Increased angiotensin II binding affinity in the nucleus tractus solitarius of spontaneously hypertensive rats

(Laura M. Plunkett and Juan M. Saavedra)

Section on Clinical Pharmacology, Laboratory of Clinical Science, National Institute of Mental Health, 9000 Rockville Pike, Building 10, Room 2D46, Bethesda, MD 20892

Communicated by Irvine H. Page, July 26, 1985

ABSTRACT Angiotensin II (Ang) binding kinetics were determined in discrete brainstem nuclei of 14-week-old spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) by a quantitative autoradiographic technique. Tissue sections were incubated with [125I]-labeled [sarcosine-1]Ang, and results were analyzed by computerized densitometry and comparison to [125I]-labeled standards. A single class of high-affinity binding sites was identified in the nucleus tractus solitarius, the area postrema, and the inferior olivary nuclei of both SHR and WKY rats. Ang binding affinity was significantly greater in the nucleus tractus solitarius of SHR compared to nonnormotensive WKY rats (0.27 ± 0.06 × 10^6 M^-1 in WKY rats vs. 0.59 ± 0.15 × 10^6 M^-1 in SHR), with no apparent changes in the maximum binding capacity of this area. There were no changes in the Ang binding kinetics of the area postrema or the inferior olivary nuclei. Our results suggest that central Ang activity is altered in established hypertension in a brainstem area of SHR associated with peripheral cardiovascular control.

After the identification of components of a central renin–angiotensin system, angiotensin II (Ang) was linked with centrally mediated cardiovascular effects (1, 2). Specific receptors for Ang have been localized to brain areas involved in cardiovascular regulation, including the nucleus tractus solitarius, area postrema, locus coeruleus, and dorsal motor nucleus of the vagus (3, 4). Additionally, Ang immunoreactive cells and fibers have been identified in the rat central nervous system, also in areas associated with peripheral cardiovascular control (5). Microinjections of Ang into the nucleus tractus solitarius have been shown to produce dose-dependent increases in blood pressure that are similar to increases seen after intraventricular injection of Ang (6). These studies link central Ang activity with cardiovascular control.

Evidence has also shown that alterations in central Ang activity occur during the pathogenesis of spontaneous hypertension. Angiotensin-converting enzyme antagonists, such as captopril, lower blood pressure in spontaneously hypertensive rats (SHR), with a greater reduction occurring after intraventricular administration (7). Ang levels have been shown to be increased in the brain of SHR, including brain areas known to affect cardiovascular function (8). These results suggest that Ang hyperactivity may contribute to the maintenance of hypertension in SHR. The purpose of this study was to determine if brainstem Ang receptor binding kinetics are altered in spontaneous hypertension, a change that could contribute to the hyperactivity of the central Ang system in these animals.

METHODS

Male SHR and Wistar-Kyoto (WKY) rats (Taconic Farms, Germantown, NY) 14 weeks of age, were housed at a constant temperature with lights on from 0600 to 1800 and given free access to food and water. Prior to sacrifice, blood pressures were measured in all animals with an indirect tail cuff method. Rats were sacrificed by decapitation between 0900 and 1100 and their brains were removed and frozen by immersion in isopentane at −30°C. Within 24 hr, tissue sections (16 μm) were cut in a cryostat at −14°C, thaw-mounted onto glass slides coated with gelatin/chrome alum, and placed under reduced pressure at 4°C until incubation.

Ang binding sites were labeled in vitro by incubation with [125I]-labeled [sarcosine-1]Ang ([125I]-[Sar]Ang; a gift from M. Khosla, Cleveland Clinic, Cleveland, OH). The ligand was iodinated by a modified chloramine-T method (Meloy Laboratories, Springfield, VA). Experiments were performed in four SHR and four WKY rats. Multiple sections for each brainstem area were incubated simultaneously to allow for complete Scatchard analysis in single nuclei from each rat. Tissue sections were first incubated at 20°C for 15 min in 10 mM sodium phosphate buffer (pH 7.4) containing 120 mM NaCl, 5 mM EDTA, 0.1 mM bacitracin, and 0.2% bovine serum albumin. The tissue sections were then incubated for 60 min in fresh buffer containing [125I]-[Sar]Ang in concentrations ranging from 80 pM to 5 nM. Nonspecific binding was determined in the presence of unlabeled Ang in concentrations ranging from 80 nM to 5 μM. All slides were washed four times (60 sec each) in 50 mM Tris-HCl buffer (pH 7.5) at 4°C and dried under a cold stream of air (9, 10).

To allow for quantification of ligand binding, sets of [125I]-labeled standards were prepared as described for [3H]-labeled standards (11). Known amounts of [125I] in increasing concentrations were thermally mixed with a brain paste, mounted as blocks on specimen holders, and frozen. Sections (16 μm) were cut on a cryostat at −14°C and thaw-mounted onto glass slides coated with gelatin/chrome alum. Adjacent sections were cut from each concentration sample of [125I] for determination of protein content (12) and radioactivity.

Incubated tissue sections and a set of [125I]-labeled standards were placed in cassettes (CGR, Baltimore, MD) and opposed against Ultrofilm 3H (LKB) at room temperature for 1–4 days, depending upon the concentration of ligand. The films were developed at 20°C for 4 min with undiluted Kodak D19 developer and optical densities were quantitated by computerized densitometry (10, 11). The optical densities observed in each brain area were related to the concentration of radioactivity contained in the [125I]-labeled standards that were processed with each autoradiograph. There was a linear

Abbreviations: Ang, angiotensin II; Sar, sarcosine; SHR, spontaneously hypertensive rats; WKY rats, Wistar-Kyoto rats; NTS, nucleus tractus solitarius; AP, area postrema; OL, inferior olivary nuclei.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
relationship between the natural logarithm of the optical densities of each standard concentration and the natural logarithm of the radioactivity contained in that section at any exposure of Ultrag film (13). This plot of In OD vs. In dpm for the standards was the basis for comparison of the brain tissue sections for determination of molar quantities of ligand bound in a particular brain area and Scatchard analysis of the ligand binding.

Binding data were analyzed and Scatchard plots were produced with the LIGAND computer program (14). All data are presented as the mean ± SEM. Statistical analysis was determined with analysis of variance using the GLM procedure of the SAS package (SAS Institute, Cary, NC) that allows for unbalanced designs and unequal cell sizes.

RESULTS

Blood Pressure Measurement. Blood pressures were elevated in 14-week-old SHR when compared to age-matched WKY rats. Mean arterial pressure was 112 ± 6 mm Hg in WKY and 171 ± 5 mm Hg in SHR (1 mm Hg = 133 Pa).

Ang Binding in Rat Brainstem. There were high-affinity binding sites for Ang in several specific areas of the brainstem, including some areas involved in cardiovascular regulation. Fig. 1A depicts a typical 16-μm tissue section labeled with 5 nM 125I-[Sar1]Ang. Specific binding was localized to the nucleus tractus solitarius (NTS), the area postrema (AP), and the inferior olivary nuclei (OL). Average densities in these areas (fmol/mg of protein) were determined with 5 nM ligand in WKY rats: NTS, 903 ± 18; AP, 963 ± 99; OL, 156 ± 15. Nonspecific binding is illustrated in Fig. 1B with an adjacent section labeled with 5 μM Ang and 5 nM 125I-[Sar1]Ang.

Ang Binding Kinetics in SHR. In addition to identifying and quantitating areas of Ang binding sites, we observed significant differences in Ang binding affinity in the NTS of SHR compared to WKY rats. The Ang binding affinity constant (Kd) increased from 0.27 ± 0.06 × 10^9 M^{-1} in WKY rats to 0.59 ± 10^9 M^{-1} in SHR (Table 1). Scatchard plots for both the NTS and the AP are illustrated in Fig. 2. There were no significant changes in the Kd for either the AP or OL areas (Table 1). Additionally, the B_{max} was not significantly altered in SHR vs. WKY rats in any of the areas (Table 1), although there was a trend towards a decreased B_{max} in SHR.

DISCUSSION

Our data provide the receptor binding kinetics in discrete brain nuclei of individual rats. A single class of high-affinity binding sites for Ang was localized in brainstem areas associated with cardiovascular control (Figs. 1 and 2). Binding was also identified in the OL, an area not known to be involved in blood pressure regulation. The density of Ang binding sites was higher in both the NTS and AP than in the OL area. The average binding site density within a particular area can be calculated from the amount of ligand bound after incubation with a saturating concentration of that ligand.

Table 1. Maximum binding capacity (B_{max}) and binding affinity (Kd) of 125I-[Sar1]Ang in brain nuclei of SHR and WKY rats

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Strain</th>
<th>n</th>
<th>Kd</th>
<th>B_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTS</td>
<td>WKY</td>
<td>4</td>
<td>0.29 ± 0.05</td>
<td>1717 ± 369</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>4</td>
<td>0.59 ± 0.15*</td>
<td>1199 ± 643</td>
</tr>
<tr>
<td>AP</td>
<td>WKY</td>
<td>4</td>
<td>0.47 ± 0.08</td>
<td>1129 ± 355</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>4</td>
<td>0.42 ± 0.05</td>
<td>1198 ± 133</td>
</tr>
</tbody>
</table>

*Significant difference from WKY (F = 5.63, P = 0.01).

By performing Scatchard analysis of the data, we determined the maximum binding capacity (B_{max}) as well as the binding affinity constant (Kd) for Ang. Changes in Ang receptor binding affinity and binding capacity have been

Fig. 1. Autoradiographic analysis of 125I-[Sar1]Ang binding sites in discrete brainstem nuclei of SHR and WKY rats. (×6.) (A) Typical 16-μm section labeled with 5 nM 125I-[Sar1]Ang, arrows indicate nucleus tractus solitarius (NTS), area postrema (AP), and inferior olivary nuclei (OL). (B) Typical 16-μm section labeled with 125I-[Sar1]Ang and 5 μM unlabelled Ang to depict nonspecific binding. (C) Section from A stained with luxol fast blue. 
Dehydration increased Ang receptor number in the organon subformicalis (13) and the anterior pituitary (10), while the $K_a$ for Ang pituitary receptors was decreased with water deprivation (10). Our results show that there were quantitative changes in Ang binding only in the NTS of SHR compared to WKY rats. The $K_a$ was greater in 14-week-old SHR than in age-matched WKY rats (Table 1). There was no significant difference in $B_{max}$ in the NTS of these rats, although there was a trend towards a lower maximum binding capacity in SHR concomitant with the higher $K_a$. Ang binding kinetics were not altered in either the AP or OL in hypertensive vs. normotensive rats.

The NTS plays a major role in central blood pressure control mechanisms in the brainstem. It can act as a relay point for sympathetic outflow rostrally to the hypothalamus and to the periphery via preganglionic neurons (16). The NTS also receives input from other brain nuclei involved in cardiovascular regulation, including the locus coeruleus, via A1 catecholaminergic cells, and the AP, a circumventricular structure directly adjacent to the NTS (16). Each of these areas is involved in central sympathetic responses, and central sympathetic hyperactivity is characteristic of SHR (17). Ablation of sympathetic nervous system function by surgical, chemical, or pharmacological manipulation has been shown to reduce blood pressure (18–20) or prevent the development of hypertension in SHR (21, 22). Further, brain nuclei that affect blood pressure control through sympathetic responses, such as the NTS and locus ceruleus, are structurally changed in spontaneous hypertension (23). This suggests that NTS activity and increased sympathetic outflow could play a role in the elevated mean arterial pressure of SHR.

In addition to sympathetic reactivity, many lines of evidence suggest that central renin–angiotensin system activity is increased in spontaneous hypertension. The Ang receptor antagonist saralasin reduced blood pressure in SHR when administered centrally, even in the absence of peripheral plasma renin activity (24). Direct measurements of components of the central renin–angiotensin system also provides evidence for central hyperactivity of the peptide. Increased central renin activity (25), elevated cerebrospinal fluid Ang levels (26), and increased brain Ang turnover (27, 28) have all been associated with SHR. A recent study by Kawasaki et al. (29) demonstrated that SHR exhibit an enhanced capacity for neurotransmission due to increased Ang actions in the mesenteric vasculature. A similar action of Ang in brain nuclei involved with cardiovascular control may explain the enhanced sympathetic activity and increased blood pressure associated with spontaneous hypertension.

Direct evidence for a role of Ang at the level of the NTS in the production of hypertension in the rat was recently reported by Casto and Phillips (6, 30). Microinjections of the peptide directly into the nucleus produced dose-dependent increases in blood pressure (6). To determine the mechanism of the Ang-induced pressor response, the researchers explored both neural and humoral factors and found that the response was mainly due to increased sympathetic activity (30). Further, their results suggest that local Ang formation occurs in the NTS (30). Immunohistochemical evidence also indicates that Ang immunoreactive cells are located in the NTS of the rat, additional support for a local Ang system (5).

Our results support the idea of a functional local Ang system in the NTS of SHR. Binding site localization and altered binding kinetics are associated with this nucleus, with no changes in AP or OL binding kinetics. Singh et al. (31) explored Ang binding kinetics in larger brain areas and failed to detect any alterations in binding affinity or maximum binding capacity in response to chronic receptor stimulation. Their results may be explained by lack of discrete localization; our data are indicative of local changes in discrete brain nuclei, which may be masked if the entire brainstem is examined. The lack of altered Ang binding kinetics in the AP, an area rich in Ang immunoreactivity (5) as well as Ang receptors (3, 4), may be attributed to the fact that this area is not of primary importance in central Ang-mediated blood pressure responses in SHR. However, the changes in NTS of SHR may be directly related to central sympathetic hyperactivity associated with the maintenance of spontaneous hypertension. It remains to be determined if other brain nuclei that participate in peripheral cardiovascular regulation are similarly altered.

We thank Dr. Hans Agren for his assistance with statistical analysis of the data, Ms. Pauline Cover for her technical assistance, and Ms. Sue O’Harra for editorial assistance.
7724 Medical Sciences: Plunkett and Saavedra