Aldosterone Binding Sites in Aortic Cell Cultures From Spontaneously Hypertensive Rats
NANCY R. NICHOLS, PH.D., CHARLES E. HALL, PH.D., AND WALTER J. MEYER, III, M.D.

SUMMARY Spontaneously hypertensive rats and rats made hypertensive by deoxycorticosterone-salt treatment have in common increased Na⁺ and K⁺ permeability and transport in their aortic cells. These changes may be important factors in the development of the hypertensive state and may be mediated by mineralocorticoid binding to intracellular sites in the aorta. Therefore, we examined 3H-aldosterone binding in aortic cell cultures from spontaneously hypertensive rats and normotensive Wistar-Kyoto rats. Vascular corticoid binding sites in the two strains were compared by Scatchard analysis of Kᵋ and B_max, pH and temperature stability, and subcellular binding. By all of these criteria we found that aldosterone binding sites in cultured aortic cells are similar in the hypertensive and normotensive rats. These results indicate that the underlying genetic defect in spontaneous hypertension is not an intrinsic cellular defect which alters mineralocorticoid binding in the aorta.

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KEY WORDS • aorta • cell culture • dexamethasone • hypertension • mineralocorticoid • vascular smooth muscle • Wistar-Kyoto rat

THE multiplicity of adrenal steroid effects on blood pressure regulation and the development of hypertension is becoming apparent. Initially, expansion of the blood volume and extracellular fluid volume, secondary to excessive salt and water retention by the kidney, was identified as a major cause of mineralocorticoid-induced hypertension. Physiological evidence derived from rat hypertension models suggests that mineralocorticoids also have a direct effect on the blood vessel wall during the prehypertensive and hypertensive states. These effects include vessel wall hypertrophy¹ and significant increases in cellular ion content and ion turnover.²³ Similar structural and functional changes have been found in various arteries of spontaneously hypertensive rats but not in normotensive control rats.¹⁴robotic recently, we have presented evidence for displaceable mineralocorticoid and glucocorticoid binding in smooth muscle cells and fibroblasts cultured from rat aorta.⁷ The data support the hypothesis that vascular smooth muscle may be a target tissue for corticoid hormones. Vascular steroid binding sites differ from classical mineralocorticoid and glucocorticoid receptors, however, and may be involved in physiological effects other than classical mineralocorticoid and glucocorticoid effects. For example, a receptor mechanism may exist for steroid actions in vascular cells that can contribute to the overall development of high blood pressure.

Previously, the aldosterone binding properties of classical renal mineralocorticoid receptors were shown to be equivalent in spontaneously hypertensive rats (SHR) and genetically related Wistar-Kyoto (WKY) normotensive control rats.⁸ In the present investigation we have compared aldosterone binding characteristics of aortic cells cultured from SHR and WKY rats.

Materials and Methods

Chemicals
The chemicals ¹H-Aldosterone (¹H-A, 57-82 Ci/m mole) and ¹H-dexamethasone (¹H-DM, 36-42 Ci/m mole) were purchased from New England Nuclear (Boston, Massachusetts). Nonradioactive aldosterone and dexamethasone were obtained from Sigma (St Louis, Missouri). Steroids were stored in absolute eth-
anol at 4°C and were checked for purity by thin layer chromatography. Tissue culture media components, Hank's balanced salt solution and trypsin were purchased from Gibco (Grand Island, New York); calf thymus DNA and human gamma globulin from Sigma (St Louis, Missouri); and Sephadex G-25 from Pharmacia (Piscataway, New Jersey).

Animals

SHR and WKY rats from Charles River Laboratories (Wilmington, Massachusetts) were matched for age (12 to 14 weeks) and sex (male) and were maintained on Ralston Purina Lab Chow and deionized water. Systolic blood pressures were measured in conscious rats by the tail-cuff method with a programmed electrophysymomanometer and physiograph (Narco Biosystems, Houston, Texas). Rats with values above 150 mm Hg were regarded as hypertensive.

Aortic Cell Cultures

Animals were exsanguinated by cardiac puncture under ether anesthesia and sections of the thoracic aorta were removed under sterile conditions to dishes containing minimal essential medium (MEM) with Earle's salts and supplemented with nonessential amino acids, 27 mM sodium bicarbonate, 15% fetal calf serum (FCS), penicillin (25 units/ml), streptomycin (25 μg/ml), and amphotericin B (2.5 μg/ml). The explants were scraped with a scalpel to remove the adventitia, washed with fresh MEM, and then finely minced in 60 mm plastic tissue culture dishes for culturing in MEM at 37°C under a humidified atmosphere of 10% carbon dioxide, 90% air. Aortic cell lines were serially subcultured and frozen for storage as previously described. These cell cultures typically contained 60-80% smooth muscle cells and 20-40% fibroblasts and were used for experimentation at passage 8 through 20.

Whole Cell Binding Assay

Replicate cultures of 2 × 10^6 cells from each rat were seeded in 100 mm tissue culture dishes. From 48 to 72 hours before an experiment, the MEM plus 15% FCS was replaced with MEM containing 10% FCS, which had been treated with charcoal to remove steroid-bound steroid determined by gel filtration chromatography on Sephadex G-25. Nonspecific binding (3H-steroid plus excess nonradioactive steroid) was proportional to the concentration of 3H-steroid. For each experiment all values of nonspecific binding were calculated from a single determination with a 1000- to 1500-fold excess of nonradioactive steroid. Nonspecific binding was subtracted from total binding to give specifically bound steroid.

Preparation of Subcellular Fractions

Aldosterone binding in subcellular fractions was examined in cells that had been harvested with trypsin, washed in ice-cold Tris-sucrose buffer (0.02 M Tris, 0.32 M sucrose, 1 mM MgSO₄, pH 7.5) containing 1 mg/ml human gamma globulin, and resuspended in phosphate-buffered Leibovitz medium containing 1 nM 3H-A with or without 1500-fold excess nonradioactive aldosterone. The cells were incubated for 30 minutes in a Dubinoff metabolic shaking incubator at 37°C, rapidly cooled at 4°C, and centrifuged for 5 minutes at 2800 × g. The cells were then lysed by hypotonic shock and a crude nuclear fraction was prepared by a previously reported method. The nuclei were suspended in 1 ml of column buffer and sonicated in an ultrasonic cleaner at 4°C to break up the nuclear membranes. After centrifugation at 1600 × g for 20 minutes, the supernatant was assayed for DNA concentration and bound radioactivity determined by the method described for whole cells. The residual pellet contained less than 5% of the nuclear binding. The crude cytoplasmic fraction (800 × g supernatant) was centrifuged at 19,000 × g for 15 minutes and the resulting supernatant assayed for specific binding by the gel filtration method described for whole cells.

pH and Temperature Stability Studies

Experimental procedures were the same as those described for the whole cell binding assay with the following exceptions: For the pH stability experiments cells from eight 100 mm dishes were pooled, vigorously suspended, and equally distributed to test tubes. After centrifugation the cells were sonicated in column buffer of varying pH; the sonicates were incubated at 4°C for 60 minutes and then assayed for bound steroid. The optimum temperature for the temperature stability studies was determined by incubating preformed complexes for 15 minutes at 2°C, 10°C, 21°C, 25°C, 33°C, 37°C, and 45°C. For the temperature stability studies the cells from four 100 mm dishes were pooled, vigorously suspended, and sonicated in column buffer, then diluted to a total volume of 4 ml with additional column buffer. Aliquots were removed and assayed for bound radioactivity at various time intervals during incubation at 25°C in a Lauda constant temperature circulating water bath.

Statistical Analysis and Sources of Error in the Binding Data

Binding and blood pressure statistics are expressed as mean ± standard deviation (SD). Data were tested for significance at the 5% level by Student's t test.

The following sources of error in the aldosterone binding assay were identified: counting error, error in the DNA determination, and error due to receptor in-
stability. The coefficients of variation were 2−10% for the sample counts and 15% for the background counts. The DNA intra-assay variation was 5−10%. Additional errors in the binding assay were due to receptor instability. These errors were introduced when assay time, assay temperature or sonication time was varied.

**Results**

**Blood Pressure Measurements**

All SHR rats had blood pressures in excess of 150 mm Hg. The highest blood pressure recorded for a normotensive WKY rat was 142.5 mm Hg. The SHR strain had a significantly (p < 0.001) elevated systolic blood pressure compared to the normotensive strain: 175 ± 11.5 mm Hg (n = 8) and 132 ± 6.8 mm Hg (n = 7) respectively.

**Scatchard Analysis of 3H-Aldosterone Binding**

A complete Scatchard plot of 3H-A binding in aortic cells cultured from WKY rats is shown in top of figure 1. The curvilinear plot in the top of figure 1 was resolved into two lines by a graphical method shown in the bottom section of figure 1: line a represents aldosterone binding to higher affinity sites, previously termed corticoid receptor I (Kd = 0.44 nM, Bmax = 56.1 moles x 10^-18/u g DNA), and line b represents aldosterone binding to lower affinity sites (corticoid receptor II; Kd = 6.08 nM, Bmax = 316.5 moles x 10^-18/u g DNA).

The variation in the binding data within the animal population is depicted geometrically in figure 1 left. The variation of binding at 0.125 nM 3H-A, a point in the corticoid I portion of the curve, was less than the variation in binding at 5 nM 3H-A, a point in the corticoid receptor II portion of the curve: 15.64 ± 1.81 and 147.70 ± 51.24 moles x 10^-18/u g DNA respectively. The coefficients of variation were 11.6% for corticoid receptor I binding and 34.7% for corticoid receptor II binding. The corticoid receptor II sites are not saturated by aldosterone under the experimental conditions; this may account for the greater variation in the data.

Scatchard plot analysis of aldosterone binding in aortic cell cultures from all WKY and SHR rats is summarized in table 1. Both strains exhibited similar dissociation constants and binding capacities for corticoid receptors I and II. The large variation in Bmax for corticoid receptor I in the SHR strain was due to a single rat cell line which exhibited 6–7 times the binding of the other seven cell lines. If this animal is left out of the analysis, then the mineralocorticoid binding capacity is 69.0 ± 27.8 moles x 10^-18/u g DNA.

**Blood Pressure and Binding Capacity**

Systolic blood pressure and the aldosterone binding capacity of both aortic corticoid receptors are poorly correlated. Blood pressure accounts for only 10% (r^2) of the variance observed in Bmax of corticoid receptors I and II in SHR aortic cells. In aortic cells of nonhypertensive rats, blood pressure accounts for 35% and 25% of the variance observed in Bmax of corticoid receptor I and corticoid receptor II respectively.
Scatchard Analysis of $^3$H-Dexamethasone Binding

Dexamethasone binds to a set of displaceable sites with the same affinity as aldosterone for corticoid receptor II sites, but with higher capacity. Therefore, binding of the potent glucocorticoid, dexamethasone, was examined in aortic cells from WKY and SHR rats. A representative saturation curve and Scatchard plot are shown in figure 2. Saturation was achieved at 10-15 nM dexamethasone (fig. 2 left). Unlike the plot of aldosterone binding in the aorta, the Scatchard plot of dexamethasone binding is linear (fig. 2 right). The results of Scatchard analysis of three WKY and three SHR experiments were compared: $K_d = 7.99 \pm 2.54$ nM and $B_m = 10,230 \pm 1,700$ moles $\times 10^{-17}$/μg DNA and $B_m = 10,370 \pm 4,800$ moles $\times 10^{-18}$/μg DNA respectively. The differences in the binding statistics were not significant.

Subcellular Binding

The subcellular aldosterone binding in WKY and SHR rats is compared in table 2. The experiments were performed on pairs of WKY and SHR aortic cell cultures incubated at 37°C for 30 minutes with 1 nM $^3$H-A with and without 1500-fold excess of nonradioactive aldosterone. At 1 nM $^3$H-A the corticoid receptor I sites were saturated, however, the ratio of occupied corticoid receptor I sites to occupied corticoid receptor II sites is approximately one. Therefore, it is not known if the binding recovered in the nuclear fraction represents steroid bound to one or both sites. Since binding was equivalent in 100,000 x g supernatants, the latter was used to estimate cytosol binding. The fractiona...

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**Table 1. Scatchard Analysis of Aldosterone Binding in WKY and SHR Rats (± se)**

<table>
<thead>
<tr>
<th>Strain (N)</th>
<th>Corticoid receptor I</th>
<th>Corticoid receptor II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (nM)</td>
<td>$B_m$ (moles $\times 10^{-17}$/μg DNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (7)</td>
<td>0.49 ± 0.20</td>
<td>10.71 ± 3.74</td>
</tr>
<tr>
<td>SHR (8)</td>
<td>0.47 ± 0.25*</td>
<td>9.26 ± 5.94*</td>
</tr>
</tbody>
</table>

*Not significant at the 5% level.

**Table 2. Percentage of Aldosterone Binding in Subcellular Fractions of WKY and SHR Rats (± se)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>19,000 x g supernatant</th>
<th>Nuclear pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>74.6 ± 7.4</td>
<td>25.4 ± 7.4</td>
</tr>
<tr>
<td>SHR</td>
<td>70.1 ± 10.3*</td>
<td>29.9 ± 10.3*</td>
</tr>
</tbody>
</table>

*Not significant at the 5% level.
tion procedure did not decrease the recovery of specifically-bound radioactivity since the nuclear binding plus the 19,000 × g supernatant binding closely approximated (101%) the whole cell binding; the sum of the binding in the two fractions was used as the denominator in table 2. Binding in the residual pellet was not included in the estimate of nuclear binding. The subcellular binding was similar for both strains with 70–75% of the binding sites recovered in the 19,000 × g supernatant and 25% to 30% recovered in the nuclear pellet. The percentage of binding in the subcellular fractions was unaffected by the number of whole cell binding sites which varied from experiment to experiment.

pH and Temperature Stability

Cells cultured from WKY and SHR rats were incubated with 1 nM [3H]-A at 37°C for 30 minutes and the pH and temperature stability of the complexes compared for the two strains. Preliminary experiments indicated that nonspecific binding did not change with buffer of varying pH. Therefore, only total binding was determined in subsequent pH stability studies. The data in figure 3 demonstrate that the total binding for both strains was very stable from pH 5 to pH 10, but that some loss of binding did occur at pH 11. The results at each pH were compared and all differences between the two strains were not statistically significant.

In contrast to their marked pH stability, aortic aldosterone binding sites are extremely thermolabile. When preformed complexes were incubated at various temperatures for 15 minutes and then assayed for bound radioactivity, a sharp decline in activity was apparent from 21°C to 37°C for both strains (not shown). Temperature stability experiments performed at 25°C for WKY and SHR rats showed similar time- and temperature-dependent dissociation of bound aldosterone (fig. 4). Statistical analysis of the data at each time point showed no significant difference between the two strains. The half-life of aortic aldosterone binding is 30–35 minutes at 25°C.

Discussion

In spite of highly significant differences in systolic blood pressure between the two strains, aldosterone binding sites in SHR and WKY aortic cells were similar in terms of affinity, capacity, subcellular binding, and pH- and temperature-stability. Differences in aldosterone binding statistics between the strains were not obscured by variation in the binding data within an animal population (fig. 1 left), since a similar pattern of variation was observed for seven repetitive experiments on the same subculture of a rat aortic cell line. The steroid specificity of aldosterone binding was not compared; these studies are difficult to interpret in the aortic cell system because of the relative lack of specificity between hormones with classical glucocorticoid and mineralocorticoid effects in competing for corticoid I or corticoid II sites. The affinity and capacity of dexamethasone binding sites were also indistinguishable in the genetically hypertensive rats and their normotensive controls. In fact, aldosterone and dexamethasone binding statistics were the same as those

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** The pH stability of [3H]-aldosterone binding in WKY and spontaneously hypertensive (SHR) rats. Preformed complexes were incubated for 60 minutes at 4°C in column buffer of varying pH. The assay is normally done at pH 7.5 and the results are expressed as the percentage of total binding at pH 7.5. Open circles (SHR) and triangles (WKY) indicate the mean of three experiments and the bars indicate the sd.

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Temperature stability at 25°C of [3H]-aldosterone binding in WKY and SHR rats. At zero time the preformed complexes were at 4°C. Specific aldosterone binding at each time point is expressed as the percentage of specific binding at zero time. Open circles (SHR) and triangles (WKY) indicate the mean of three experiments and the bars indicate the sd.
previously reported for Sprague-Dawley rats, which were neither matched for age nor monitored for blood pressure.7

The cell morphology and growth pattern of SHR and WKY aortic cell lines were indistinguishable and were similar to those already described for aortic smooth muscle cell cultures selected from SHR and WKY rats by Sands et al.15 The proportion of smooth muscle cells and fibroblasts in the aortic cultures did not contribute to error in the binding data since the Kd and Bmax of predominantly smooth muscle cell cultures were not significantly different from predominantly fibroblast cultures.7

A comparison of mineralocorticoid binding in the aorta of SHR and WKY rats was of particular interest because similar ionic disturbances have been found in aortic smooth muscle of SHR and DOCA-salt treated rats.4–6 Mineralocorticoid involvement is also suggested by the fact that adrenalectomy delays the development of hypertension in SHR rats.16 Several studies indicate that mineralocorticoids and glucocorticoids increase vascular reactivity to catecholamines17–19 and hyperreactivity of the sympathetic nervous system has been implicated in the pathophysiology of hypertension in the SHR strain.20

Feldman examined SHR rats for defects in binding to classical renal mineralocorticoid receptors.8 Mineralocorticoid receptors in kidney slices of SHR and WKY rats exhibited similar properties of affinity, capacity, specificity, and nuclear uptake. These studies on aortic aldosterone binding differ from the renal experiments in two ways. First, on the basis of steroid specificity, aortic aldosterone binding sites differ from classical mineralocorticoid and glucocorticoid receptors.7 Aortic binding sites may represent a new class of steroid receptors with physiological effects different from classical renal receptors. Second, aortic cells were in culture for 8–20 passages. Therefore, the results exclude intrinsic genetic differences in aortic cell aldosterone binding, but do not exclude in vivo differences due to extrinsic factors present in hypertensive and normotensive rats.

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Aldosterone binding sites in aortic cell cultures from spontaneously hypertensive rats.
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