Hormone Receptors of the Baboon Cardiovascular System

Biochemical Characterization of Aortic Cytoplasmic Androgen Receptors

Alan L. Lin, Henry C. McGill, Jr., and Sydney A. Shain

Cytoplasmic androgen receptors were identified in the aorta of the baboon (Papio sp.). Using the synthetic androgen R1881 (17β-hydroxy-17a-methyl-11-trien-3-one) as probe, androgen receptors were demonstrated only when the incubation mixtures contained 1.0 μM triamcinolone acetonide (TA). The relative binding affinity of selected steroids for the aortic androgen receptor was R1881, 100%; 5α-dihydrotestosterone, 46.1%; testosterone, 60.7%; progesterone, 9.1%; R5020, 3.1%; and estradiol-17β, 7.5%. The androgen receptor migrated on low-ionic-strength linear sucrose density gradients as a macromolecule with a sedimentation coefficient of 8.0S. Saturation analysis performed at 2°C (available sites) showed that the androgen receptor content of baboon aortic cytoplasmic extracts was 12.9 ± 2.4 fmols/mg protein and that the dissociation constant for R1881 was 1.47 ± 0.07 nM. The observation that TA was required in the cytoplasmic extracts in order to identify androgen receptor indicates the presence of progesterone receptor-like binding components. These aortic cytoplasmic androgen receptors are indicated to be physiologically functional by previous autoradiographic studies that showed localization of radiolabeled estradiol in the nuclei of aortic smooth muscle cells following injection of baboons with 5α-dihydrotestosterone. (Arteriosclerosis 1981; 1:257-264)

Estrogens and androgens alter blood flow rates in accessory reproductive organs. Clinical observations and epidemiologic studies have repeatedly confirmed that women have lower rates of coronary heart disease (CHD) and less severe coronary atherosclerosis than do males of equivalent age. These studies imply a possible role for sex steroid hormones as modulators of vascular tissue cell function.

From the Department of Pathology, University of Texas Health Science Center, and the Departments of Cardiopulmonary Disease and Cellular and Molecular Biology, Southwest Foundation For Research and Education, San Antonio, Texas.

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Address for reprints: Dr. Sydney A. Shain, Southwest Foundation for Research and Education, P. O. Box 28147, San Antonio, Texas 78284.

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cardiovascular system were steroid hormone target tissues.

Recent autoradiographic studies have shown limited capacity nuclear localization of radioisotope in the media of the pulmonary artery and aorta of the rat following injection of tritiated estradiol. Limited capacity localization of radioisotope in the nuclei of smooth muscle cells of the anterior and middle cerebral arteries of the rat following injection of tritiated 5α-dihydrotestosterone also has been reported. Biochemical studies have provided evidence of limited capacity, high affinity estrrophiles in extracts prepared from either canine coronary arteries or a vascular endothelial cell line derived from rabbit aorta. Cell culture studies have shown that testosterone causes an increase in the labeling index of muscle cells and that estradiol increases prostacyclin biosynthesis in aortic smooth muscle cells. These biochemical and autoradiographic studies provide evidence that vessels of the rodent cardiovascular system are androgen and estrogen target tissues.

The first direct evidence that vessels of the nonhuman primates (Papio sp.) cardiovascular system may be sex hormone target tissues was the report of McGill and Sheridan. These investigators showed limited capacity nuclear localization of radioisotope in smooth muscle cells of the major elastic and muscular arteries following injection of either tritiated 5α-dihydrotestosterone or tritiated estradiol. We now describe detailed biochemical characterization of baboon aortic cytoplasmic androphiles. In this report we demonstrate that baboon aorta contains cytoplasmic androphiles that have the properties of classical androgen receptors.

Methods

Animals

Mature female baboons (Papio sp.) were obtained from the baboon colony at Southwest Foundation for Research and Education. These baboons had been used for studies in reproductive physiology and had undergone bilateral oophorectomy 3 to 6 months prior to autopsy.

Chemicals Used

The following chemicals were obtained from New England Nuclear Corporation (Boston, Massachusetts): [17α-Methyl-3H]R1881 (17β-hydroxy-17α-methyl-estro-4,9,11-trien-3-one, specific activity 87 Ci/mmol), [1,2-3H] 5α-dihydrotestosterone (specific activity 60 Ci/mmol), [1,2,6-3H] testosterone (specific activity 92 Ci/mmol); radioinert R5020 (17α,21-dimethyl-19-norpregna-4,9-diene-3,20-dione), and radioinert R1881. Before using radiolabeled testosterone or 5α-dihydrotestosterone, we purified them by high performance liquid chromatography (see subsequent section). We used radiolabeled R1881 as obtained from the manufacturer. Triamcinolone acetonide and bovine serum albumin (Fraction 5) were obtained from Sigma Chemical Company (St. Louis, Missouri). Additional radioinert steroids were obtained from Steraloids, Inc. (Wilton, New Hampshire), and purity was established as previously described. Human γ-globulin was from Calbiochem Behring Corporation (La Jolla, California) and Dextran T70 was from Pharmacia Fine Chemicals, Inc. (Piscataway, New Jersey). Other materials were the highest quality reagent grade available from the manufacturer. All solutions were prepared in water that had been distilled, deionized, and redistilled from glass.

Preparation of Cytoplasmic Extracts and Quantitation of Cytoplasmic Androphiles by Saturation Analysis

We sedated the baboons with ketamine (15 mg/kg) and killed them by pneumothorax. We removed the major arteries and cut them into blocks of 0.8 to 1.0 g. Tissue samples were immediately placed into polypropylene screw top cryotubes (Vanguard International Inc., Neptune, New Jersey) and frozen by immersion in liquid nitrogen. Tissues were transferred, without delay, to a −90°C freezer where they were stored until used.

Cytoplasmic extracts were prepared by a slight modification of methods previously described. In brief, tissue (only the aortic arch was used in the current studies) was allowed to thaw on ice and subsequent procedures were performed at 2°C. Tissue was minced with fine surgical scissors and homogenized in a Duali (Kontes, Vineland, New Jersey) glass-glass homogenizer in 4 to 5 volumes of Buffer T^E^M^S.^ containing either 200 mM sodium molybdate or 22 μM TA. Saturation analysis was performed at 2°C using methods previously described. Incubation was performed in a final volume of 220 μl Buffer T^E^M^S.^ containing 100 μl of cytosol and radiolabeled R1881 (0.5 to 10 nM). Nonspecific binding was determined in a parallel series of
identical incubations which additionally contained 1.0 \( \mu \text{M} \) radioinert R1881. Separation of bound and free radioligand was by the dextran-\( \gamma \)-globulin coated charcoal/ethanol (DGCC/EtOH) procedure previously described by us.\textsuperscript{22, 23} After correction for nonspecific binding,\textsuperscript{24} the data were analyzed by the method of Scatchard.\textsuperscript{25}

**Characterization of the Specificity of Steroid Binding to Aortic Cytoplasmic Androphiles**

The conditions of incubation and determination of bound radiosteroid were as described in the preceding section. Survey determinations to assess qualitatively relative steroid specificity used the single concentration assay.\textsuperscript{24} Quantitative determinations of relative steroid specificity were performed by using procedures previously described\textsuperscript{22, 23, 26} which are based upon the original protocols reported by Liao et al.\textsuperscript{27}

**Purification of Radiosteroids and Separation of Steroid Metabolites**

We separated radiometabolites by high performance liquid chromatography (HPLC) using the Waters (Waters Associates, Medford, Massachusetts) Model 440 liquid chromatograph equipped with Model 660 solvent programmer and Model 401 refractometer. We employed isocratic chromatography on a C-18 reverse phase analytical column (ES Industries, Marlton, New Jersey) using methanol:water (55:45) as eluent to achieve separation of metabolites.\textsuperscript{28} Radiolabeled testosterone or 5a-dihydrotestosterone (5a-DHT) was purified by chromatography using the chromatographic system employed for separation of metabolites.

**Testosterone and 5a-Dihydrotestosterone Metabolism by Aortic Extracts**

We prepared 220 \( \mu \text{l} \) incubation mixtures which contained 100 \( \mu \text{l} \) cytosol and either 10 nM radiolabeled testosterone or 10 nM radiolabeled 5a-dihydrotestosterone. After incubation at 2\( ^\circ \)C for 2 hours, incubation was terminated by addition of ice-cold acetone. We performed acetone extraction and recovered steroid metabolites by dichloromethane extraction, as previously described.\textsuperscript{29} The dichloromethane extracts were dried under nitrogen, and the resultant residue was immediately dissolved in 1.0 ml methanol containing each selected radioinert androgen at a concentration of 500 \( \mu \text{g} / \text{ml} \). A 200 \( \mu \text{l} \) sample of this methanol solution was used for analysis. Control incubations (cytosol deleted) were performed and analyzed in the same manner.

**Other Methods**

Sucrose density gradient centrifugation was performed on linear 10% to 30% gradients in the Sorvall vertical tube rotor (TV 865).\textsuperscript{22, 23} Protein was determined by the procedure of Lowry et al.\textsuperscript{30} using bovine serum albumin as standard. We prepared samples for quantitation of radioisotope, as we have previously described.\textsuperscript{23} Tritium was quantitated in a Beckman 7500 scintillation spectrometer, and most samples were counted to a precision of 2\( \sigma \) equal to 2.0 or less. Samples containing low levels of radioisotope were counted to a precision of 2\( \sigma \) equal to 5.0 or less.

### Results

**Characterization of the Relative Steroid Specificity of Aortic Cytoplasmic Androphiles**

We chose R1881 as a probe of baboon aortic androphiles because it has limited affinity for plasma steroid binding proteins such as sex hormone binding globulin.\textsuperscript{31, 32} Since R1881 does not distinguish between androgen receptors and progesterone receptors during incubation at 2\( ^\circ \)C,\textsuperscript{32-35} it was necessary to establish steroid specificity before other studies were undertaken.

Single concentration survey determinations to examine the ability of a variety of steroids to inhibit R1881 binding to baboon aortic cytoplasmic binding components showed that androgens (5a-dihydrotestosterone, testosterone, 19-nortestosterone), progestins (progesterone, R5020), estriadiol-17\( \beta \), and synthetic corticosteroids (TA) but not natural corticosteroids (cortisol) were effective inhibitors of R1881 binding (table 1). This result is reminiscent of R1881 binding to cytoplasmic androphiles of tissue, such as human prostate, which contains both androgen receptors and progesterone receptor-like cyto-

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Specific binding remaining (%)*</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>R1881</td>
<td>0</td>
</tr>
<tr>
<td>5a-Dihydrotestosterone</td>
<td>63</td>
</tr>
<tr>
<td>Testosterone</td>
<td>73</td>
</tr>
<tr>
<td>19-Nortestosterone</td>
<td>9</td>
</tr>
<tr>
<td>Progesterone</td>
<td>7</td>
</tr>
<tr>
<td>R5020</td>
<td>30</td>
</tr>
<tr>
<td>Triamcinolone acetonide</td>
<td>51</td>
</tr>
<tr>
<td>Estradiol-17( \beta )</td>
<td>51</td>
</tr>
<tr>
<td>Cortisol</td>
<td>109</td>
</tr>
</tbody>
</table>

*Data are mean values (n = 2) ±TA. or the result of a single assay. +TA. Probe, \( ^{3} \text{H}-\text{R1881}, \) concentration was 10 nM; competitor concentration was 500 nM.

†TA, triamcinolone acetonide, concentration was 1.0 \( \mu \text{M} \).
plasmic binding components. Other investigators have shown that it is possible to eliminate the progesterone receptor-like contribution to measured R1881 binding by including TA in the incubation mixture when measuring androgen receptors in human prostate or human breast cancer cells in culture. When we surveyed steroid specificity using incubations that contained radiolabeled R1881, radioinert TA (1.0 μM), and the test competitor, the effectiveness of androgens as inhibitors of R1881 binding was markedly enhanced, whereas the effectiveness of progestins and estradiol-17β was reduced (table 1).

We then prepared baboon aortic cytosols which contained TA, and we performed multiple concentration inhibition studies. This procedure allowed us to examine the relative ability of selected steroids to inhibit R1881 binding to baboon aortic cytoplasmic R1881 binding components under conditions which eliminated the contribution of progesterone receptor-like binding components. When baboon aortic cytosols contained TA (final concentration 1.0 μM), the relative binding affinity (RBA) of selected steroids was as follows: R1881, 100%; 5α-DHT, 46.1%; testosterone, 60.7%; progesterone, 9.1%; R5020, 3.1%; and estradiol-17β, 7.5% (figure 1).

The results showed that under these conditions R1881 was principally binding to putative baboon aortic cytoplasmic androgen receptors. All subsequent studies were performed with baboon aortic cytoplasmic extracts which contained 1.0 μM TA (final concentration) during incubations to characterize or quantitate binding components.

**Effect of Time and Temperature of Incubation on R1881 Binding in Aortic Cytoplasmic Extracts**

During incubation at 2°C, maximum binding of R1881 to baboon aortic cytoplasmic androphiles occurred between 2 and 6 hours and was maintained through 24 hours of incubation (figure 2). Between 24 and 48 hours of incubation at 2°C, the quantity of bound R1881 was modestly decreased. During incubation at 15°C, maximum binding occurred after 1 hour of incubation (figure 2). Between 24 and 48 hours of incubation at 15°C, R1881 binding to baboon aortic cytoplasmic androphiles decreased to 35% of that detected after 1 hour of incubation (figure 2). During 1 hour of incubation at 37°C in the absence of radiolabeled probe and sodium molybdate, R1881 binding activity in baboon aortic cytosols was completely inactivated (data not shown).
Sucrose Density Gradient Characterization of Aortic Cytoplasmic Androphiles

Sucrose density gradient analysis showed that baboon aortic cytosol contains two areas of radioactivity (figure 3). One region of radioactivity appears as a well-defined peak containing macromolecule(s) with a sedimentation coefficient of 8.0S. The second region of radioactivity is diffuse and appears to contain macromolecular component(s) with a sedimentation coefficient of 2.2S. Competition experiments showed that 8.0S binding was effectively eliminated, while 2.2S binding was unaffected by 10-fold excess radioinert R1881 (figure 3). Similarly, 5a-DHT was a highly effective inhibitor of 8.0S binding and caused no reduction of 2.2S binding. Both R5020 and estradiol-17β (10-fold excess) caused the expected minor degree of inhibition of 8.0S binding (see figure 2); neither steroid caused any displacement of 2.2S binding (figure 3).

Quantitation of Aortic Cytoplasmic Androphiles by Saturation Analysis at 2°C

Typical saturation data are presented as observed binding (figure 4 A) and as the resultant Scatchard plot (figure 4 B). These demonstrate that R1881 is binding to limited capacity, high affinity binding sites that are saturated at 3 to 5 nM ligand. The concentration of cytoplasmic androphiles was 12.9 ± 2.4 fmoles/mg of cytosol protein (mean ± sd, n = 10; 8, single saturating dose (10 nM); 2, full saturation analyses), and the dissociation constant was 1.47 ± 0.07 nM (mean ± range, n = 2).

Testosterone and 5α-Dihydrotestosterone Metabolism by Aortic Cytosol

To assess the possibility that the difference in the relative affinity of R1881, testosterone, and 5α-dihydrotestosterone for baboon aortic cytoplasmic androphiles may be due in part to metabolism of testosterone or 5α-dihydrotestosterone during incubation at 2°C, we identified the steroids obtained following 2 hours of incubation of either 10 nM radiolabeled testosterone or 10 nM radiolabeled 5α-dihydrotestosterone with baboon aortic cytosol. Control determinations showed that neither testosterone nor 5α-dihydrotestosterone was affected by incubation in the buffer for 2 hours at 2°C. Inclusion of aortic cytosol did not result in measurable metabolism of either testosterone or 5α-dihydrotestosterone (data not shown).

Figure 3. Sucrose density gradient characterization of baboon aortic cytoplasmic androgen receptors. Cytoplasmic extracts were labeled by incubation at 2°C and treated with DGCC/ EtOH before application to linear gradients (10% to 30% sucrose) which were developed by centrifugation at 370 K X g (TV 865 rotor) at 2°C for 2 hours. Incubations contained radiolabeled R1881 (10 nM), radioinert TA (1.0 μM), and radioinert competitor (100 nM). Sedimentation is from left to right. The sedimentation coefficient of human γ-globulin is 7S.
Figure 4. Typical saturation data for incubation of baboon aortic cytoplasmic extracts with various concentrations of radiolabeled R1881. All incubates contained 1.0 μM TA. A. Observed saturation data: (A—A), total binding; (●—●), nonspecific binding; (■—■), specific binding. B. Scatchard representation of the data in A.

Discussion

In this study we identified two classes of limited capacity, high affinity R1881 binding sites in baboon aortic cytoplasmic extracts. One class of R1881 binding sites is inhibited by triamcinolone acetonide (TA) and the synthetic progestin R5020. We have not yet further characterized this class of aortic R1881 binding sites; however, this binding component appears identical to the TA inhibitable R1881 binding sites that we have characterized in baboon myocardial cytosols.28 Our current data for aortic cytosol are characteristic of R1881 binding to progesterone receptor-like components in extracts of tissues which contain both androgen receptors and progesterone receptor-like cytoplasmic binding components.37 39

We characterized the second class of limited capacity, high-affinity binding sites of baboon aortic cytosol by incubating with radiolabeled R1881 in the presence of 1.0 μM TA. This baboon aortic cytoplasmic R1881 binding component is a cytoplasmic androgen receptor as established by the following evidence: 1) relative steroid specificity (table 1 and figure 1), which shows that androgens are effective inhibitors, whereas progestins, estradiol-17β, and cortisol are ineffective or only modestly effective inhibitors of R1881 binding; 2) sedimentation properties on linear sucrose density gradients (figure 3); 3) temperature instability; and 4) high-affinity limited-capacity binding.

When R1881 is used as probe, measurement of total androgen receptors generally requires either incubation at 15°C22-23 or prolonged incubation (24 hours) at 2°C. Since we have used brief incubation at 2°C, our determinations most likely only represent quantitation of steroid unoccupied receptors. The concentration of baboon aortic available cytoplasmic androgen receptors, 12.9 fmoles/mg protein, is comparable to that of rat myocardium40 and striated muscle41-44 and to that which we reported for baboon myocardium.28 It should be noted that the number of sites quantitated may be a minimal estimate because neither the effects of oophorectomy nor the effect of freezing aortic tissue on detected androgen receptor content have been evaluated.

In a previous study we reported that baboon myocardial cytosols rapidly metabolize 5α-dihydrotestosterone to 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol, whereas testosterone is not metabolized.28 Baboon aortic cytosols differed from baboon myocardial cytosols in that neither testosterone nor 5α-dihydrotestosterone was metabolized during incubation at 2°C. These observations suggest that the differences in relative affinity of R1881, testosterone, and 5α-dihydrotestosterone for baboon aortic cytoplasmic androgen receptors probably reflect actual differences in binding properties.

This study represents the first definitive biochemical identification of cytoplasmic androgen
receptors in the aorta of a nonhuman primate (Papio sp.). The previously reported autoradiographic localization of radioisotope in the nuclei of smooth muscle cells of the aortic media following injection of radiolabeled 5α-dihydrotestosterone indicates that the aortic androgen receptors detected by biochemical analyses may be physiologically functional. In aggregate, these independent observations appear to establish that baboon arteries are androgen target tissues. Consideration of the extensive homologies between baboon and human endocrinology leads us to suggest that the baboon may be useful for investigating the role of sex steroid hormones in arterial physiology and their role in the pathogenesis of arterial disease.

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