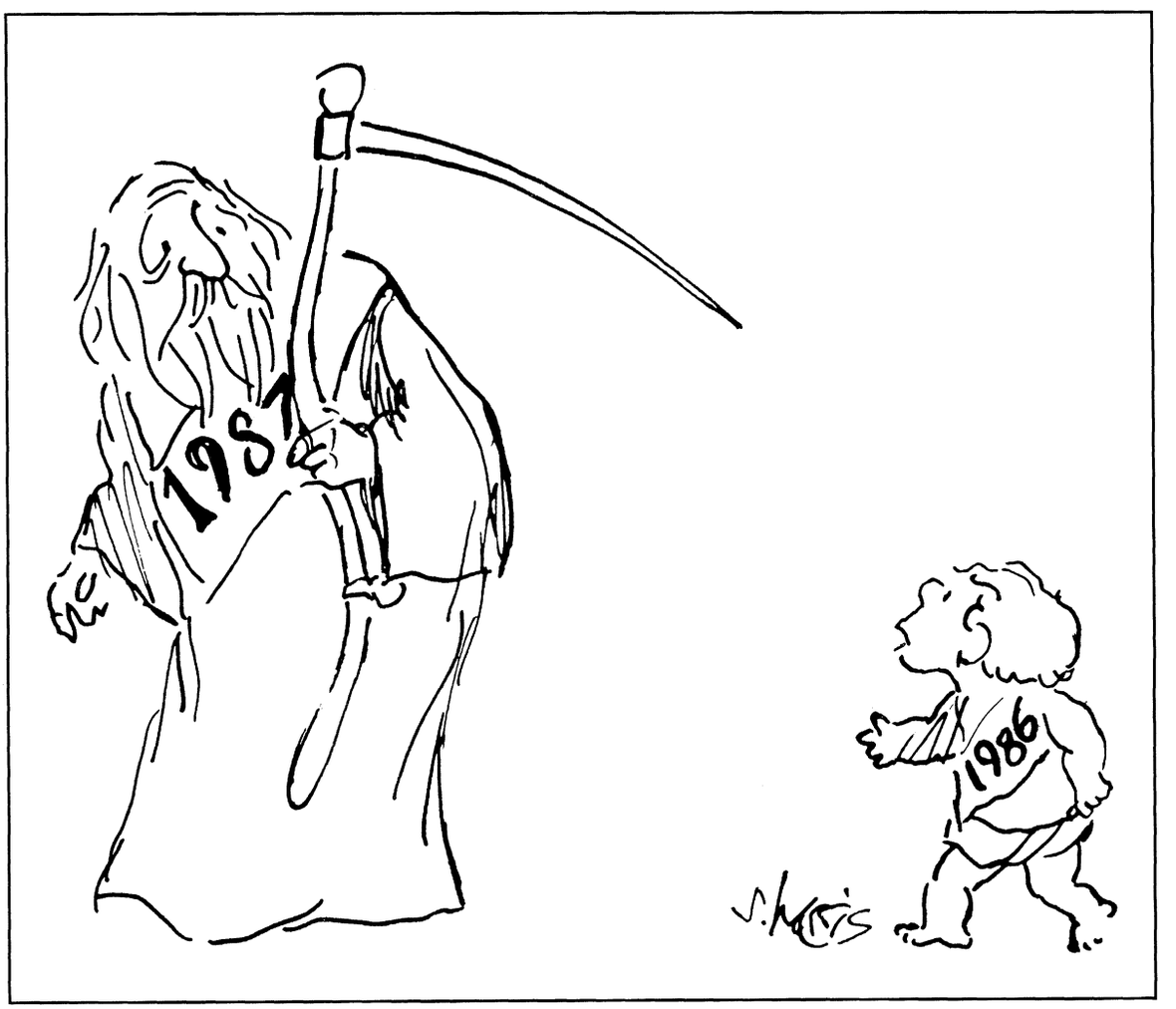

"Nutrinos have mass. The universe is contracting, and therefore time is running backward."