Intrauterine Position Effects on Steroid Metabolism and Steroid Receptors of Reproductive Organs in Male Mice

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ABSTRACT

Mice differ in their adult reproductive characteristics as a function of whether they developed in utero between two male fetuses (2M mice), which have higher testosterones levels, or between two female fetuses (0M males), which have higher estradiol levels. The present study was designed to further characterize biochemical parameters of 2M and 0M adult male mice. Activities of several steroidogenic enzymes, namely A^-beta-hydroxysteroid dehydrogenase/17,20-lyase, 11-beta-hydroxylase, and C21-dehydrogenase (3P, 5P), were measured by means of radiometric assays and HPLC fractionation of substrates and products. Activity of 5a-reductase in both seminal vesicle and prostate was measured by similar techniques. Estradiol and androgen receptor concentrations, which indicate capacity to respond to sex hormones, were also examined in the accessory sex organs. For both seminal vesicle and prostate, 5a-reductase activity was approximately 60% greater in 2M mice than in 0M mice, indicating greater capacity to form dihydrotestosterone from testosterone in organs from 2M mice. No significant differences were found in several steroidogenic enzymes between 2M and 0M animals, whereas the trend for all three activities was higher for 2M mice than for 0M males. While no differences were found in estrogen receptor concentrations, 0M prostates had three times the concentration of androgen receptors (occupied receptors) compared to 2M prostates. Our findings suggest that intrauterine fetal position exerts a significant influence on subsequent adult male reproductive androgen responsiveness in reproductive organs of adult male mice.

INTRODUCTION

In mice and rats, a significant component of phenotypic variation among adult individuals of the same sex is due to the contiguity of an animal to siblings of the same or opposite sex during intrauterine development [1, 2]. This naturally occurring phenotypic variation due to random arrangement of fetuses is referred to as the intrauterine position phenomenon [2]. Fetal mouse testes differentiate at approximately Day 15 of gestation and begin to secrete testosterone (T) [3], which remains elevated until birth at approximately Day 19 in CF1 mice [2]. Amniotic fluid and blood of animals that develop between two males (2M) have greater concentrations of T, but lesser concentrations of estradiol, than those of animals that develop between two females (0M) [4-6]. Intrauterine fetal position effects on female development have been studied extensively. Females that develop between two males in utero have a larger anogenital distance than females that develop between two females, i.e., 2M females are slightly masculinized [2]. As adults, 2M female mice are more aggressive, have longer (6-day vs. 4-day) estrous cycles, and cease producing young at an earlier age than do 0M females [4-6]. Intrauterine fetal position also influences male development; however, this has been less extensively described. Gonadally intact 0M males exhibit more mammary and more intromissions when paired with females [6]. When male 2M and 0M mice were castrated at birth to eliminate any postnatal androgen exposure, and then treated with T by implants in adulthood, the 2M males were found to be more aggressive towards other males than were the 0M males [5, 6]. For these reasons, the naturally occurring phenomenon of random intrauterine positioning of males and female fetuses affords a unique model for studying the effects of small physiological differences in fetal hormone concentrations on later development.

In the present study, we investigated certain biochemical parameters that would allow us to further characterize the effects of intrauterine position in adult male mice. We measured activities of three important steroidogenic enzymes to monitor the steroidogenic potential. 5a-Reductase activity was measured in accessory sex tissues (i.e., seminal vesicles and prostate) to determine the ability of 2M and 0M mice to convert T to dihydrotestosterone (DHT), a more potent androgen. In addition, we measured steroid hormone receptors in the accessory sex organs to assess the ability of these tissues to respond to androgens and estrogens.

MATERIALS AND METHODS

Materials
Steroid standards were purchased from Sigma Chemical Co. (St. Louis, MO) and reconstituted from aqueous ethanol prior to use. New England Nuclear (Boston, MA) was the source for [1,2,6,7,16,17]-Htestosterone (H-testosterone) ([1,2,6,7,16]-H-DHT (148 Ci/mmol), [2,4,6,7]-Hestradiol (1069 Ci/mmol), [1,2,6,7]-Hprogesterone (115 Ci/mmol), [2,4,6,7]-Hthi-}

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After the incubation, the reaction was terminated by placing the incubation vials on ice. The sample preparation procedure, using C<sub>60</sub> solid-phase extraction (SPE) columns, was conducted as described and analyzed in detail elsewhere [8]. Briefly, the C<sub>60</sub> SPE columns were activated by diantis 2.0 ml of methanol followed by 5.0 ml of water through the column with positive air pressure. Incubation vial contents were transferred to the column and washed with 8.0 ml methanol/water (1:5 v/v) and 20.0 ml of n-hexane. Steroids were eluted from the column with 4.0 ml of benzene into glass conical tubes. The benzene was evaporated, and the conical tube contents were concentrated to the tip.

The activity of 17α-hydroxylase was calculated by using the value of 5 μM progesterone initially present in each incubation flask and the percentage of the total radioactive metabolites formed, i.e., 17α-hydroxyprogesterone, 4-androsten-3α,17α-dione, and T. Data were expressed as specific activity in nanomoles of product formed in 30 minutes per milligram of protein. Tissue side-chain cleavage activity was determined in exactly the same manner except that 1α-[3H]hydroxyprogesterone was used as substrate. The percentage of conversion to 4-androsten-3α,17α-dione and T was used to calculate specific activity. The enzymatic activity of Δ<sup>5</sup>-3β-hydroxysteroid dehydrogenase/sorase was similarly determined except that 1α-[3H]progesterone was used as substrate and 0.5 mm NAD<sup>+</sup> was used as the cosubstrate. The percentage of conversion to progesterone was used to calculate specific activity.

Quantiﬁcation of Steroid Metabolites by Isocratic HPLC

We employed the use of an isocratic ternary solvent system and a narrow-bore reversed-phase HPLC column to accomplish the separation of progesterone, progesterone, 17α

hydroxyprogesterone, 4-androsten-3α,17α-dione, and T. Steroids were judged to be completely separated from each other on the basis of the resolution of adjacent radioactive peak pairs. The resolution between peaks was always greater than 1.25, indicating complete separation of compounds [7].

A Perkin-Elmer (Norwalk, CT) Series 10 liquid chromatograph equipped with a Rheodyne model 7125 injector, a 100 μl sample loop, a Beckman-Al
tex (Fullerton, CA) × 250-mm Ultrasphere ODS (5 μm) reversed-phase C<sub>8</sub> column, and an Upchurch (Oak Harbor, WA) 2-μm precolumn filter, was used for the chromatography. The elution of 4 and 5 ketosteroids was monitored through UV absorbance detection at 240 nm; the elution of pregnenolone (2 5-mec-3β-hydroxy steroid) was monitored at 215 nm with a Perkin-Elmer model LC-95 11 V/ VIS spectrophotometric detector equipped with an 18-μl flow cell. Detection and quantification of 4-mec-3β-hydroxy steroid was accomplished by use of a Beckman Model 117 on-line radiochromatographic detector equipped with a 500-μl flow cell. An isocratic acetonitrile:methanol:water (53:24:40, v/v/v)
tertiary solvent system at a flow rate of 0.5 ml/min (2000 psi) was employed for the 20-min analysis.

5α-Reductase Radiometric Assay

Prostate and seminal vesicle tissues were transferred to polypropylene homogenizing cylinders and homogenized in an amount of KRBP equal to 10 mg/10 mg tissue wet weight. Homogenization was done using a type PT-10/55 Poltron (Brinkman Instruments, Westbury, NY) was performed on ice so that tissues remained at 0°C. Ten milligrams of tissue were placed into separate incubation vials containing 0.05 μl of 1H-7NADPH [100,000 cpm] and enough reconstituted nonradioactive T so that the final T concentration was 5 × 10^{-7} M in a 1.2-mM incubation volume. The final concentration of β-NADPH, prepared with KRBP in each incubation vial, was 0.5 mM. Incubations were conducted at 22°C for 1 h [8]. Assay conditions for the enzyme reactions were designed to give a substrate conversion rate of no less than 1% and not more than 15% to ensure linearity of the re-action with respect to time and tissue concentration. In or-der to account for any endogenous reducing ability in the homogenates, control tissues were processed in exactly the same manner except that they did not contain β-NADPH. We did not see any measurable endogenous reducing ability in the tissue homogenate. Our preliminary control ex-periments using boiled prostatic and seminal vesicular tissue failed to detect 5α-reduced T metabolites (e.g., 5α-androstane-3α,5β-diol [5α], 5α-androstane-3β,11β-diol [3β], and 5α-DHT), suggesting that little spontaneous reduction of T occurs un-der the above experimental conditions through the 1-h in-cubation period. A C57K/SW steroid elution procedure was used to isolate the androgens from the biological matrix, as described above.

Samples were resuspended in 50 μl HPLC-grade ethanol containing acetylated deoxyribonucleic standards 5α, 3β, and DHT and filtered to aid in UV visualization at 210 nm. A Rainin 0.66 × 25 cm Micro VODS reverse-phase C18 column (5 μm packing) and a Brownlee (Rainin, Woburn, MA) 5.2 mm × 1.5 cm HPLC C18 guard column were used. All other chromatographic conditions were the same as above. An isocratic acetonitrile/water (45:55 v/v) solvent system at a flow rate of 1.0 ml/min (3000 psi) was used for the 20-min analysis. Detection and quantification of 1H-estrone elution was accomplished by use of the Beckman Model 171 on-line radiochromatographic detector equipped with a 1.0-mL flow cell [9]. The counting efficiency of on-line radiochromatographic detection was 20% when scintillation cocktail (Squibb/LE Fisher Scientific, Pittsburgh, PA) was mixed with HPLC effluent (5:1 ratio) in a high efficiency mixer. The formation of 5α-reduced androgens was calculated with use of the value of 5 × 10^{19} M T initially present in the incubation and the percentage of total radioactive 3β, 5α, and DHT formed.

Estrogen Receptor Assay

Estrogen receptors were measured by the hydroxylap-a-tite (HAP) method of Williams and Gorski [14]. Briefly, tis-sues were excised and placed immediately on ice. Tissues were homogenized in 10 mM Tris-HCI, 1.5 mM sodium EDTA, and 1 mM di-thiothreitol (pH 7.5, 25°C) (TED) at a ratio of 1:20 (w/v). Aliquots of the cytosol (100,000 × g for 1 h) containing 0.5–2.0 mg/ml of protein were incubated with 1–10 nM [3H]estradiol overnight at 4°C. Non-specific binding was determined by using 10-fold excess of non-radioabeled steroid in parallel incubations. To separate bound from free hormone, 200 μl of cytosol was added to a 60% slurry of HAP in 50 mM Tris + 1 mM KCl, and allowed to stand on ice with several mixings for 50 min. The HAP pellet was washed five times in 50 mM Tris-HCl, pH 7.5. After the final wash, radioactivity was extracted from the pellet with 2 ml of ethanol and counted in 10 ml of scintillation cocktail.

Androgen Receptor Assay

Tissues were immersed in ice-cold TEDG buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol (v/v), and 1 mM each of di-thiothreitol, phenylenediamine, and sodium molybdate; pH 7.4) [11]. Tissue was homogenized on ice (1 g tissue/5 ml buffer) with 5 sec bursts of a polytron. The homogenate was centrifuged at 20,000 × g for 50 min. This supernatant was used as the low-salt extract and contained the unoccupied cytosolic receptors [12]. The pellet was ho-mogenized every 15 min for 1 h in high-salt buffer (TEDG buffer with 0.4 M KCl) and centrifuged as before. This supernatant was used as the high-salt extract and contained those receptors that were occupied by endogenous hormone [12].

Before incubation with labeled hormone for the binding assay, endogenous steroids were removed from the low-salt extract by incubation with deionized charcoal (DCC: 12.5 mg charcoal plus 1.25 mg deionized/ml of extract). After 1 h at 0–4°C, DCC was removed by centrifugation at 10,000 × g for 10 min. Specific binding of [3H]DHT by the extracts was estimated by competitive inhibition using nonlabeled DHT. An aliquot of extract (500 μl) was incubated with 0.5–200 nM [3H]DHT. Parallel tubes contained 100-fold excess nonradioactive DHT to determine non specific binding. The extracts were incubated for 2 h at 4°C, during which la-beled DHT bound to the unoccupied receptors in the low-salt extract or exchanged with endogenous hormone bound to the occupied receptors in the high-salt extract. Free ster-oid was separated from bound steroid by adding, in du-plicate, 200 μl of extract to 0.5–0.95 ml of packed HAP in 0.5 ml buffer and incubating this mixture for 20 min with several mixings. The samples were then centrifuged for 2 min at 600 × g and 4°C. The supernatant was aspirated, the packed HAP was washed four times with 2 ml ice-cold 10 mM Tris buffer (pH 7.5), and bound hormone was ex-tracted with ethanol as described above.
TABLE 1. Effects of intratesticular position on testicular steroidogenic enzyme activities*  

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>0M</th>
<th>2M</th>
</tr>
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<tbody>
<tr>
<td>Δ⁵-3β-Hydehydroxysterol Dehydrogenase/Isomerase</td>
<td>7.68 ± 0.64</td>
<td>7.89 ± 0.89</td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td>1.34 ± 0.11</td>
<td>1.60 ± 0.30</td>
</tr>
<tr>
<td>C₁₇β-Hydroxysterol Dehydrogenase/Isomerase</td>
<td>2.54 ± 0.13</td>
<td>2.62 ± 0.33</td>
</tr>
</tbody>
</table>

*Data are expressed as nmol product formed/30 min/mg protein (mean ± SEM, n = 7 tissues/group). The designations 0M and 2M represent pups excised in utero for few hours or two males, respectively.

Protein Determination

Protein determinations of tissue homogenates were carried out according to Bradford [13]. Briefly, homogenates were digested with 0.1 N NaOH at 60°C for 1 h, diluted in 1 M HCl buffer (pH 7.5), and assayed in duplicate for protein concentration against a standard curve of 5-50 μg/ml of BSA.

Data Analysis

Data were expressed as enzyme activity or receptor number per milligram protein. Data for enzyme activities and receptor numbers were subjected to one-way ANOVA. For all organ weights, body weight was used as the covariate in covariance analysis. The level of significance was set at p < 0.05.

RESULTS

Testicular Steroidogenic Enzymes

We examined the activities of three enzymes, namely Δ⁵-3β-hydroxysterol dehydrogenase/isomerase, 17α-hydroxylase, and C₁₇β-hydroxysterol dehydrogenase/isomerase. Activities were determined radioisotopically and are expressed as specific activity in nmol/mg protein. All activities were not different between 2M and 0M adult mice (1243 ± 53 vs. 1212 ± 59 mg, respectively) total activity paralleled the values seen for specific activity.

Δ⁵-3β-Hydroxysterol Dehydrogenase/Isomerase

With pregnenolone as substrate and NAD⁺ as the cofactor, progesterone was the single product detected when Δ⁵-3β-hydroxysterol dehydrogenase/Isomerase activity was measured in whole testicular homogenates. No significant differences were seen between 2M and 0M adult mice (Table 1).

17α-Hydroxylase and C₁₇β-Lyase Activity

The conversion of progesterone to androstenedione occurs through two activities of one testicular enzyme [14]. Both activities were measured in 0M and 2M testicular homogenates to evaluate their ability to form androgens from progesterone (Table 1). With progesterone as substrate and NADPH as the cofactor for 17α-hydroxylase activity, whole homogenates converted about 4.5 nmol of progesterone to 17α-hydroxyprogesterone and androstenedione per milligram of protein, with 17α-hydroxyprogesterone predominating throughout 17α-hydroxylase activity. In the 55-min incubation, C₁₇β-lyase activity yielded an average of 2.0 nmol of C₁₇β-steroids from 17α-hydroxyprogesterone.

5α-Reductase Activity

Seminal vesicle. At 5 × 10⁻⁷ M T, conversion rates of 2-5% were obtained under the conditions described in Materials and Methods. Seminal vesicles of 2M mice were

TABLE 2. Effects of intratesticular position on reproductive organ weights and 5α-reductase activity.*

<table>
<thead>
<tr>
<th>Reproductive Organ</th>
<th>0M</th>
<th>2M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>85.0 ± 10.4</td>
<td>131.0 ± 9.9*</td>
</tr>
<tr>
<td>5α-Reductase activity</td>
<td>0.17 ± 0.03</td>
<td>0.25 ± 0.04*</td>
</tr>
</tbody>
</table>

*Data are expressed as nmol product formed/30 min/mg protein (mean ± SEM, n = 7 tissues/group). The designations 0M and 2M represent pups excised in utero for few hours or two males, respectively.

*Significantly different, p < 0.05.
significantly lower and had higher 5α-reductase activity ($p < 0.05$) than seminal vesicles of 0M mice (Table 2). Analysis of variance showed that body weights accounted for virtually none of the variance ($F = 0.1, p = 0.75$) in seminal vesicle weight.

**Prostate**: The mean specific activity of 5α-reductase was significantly greater ($p < 0.05$) in prostates from 2M males than from 0M males (Table 2). The specific activity of 5α-reductase ranged from 0.195 to 0.428 pmol/h/mg protein in 2M males and from 0.079 to 0.267 pmol/h/mg protein in 0M males. Prostatic weight, however, varied significantly ($p < 0.05$) as a function of this ratervative position in that the 0X prostates were heavier than prostates obtained from 2M animals (Table 2). This difference was not due to variation in body weights ($F = 0.7, p = 0.45$). Total 5α-reductase activity was not different since 0M prostates were heavier than 2M prostates.

**Estrogen Receptor Studies**

Specific estrogen receptors were found in cytosols prepared from mouse prostate, whereas cytosols prepared from seminal vesicles exhibited very low binding of [17β-3H]estradiol. Estrogen binding studies using mouse prostate cytosols showed saturable binding by 2 nM estradiol. Scatchard analysis revealed two components: a high-affinity component with low capacity and a lower-affinity component. The addition of 10 nM DHT (to reduce any cross-binding of labeled estrogen to androgen receptors) reduced the lower-affinity component. The main component showed high-affinity binding and low capacity. $K_d = 0.15$ nM and $B_{max} = 30$ fmol/mg protein (Fig. 1). Competition studies carried out as previously described [15], i.e. using 100-fold excess of estradiol, estradiol, T, DHT, progesterone, and cortisol against 2 nM labeled estradiol showed that, in addition to estrogens, only DHT competed with binding of estradiol.

Estrogen binding assays were performed on prostatic tissue in duplicate, with 2 nM [17β]estradiol in the presence of nonradioactive 10 nM DHT. Prostate estrogen receptor concentrations expressed as fmol/mg protein were not significantly different ($p > 0.05$) between 0M and 2M mice (18.66 ± 3.80 vs. 15.77 ± 2.00 fmol/mg protein, respectively; $n = 7$ per group).

**Androgen Receptor Studies**

Androgen binding was demonstrated in both high-salt and low-salt extracts (occupied and unoccupied receptors, respectively) of mouse prostate and seminal vesicle. An equilibrium saturation was reached near 20 nM DHT (Fig. 3). The observed affinity of DHT binding ($K_d = 3.6$ nM) was comparable to values previously reported [16, 17]. Time course incubations showed that androgen binding was maximal from 8 to 24 h of incubation at 4°C. Competition studies using DHT, T, estradiol, progesterone, and cortisol showed that DHT and T were the most effective competitors for binding in the mouse prostate. However, a 100-fold excess of estradiol competed partially with DHT for binding. Low-salt extracts (unoccupied receptors) of the seminal vesicle and prostate gave low binding values, so only high-salt extracts (occupied receptors) were used for comparisons of androgen receptor concentrations in these tissues from 0M and 2M males.

Androgen binding in the extracts of 0M and 2M seminal vesicles did not differ significantly ($p > 0.05$) at 15.99 ± 7.52 vs. 16.90 ± 4.33 fmol/mg protein, respectively (n = 7/group). However, in the prostate, androgen receptors in high-salt extracts (occupied receptors) were significantly lower ($p < 0.05$).
higher (p < 0.01) in OM males than in 2M males (Fig. 3). The values in 2M males ranged from 10.9 to 35.6 fmol/mg protein (22.5 ± 3.5, mean ± SE, n = 7). In 2M males they ranged from 4.0 to 9.5 fmol/mg protein (7.4 ± 0.8, mean ± SE, n = 7).

DISCUSSION

The integrity of the accessory sex organs and their secretions is an important factor in the reproductive success of mice [18]. Either neotalon or prepubertal administration of androgens or estrogen is known to drastically alter the normal development (i.e., morphogenesis and differentiation) and function of the seminal vesicles and prostate [19–21]. Since OM and 2M male mouse fetuses differ in their circulating levels of T and estradiol, it was of interest to evaluate the developmental consequences (i.e., effects of intraperinatal position) on the male accessory sex tissues in adulthood.

Measuring the activity of testicular steroidogenic enzymes, namely 3β-hydroxysteroid dehydrogenase/iso- merase, 17α-hydroxylase, and CYP11A1, provided an index of the relative abilities of testes from OM and 2M males to produce androgens. Although adult levels of circulating T and estradiol have not been reported in OM and 2M mice, our findings suggest that the observed levels of steroidogenic enzymes alone are probably not responsible for the differences seen in accessory sex organs of OM and 2M male mice, especially since the mean activities of all three enzymes between the two groups were not significantly different (Table 1).

Prior intraperinatal fetal position had dramatic effects on both seminal vesicle and prostate weights in adult mice (Table 2). The increase in 3α-reductase enzyme activity observed in 2M animals was consistent with the significantly heavier (p < 0.05) seminal vesicles in these animals compared to those in OM mice. Adult 2M male mice, either castrated or intact, that had received a silicone capsule implant containing T had significantly greater 3α-reductase in seminal vesicles than did OM males that had received the same treatment (our unpublished observations). Differences between adult OM and 2M mice in seminal vesicle weight may have been due to induction of greater concentrations of 3α-reductase by elevated concentrations of T in 2M male fetuses, rather than to differences in circulating T in adult males.

In contrast, 0% prostates were heavier although 2M prostates had higher 3α-reductase levels (Table 2). Since androgens and estrogens are important for prostatic growth, both hormone receptors were measured to further investigate the basis of these differences in organ weights. Estrogen receptors reside in both the stromal and epithelial compartments of the prostate in rats [22, 23], but are probably more concentrated in the stroma. It is possible that differences in stroma might account for the differences in prostate weight between OM and 2M males. Since fetal OM mouse prostates are exposed to higher levels of circulating estradiol [5], heavier prostates in OM males could be due to stromal growth induced by estrogen [22]. We had thus predicted that prostates in OM males would show higher estrogen receptor concentrations than estrogens in 2M males. We failed to observe any effect of prior intraperinatal position on prostatic estrogen binding in OM and 2M male mice (data not shown).

However, OM testes had significantly higher (p < 0.01) androgen receptor concentrations (occupied receptor) compared to 2M prostates (Fig. 3), and this could sufficiently explain the androgen responsiveness/sensitivity to ac- count for the observation of larger prostates in OM adults. Whether this striking difference was the result of greater estrogen exposure and/or lesser androgen exposure in the fetus is unclear. The organization of OM prostates by estradiol may act through stromal instruction and permissive effects in prostatic development, thus leading to the observation of elevation in androgen receptor in our studies [24]. On the other hand, fetal androgens may also play a role in "programming" prostate androgen responsiveness, perhaps through relapse down-regulation of androgen receptors in 2M mice, which have higher serum concentrations of T than do OM males during fetal life.

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