Microheterogeneity of pituitary follicle-stimulating hormone in male rats: differential effects of the chronic androgen deprivation induced by castration or androgen blockade

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ABSTRACT

Testicular androgens are known to influence not only the secretion but also the bioactivity and molecular composition of pituitary FSH. In the present study, we investigated the effects of chronic androgen blockade and castration on the molecular heterogeneity of the gonadotrophin. Groups of male adult rats (five animals per group) received one of the following treatments: vehicle, the non-steroidal anti-androgens casodex (20 mg/kg per day) or flutamide (20 mg/kg per day), or castration. After 8 weeks, the animals were killed and individual pituitary homogenates fractionated by isoelectric focusing (IEF) on sucrose density gradients in the pH range 2.5-8. FSH was measured by radioimmunoassay (RIA) in the individual fractions and by in vitro bioassay (Sertoli cell aromatase bioassay) in pools of fractions which were combined according to pH intervals of 0.5 units. Bioactive and immunoreactive FSH were also measured in sera and unfractionated pituitary extracts. Testosterone and inhibin were assayed in sera by RIA.

A significant increase in serum immunoreactive and bioactive FSH was demonstrated in flutamide-treated and castrated animals, whereas the pituitary content of bioactive FSH remained unchanged in the four groups. Serum testosterone and inhibin were undetectable in castrated animals and significantly increased in those treated with flutamide. By RIA, the IEF profiles of the flutamide-treated and castrated rats showed a significant reduction of the FSH isoforms with 3.5<pI<4, with a significant increase in the isoforms with pI>4 only in the castrated group. By bioassay, there was a significant decrease in the isoforms with 3.5<pI<4 in both casodex- and flutamide-treated animals, with no significant differences between the two groups. Castration caused a further significant shift in the relative distribution of FSH isoforms towards the less acidic components, with a significant increase in the isoforms with 5<pI<5.5 not attained by androgen blockade alone.

These results suggest that the effects of long-lasting castration on pituitary FSH heterogeneity cannot be entirely reproduced by the androgen blockade. Since inhibin, eliminated by castration but not by androgen blockade, is a major regulator of FSH in the male rat, we speculate that it might not only influence FSH secretion but also modulate its qualitative properties.

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INTRODUCTION

Follicle-stimulating hormone (FSH) exists in many molecular forms within the anterior pituitary gland. The study of immunoreactivity and in-vitro bioactivity of the different isoforms of pituitary FSH suggests that FSH microheterogeneity is regulated by gonadal and hypothalamic factors, both in humans and in experimental animals (for review see Ulloa-Aguirre et al. 1988a). Testicular androgens are known to influence the characteristics of pituitary FSH (Diebel et al. 1973; Bogdanove et al. 1974;
Kennedy & Chappel, 1985). In the male rat, microheterogeneity of pituitary FSH changes during sexual maturation; at the inception of spermatogenesis (45 days of age) there is a shift in the relative distribution of the different molecular isoforms towards the more acidic components, a situation which is partially reversed 15 days after orchidectomy (Uloa-
Aguirre et al. 1986). Moreover, in FSH-suppressed rats, testosterone can stimulate pituitary and serum FSH (Rea et al. 1986a,b) and also influences the molecular composition of the gonadotrophin via a mechanism which probably does not involve hypothalamic gonadotrophin-releasing hormone (GnRH; Sharma et al. 1990).

Although the role of gonadal steroids is suggested by several studies, the possibility that testicular factors other than testosterone may influence the quality of pituitary FSH has not received much attention. In this study, we reasoned that if other testicular factors are not involved in the regulation of FSH heterogeneity, the selective blockade of androgen action should be sufficient to reproduce the qualitative effect of castration. However, the pure antiandrogen flutamide (Neumann & Töpert, 1986) also prevents the negative feedback action of androgens on gonadotrophin secretion, resulting in an increase of serum testosterone which could override the androgen blockade (Peets et al. 1974). We took advantage of the recent demonstration that the new nonsteroidal antiandrogen, casodex, can effectively block androgen action at the peripheral level, without substantially increasing serum testosterone concentrations (Chandolia et al. 1991a,b). Casodex preferentially, if not selectively, acts on the androgen receptors located outside the blood–brain barrier (Furr et al. 1987; Furr, 1988). Using these two antiandrogens, we challenged the idea that the effects of castration on the molecular heterogeneity of the gonadotrophin, as measured by radioimmunoassay (RIA) and by a sensitive in-vitro FSH bioassay, are solely due to androgen withdrawal, with the aim of further clarifying the exact role of androgens in the regulation of FSH pleomorphism. The molecular heterogeneity of pituitary FSH from animals chronically treated with casodex or flutamide was analysed by isoelectric focusing (IEF) and compared with the effects observed in an analogous group of castrated animals.

**MATERIALS AND METHODS**

**Animals**

The pituitaries analysed in the current study were obtained from animals described in another investigation (Chandolia et al. 1991b). For the purposes of the present study, four groups of five animals (adult male Wistar rats, aged 15–18 weeks, 380–400 g each) were utilized. The animals were obtained from the Central Institute for Laboratory Animal Breeding (Hannover, Germany) and were kept in groups of two and three under conditions of controlled temperature and a cycle of 12 h light : 12 h darkness, with free access to rat chow and tap water. Handling and treatment of the experimental animals were performed according to the regulations of the German Federal Law on the Care and Use of Laboratory Animals.

Three groups of five rats each received one of the following treatments subcutaneously: vehicle (controls); casodex (20 mg/kg per day; IC1, Macclesfield, Cheshire, U.K.); flutamide (20 mg/kg per day; Essex Pharma, München, Germany). Moreover, an additional group of five animals was castrated under ether anaesthesia. Eight weeks after the beginning of the treatment or after castration, the animals were killed and the pituitary glands were dissected out, weighed, sonicated to homogeneity in 1 ml ice-cold 0.01 M phosphate-buffered saline (pH 7.4) and stored at −80 °C until analysed. Trunk blood was collected and serum stored at −20 °C for hormone measurements. Testicular histology and flow-cytometric analysis of the testicular cells were performed in the control animals and in the rats treated with antiandrogens, and these results have been published elsewhere (Chandolia et al. 1991b).

**Isoelectric focusing**

The individual pituitary homogenates were fractionated by IEF on sucrose density gradients, using a 110 ml column (No. 8100; LKB Produkter, Bromma, Sweden). To create the pH gradient, ampholites (Ampholine; Pharmacia, Freiburg, Germany) were used at a final concentration of 1% in the pH range 2.5–8. At the end of each run (16–18 h), 46–47 fractions (≈2.4 ml) were collected from the bottom of the column and pH was measured in the individual fractions using a digital pH meter (pH 82; Radiometer, Copenhagen, Denmark). The fractions were then passed through small columns of Sephadex G-25 (PD-10; Pharmacia) to eliminate sucrose and ampholites and stored at −20 °C until analysed.

The reproducibility of the electrofocusing procedure was monitored by assessing the pI of horse heart myoglobin (Sigma, Deisenhofen, Germany) in each run; the mean pI value (±s.d.) from 20 IEF runs was 7.88±0.15, with a reproducibility higher than 98%. The total recovery after IEF and gel filtration was monitored by measuring immunoreactive and bioactive FSH in pooled aliquots from the
individual fractions of each IEF run, along with an aliquot of unfractionated pituitary extract, both analysed at two to four dose levels. The overall recovery of FSH immunoreactivity was markedly lower (33 ± 2%) than that of FSH bioactivity (86 ± 9%).

Radioimmunoassays

Immunoreactive FSH was measured in duplicate in serum samples, in the individual IEF fractions at single dose levels and in the pituitary extracts at three to four dose levels. The method employed was a double-antibody RIA using reagents for rat FSH provided by NIDDK (Bethesda, MD, U.S.A.). The standard preparation used was rFSH-RP2, the tracer was prepared from FSH-1–6 and the antiserum was anti-rFSH-S-11. The sensitivity was 1-6 ng/ml. The intra- and interassay coefficients of variation were < 6% and 7.1% respectively for a pool of sera from normal rats and < 10% and 12.3% respectively for a pool of sera from castrated rats.

Serum testosterone was measured by a solid-phase double-antibody RIA method, after ether extraction, as previously described (Chandolia et al. 1991b). The sensitivity was 2 pg/tube (0-07 nmol/l). The mean intra-assay and interassay coefficients of variation were 5.7% and 8.9% respectively.

Serum inhibin was measured in a double-antibody RIA as described for male rats (Robertson et al. 1988) using an antiserum raised in rabbits against highly purified bovine 31 kDa inhibin and the standard described previously (Weinbauer et al. 1989). The sensitivity and the intra-assay coefficient of variation were 270 U/l and 3.5% respectively.

In-vitro FSH bioassay

Aliquots of the individual fractions were combined according to pH value in 13 pools, with a pH interval of 0-5 units. Bioactive FSH was measured in the IEF pools, in pituitary homogenates and in serum samples using a highly sensitive and specific in-vitro bioassay based on the conversion of androgen substrates to oestradiol by immature rat Sertoli cells (Van Damme et al. 1979; Padmanabhan et al. 1987), as previously described (Jockenhövel et al. 1990; Sharma et al. 1990). Sertoli cells from 7–10-day-old male Sprague–Dawley rats were cultured for 72 h in medium (Ham’s F12: Dulbecco’s modification of Eagle’s medium, 1:1; Flow Laboratories, Irvine, Strathclyde, U.K.) enriched with antibiotics and hormones in 96 microwell plates, in an atmosphere of 95% air and 5% CO₂ at 32 °C. The attached cells were washed twice in medium and were cultured for an additional 24 h in the presence of 19-hydroxyandrostenedione (2-5 × 10⁻⁶ mol/l), methylisobutylxanthine (0–1 mmol/l), FSH standard (rFSH-RP2) or the unknown samples. Oestadiol was measured in the culture media using a solid-phase, double-antibody RIA technique, employing a commercially available iodinated tracer (oestadiol 6-(O-carboxymethyl)oximino-(2-[¹²⁵I]iodohistamine); Amer sham International, Braunshweig, Germany) and an antiserum raised in rabbit against 17β-oestadiol 6-(O-carboxymethyl)oxime–bovine serum albumin, purchased from Steranti Research Ltd, St Albans, Herts, U.K. After overnight incubation at 4 °C, the bound/free separation was performed by the addition of a solution of solid-phase antirabbit immunoglobulins (Immunobead Second Antibody; Biorad, München, Germany). Using a control pool prepared with the enriched medium used in the bioassay supplemented with oestadiol standard the intra- and interassay coefficients of variation of the oestadiol RIA were < 10% and 15% respectively.

The FSH standard employed in the bioassay was the same as that used in the RIA and gave linear responses in the range 11–1440 pg/well. The sensitivity (0-02 ng/tube) and the slope of the dose-response curve (–1.80) were consistently different from the corresponding parameters obtained in the RIA (0-16 ng/tube and –2.65 respectively), making the direct comparison of the values obtained in the two assays inappropriate (Jockenhövel et al. 1990). Therefore bioactive/immunoreactive FSH ratios were not considered.

Bioactive FSH was determined in each IEF pool, in sera and in the pituitary extracts at four to six dose levels in triplicate. The results were calculated from the mean values from two or three dose levels which gave responses parallel to the standard curve. When non-parallelism was repeatedly observed between the dose–response line of the test sample and that of the standard after logit–log transformation (which happened in the case of some pituitary extracts), the bioactivity was approximated by averaging the values obtained for two dilutions of the sample within the logit range –1-5 to –0-1 (Robertson et al. 1982). The intra- and interassay coefficients of variation were < 20% and 24-18% respectively for a pool of sera obtained from normal rats, and < 15% and 16-61% respectively for a pool of sera from castrated rats.

Statistical analysis

Data are expressed as means ± s.e.m. Prior to statistical evaluation the data were log-normalized (pituitary and serum hormone levels) or subjected to arc sine transformation (relative distribution of the different isoforms). The transformed data were then
analysed by ANOVA followed by Tukey’s multiple comparison test.

RESULTS

Serum and pituitary concentrations of immunoreactive and bioactive FSH are shown in Tables 1 and 2 respectively. A significant increase in immunoreactive FSH and bioactive FSH secretion was documented in flutamide-treated and castrated animals. At the pituitary level, immunoreactive FSH was slightly, but significantly, increased in the casodex-treated group (Chandolia et al. 1991b) and in the castrated animals, but no substantial modifications of bioactive FSH could be demonstrated.

After fractionation of the individual pituitary extracts by IEF, FSH was measured by RIA and by in-vitro bioassay in the individual fractions and in 0.5 pH unit pools respectively. The amounts of FSH detected by the two methods in the different pH ranges, expressed as percentages of the total activity recovered, are shown in Figs 1 and 2. By RIA (Fig. 1), no significant differences between the control group and casodex-treated animals were evident. In the flutamide-treated group, a significant decrease in the molecular species with 3.5 < pI < 4 and a significant increase in the isoforms with pI > 8 could be demonstrated. Similar variations were shown in the castrated group, where the isoforms with 4 < pI < 4.5 and 5 < pI < 5.5 also increased significantly. By bioassay (Fig. 2), castration was shown to induce a clear-cut shift of the molecular isoforms towards the less acidic components, with a statistically significant decrease in the isoforms with 3.5 < pI < 4 in all the androgen-deprived animals, but an increase in the isoforms with 5 < pI < 5.5 only in the castrated group. Thus, qualitatively, the effect of castration was only partially reproduced by the treatment with antiandrogens, with no significant difference between the casodex- and the flutamide-treated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Immunoactive FSH (ng/ml)</th>
<th>Bioactive FSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.010 ± 0.025</td>
<td>0.950 ± 0.335</td>
</tr>
<tr>
<td>Casodex</td>
<td>1.166 ± 0.549</td>
<td>1.111 ± 0.585</td>
</tr>
<tr>
<td>Flutamide</td>
<td>1.393 ± 0.049</td>
<td>1.416 ± 0.550</td>
</tr>
<tr>
<td>Castration</td>
<td>2.025 ± 0.160*</td>
<td>1.748 ± 0.373*</td>
</tr>
</tbody>
</table>

*P < 0.05 versus control group by Tukey’s test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Immunoactive FSH (ng/ml)</th>
<th>Bioactive FSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>2.463 ± 0.016</td>
<td>2.022 ± 0.103</td>
</tr>
<tr>
<td>Casodex</td>
<td>2.766 ± 0.777*</td>
<td>1.998 ± 0.059</td>
</tr>
<tr>
<td>Flutamide</td>
<td>2.618 ± 0.055</td>
<td>2.050 ± 0.053</td>
</tr>
<tr>
<td>Castration</td>
<td>2.883 ± 0.019*</td>
<td>2.207 ± 0.095</td>
</tr>
</tbody>
</table>

*P < 0.05 versus control group by Tukey’s test.

Serum inhibin was undetectable in the castrated group and significantly increased in the flutamide-treated animals (control: 1257 ± 44 U/l; casodex-treated: 1223 ± 152 U/l; flutamide-treated: 2360 ± 169 U/l, P < 0.05). Similarly, serum testosterone was undetectable in the castrated group, whereas the values obtained in the other groups showed a significant increase only in the flutamide-treated animals (control: 1489 ± 2.10 nmol/l; casodex-treated: 40.45 ± 0.66 nmol/l; flutamide-treated: 156.82 ± 20.50 nmol/l, P < 0.05), as reported previously (Chandolia et al. 1991b). The antiandrogenic effectiveness of the treatments has also been reported in detail in the previous paper; in particular, it was shown that both antiandrogens could markedly suppress the androgen action indicated by the weight of the seminal vesicles and coagulating glands. In the case of casodex, this effect occurred without increasing the intratesticular concentration of androgens and was similar to that obtained by the administration of a potent GnRH antagonist (Chandolia et al. 1991b).

DISCUSSION

The results presented here show that in male adult rats subjected to chronic androgen deprivation there is a significant relative decrease in the acidic isoforms of pituitary FSH. This could be demonstrated both by RIA and by bioassay, but important differences emerged between the two methods. Most of the immunoreactive material recovered in the fractions of pH < 3.5 was poorly bioactive, indicating that it consisted of molecular forms or fragments which cross-react in the RIA without contributing significantly to the bioactivity of the samples. This probably led to an important overestimation of the proportion of acidic FSH in all the groups, since the relative amount of this immunoreactive material was almost unaffected by the different treatments. The overestimation of immunoreactive FSH is also suggested by the lower recovery of immunoreactivity, compared with bioactivity, after IEF. A similarly

*P < 0.05 versus control group by Tukey’s test.

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marked preferential loss of immunoreactivity after IEF has been reported before for preparations of rat (Robertson et al. 1982) and human pituitary FSH (Zaidi et al. 1981), and related to the presence of a considerable amount of immunoreactive material devoid of bioactivity. On the other hand, it is known that the less acidic isoforms are less glycosylated and more biopotent in vitro (Miller et al. 1983; Blum et al. 1985). For these reasons, the bioassay seems to be more sensitive and accurate than the RIA in detecting qualitative changes in the isoform pattern of pituitary FSH.

This study clearly shows that long-lasting castration significantly modifies the qualitative pattern of pituitary FSH in male adult rats. Although the effects of castration and steroid substitution have been extensively studied in hamsters (Chappel et al. 1982; Ulloa-Aguirre & Chappel, 1982) and female rats (Ulloa-Aguirre et al. 1988b, 1990), only a few studies have dealt with male rats, producing

FIGURE 1. Recovery of FSH immunoreactivity (as a percentage of total recovered) in various pH ranges after isoelectric focusing of pituitary homogenates from four groups of male rats under different conditions of androgen deprivation. Each bar represents the mean ± S.E.M. for five individual pituitaries. Asterisks denote a significant difference (P<0.05, by Tukey's test on arcsine-transformed data) from the corresponding pH range in the control group. (a) Control, (b) casodex-treated, (c) flutamide-treated and (d) castrated.

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conflicting results. In fact, Robertson et al. (1982) could not demonstrate any significant variations of the isoform distribution of pituitary FSH 28 days after castration. In contrast, Ulloa-Aguirre et al. (1986) reported a significant increase in the less acidic gonadotrophin isoforms 2 weeks after orchiectomy. Apart from differences in the strain of the animals and/or the duration of gonadectomy, the assay method seems to be important as well. In the present investigation we have employed a very sensitive and specific homologous bioassay in vitro, which probably allowed clear-cut detection of the qualitative modifications of the gonadotrophin which would be difficult to demonstrate by RIA or radioligand receptor assay. Moreover, our data demonstrate that the interruption of androgen action at the receptor level can modify the relative distribution of FSH isoforms, irrespective of the actual serum testosterone levels and in the presence of normal spermatogenesis, as documented by histological
examination and flow cytometric analysis (Chandolia et al. 1991b). Qualitatively, this effect was already evident when the blockade of androgen action was achieved by casodex, a new non-steroidal peripherally selective antiandrogen, and no significant differences with the flutamide-treated group could be demonstrated. Since the pituitary gland is situated outside the blood–brain barrier and casodex is believed not to enter the central brain tissue (Freeman et al. 1986), the androgen blockade should occur only (or mainly) at the pituitary level. On this basis, our data suggest that the effect of androgen deprivation on the molecular heterogeneity of pituitary FSH does not involve hypothalamic GnRH, in accordance with previous evidence obtained in GnRH antagonist-treated rats supplemented with testosterone (Sharma et al. 1990).

In the 8-week castrated animals, the pattern of the relative amounts of FSH isoforms was further and significantly shifted towards the less acidic components, suggesting that androgen withdrawal alone is not sufficient to reproduce the effects of castration on pituitary FSH heterogeneity fully. This difference is difficult to explain on the basis of a presumably higher production of endogenous GnRH in castrated animals. Studies in vitro have shown that GnRH stimulates the glycosylation of luteinizing hormone subunits (Vogel et al. 1986), effecting the relative glycosylation only of the secreted gonadotrophin, without altering the intracellular subunits (Krummen & Baldwin, 1988). Moreover, the exposure of rat pituitary cells to testosterone and GnRH increases the secretion of FSH isoforms with lower and not higher isoelectric points when compared with control cultures, as a result of an increased glycosylation rate of the FSH molecule (Kennedy & Chappel, 1985).

The difference between the qualitative patterns of pituitary FSH in castrated and antiandrogen-treated animals could also be related, at least in part, to the androgen withdrawal. It is quite possible that in the flutamide-treated group, the androgen blockade has been partially counteracted by the high testosterone levels. However, the same qualitative effect was recorded in the casodex-treated group, where the androgen blockade on the target organs was shown to be more profound and complete. Alternatively, testicular factors other than androgens, eliminated by castration but not by androgen blockade, could be important in modulating the molecular heterogeneity of pituitary FSH. It is well accepted that, in the rat, inhibin plays a major role in the feedback control of FSH secretion (Tsonis & Sharpe, 1986; Au et al. 1987; Weinbauer et al. 1989). In the present study, inhibin production was completely abolished by castration but maintained or even increased in the antiandrogen-treated animals. This might indicate that inhibin could also play a role in the modulation of the qualitative characteristics of pituitary FSH.

The role of the different isoforms of FSH is not known. Although more biopotent in vitro, the less acidic components of FSH have a shorter plasma half-life and the modulation of the relative amounts of the different isoforms could be a mechanism through which not only the quality but also the concentration of circulating FSH is regulated (Blum & Gupta, 1985). Recent data, however, indicate that deglycosylated molecular species of ovine FSH can induce aromatase stimulation in Sertoli cells in vitro, apparently without increasing cyclic AMP production (Padmanabhan et al. 1991), and it is tempting to speculate that different isoforms could variably activate different intracellular events and cellular responses. If this is the case, one would reasonably expect that different testicular products, like androgens and perhaps peptides of the inhibin family, might in turn preferentially regulate some gonadotrophin isoforms.

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