Effects of gonadotropin-releasing hormone on bioactivity of follicle-stimulating hormone (FSH) and microstructure of FSH, luteinizing hormone and sex hormone-binding globulin in a testosterone-based contraceptive trial: evaluation of responders and non-responders

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Only a proportion of normal men participating in testosterone-based contraceptive trials develop azoospermia (responders). This study analyzed whether serum follicle-stimulating hormone (FSH), luteinizing hormone (LH) and sex hormone-binding globulin (SHBG) are qualitatively different between responders and non-responders. Determination of in vitro bioactive FSH after stimulation with gonadotropin-releasing hormone (GnRH) and analysis of molecular heterogeneity of serum FSH, LH and SHBG was carried out by chromatofocusing and concanavalin-A affinity chromatography in eight men who had participated in a previous contraceptive study with testosterone bucillate. Blood was withdrawn at 15-min intervals on two basal occasions and 30, 45 and 60 min after iv administration of GnRH (100 μg). Pools of sera were separated by chromatofocusing in the pH range 3–6 and by lectin chromatography on concanavalin A. Immunoactive FSH, LH and SHBG were assayed in the eluates. Bioactive FSH was analyzed by the rat Sertoli cell bioassay. Serum bioactive FSH increased after GnRH stimulation, without significant differences between responders and non-responders. The chromatofocusing profiles of serum FSH showed a significant shift towards the less acidic region after GnRH. The isofrom distribution was similar in responders and non-responders. No significant differences were found in the relative proportion of FSH, LH and SHBG retained by concanavalin A. It is concluded that the extent of suppression of sperm production by androgen administration cannot be foreseen either on the basis of the response of bioactive FSH to GnRH administration or from the glycosylation pattern of serum FSH, LH and SHBG.

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Although its specific role in spermatogenesis remains to be elucidated fully, follicle-stimulating hormone (FSH) is a key hormone in male reproduction (1). The most recent studies employing hormonal methods for male contraception have shown that suppression of the gonadotropins luteinizing hormone (LH) and FSH is necessary to arrest sperm production and achieve azoospermia (2). However, not all men treated with testosterone esters become azoospermic (3), a phenomenon in which individual differences in the qualitative characteristics of gonadotropins might be involved. In particular, FSH is known to be highly heterogeneous, due to a certain pleomorphism in its carbohydrate composition that results in many molecular isoforms differing in their electric charge, survival in the circulation, binding properties and bioactivity (4). Several events can influence FSH heterogeneity, and its charge characteristics have been shown to vary in a number of pathophysiological conditions, especially in women (5–8).

Follicle-stimulating hormone heterogeneity in man has been investigated less thoroughly and the effects of steroid hormones are more controversial. We have observed that testosterone administration to androgen-deficient patients with Klinefelter’s syndrome for up to 3 months does not significantly influence FSH heterogeneity (9), a finding at variance with the well-known effects of androgens on the pituitary FSH isoforms in experimental animals (4, 10).

In a group of young healthy men participating in a contraceptive trial with testosterone enanathate (TE), Dahl et al. (11, 12) found that the androgen treatment results in a significant change of the FSH microheterogeneity, with a shift to the preponderance of more
basic isoforms with higher in vitro bioactivity/immunoreactivity (b/i) ratios. These authors also suggested that this change in the qualitative characteristics of the gonadotropin could be involved in the lack of achievement of consistent azoospermia following high-dose TE administration in normal men (2, 3). In a recent study where testosterone buciclate (TB) was evaluated as a potential male contraceptive, we could show that serum gonadotropins were better suppressed in those men who achieved azoospermia (responders) compared to those who did not (non-responders). The former group also had significantly lower serum concentrations of SHBG.

However, both subgroups showed similar increases in serum immunoreactive LH and FSH after stimulation with gonadotropin-releasing hormone (GnRH) (2).

Predicting which men will develop azoospermia under testosterone treatment would be of help in the identification of those subjects for which hormonal contraception can become a real option. This study was undertaken in the attempt to identify physiological features of FSH heterogeneity that could possibly be related to a different propensity towards gonadotropin suppression upon testosterone administration in normal men. In particular, we have investigated the effects of GnRH on bioactive FSH and whether the molecular pattern of serum FSH varies spontaneously under basal conditions. Moreover, we have analyzed the occurrence of different patterns of FSH heterogeneity after chromatofocusing in men who developed azoospermia when treated with TB (responders) compared to non-responders (2), as well as the elution patterns of their serum FSH, LH and sex hormone-binding globulin (SHBG) after lectin chromatography.

Materials and methods

Subjects and blood samples

The eight healthy males participating in this study correspond to study group II described in detail by Behre et al. (2). Briefly, the group consisted of eight male volunteers (mean age 25.1 years) who had received a single im injection of 1200 mg of TB. Following this treatment, three of the subjects developed azoospermia within 10 weeks and had gonadotropin levels suppressed to the assay detection limits (responders). Sperm concentrations were variably affected in the remaining five subjects, but none of them developed azoospermia (non-responders) (2). Six months after their participation in the contraceptive trial, the volunteers were recalled for the evaluation of possible differential parameters of pituitary responsiveness between responders and non-responders and gave their informed consent for this study, performed when the subjects were free of medication. At the time of the present investigation serum testosterone levels were 17.3 ± 0.4 and 24.3 ± 2.5 nmol/l in responders and non-responders, respectively. Three blood sample were taken from an indwelling catheter inserted in the antecubital vein at 15-min intervals on two separate occasions at least 1 week apart. On one of the two occasions a bolus of 100 μg of GnRH (Relefact LH-RH, Hoechst, Frankfurt am Main, Germany) was given iv immediately after collecting the third basal blood sample, and additional samples were taken 30, 45 and 60 min thereafter.

Chromatofocusing

Serum FSH was fractionated by chromatofocusing as described previously, (9) with minor modifications. Three samples from each subject were analyzed. The samples were obtained by pooling together equal aliquots of serum obtained at 15-min intervals under the two basal conditions and after GnRH stimulation, respectively. A 10–15 ml aliquot of each serum pool was dialyzed overnight against starting buffer (25 mmol of imidazol - HCl, pH 6.2) and applied to a 1 × 20 cm column of exchanger gel (PBE 94, Pharmacia, Freiburg, Germany). The column was equilibrated to pH 6.2 with starting buffer, loaded and the sample eluted with eluting buffer (Polybuffer 74, Pharmacia; dilution 1:6 v/v in distilled water, adjusted to pH 3 with 1 mol/l HCl) at the flow rate of 2 ml/min. Fifty 2-ml fractions were collected, pH was measured and 15 additional fractions were collected after application of 1 mol of NaCl. The column was then regenerated by extensive washing with starting buffer. All chromatofocusing runs were performed at 10°C and the recovery after dialysis and chromatofocusing ranged between 91 and 138%. The reproducibility of the procedure has been reported previously (9).

Concanavalin A (Con-A) chromatography

Concanavalin-A affinity chromatography was performed according to Manjunath et al. (13). Lectin columns were prepared in 7 × 0.65 cm glass columns (Sigma, Deisenhofen, Germany) with 4 ml of Con-A Sepharose 4B (Pharmacia) washed previously with Con-A buffer (25 mmol/l TRIS - HCl, pH 7.4, 10 mmol/l MgCl₂, 10 mmol/l CaCl₂, 10 mmol/l MnCl₂, 0.5 mol/l NaCl, 0.02% NaN₃). The columns had a void volume of about 2 ml, but protein overloading was observed when serum volumes of >0.5 ml were used. 0.5 ml serum samples (pools of equal aliquots of three basal samples obtained at 15-min intervals) were equilibrated with 1.5 ml of Con-A buffer, loaded onto the column and allowed to interact with the lectin for 1 h. The column was eluted sequentially at the flow rate of 1 ml/min with 20 ml of Con-A buffer, 20 ml of 10 mmol/l α-methylglucopyranoside (MG, Sigma) and 20 ml of 500 mmol/l MG in Con-A buffer to collect unbound, weekly bound and firmly bound glycoproteins, respectively. Columns were used only once. Because of the sample dilution, aliquots of eluates were concentrated 8–10 times by vacuum evaporation, using the Maxi dry plus
equipment (Heto-Holten, Wettemberg, Germany), prior to FSH and LH determination. The sample concentration brought the hormonal levels well into the assay range, without interfering with assay performance. Sample concentration was not necessary for SHBG determination. In order to assess reproducibility, aliquots of the same serum pool were run three times and FSH, LH and SHBG determined in individual 2-ml fractions. The separate runs gave superimposable elution profiles. The amounts of hormones loaded and recovered after Con-A chromatography were: FSH: 0.68–2.9 mIU loaded, 1.03–2.08 recovered (94 ± 8.03%); LH: 0.79–2.65 mIU loaded, 0.27–1.33 mIU recovered (61.87 ± 9.32%); SHBG: 6.7–32.7 pmol loaded, 6.49–41.8 pmol recovered (98.25 ± 7.74%)

Hormone assays

Immunoreactive FSH (iFSH), LH and SHBG were measured in duplicate by immunofluorimetric assay (IFMA) using commercial kits (Delfia hFSH, Delfia hLH, Spec. Delfia SHBG, Pharmacia). In order to increase the sensitivity, the sample volumes in the FSH and LH IFMAs were increased to 50 µL. In preliminary experiments we could show that this increase in assay volume did not result in substantial deviation from parallelism. Serial dilutions of standard FSH in chromatofocusing fractions with different pH and from the salt peak gave responses parallel to the standard curve, as did serial dilutions of Con-A buffer and 10 and 500 mmol/l MgCl2. The sensitivity of the FSH IFMA was 0.025 IU/l. The assay precision was controlled according to the WHO guidelines (14). The Intra-assay coefficient of variation (CV) for iFSH was 3.5 ± 2.6%. The interassay variability was checked by running, in every assay, a control sample obtained by pooling together chromatofocusing fractions with low FSH concentration (about 1.75 IU/l). The interassay CV was 6.7%. All the samples obtained from Con-A chromatography were measured in the same assay, with intra-assay CVs of 8% and 5% for LH and SHBG, respectively. The sensitivities of the LH and SHBG IFMAs were 0.05 IU/l and 2 nmol/l, respectively.

Bioactive FSH was measured in the individual serum samples by an in vitro bioassay based on FSH-dependent aromatase activity of immature rat Sertoli cells as described previously (10, 15). Each sample was measured in triplicate at three dose levels. The sensitivity of the bioassay, defined as the minimal detectable aromatase-stimulating activity in serum, is 2IU/l (WHO standard 2nd IRP 78/549). However, we noticed that a discrete amount of aromatase-stimulating activity could be quantified also in the presence of FSH-free serum or 7% serum protein solution (not shown). In order to account for this specific aromatase-stimulating effect, which could lead to the overestimation of bioactive FSH in sera from normal, eugonadal men with low FSH concentrations, serial dilutions of a 7% protein mixture containing serum albumin and globulins (blank) were run in each bioassay at two dose levels and the corresponding bioFSH-like activity subtracted from the bioFSH values measured in the serum samples. BioFSH was considered undetectable when its concentration resulted ≤ to the blank value and, for statistical purposes, was assigned a value of 0.1 IU/l, i.e. the minimal value that could be differentiated from the blank. The interassay CVs were 28.3% and 14.1% for control sera with bioFSH concentrations of 4.8 and 24.6 IU/l, respectively. The corresponding intra-assay CVs were 25.9% and 12.9%.

Statistical analyses

All data are expressed as means ± SEM. For statistical purposes, samples with undetectable immunoreactive hormone levels were arbitrarily assigned a value corresponding to the assay sensitivity. The statistical evaluations were performed by ANOVA and Tukey’s test and by a paired Student’s t-test. The chromatofocusing data were analyzed after grouping the FSH values expressed as a percentage of total FSH recovered in eight segments of 0.5 pH units. The percentage values were arcsine-transformed prior to statistical analysis. The analysis of the serum hormones was performed after log transformation.

Results

The serum iFSH and bioFSH concentrations in the eight subjects are shown in Table 1. The GnRH induced a significant increase of both iFSH and bioFSH, whereas the b/i ratio did not increase significantly. The lack of significant difference in the iFSH response to GnRH between the subgroup of three subjects who became azoospermic (responders) and the five subjects who did not reach azoospermia (non-responders) was reported previously (2). Here we show that also the response of bioFSH to GnRH was not significantly different between responders and non-responders (Fig. 1).

Figure 2 shows representative chromatofocusing profiles of iFSH in one subject, demonstrating a rather constant FSH elution pattern on the two separate basal occasions with a significant shift towards the less acidic region after GnRH stimulation. Figure 3 summarizes the results obtained in the eight subjects and shows a

<table>
<thead>
<tr>
<th></th>
<th>iFSH (IU/l)</th>
<th>bioFSH (IU/l)</th>
<th>b/i ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal 1</td>
<td>2.92 ± 0.24</td>
<td>2.25 ± 0.50</td>
<td>0.76 ± 0.16</td>
</tr>
<tr>
<td>Basal 2</td>
<td>2.62 ± 0.32</td>
<td>1.81 ± 0.60</td>
<td>0.63 ± 0.18</td>
</tr>
<tr>
<td>GnRH</td>
<td>4.52 ± 0.58**</td>
<td>5.51 ± 1.22*</td>
<td>1.11 ± 0.19</td>
</tr>
</tbody>
</table>

* p < 0.05 and ** p < 0.01 vs basal 2. Paired t-test.
significant increase in the less acidic molecular species and a significant decrease in the very acidic portion of FSH recovered in the salt peak after GnRH stimulation. It is noteworthy that this significant shift towards a preponderance of more basic FSH isoforms was documented in the absence of a significant increase in the b/i ratio (Table 1). Tables 2 and 3 show the relative distribution of iFSH isoforms in the three responders and in the five non-responders. In both subgroups, GnRH stimulation led to a significant increase in the more basic isoforms and no obvious differences in the charge properties of the gonadotropin were evident.

In our previous report we have shown that the only endocrine differences between responders and non-responders in this group of men lay in the susceptibility to suppress gonadotrophins and serum levels of SHBG (2). It is known that genetic variants of SHBG are present in the population worldwide (16) and may differ in their carbohydrate composition (17). We have, therefore, analyzed the binding properties to Con-A of serum FSH, LH and SHBG. The FSH, LH and SHBG concentrations measured in the Con-A fractions were, respectively, in the following ranges: 0.03–2.84 IU/l, 0.03–1.21 IU/l and 2–55 nmol/l. The percentage elution patterns of FSH, LH and SHBG after Con-A chromatography are shown in Table 4. No significant differences between responders and non-responders could be documented, again suggesting that differences in the glycosylation pattern of these glycoproteins are not a hallmark of the different responsiveness to testosterone.

Discussion
The recent availability of very sensitive immunometric methods for FSH determination permits the molecular heterogeneity of the gonadotropin to be characterized in serum from normal, eugonadal men. The chromatographic procedures used were formerly applicable only to pituitary preparations and sera from subjects with primary hypogonadism because they involve extensive sample dilution. It has now been shown that serum FSH is highly heterogeneous (5, 9, 18) and more negatively charged, i.e. more acidic than pituitary FSH (18, 19). Many studies suggested that pleomorphism of circulating FSH is modulated by age, sex and endocrine status of the subject. In this paper we have analyzed molecular heterogeneity of serum FSH in normal men, showing that the pattern of isoform distribution remains substantially constant within the same individuals when multiple blood samples are taken on separate
occasions. Although the variability observed in two men investigated previously (9) was also detected in one subject in this study, overall the isoform distribution of serum FSH appears rather stable under basal conditions.

Administration of GnRH results in the release of less acidic FSH, possibly because the neurohormone stimulates rapid secretion of FSH isoforms that have not completely undergone the glycosylation process due to the acute stimulation. The release of more basic FSH following GnRH administration has been described previously using other chromatographic techniques in normal adults (20), in girls with Turner’s syndrome (21) and in pubertal children (7). However, all these studies, including the present one, have employed a pharmacological dose of GnRH, and the question remains whether endogenous GnRH secretion also results in release of less-acidic GnRH. Pulsatile administration of physiological doses of GnRH does not change the isoform distribution of serum FSH in ovariectomized, nutritionally restricted lambs, a model for hypogonadotropic hypogonadism (22). The secretion of less-mature FSH could therefore occur when the secretion rate of the gonadotropin is artificially raised, whereas under physiological, basal conditions the endogenous GnRH pulsatility results only in a modestly pulsatile secretion of long-lived FSH with a rather constant isoform composition. It is interesting to note that these qualitative modifications of FSH after GnRH do not result in changes in the b:i ratio, yet confirming that this parameter depends on the parallelism between bio- and immunoassay (23) and type of immunoassay (24, 25), rather than reflecting real qualitative variations.

Glycosylation is an intrinsic property of the molecule, dictated by its primary structure, and strongly influences intracellular storage and release of the gonadotropin (26), as well as its receptor binding and signal transduction (27, 28). In a group of men participating in a contraceptive trial with TB, we observed that gonadotropin suppression was more pronounced in men who achieved azoospermia (2). In a previous, preliminary report, Dahl et al. reported that serum FSH microheterogeneity is changed after suppression with TE

### Table 3. Relative distribution of serum immunoreactive FSH isoforms (% of total recovered) after chromatofocusing in five normal men who did not achieve azoospermia upon treatment with testosterone bucillate (non-responders).

<table>
<thead>
<tr>
<th>pH region</th>
<th>Basal 1</th>
<th>Basal 2</th>
<th>GnRH</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;3</td>
<td>16.84 ± 2.4</td>
<td>18.64 ± 1.78</td>
<td>9.11 ± 2.03</td>
</tr>
<tr>
<td>3.00-3.49</td>
<td>6.77 ± 1.21</td>
<td>4.09 ± 1.55</td>
<td>4.48 ± 0.90</td>
</tr>
<tr>
<td>3.50-3.99</td>
<td>20.5 ± 1.26</td>
<td>14.01 ± 3.37</td>
<td>13.90 ± 2.47</td>
</tr>
<tr>
<td>4.00-4.49</td>
<td>17.42 ± 1.26</td>
<td>18.10 ± 2.20</td>
<td>16.15 ± 1.50</td>
</tr>
<tr>
<td>4.50-4.99</td>
<td>7.67 ± 0.70</td>
<td>9.00 ± 1.02</td>
<td>10.12 ± 0.89</td>
</tr>
<tr>
<td>5.00-5.49</td>
<td>8.50 ± 0.63</td>
<td>10.40 ± 0.92</td>
<td>12.75 ± 2.11</td>
</tr>
<tr>
<td>5.50-5.99</td>
<td>13.77 ± 2.01</td>
<td>13.32 ± 1.86</td>
<td>18.25 ± 2.94</td>
</tr>
</tbody>
</table>

*p < 0.05 vs basal 2, paired t-test.

### Table 4. Elution pattern of serum immunoreactive FSH, LH and SHBG after concanavalin A chromatography in eight normal men. subdivided into subjects who achieved azoospermia or not (responders/non-responders) upon treatment with testosterone bucillate.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unbound (%)</th>
<th>Weakly bound (%)</th>
<th>Firmly bound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All the subjects</td>
<td>66.55 ± 2.30</td>
<td>30.01 ± 2.49</td>
<td>3.43 ± 0.41</td>
</tr>
<tr>
<td>Responders</td>
<td>65.11 ± 5.46</td>
<td>32.23 ± 5.69</td>
<td>2.65 ± 0.81</td>
</tr>
<tr>
<td>Non-responders</td>
<td>67.42 ± 2.32</td>
<td>28.67 ± 2.53</td>
<td>3.91 ± 0.36</td>
</tr>
<tr>
<td>LH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All the subjects</td>
<td>33.75 ± 3.80</td>
<td>45.51 ± 2.67</td>
<td>20.73 ± 3.49</td>
</tr>
<tr>
<td>Responders</td>
<td>39.21 ± 6.98</td>
<td>42.80 ± 3.26</td>
<td>17.97 ± 7.31</td>
</tr>
<tr>
<td>Non-responders</td>
<td>30.47 ± 4.31</td>
<td>47.14 ± 4.32</td>
<td>22.38 ± 4.04</td>
</tr>
<tr>
<td>SHBG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All the subjects</td>
<td>8.38 ± 0.69</td>
<td>42.42 ± 1.74</td>
<td>49.20 ± 1.47</td>
</tr>
<tr>
<td>Responders</td>
<td>9.68 ± 1.09</td>
<td>41.48 ± 5.04</td>
<td>48.85 ± 4.2</td>
</tr>
<tr>
<td>Non-responders</td>
<td>7.60 ± 0.77</td>
<td>42.99 ± 0.77</td>
<td>49.41 ± 0.85</td>
</tr>
</tbody>
</table>
and the b/i ratio of the gonadotropin is significantly increased (11). In our hands, serum FSH concentrations during testosterone administration are too low to be determined accurately by bioassay and/or analyzed after chromatofocusing separation, a procedure involving extensive sample dilution. Therefore, we have chosen a different approach and, in this study, we have analyzed the qualitative characteristics of FSH in the absence of testosterone treatment. In an attempt to identify differences in its biological properties between responders and non-responders. Our data show that bioFSH reacts to GnRH stimulation in a similar way in the two groups. Moreover, we could not identify differences in the glycosylation pattern of FSH and LH, therefore excluding that dissimilar half-lives and in vivo bioactivities of the gonadotropins are involved in the suppression of sperm production upon treatment with testosterone. Together with the data presented earlier, the results of this study suggest that it is the extent of gonadotropin suppression and not its quality that determines the degree of inhibition of spermatogenesis (2).

The other peculiarity observed in our previous contraceptive study was a clear-cut difference in the serum levels of SHBG between responders and non-responders. Sex hormone-binding globulin is a glycoprotein known to consist of glycosylation variants differently retained by Con-A (17, 29). Because the sugar moieties determine the half-life of glycoproteins, we argued that the distinction between the two groups could lie in the glycosylation patterns of SHBG, but this hypothesis could not be confirmed.

To summarize, the present study shows that FSH molecular heterogeneity is modified by GnRH administration, but this parameter, the response of in vitro bioactive FSH to GnRH and the glycosylation pattern of FSH, LH and SHBG are not involved in the susceptibility to develop azoospermia in men treated with testosterone for contraceptive purposes.

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References
6. Wide L, Bakos O. More basic forms of both human follicle-stimulating hormone and luteinizing hormone in serum at midcycle compared with the follicular and luteal phase. J Clin Endocrinol Metab 1993;75:885–9

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