Alterations of Gene Expression in Adult Male Rat Testis and Pituitary Shortly After Subacute Administration of the Antiandrogen Flutamide

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Abstract. In the course of profiling alterations of gene expression in the male reproductive system induced by anti-androgenic agents, 28 genes expressed in the testis or pituitary of adult rats were examined shortly after subacute administration of the well-known anti-androgen, flutamide (FM). FM (25 mg/kg/day) was orally administered to male rats for six days. On day 8 (D8) after the first dose of FM, intratesticular testosterone (T) levels had dramatically increased, but daily sperm production on D36 was significantly decreased. The mRNA levels of testicular and pituitary genes on D8 were measured by semiquantitative RT-PCR. Among the six testicular steroidogenic enzyme genes, the mRNAs of the P450 side chain cleavage, P450 17α/C17–20 lyase, and 3β-hydroxysteroid dehydrogenase type I (3βHSD) genes significantly increased, whereas 17β-hydroxysteroid dehydrogenase type III slightly decreased. Among the three steroid receptors examined, androgen receptor (AR) and glucocorticoid receptor (GR) mRNAs were significantly down-regulated (29% and 35%, respectively) in the testis, but there was no change in estrogen receptor α. There were no clear changes in expression of the gonadotropin receptors and Sertoli cell specific genes, but a slight increase was observed in expression of the lactose dehydrogenase-c mRNA, a germ cell specific gene. Among the three immediate early genes, c-myc mRNA was increased approximately 1.4-fold. In the pituitary, on the other hand, mRNAs for LHβ and FSHβ subunits and gonadotropin releasing hormone receptor had increased significantly. These results show that subacute FM administration first affected hypothalamus/pituitary hormone gene expression, then altered gonadotropin secretion, and subsequently induced over-expression of testicular steroidogenic enzyme genes. However, the significant up-regulation of 3βHSD and down-regulation of AR mRNAs, despite the higher level of intratesticular T, might be explained by an antagonistic action of hydroxyflutamide retained in the testis. The profiles of alterations in gene expression observed will provide important information for the screening of adult male animals for anti-androgenic chemicals.

Key words: Antiandrogen, Flutamide, Testis, Pituitary, Androgen receptor


The anti-androgen, flutamide (FM), is a non-steroidal androgen receptor (AR) antagonist, that interferes with endogenous androgen binding to ARs in target organs [1]. Several experiments in intact male rats have shown that FM increases...
serum gonadotropin and testosterone (T) concentrations and intratesticular T levels shortly after acute administration [2–4], and a 4-fold increase in serum T concentration was reported in male hamsters [5]. FM appears to first block the negative feedback of T in the hypothalamus and pituitary, and increased LH secretion then enhances T production in the testis [6]. FM administration has also been found to affect the initial step of spermatogenesis and cause a reduced sperm count due to inhibition of differentiation of spermatogonia to spermatocytes [4, 6, 7]. However, the mechanism by which FM inhibits spermatogenesis is still unclear.

To screen for and assess the health risks of exogenous endocrine-active chemicals (EACs), a ‘Tiered-testing scheme’ has been proposed [8, 9]. The Hershberger assay is generally used as an in vivo Tier I screening battery to screen for anti-androgenic EACs [10], but it requires that castration be performed, and the effects of test compounds are evaluated on the basis of accessory sex gland’s weight alone. In addition to the Hershberger assay, a 15 days intact rat sub-chronic in vivo assay has been proposed [11]. This allows observation of the effects of test compounds in a relatively short time and a wide variety of organs, including the testis and pituitary. It permits evaluation of the effects of test compounds on the hypothalamo-pituitary-gonadal axis, which is the most important mechanism in the male reproductive system. However, the endpoints of this assay are still limited to only the weight data for the gonads and accessory sex glands and serum hormone levels (T, 17β-estradiol (E2), 5α-dihydrotestosterone (5αDHT), PRL, FSH, and LH) [12]. Although quantification of mRNA levels is still laborious, advances in biotechnology, especially in gene expression profiling systems, such as gene arrays or DNA chips, have made it easier and more convenient to obtain data on alterations of gene expressions in a variety of tissues exposed to test compounds. In terms of toxicogenomics [13, 14], it will be also necessary to provide precise information for databases concerning the alterations of gene expression when test compounds are administered to laboratory animals. Profiles of alterations of gene expression when an anti-androgen is administered to intact animals will be more important for researches of anti-androgenic EACs. However, precise information on alterations of gene expression, especially in the testes, is still limited for anti-androgenic compounds administered to intact laboratory animals.

In the present study, we analyzed the effects of subacute FM administration to adult male rats on the efficiency of spermatogenesis, serum and testicular hormone levels, and gene expression profiles for the establishment of a more reliable method of evaluating the effects of anti-androgenic compounds. The specific targets of this study were the alterations of gene expression in the testis and pituitary. Dramatic reductions in androgen and glucocorticoid receptor mRNA levels in the testis were found.

## Materials and Methods

### Materials

Flutamide (FM; 2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]propamide) was purchased from Sigma (St. Louis, MO). The corn oil used to dissolve FM and used as the vehicle control was also bought from Sigma. Serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were determined with an enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Serum and intratesticular T levels were determined with an enzyme immunoassay (EIA) system (Cayman Chemical Co., Ann Arbor, MI). SuperScript™ II RNase H- Reverse Transcriptase and oligo(dT)12-18 primer were purchased from TaKaRa Biomedicals (Otsu, Shiga, Japan). The plasmid pGEM-T Easy vector was obtained from Promega Corp. (Madison, WI).

### Animals and treatment

Male Holtzman rats were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN) and bred in our own facility. They were maintained in a controlled environment at a temperature of 24 ± 1 C, humidity of 45 ± 5%, and on a 12/12 h light/dark cycle. The animals were given access to food and distilled water ad libitum. At 13 weeks of age males were randomly selected and divided into four groups (n=8), so that average body weight was the same in each group. The rats were given
vehicle (corn oil) or FM (25 mg/kg/day, in corn oil) orally for 6 days. At 8 days (D8) and 36 days (D36) after the first dose, rats were sacrificed by exsanguination from the aorta under ether anesthesia. The serum was separated and stored at −20°C in a freezer. Both testes were excised, and the surrounding adipose tissue was carefully removed. The pituitary was also quickly removed. The specimens were frozen in liquid nitrogen immediately after excision, and maintained at −80°C until daily sperm production and intratesticular T concentration were measured and RNA was extracted.

**Daily sperm production**

Testes were homogenized in 10 mM phosphate-buffered saline (PBS, pH 7.4) in a polytron homogenizer, and the homogenization-resistant spermatid nuclei were counted with a hemocytometer. The numbers of homogenization-resistant spermatid nuclei per testis were calculated and then divided by 6.1 days to convert them to testicular daily sperm production (DSP) values [15].

**Hormone assay**

Serum LH, FSH, and T and intratesticular T levels were determined by using the corresponding EIA systems. The serum samples collected were directly applied to the well in the kit, and measurements were made according to the procedure described by the manufacturer. To measure intratesticular T levels, the frozen right testis was homogenized in 8 ml of 10 mM PBS with a polytron homogenizer. The homogenate (2 ml) was then extracted with diethyl ether, and the ether phase was dried in air. The serum was also extracted with diethyl ether, and the ether phase was dried in air. The dried lipophilic substances were resuspended with the proper volume of the buffer in the kit, and the measurements were made according to the procedure described by the manufacturer.

**Semiquantitative RT-PCR**

Total RNA was extracted from the testes and pituitaries (n = 8) by the basic protocol of Chomczynski and Sacchi [16]. RNA samples (testis, 8 µg; pituitary, 1.25 µg) were reverse-transcribed for 50 min at 42°C in a 40-µl reaction mixture with 400 units of SuperScriptTM II reverse transcriptase and 1 µg of oligo(dT)12–18 primer according to the standard protocol of the supplier. Each PCR reaction mixture (50 µl) contained 0.2 mM of each dNTP mixture, 0.4 µM of each primer, and 1 µl of the cDNA reverse-transcribed as described above. Taq polymerase was added to the reaction tube as 2.5 units of TaKaRa LA Taq™ polymerase in 1 × GC Buffer I or 1.25 units of TaKaRa Ex Taq™ polymerase in 1 × Ex Taq™ Buffer. PCR was performed by denaturing at 94°C for 30 sec, annealing for 30 sec, and extension at 72°C for 45 sec. Table 1 shows the primer sequences, PCR product sizes, optimized cycles, annealing temperatures, Taq polymerase used, and GenBank accession numbers for all genes examined in this study: the genes coding cytochrome P450 side chain cleavage (P450scc), cytochrome P450 17α/C17–20 lyase (P450c17), 3β-hydroxysteroid dehydrogenase type I (3βHSD), 17β-hydroxysteroid dehydrogenase type III (17βHSD), cytochrome P450 aromatase (P450arom), 5α-reductase type I (5αR-I), androgen receptor (AR), estrogen receptor α (ERα), glucocorticoid receptor (GR), LH receptor (LHR), FSH receptor (FSHR), protamine-2 (Pm2), lactose dehydrogenase-c (LDH-C), acrosin, low affinity nerve growth factor receptor (LNGFR), androgen binding protein (ABP), transferrin (Tf), Sertoli glycoprotein-1 (SGP1), α subunit of LH and FSH (Gna), β subunit of LH (LHβ), β subunit of FSH (FSHβ), gonadotropin releasing hormone receptor (GnRHR), β subunit of thyrotropin stimulating hormone (TSHβ), prolactin (PRL), c-fos, c-jun, c-myc, Egr1, cyclophilin (CP), and β-actin. The PCR products (5 µl) were then separated on 2% agarose gel. The bands on the UV-transilluminated gel were converted into digital images with a gel analyzer (ATTO Inc., Bunkyo-ku, Tokyo, Japan), and the amounts of RT-PCR products were quantified with Scion Images software (Scion Corporation, Frederick, MD). PCR products for CP or β-actin were used as an internal standard. To determine the sequences, the PCR product for each gene was subcloned into pGEM-T Easy vectors and sequenced by the dideoxynucleotide chain termination method using the ABI Prism BigDye terminator cycle sequencing kit (PE-Biosystems, Foster City, CA).

**Statistical analysis**

StatView for Windows version 5.0 (SAS Institute Inc., Cary, NC) was used for the statistical analysis.
All results are shown as means ± SE. Data were analyzed by the two-tailed Student’s *t*-test. Significance was accepted at *p* < 0.05.

Results

Testicular weight and daily sperm production

Table 2 shows the body weights, testicular weights, daily sperm production (DSP), and efficiency of testicular sperm production calculated by dividing the DSP number by testicular weight (DSP/gt). Administration of FM for 6 days did not result in any changes in body weight on either D8 or D36 compared to the vehicle-treated control. The testicular weight of the FM-treated animals on D8 was significantly higher than in the control group, but there was no difference on D36. DSP and DSP/gt, on the other hand, were significantly lower on D36 (79.5% and 76.9% of the control, respectively), but there was no difference on D8.

Serum and testicular hormone concentrations

Table 3 shows the changes in serum LH, FSH, and T levels. The LH level was significantly increased (1.2-fold) by FM on D8, but there was no change on D36. The FSH levels on both D8 and D36 were slightly higher than in the control, but the difference was not statistically significant. Serum T was increased 3.4-fold over control on D8 (*p* < 0.001), but there was no significant difference on D36. Similarly, there was a 4-fold increase in intratesticular T level on D8 (FM, 720 ng/g testis; control, 170 ng/g testis; Fig. 1). However, no difference between FM-treated and vehicle-treated animals was observed in intratesticular T levels on D36, which was slightly lower than that of control (Fig. 1).

Table 1. Primers used for semiquantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence Product size (bp)</th>
<th>Cycle used</th>
<th>Anneal Temp (C)</th>
<th>Taq used</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primer (5’ to 3’)</strong></td>
<td><strong>Reverse primer (5’ to 3’)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450sc</td>
<td>CGCTCACTGCTGCTGTCAAAA</td>
<td>TCTGGTAGACCGCCGTCGAT</td>
<td>688</td>
<td>23</td>
<td>55</td>
</tr>
<tr>
<td>P450c17</td>
<td>GACCAAGGCCAAGACGCGT</td>
<td>GCATTCCAGATACCTTCTC</td>
<td>302</td>
<td>24</td>
<td>55</td>
</tr>
<tr>
<td>3βHSD</td>
<td>TTGGTCAGGAGAGAAGAAC</td>
<td>CGGCAAGTATCATGAGACGA</td>
<td>547</td>
<td>24</td>
<td>55</td>
</tr>
<tr>
<td>17βHSD</td>
<td>TTCTCAGGAGGCCTTACAGGGG</td>
<td>AACAACTCAGCCGCGCTTCT</td>
<td>653</td>
<td>26</td>
<td>55</td>
</tr>
<tr>
<td>P450arom</td>
<td>GTGCCGTCAACTACTACAATAAAG</td>
<td>CTTAATTTTGCATTAGGCAAGCAG</td>
<td>521</td>
<td>32</td>
<td>60</td>
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<tr>
<td>5αR-I</td>
<td>ATGGAGTGGAGTGAGTCTCTG</td>
<td>TTCAAGCTACTGTGAGTCAAGCAG</td>
<td>508</td>
<td>25</td>
<td>60</td>
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<tr>
<td>AR</td>
<td>TGGCCCTTGTGTTATCTACTGCTCA</td>
<td>ACCATTAGGGAGTAGTTTAGCACGCA</td>
<td>570</td>
<td>26</td>
<td>60</td>
</tr>
<tr>
<td>ERez</td>
<td>TTACGAAGTGCGATGATGA</td>
<td>ATCTTCCAGAGCTGGTGTGATG</td>
<td>711</td>
<td>26</td>
<td>60</td>
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<tr>
<td>GR</td>
<td>GGAAAAATTATGACACACATCAAC</td>
<td>GCAGTGGTAAGGAGATCTTCAA</td>
<td>539</td>
<td>26</td>
<td>60</td>
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<tr>
<td>LHR</td>
<td>CTCATCTATTCCTCTGGTCAAGAT</td>
<td>ACAGACTGCTGCTTCAATCCCTTG</td>
<td>365</td>
<td>25</td>
<td>60</td>
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<tr>
<td>FSHR</td>
<td>GGTCTTCATGTGACCTTGGG</td>
<td>AACTATTGITTGAGCTTGAGGCTG</td>
<td>652</td>
<td>26</td>
<td>60</td>
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<tr>
<td>Pn2</td>
<td>GGTGGCGTGAAGGAAGATGA</td>
<td>CAATTCTTCTCCTGAGCTGCAG ATC</td>
<td>265</td>
<td>25</td>
<td>55</td>
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<tr>
<td>LDH-C</td>
<td>CAAGGAGCACTTAAATTGACAAAGCACAGAGTGAAAG</td>
<td>CTTAATTTTGCATTAGGCAAGCAG</td>
<td>522</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>Acerin</td>
<td>TCAGCGGCTCAGCTGCTGCTGGT</td>
<td>CGACCGCTTCTTGCACATGGAG</td>
<td>502</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>LNGFR</td>
<td>CAAACCTTCTGCAAGAGAAGTCTATTG</td>
<td>CTGAAATGAGGGGTTGCCACTTG</td>
<td>732</td>
<td>27</td>
<td>60</td>
</tr>
<tr>
<td>ABT</td>
<td>CTATGCCTGAGAAGATGTCGGGGG</td>
<td>CTGAAATGAGGGGTTGCCACTTG</td>
<td>533</td>
<td>21</td>
<td>60</td>
</tr>
<tr>
<td>FSHR</td>
<td>TGCAGCTGCTGCTGCTGCTGCT</td>
<td>TCTGCTCAGCTGCTGCTGCTG</td>
<td>428</td>
<td>22</td>
<td>60</td>
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<tr>
<td>SGPI</td>
<td>GTGCAGCAACGGCGAAGTATGCTG</td>
<td>GAATTCGAGACTGCTGAGCTG</td>
<td>484</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>Gna</td>
<td>ATGGGATGCTACAGAACAGT</td>
<td>TGGAAATGAGGGGTTGCCACTTG</td>
<td>501</td>
<td>19</td>
<td>60</td>
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<tr>
<td>LHβ</td>
<td>TGTGCTGCTCAGGAGAAGATGA</td>
<td>GAAATGAGGGGTTGCCACTTG</td>
<td>292</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>FSHR</td>
<td>TGTTGCTCAGCTGCTGCTGCT</td>
<td>TGGAGTATCTTCTGAGCTG</td>
<td>757</td>
<td>22</td>
<td>60</td>
</tr>
<tr>
<td>GnRHR</td>
<td>ATGGCTAACATGCTGCTGCTG</td>
<td>ATATAATGGTGCTGCCCTG</td>
<td>877</td>
<td>26</td>
<td>50</td>
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<tr>
<td>TSHβ</td>
<td>ATGGCTGCATGCTGCTGCTG</td>
<td>CACCTCAGCTGCTGCTG</td>
<td>358</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>PRL</td>
<td>ACACCTCATTCTGCTGCTGCTG</td>
<td>GAAATGAGGGGTTGCCACTTG</td>
<td>630</td>
<td>17</td>
<td>60</td>
</tr>
<tr>
<td>c-fos</td>
<td>GAAGATGCGCATATGACGAAAG</td>
<td>AGGAAATGAGGGGTTGCCACTTG</td>
<td>484</td>
<td>31(27)</td>
<td>58</td>
</tr>
<tr>
<td>c-jun</td>
<td>GAGTACGCACTGCTGCTGCTG</td>
<td>AGGAAATGAGGGGTTGCCACTTG</td>
<td>306</td>
<td>31,28(25)</td>
<td>65</td>
</tr>
<tr>
<td>c-myc</td>
<td>CACCCAGGAGCCGTTAGAGGAAGAG</td>
<td>AGTCCCGCAGCTCATTCCCTTCTG</td>
<td>593</td>
<td>31,29(25)</td>
<td>65</td>
</tr>
<tr>
<td>Egr1</td>
<td>CCCATCACTGCTGCTGCTGCTG</td>
<td>GGGGTTGATGAGGGCTGTGCTG</td>
<td>569</td>
<td>30</td>
<td>65</td>
</tr>
<tr>
<td>CP</td>
<td>TGGAGGAGGGGTTGCCACTTG</td>
<td>AGGAAATGAGGGGTTGCCACTTG</td>
<td>524</td>
<td>18,21(61)</td>
<td>60</td>
</tr>
<tr>
<td>β-actin</td>
<td>CTTGAGGCTCTGCTGCTGCTG</td>
<td>AATCTCAGACAGACTGCTGCTG</td>
<td>359</td>
<td>22</td>
<td>60</td>
</tr>
</tbody>
</table>

a used for the testis samples.
b used for the pituitary samples.
Gene expression profiles

Since the samples on D8 were expected to show more drastic changes by FM than on D36, total RNAs from testis and pituitary on D8 were subjected to semiquantitative RT-PCR. Figure 2 shows the amplification curves of all genes analyzed in this study to optimize the cycles for quantification. In the testis sample from a control rat, the PCR product for cyclophilin (CP) mRNA was first detected in the early cycles, suggesting that CP was the most abundant gene in the testis. The PCR product for P450arom mRNA, on the other hand, could not be detected at less than 30 cycles, suggesting an extremely low level in the testis. Actually, we used two sets of primers for AR with DNA sequences located in different regions in the AR open reading frame. The band intensities of the two PCR products of AR were the same on the corresponding cycles (data not shown).

Steroidogenic enzymes: Among the six steroidogenic enzymes examined, marked up-regulations, as large as 2-fold, of P450scc and P450c17 mRNAs were detected (Fig. 3). When standardized by CP, the 3βHSD mRNA level was 2.4-fold higher than the control (Fig. 3). 17βHSD mRNA was slightly reduced by FM (statistically significant when corrected by CP, but not by β-actin). The 5αR-I mRNA level was reduced to 79% (by CP), but no significant change was observed in P450arom (Fig. 3). Other steroidogenic enzyme genes, 17β-HSD-type I, 17β-HSD-type II, and 5α-reductase type II, were examined in testis samples, but no signals were detected even after 40 cycles of the PCR reaction (data not shown).

Steroid hormone receptors: In the testis, significant decreases in the mRNAs of two of the

Table 2. Effects of subacute FM administration on the reproductive system of the adult male rat

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>491.8 ± 11.9</td>
<td>470.9 ± 11.5</td>
</tr>
<tr>
<td>Paired testicular weight (g)</td>
<td>3.71 ± 0.07</td>
<td>3.95 ± 0.09*</td>
</tr>
<tr>
<td>DSP (× 10⁶)</td>
<td>4.20 ± 0.01</td>
<td>4.41 ± 0.12</td>
</tr>
<tr>
<td>DSP/gt (× 10⁶)</td>
<td>2.27 ± 0.07</td>
<td>2.23 ± 0.07</td>
</tr>
<tr>
<td>D36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>518.4 ± 19.3</td>
<td>528.3 ± 12.8</td>
</tr>
<tr>
<td>Paired testicular weight (g)</td>
<td>3.82 ± 0.08</td>
<td>3.78 ± 0.27</td>
</tr>
<tr>
<td>DSP (× 10⁶)</td>
<td>4.10 ± 0.11</td>
<td>3.26 ± 0.10**</td>
</tr>
<tr>
<td>DSP/gt (× 10⁶)</td>
<td>2.12 ± 0.05</td>
<td>1.63 ± 0.04**</td>
</tr>
</tbody>
</table>

Male rats 13 weeks old were daily dosed vehicle or FM (25 mg/kg) for six days. Data were expressed as means ± SE (n=8; * p<0.01; ** p<0.001).

Table 3. Effects of subacute FM administration on the serum hormone levels of the adult male rat

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>19.21 ± 0.34</td>
<td>23.62 ± 0.28**</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>129.02 ± 13.50</td>
<td>137.88 ± 7.25</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>1.40 ± 0.78</td>
<td>4.73 ± 0.59**</td>
</tr>
<tr>
<td>D36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>22.56 ± 1.31</td>
<td>22.25 ± 2.40</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>88.69 ± 10.51</td>
<td>109.37 ± 10.45</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>0.42 ± 0.20</td>
<td>0.68 ± 0.15</td>
</tr>
</tbody>
</table>

Male rats 13 weeks old were daily dosed vehicle or FM (25 mg/kg) for six days. Data were expressed as means ± SE (n=8; ** p<0.01).
Fig. 2. Optimization of amplification cycles for each gene in the semiquantitative RT-PCR analysis. A, Amplification curves of PCR-products of 23 genes from the testicular cDNA sample of a vehicle-treated rat. B, Amplification curves of PCR-products of 15 genes in the pituitary. The PCR cycles used for semiquantitative analysis were determined from the linear regression phase of these curves and are indicated in Table 1.

Fig. 3. Semiquantitative RT-PCR analysis of the effect of FM on testicular steroidogenic enzyme mRNA levels. Relative amounts of RT-PCR products from 6 steroidogenic enzyme mRNAs (P450scc, P450c17, 3βHSD, 17βHSD, 5αR-I, P450arom) were calculated by dividing by one of the two internal controls, CP (C) or β-actin (β). The values expressed are means ± SE (n=8). Note the significant increases of P450scc, P450c17, and 3βHSD mRNA levels. Significant differences from means in the control were analyzed with Student’s t-test (a: P<0.05, b: P<0.01, c: P<0.001).
three steroid receptors, AR and GR, were found (29% and 35%, respectively, of the control by β-actin standardization), but there was no significant change in ERα mRNA (Fig. 4A). There were no such decreases in AR or GR mRNA levels in the pituitary from the same individuals on D8 (Fig. 4B). The ERα level in the pituitary was somewhat decreased, but the difference was not statistically significant. Other steroid receptor genes, the genes for ERβ and progesterone receptor, were examined in the testis samples, but no signals were detected even at 40 cycles of the PCR reaction (data not shown).

Gonadotropin receptors, Sertoli cell proteins and spermatogenic cell-specific genes: The levels of gonadotropin receptors, LHR and FSHR, expressed in Leydig cells and Sertoli cells, respectively, were somewhat lower than in the control, but the differences were not statistically significant (Fig. 5). LNGFR, also expressed in Sertoli cells, was unchanged (Fig. 5). Although a slight but not statistically significant increase was seen in ABP, transferrin and SGP1, both major secretory proteins of Sertoli cells, were unaltered by FM administration. The mRNAs of germ cell specific genes, i.e., the gene for protamine-2, specifically expressed in haploid germ cells, and the gene for LDH-C, which is transiently expressed in spermatocytes and early phase spermatids, were slightly increased, but statistical significance was only detected for LDH-C mRNA (Fig. 5). The mRNA of acrosin, a pachytene stage specific protein, was completely unchanged by FM.

Gonadotropin subunits and other pituitary genes: Among the three genes for gonadotropin subunits expressed in the pituitary, significantly increased mRNA levels of LHβ and FSHβ subunit were detected (2.9-fold and 2.2-fold, respectively, the control levels by β-actin-standardization), and a 1.5-fold increase in LH/FSHα (Gnα) subunit mRNA was seen, but the difference was not statistically significant (Fig. 6). GnRHR mRNA increased significantly (2.1-fold the control value by β-actin-standardization), but there were no changes in TSHβ subunit or PRL mRNA levels.

Immediate early genes: No significant increases in the mRNAs of the immediate early genes c-fos and c-jun, were detected in the testis, but a 1.4-fold increase in c-myc mRNA (by CP-standardization) was observed (Fig. 7A). In the pituitary, c-fos, c-jun, and Egr1 mRNAs had decreased to 74%, 67%, and 77%, respectively, of control levels (by CP-standardization), but only the difference in c-jun mRNA was statistically significant (Fig. 7B).
The present study showed a slight but statistically significant increase in testicular weight in the FM-treated rats on D8, but no changes in daily sperm production (DSP). By contrast, on D36, no change in testicular weight was detected, but DSP was significantly reduced. Since there was no change in DSP, the increase in testicular weight appeared to be attributable to an increase in volume of other compartments, not the homogenization-resistant spermatid heads. The semiquantitative RT-PCR analysis revealed slight increases in the mRNA levels (not significant) of ABP, a Sertoli cell-secreted protein [17] and protamine-2, a haploid germ cell specific protein [18], and a significant increase in the mRNA level of LDH-C, a germ cell specific glycolytic enzyme [19]. The increase in testicular weight on D8 may be explained by the increased levels of these relatively abundant testicular proteins. Actually their genes should have been stimulated by the increased level of gonadotropins or T, as described below.

It has been well documented that it takes about 49 days for type A spermatogonia to develop to step 19 spermatids, which are homogenization-resistant, in the rat seminiferous tubule [20]. Thus, the reduction of DSP on D36, but not on D8, means that FM administration suppressed the early stage of spermatogenesis, that is, differentiation of spermatogonia or early phase spermatocytes, and the effect appears to be transient. Our method, i.e., measuring DSP 36 days after the first dose of a test compound, would be useful for detecting slight changes in numbers of elongated spermatids, if the test compounds affected the proliferation and differentiation of spermatogonia or spermatocytes [21]. Consistent with our data, early histopathological studies in male rats given FM for 15 days clearly demonstrated that FM affected spermatogenesis: the ratio of spermatogonia to spermatids was reduced on the day of the final injection, indicating that FM affected the early stages of spermatogenesis [4, 6, 7].

**Discussion**

**Subacute FM administration affects the early stage of rat spermatogenesis**

The dramatic increase in serum and intratesticular T levels shortly after FM administration blocks negative feedback of T to the hypothalamus and pituitary

The dramatic increase in serum and intratesticular T levels shortly after FM
administration (on D8) must have been due to an increase in LH secretion, and this was corroborated by the increased level of serum LH and pituitary LHβ mRNA levels. It can be explained by an inhibitory action of FM on the negative feedback effects of T on the hypothalamus and pituitary, and subsequent enhancement of the LH or GnRH pulse [6, 7]. In the present study, there was no significant increase in LH/FSHα (Gnα) subunit mRNA. Gnα expression has been documented to also be regulated by GnRH pulse, because it has been reported to increase 3-fold in male rats one week after castration [22, 23]. FM administration also significantly increased the GnRHR mRNA level (2.1-fold) in the pituitary (Fig. 6). In the male rats, GnRHR mRNA has been reported to increase 5-fold in male rats 21 days after castration [24, 25], and this increase is presumably due to an increased GnRH pulse caused by termination of the negative feedback effect of T. Thus, the above result also supports the contention that FM administration inhibits the negative feedback effects of T on the hypothalamus and pituitary enhancing the GnRH pulse.

However, Dalkin and coworkers [22, 23] reported that 6-fold increases in LHβ mRNA and 4-fold increases in FSHβ mRNA were observed in castrated rats stimulated by exogenous GnRH pulses with an implanted silastic tube containing T, whereas the increase in Gnα was not statistically significant. We detected a 1.5-fold increase in Gnα mRNA level, although the increase was not statistically significant, which seems similar to the findings in their report, suggesting that endogenous T was still effective in the pituitary of rats shortly after FM administration. In addition, there was no significant change in serum FSH level, whereas the FSHβ mRNA content in the pituitary was 2.2-fold higher than in the control (Fig. 6). This difference might be due to the sensitivity of the FSH assay system. However, we did not detect any significant increase in mRNA levels of testicular ABP or Tf, the Sertoli cell secretory proteins, which are well known to be up-regulated by FSH.

Fig. 6. Semiquantitative RT-PCR analysis of the effect of FM on pituitary hormone and receptor gene mRNA levels. Relative amounts of RT-PCR products from gonadotropin subunit (Gnα LHβ, FSHβ), gonadotropin releasing hormone receptor (GnRHR), and other hormone (TSHβ, PRL) mRNAs were calculated by dividing by one of the two internal controls, CP (C) or β-actin (β). The values expressed are means ± SE (n=8). Note the significant increases in LHβ and FSHβ subunit mRNA levels. Differences between the means and the control were analyzed for significance with Student’s t-test (a: P<0.05, b: P<0.01, c: P<0.001).
stimulation [26]. Testicular and epididymal ABP and plasma Tf were found to be up-regulated in hamsters and humans after FM administration, and the FSH level increased simultaneously [5, 27]. These findings strongly suggest that the serum FSH level on D8 (2 days after the final dose) was already reduced in our study. There is a report, on the other hand, that T increases the FSHβ mRNA content in the pituitary selectively by post-transcriptional mechanisms, independent of the GnRH action, based on the result that the addition of T to rats castrated and implanted with GnRH

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**Fig. 7.** Semiquantitative RT-PCR analysis of the effect of FM on testicular (A) and pituitary (B) immediate early gene mRNA levels. Relative amounts of RT-PCR products of immediate early genes (c-myc, c-fos, c-jun, Egr1) were calculated by dividing by one of the two internal controls, CP (C) or β-actin (β). The values expressed are means ± SE (n=8). Note the significant increase in c-myc mRNA levels in the testis. Differences between the means and the control were analyzed for significance with Student’s t-test (a: P<0.05, b: P<0.01, c: P<0.001).
The up-regulation of the three steroidogenic enzyme genes must be due to an inhibitory action of FM on the negative feedback effect of T on the hypothalamus and pituitary, and subsequent increase in LH secretion.

An early endocrinological study reported an increase in P450scc enzyme activity in response to LH or hCG injection of intact male rats [35]. However, a single high dose of LH/hCG decreased testicular T synthesis, a phenomenon referred to as Leydig cell desensitization [36], which was revealed to be due to reduction of LHR expression and decreased levels of P450c17 enzyme activity [37]. In our study, there was no significant change in LHR mRNA expression, suggesting that an increased level of LH is insufficient to cause the desensitization. By contrast, repeated administration of a relatively low dose of LH/hCG for 6 days was reported to increase T synthesis and enzyme activities of P450c17 and 3βHSD with a slight decrease of 17βHSD [36]. The changes observed in mRNA expression levels of P450c17, 3βHSD, and 17βHSD in the present study strikingly resemble the changes in enzyme activity described in the earlier report, suggesting that the subacute FM administration caused medium amplification of pulsatile LH secretion.

It has been well-documented in a study that used a cultured Leydig cell line and promoter analysis that transcription of P450scc, P450c17, and 3βHSD genes, all of which were up-regulated by FM in our study, is mediated by cAMP, mostly due to LH signaling [38]. However, the nuclear factors modulating the steady-state or induced level of P450scc and P450c17 expression differ from each other [39]. Moreover, expression of the 3βHSD gene was found to be inhibited by T via AR in a trans-acting manner [40]. Based on these findings, a significant increase in 3βHSD, despite the 4-fold intratesticular T level in the present study, strongly suggests that the level of FM, probably hydroxyflutamide (OH-FM), was still high enough in the testis on D8 to antagonize T binding to AR and then blocked the inhibition of 3βHSD gene expression. Presumably, this inhibitory effect on the blockade of 3βHSD gene expression and enhancements of P450scc and P450c17 gene expression by increased levels of LH synergistically amplified T production in the FM-administered rats.

The immediate early genes, c-fos and c-jun, encode two components forming activator protein-1 (AP1), a potential transcription factor, and their transcription is rapidly up-regulated by activation of cytoplasmic kinase [29, 30]. Expression of both genes is induced in the pituitary by GnRH stimulation, and they up-regulate LHβ and FSHβ gene transcription. In the present study, the mRNA levels of c-fos and c-jun on D8 were lower than in the control (although the difference in c-fos mRNA was not significant), suggesting that LHβ and FSHβ up-regulation may have already been reduced 2 days after the final FM injection. Egr1, another immediate early gene, has been reported to be rapidly induced in the pituitary by GnRH stimulation and has a responsive element in the LHβ gene promoter [31]. The somewhat lower Egr1 mRNA level (not significant) than in the control appears to be caused by the same reason as the c-jun reduction.

The increase in intratesticular T level is due to up-regulation of the 3β-hydroxysteroid dehydrogenase gene

Among the six steroidogenic enzyme genes assessed in this study (Fig. 3), the most obvious effect of FM was up-regulation (2.5-fold) of the 3β-hydroxysteroid dehydrogenase type I gene, an isoform showing the most dominant expression of all 3βHSD isoforms [32]. P450scc and P450c17 were also up-regulated, 2-fold and 1.8-fold, respectively, over the control, whereas 17β-hydroxysteroid dehydrogenase type III, the only 17βHSD isoform expressed in the testis [33], was reduced slightly, but with statistical significance. The 3βHSD-type I up-regulation appeared to cause the extraordinary increase in intratesticular T level. Consistent with the present study, early studies of FM reported that 3βHSD enzyme activity increased more than any of the other steroidogenic enzymes in male rats [34].
Marked decrease in AR and GR mRNA levels in the testis of rats subacutely administered FM

Although there was no change in ERα mRNA, both AR and GR mRNAs were down-regulated in the testis by FM (29% and 35%, respectively, of the control by β-actin standardization). The magnitude of AR mRNA down-regulation was the most dramatic change among the genes examined in the present study, and this is the first report of AR gene alteration by an anti-androgenic compound. In the adult rat testis, AR protein has been localized to Sertoli cells, peritubular myoid cells, and Leydig cells [41]. An *in situ* hybridization study showed that the signal for AR mRNA was most intense in the Sertoli cells at stage VII-VIII of the seminiferous tubule [42]. The AR gene itself is a target for androgen, and AR mRNA levels in the ventral prostate of adult rats have been shown to be down-regulated by androgens, suggesting the presence of an autoregulation system on the AR gene [43, 44]. There have been several studies on AR-autoregulation in the testis. In rats pretreated with ethane dimethane sulfonate (EDS), which specifically inhibits Leydig cell function subsequently inhibiting T production, the AR mRNA level in the testis was unchanged, suggesting that there is no autologous regulation by androgen in the testis [45]. However, in the GnRH-analogue-treated rat model, in which LH secretion and T production are inhibited, exogenous LH or synthetic androgen R1881 restored immunoexpression of AR protein in Sertoli cells [46]. On the other hand, a 4-fold increased in testicular AR mRNA level has been reported 15 days after hypophysectomy [41]. As the above findings suggest, autologous regulation of the AR gene in the testis is still a matter of controversy.

The AR mRNA down-regulation observed in the present study appears to be the result of the markedly higher level of intratesticular T, which seems to be due to the same mechanism as in the prostate. However, as described above, based on the trans-acting inhibitory effect of T on the 3βHSD gene, it is likely that the OH-FM level in the testis on D8 was still high enough to antagonize androgen-binding to AR [40]. Moreover, we could not detect any changes in the mRNA level of LNGFR, a subunit of NGF-receptor expressed on the Sertoli cell surface. The LNGFR gene has been well-documented to be directly down-regulated by T [47, 48]. A more than 10-fold increase in LNGFR mRNA level has been shown in EDS-treated (T free) or hypophysectomized rats [47], and the LNGFR gene has a promoter region that is responsive to T [48]. The absence of any significant change of LNGFR in the FM treated testis, in spite of the markedly higher level of intratesticular T, strongly suggests that T did not suppress the LNGFR gene. This appears to mean that an antagonistic activity of the higher level of OH-FM retained in the testis on D8 blocked the inhibitory effect of T on LNGFR expression. If the OH-FM level in the testis on D8 was still high enough to antagonize androgen-binding to AR, T probably maintains AR gene expression in the rat testis, rather than down-regulating it, as in the prostate.

In the testis, GR has been reported to be localized to Leydig cells [49]. Although there have been no reports on the regulation of GR gene expression by androgen in the testis, a glucocorticoid and GR complex has been reported to inhibit cAMP-dependent up-regulation of P450scC gene expression [50], suggesting that GR is one of the regulatory factors for T production in the testis. Therefore, down-regulation of GR mRNA by FM to the same level as AR mRNA seems to be implicated in the markedly higher level of T production after FM treatment.

Burnstein and coworkers [51, 52] are trying to identify the AR gene transcription machinery on a molecular basis. Using a prostate cancer cell line PC3, they identified two distinct AR responsive elements in exon D and exon E of the AR gene itself, that were found to be involved in the up-regulation of AR gene transcription by androgen [51]. They subsequently discovered that the elements contain specific DNA sequences bound to the proto-oncogene: immediate early gene products Myc and Max, suggesting that these proteins might be involved in the AR gene autoregulation system [52]. The significant up-regulation of c-myc mRNA by FM in the present study might also implicate Myc in the AR gene down-regulation.

*Overproduction of T or down-regulation of AR gene expression may be involved in the reduced sperm production*

It has recently been pointed out that abnormally high intratesticular T levels inhibit spermatogenesis [53–55]. Rats exposed to 6 Gy medium strength gamma irradiation showed
markedly increased levels of intratesticular T (3-fold) with complete loss of spermatogenesis. GnRH antagonist administration to these rats decreased the LH and intratesticular T levels and then stimulated spermatogonial reproliferation [54]. A mutant mouse, juvenile spermatogonial depletion (jsd), also showed a higher level of intratesticular T (3-fold) with complete loss of spermatogenesis. In this model, spermatogonial reproliferation was also stimulated by GnRH analogue [55]. The results of these series of studies by Meistrich and coworkers strongly suggest a greatly increased level of intratesticular T directly inhibits spermatogenesis. They hypothesized that the inhibition is due to apoptosis of spermatogonia by high T levels unrelated to gonadotropin [54]. More recently, they showed that this spermatogenic inhibition by high T levels is mediated by AR [56, 57]. In the present study, subacute FM administration produced a 4-fold intratesticular T level 2 days after the final administration (D8), but not one month later (D36). This temporary increase in the T level may affect the early stage of spermatogenesis through the same mechanism described in the studies by Meistrich and coworkers, and then reduce DSP on D36. Since we did not measure the concentration of OH-FM in the testis on D8 in the present study, we could not compare the testicular OH-FM with the T level. A study on FM metabolism in 6-week-old male rats showed that the plasma OH-FM level after a 10 mg/kg single dose remained above approximately 100 ng/ml for 24 hrs [58]. As described above, based on the report by Paul et al. [28] our finding that the pituitary FSHβ mRNA level had increased 2-fold despite the absence of any difference in serum FSH level suggested that the pituitary T level was higher than the OH-FM level. Based on the reports by Stalvey and Clavey [40] and Shan et al. [46], however, the results showing up-regulation of 3βHSD and down-regulation of AR suggest that the testicular T level is likely to be lower than the OH-FM level. Further investigations will be required to clarify this discrepancy. A possible explanation is that ABP secreted by Sertoli cells may neutralize intratesticular T, making the effective level of intratesticular T much lower than the measured value.

The gene expression profiles presented in this study should provide useful data for initial screening for anti-androgenic compounds in vivo. Future studies with other anti-androgenic drugs, such as Casodex, will be needed to compare the effects of FM and elucidate the differences between the mechanisms of action of different anti-androgenic compounds.

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