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Cytochrome P450 Aromatase in Testis and Epididymis of Male Rhesus Monkeys

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To understand the role of estrogen in testicular and epididymal function of rhesus monkeys, we measured steroids in the spermatic and peripheral venus circulation and aromatase activity and its mRNA in testis and epididymis. Testosterone, estradiol-17β, and estrone, but not androstenedione, were elevated in the spermatic vein serum compared to the peripheral circulation. Aromatase activity in testis and in caput epididymis (259 ± 16 [SEM] vs 274 ± 47 fmol of ^3H_2O/mg of protein/h [*n* = 10], respectively) was significantly higher (*p* < 0.01) than in corpus and cauda (124 ± 28 and 113 ± 33 fmol of ^3H_2O/mg of protein/h [*n* = 10], respectively). In the ribonuclease protection assay, two $P450_{arom}$ mRNA transcripts were identified in testis and epididymis. One corresponded with the aromatase full-length transcript and the other was a truncated isoform. The latter was significantly more abundant than the former (*p* < 0.01). Our results demonstrate that the monkey testis and, to a lesser extent, the epididymis can aromatize androgens. However, in the epididymis, like in some areas of the brain, there was a discrepancy between the aromatase activity and the mRNA. The fact that $P450_{arom}$ mRNA and aromatase activity do not correlate in the epididymis may indicate that aromatase activity is not strictly regulated at the level of RNA expression and that other mechanisms for this regulation should be considered.

Key Words: Messenger RNA; aromatase; rhesus monkey; testis; epididymis.

Introduction

Estrogens are synthesized in tissues of many vertebrates including mammals, birds, reptiles, amphibians and fish *(1)*. The synthesis of estrogen from androgen is catalized by an enzyme complex that is comprised of a cytochrome P450 aromatase ($P450_{arom}$) and a flavoprotein NADPH–cytochrome P450 reductase. In those species in which it has been studied, aromatase activity (AA) and its messenger RNA have been found in many different tissues [reviewed by Simpson et al. *(2)*], including adipose tissue, placenta, granulosa cells of the ovaries, different areas of the brain, and Sertoli and Leydig cells of the testes *(3–5)*. Recent studies have also demonstrated immunoreactive cytochrome $P450_{arom}$ in testicular germ cells from several animal species *(4,6,7)*. Immunostaining first appeared in pachytene spermatocytes *(4,7)* and in round spermatids, elongated spermatids, and flagella of late spermatids *(6,7)*. Immunostaining was also found in rat testicular spermatozoa *(4)* and avian epididymal sperm *(7)*.

In addition to the above, male mice which lack a functional aromatase gene (*ArKO*) *(8)* or α-estrogen receptors (ERα-KO) *(9)* have impaired spermatogenesis, which is age dependent *(10)*. Other direct actions of estrogen mediated through β-receptors that are found in spermatogonia, pachytene spermatocytes, and Sertoli Cells may also be involved *(11)*. Thus, it appears that local expression of aromatase and the presence estrogen receptors both α and β are necessary for germ cell development. In a recent study, it has been shown that estrogen action in efferent ductules is, also, necessary for normal testicular function *(12)*.

Although the capacity of the testis to produce estrogens has been demonstrated in many different animal species, including men *(13)*, the distribution of $P450_{arom}$ among male reproductive tract tissues has not been reported in nonhuman primates. The purpose of this work was to investigate the capacity of the testis and three segments of the epididymis (caput, corpus, and cauda) to aromatize androgens in nonhuman primates; to compare aromatase activity with the expression of $P450_{arom}$ mRNA in the rhesus monkey testis and epididymis; and to quantify estrogens and their precursors in serum from the spermatic vein and compare these quantities with amounts found in blood obtained through a cardiac catheter (peripheral blood).

Results

Aromatase activity (fmol of ^3H_2O/mg of protein/h) was similar in the testis and in the caput epididymis (259 ± 16 [SEM] vs 274 ± 47 fmol of ^3H_2O/mg of protein/h [*n* = 10], respectively), but it was significantly higher (*p* < 0.01) in both of these tissues compared to the corpus and

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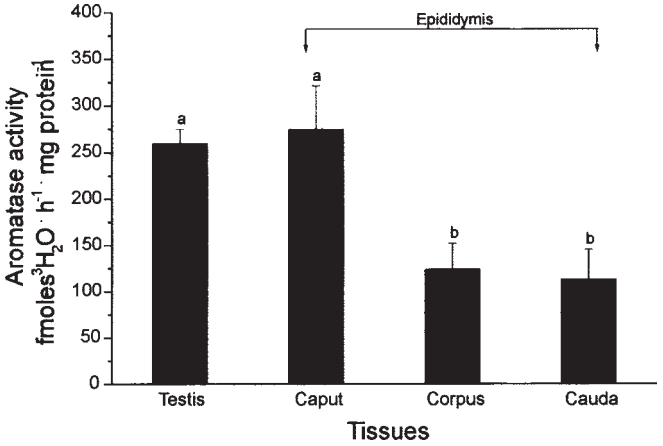

Fig. 1. Aromatase activity in testes and regions of epididymis (caput, corpus, and cauda) from rhesus monkeys ($n = 10$). Data are presented as means (bars) ± SEM (vertical lines). Bars marked with dissimilar letters differed significantly ($p < 0.01$, analysis of variance [ANOVA], followed by a Newman-Keuls multiple range test). The ^{3}H-water assay was performed on sonicated microsomal pellets (diluted 1/100 for testicular and 1/200 for epididymal tissues) derived from homogenized tissues. See Materials and Methods for other details.

cauda epididymis (124 ± 28 and 113 ± 33 fmol of ^3H_2O/mg of protein/h [$n = 10$], respectively) (Fig. 1).

Figure 2A is a representative phosphoimage of the amount of $P450_{arom}$ mRNA in the testis and epididymis as well as in the placenta (positive control) and muscle (negative control). This assay in which we used 10 µg of total RNA protected two mRNA transcripts in testis, one transcipt was the expected size of 455 nucleotides (nt) and a second transcript was approx 300 nt long. In the epididymis, however, the 455-nt transcript was not detectable on the phosphoimage even after exposure for 78 h (data not shown), but small amounts of the truncated form appeared on the gels. Because of these results, in a second experiment, we analyzed 50 µg of total RNA in various parts of the epididymis obtained from 4 of the 10 males used in Fig. 2A; these results are shown in Fig. 2B. When larger amounts of RNA were used, both transcripts (455 and 300 nt) were detectable.

Table 1 gives the data for $P450_{arom}$ mRNA (10 µg of total RNA analyzed) in testis and epididymis. The mean concentration of the 455-nt transcript in testis was significantly lower than that observed for the 300-nt one (40.1 ± 1.9 [SEM] vs 51.0 ± 5.8 fg/µg of total RNA, respectively) ($p < 0.01$, $n = 10$). In the epididymis, only the 300-nt transcript was protected. The mean concentrations of this transcript/µg of total RNA were higher in the corpus and cauda epididymis compared to the caput ($p < 0.01$).

In a second experiment, we increased the amounts of total RNA used in the protection assay from 10 to 50 µg. The data obtained from this experiment clearly showed that both the 455- and 300-nt transcripts were present in all segments of the epididymis when greater amounts of total RNA were used in the assay (Fig. 2B).

Table 2 gives the mean concentrations of estradiol-17β (E_2), estrone (E_1), androstenedione, and testosterone in sera from the spermatic vein and the heart ($n = 10$). Significantly higher concentrations of these steroids were found in the spermatic vein compared to the peripheral circulation ($p < 0.01$), except for androstenedione.

Discussion

Our results clearly demonstrate that the testes of rhesus monkeys secrete estrogens because the concentrations of both E_1 and E_2 were significantly higher in serum from the spermatic veins, which drain the testes, compared to the peripheral venous circulation.

Our data also show that both the testis and epididymis are capable of aromatization. The physiologic role of estrogen synthesized in the epididymis is not known, especially in nonhuman primates. However, in rhesus males, estrogen receptors (ERs) are found in high concentrations in the nonciliated absorptive cells of the ductuli efferentes and are present in relatively low amounts in the epididymis *(14)*. The presence of ERs in these tissues could mean that estrogen serves some function in the testis and epididymis of nonhuman primates as it does in other species. For example, mice in which the functional aromatase gene has been experimentally deleted have impaired spermatogenesis *(8)*. A similar result has been observed in mice in which the ERα has been deleted *(9)*. It appears that estrogens regulate reabsorption of luminal fluids in the caput epididymis of mice *(12)*. Males lacking the ERα cannot reabsorb luminal fluids; hence, there is back pressure associated with testicular atrophy *(12)*. In addition, testicular cells of rats, such as spermatogonia, pachytene spermatocytes, and Sertoli cells, all contain ERβ, which suggests that estrogens are involved in sperm maturation *(11)*.

In men, naturally occurring mutations of ER and aromatase genes have been reported *(15–17)*. In one man with a mutated ER, gonadotropins and estrogen concentrations were elevated in the systemic circulation *(15)*. Even though sperm counts were normal in this case, infertility occurred because of low sperm viability *(15)*. Aromatase deficiency in a second man was accompanied by low levels of estrogen in the systemic circulation and low sperm count, and spermatogenesis was arrested at the level of the primary spermatocyte *(16)*. These data suggest that the action of estrogen is required for human spermatogenesis.

We also found $P450_{arom}$ mRNA in the testis in which our 455-bp monkey probe protected two mRNA transcripts 455 and 300 nt in length, respectively. The 455-nt transcript is probably the active one because it correlates with aromatase activity in the brain *(23)*. The alternate transcript, which is found in relatively large amounts in the monkey testis, is also found in the monkey brain. This transcript does not correlate with the capacity of monkey brain to aromatize androgens *(23)*. A truncated form of human aromatase

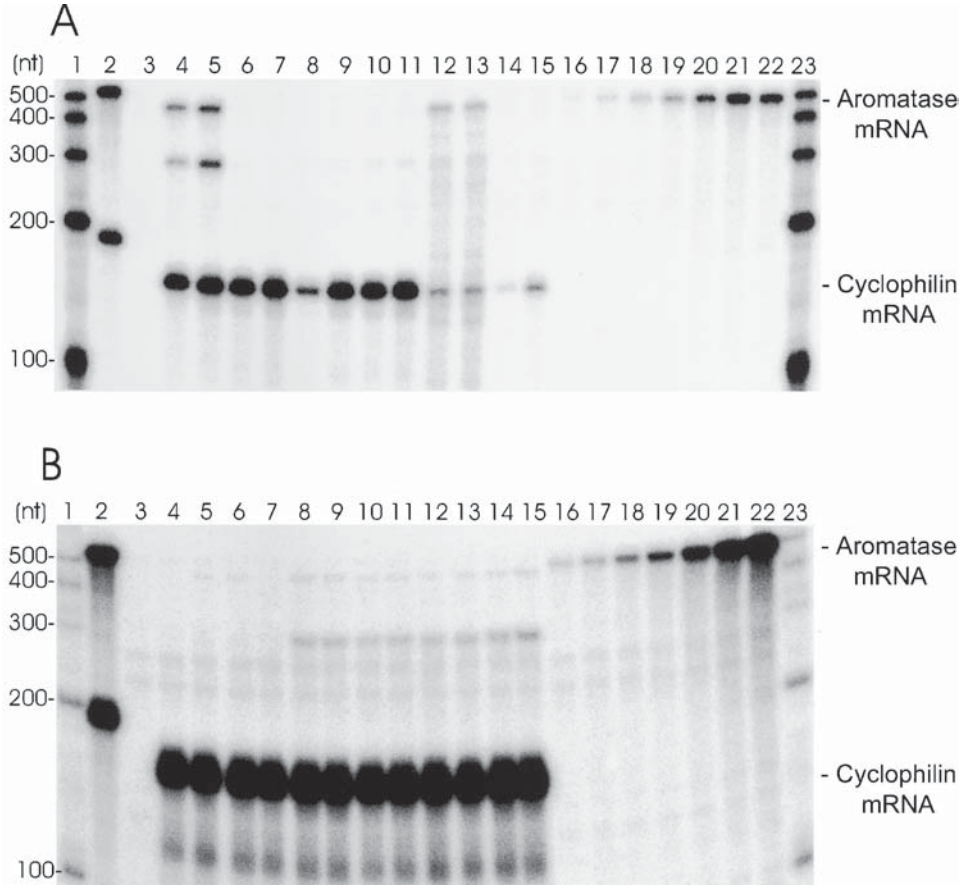

Fig. 2. (**A**) Representative ribonuclease protection assay phosphoimage showing distribution of $P450_{arom}$ mRNA (10 µg of total RNA/lane) in testis, epididymis, placenta (positive control), and femoral muscle (negative control). Lanes 1 and 23, nucleotide ladder; lane 2, incubated undigested probe; lane 3, incubated ribonuclease digested probe; lanes 4 and 5, testis; lanes 6 and 7, caput epididymis; lanes 8 and 9, corpus epididymis; lanes 10 and 11, cauda epididymis; lanes 12 and 13, placenta (139 d of gestation, rhesus monkey); lanes 14 and 15, muscle (rhesus monkey femoral); lanes 16–22, $P450_{arom}$ mRNA sense RNA standard curve (31.2, 62.4, 125, 250, 500, 1000, and 2000 fg, respectively). Exposure time was 15 h. (**B**) Representative ribonuclease protection assay phosphoimage showing the distribution of $P450_{arom}$ mRNA (50 µg of total RNA/lane) in epididymis. Lanes 1 and 23, nucleotide ladder; lane 2, incubated undigested probe; lane 3, incubated ribonuclease digested probe; lanes 4–7, caput epididymis of four males; lanes 8–11, corpus epididymis of the same four males; lanes 12–15, cauda epididymis of the same four males; lanes 16–22) $P450_{arom}$ mRNA sense RNA standard curve (31.2, 62.4, 125, 250, 500, 1000, and 2000 fg, respectively). Exposure time was 71 h.

Table 1
Distribution of $P450_{arom}$ mRNA in Testes and Epididymides of Rhesus Monkeys as Determined by Ribonuclease Protection Assays

			fg/µg of total RNA ± SEM[b]	
Tissue	RNA assayed (µg)	N^a	455 nt transcript	300 nt transcript
Testis	10	10	40.1 ± 1.9*	51.0 ± 5.8†
Epididymis				
Caput	10	10	ND	4.4 ± 0.3‡
Corpus	10	10	ND	13.2 ± 0.3§
Cauda	10	10	ND	13.9 ± 1.0§

[a] *N*, number of animals from which tissues were obtained.
[b] ND, not detectable. * and †, Differed significantly from one another ($p < 0.01$, by Student's *t*-test). Different superscript symbols for epididymis indicate that those means differ significantly ($p < 0.01$, ANOVA followed by Neuman-Keuls, multiple range test).

Table 2
Steroid Concentrations in Sera from Spermatic Veins and Heart of Rhesus Macaques

		Mean ± SEM	
Steroid	N^a	Spermatic vein	Heart
Testosterone	10	22.7 ± 7.0 (ng/mL)	8.5 ± 1.3 (ng/mL)[b]
Androstenedione	10	1.7 ± 0.6 (ng/mL)	0.5 ± 0.1 (ng/mL)
Estradiol-17β	10	66.0 ± 20.0 (pg/mL)	12.0 ± 2.0 (pg/mL)[b]
Estrone	10	46.0 ± 11.0 (pg/mL)	11.3 ± 3.0 (pg/mL)[b]

[a] *N*, number of animals from which sera were obtained.
[b] Sera concentrations differed significantly ($p < 0.01$, Student's *t*-test) between spermatic veins and blood obtained through a cardiac catheter.

when expressed in Chinese hamster ovary cells and incubated with an excess of NADPH cytochrome P450 reductase and dilauroyl L-α-phosphatidylcholine can aromatize androgen in vitro in a time-dependent manner *(18)*. It has been postulated that the truncated form of aromatase may no longer be associated with the endoplasmic reticulum; thus, it is disconnected from its redox partner, NADPH-cytochrome P450 reductase, an association that is necessary for aromatization to occur *(18)*. We do not currently know what the relationship is between the truncated form of aromatase and NADPH-cytochrome reductase in the monkey testis and epididymis. If it is coupled to the reductase, it could be active. Perhaps the relative amounts of the two transcripts and their respective encoded proteins in the primate testis and epididymis are not static but constantly changing. In this way, the ability to couple with cytochrome P450 reductase could be part of a regulatory mechanism for limiting local estrogen formation. This hypothesis is compatible with the idea that small amounts of estrogen are necessary for gamete formation but that overexpression of aromatase leads to pathology (e.g., development of testicular Leydig cell tumors) *(19)*.

The fact that $P450_{arom}$ mRNA and aromatase activity do not correlate in the epididymis may indicate that aromatase activity is not strictly regulated at the level of RNA expression and that other mechanisms for this regulation should be considered.

In summary, we have shown that several important elements for the biosynthesis of estrogen, such as the ability to aromatize androgens and the presence of $P450_{arom}$ mRNA, are present in the testes and epididymis of rhesus monkeys and that the testes secrete both E_1 and E_2 into the systemic circulation. Local production of estrogen may be important for sperm maturation in the testis and fluid transport in the epididymis, as it appears to be in other species such as the mouse.

Materials and Methods

Animals and Tissue Preparation

Ten healthy adult male rhesus monkeys (*Macaca mulatta*, 5–10 yr of age, weighing on average 8.8 ± 2.0 kg) were used. Animals were housed in individual cages and the temperature of the room where the animals were kept was maintained at 22ºC. During the experiment, they were exposed to 12 h of light from 7:00 AM to 7:00 PM and 12 h of darkness from 7:00 PM to 7:00 AM. The males were fed a diet of Purina Lab Monkey Chow supplemented with fresh fruit and provided with water ad libitum. All experimental procedures and animal care were conducted under the supervision of the Veterinary Staff of the Oregon Health Science University in accordance with the principles and procedures outlined by the NIH Guidelines for the Care and Use of Laboratory Animals. Following anesthesia with ketamine hydrochloride (10 mg/kg/intravenously), venous blood samples were obtained from the spermatic vein by venipuncture and the heart through a cardiac catheter. Placement of the catheter to obtain blood remotely has been described previously *(20)*. The serum was collected and stored at –20°C until assayed for steroid hormones by radioimmunoassay (RIA). Castration was then performed through a suprascrotal midline incision. After castration, the testes were rapidly decapsulated, cut into approx 0.5-cm^2 pieces, immediately frozen on dry ice, and stored at –80ºC until they were assayed. The epididymis was obtained at the time of castration, cleaned of connective tissue, rinsed with saline, and blotted, and the caput, corpus, and cauda were dissected before being stored at –80ºC for later use in the various assays.

Radiometric ^3H_2O Assay for Aromatase Activity

Aromatase activity was measured by a radiometric ^3H_2O assay. This assay quantifies the stereospecific production of ^3H_2O from [1β-^{3}H]androstenedione as an index of estrogen formation *(21)*. The details of this assay have been described previously *(21)*. The apparent Michaelis-Menton (K_m) for testicular tissue averaged 0.15 μ*M* ± 0.02 (SE) ($n = 4$). The amount of estrogen formed by testicular microsomes as determined by the water assay was compared vs the amount determined by product isolation of E_2 and E_1. The details of the procedure for product isolation have been published previously *(22)*. We found 99.1 ± 20 (SEM) fmol of estrogen produced/mg of protein/h) ($n = 10$ assays) by product isolation vs 108.0 ± 3.5 fmol of ^{3}H water produced/mg of protein/h) ($n = 10$ assays). These values did not differ significantly by a *t*-test ($p > 0.05$). In addition, aromatase activity of testicular and caput epididymis microsomes incubated with 20 μ*M* of the aromatase inhibitor 1,4, 6-androstatriene-3,17-dione, was reduced by 88 and 34%, respectively, over untreated control samples in vitro.

Ribonuclease Protection Assay

Ribonuclease protection assays were performed to identify the $P450_{arom}$ mRNA in testis, and three segments of the epididymis (caput, corpus, and cauda) using a 455-bp cDNA insert cloned from first-trimester placenta of rhesus monkey. Details of cloning have been described previously *(23)*. Total RNA was extracted from testis and the caput, corpus, and cauda regions of the epididymis by the guanidinium thiocyanate method followed by centrifugation through a 5.7 *M* cesium chloride gradient *(24)*. An antisense ^{32}P complementary $P450_{arom}$ RNA ([^{32}P]cRNA) probe was synthesized in vitro from the DNA template by using SP6 RNA polymerase. Purification of the [^{32}P]cRNA probe and other procedures used in preparing the samples for analysis in ribonuclease protection assays have been published previously *(23)*. Third-trimester placenta and femoral muscle of monkeys were used as positive and negative controls, respectively. Cyclophilin mRNA, which was used as a control for RNA loading on gels, was measured using a 185-nt [^{32}P] cRNA probe transcribed from a rhesus monkey p1B15 cyclo-

philin cDNA cloned into pGEM-3Z vector (provided by Dr. Sergio Ojeda at the Oregon Regional Primate Research Center). The protected cyclophilin mRNA fragment was 158 nt long.

We used a Molecular Imaging System (Bio-Rad GS-525; Bio-Rad, Hercules, CA) to quantify protected bands on the polyacrylamide gels as described previously *(25)*. The amount of $P450_{arom}$ mRNA in each sample was determined from the ribonuclease protection assay standard curve and normalized to the cyclophilin mRNA value *(25)*.

Steroid RIA

Serum steroid concentrations were measured by RIA. Sera were extracted with ether and partitioned by chromatography on Sephadex LH-20 columns. E_1, E_2, androstenedione, and testosterone were assayed as previously described *(26)*.

Statistical Analyses

Statistical comparisons of aromatase activity and $P450_{arom}$ mRNA in the testes and the caput, corpus, and cauda regions of the epididymis were performed by an ANOVA followed by Newman-Keul's multiple range tests if the ANOVA was statistically significant. Differences in concentrations between the 455- and 300-nt transcripts of the testis and of steroids in peripheral vs spermatic vein serum were determined by *t*-tests. Before analysis, the groups to be compared were tested for homogeneity of variances. If the variances were not the same, the data were log transformed and the transformed data used in the analysis. The statistical program GB STAT (Dynamic Microsystems, Inc., Silver Spring, MD) was used for these analyses.

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