Angiotensin-Like Immunoreactivity in the Brain of the Spontaneously Hypertensive Rat

JAMES A. WEYHENMEYER, PH.D., AND M. IAN PHILLIPS, PH.D.

SUMMARY Evidence for the brain renin-angiotensin system being involved in the hypertension of the spontaneously hypertensive rat (SHR) includes central administration of angiotensin II (All) antagonists and converting enzyme inhibitors that lower blood pressure in SHR. Using the unlabeled antibody enzyme method, we have found a significant difference in the distribution of brain angiotensin in SHR and Wistar-Kyoto controls (WKY). Six rats of each group were perfused with buffered picric acid-paraformaldehyde, and their brains sectioned at 50 and 100 μ. The sections were reacted with a 1:1000 dilution of All antiserum for 36 hours followed by goat antirabbit immunoglobulin G and rabbit peroxidase antiperoxidase. For controls, preabsorption with All, arginine vasopressin or preimmune serum were evaluated. The results showed over twice as many cells and fibers staining for All-like immunoreactivity in SHR. The All immunoreactive cell bodies were localized, in the order of their relative preponderance, in supraoptic and paraventricular nuclei of the hypothalamus, hippocampus, and cortex. The most prominent demonstration of All-like immunoreactivity was observed in fiber profiles containing densely stained varicosities, which were present in many neuroanatomical subdivisions of the brain and brain stem including anterior and middle hypothalamus, basal ganglia, thalamus, locus coeruleus, nucleus of the solitary tract, limbic structures, and reticular formation. The increased fiber staining in the SHR was particularly evident in the frontal hypothalamic region, medial preoptic, and stria terminalis. We conclude that the results support the hypothesis of brain All involvement in hypertension. (Hypertension 4:514-523, 1982)

KEY WORDS • angiotensin II • immunochemistry • brain • spontaneously hypertensive rat

SPONTANEOUSLY hypertensive rats (SHR) begin to develop high blood pressure in the first few weeks of life. By 10 weeks, the SHR have established hypertension reflected in blood pressure levels significantly higher than their appropriate Wistar-Kyoto controls (WKY). Numerous mechanisms for the development and maintenance of hypertension in SHR have been hypothesized. These include high renin at birth, elevated brain catecholamines in development, kidney dysfunction, altered baroreceptor reflex, increased vascular reactivity, and elevated vasopressin levels, but no unifying concept has been accepted.

The central nervous system could play a role in this pathophysiology, and one possible cause of maintained hypertension in these rats is an increased amount of angiotensin II (All) in the brain. Although the rats have low or normal plasma renin-angiotensin levels, there are indications that angiotensin in the brain has more powerful effects than angiotensin in the periphery. When the kidneys are removed in SHR, no change in blood pressure is seen, thereby eliminating the role of peripheral angiotensin in the maintenance of the hypertension. However, when angiotensin is injected directly into the brain of SHR, there is a marked increase in responsiveness compared to the same dose injected into the brains of WKY. In addition, central infusion of saralasin, the angiotensin II antagonist, consistently reduces blood pressure in SHR, although it is without effect in the normotensive WKY.

The brain renin-angiotensin system is poorly understood, but recently we carried out immunocytochemical studies to localize the extent of the angiotensin-like immunoreactivity in the brain. In this report, we have compared the distribution of All-like immunoreactivity in SHR and WKY brains. The finding of a
denser and broader distribution of the All-like substance in SHR supports the concept of the brain renin-angiotensin system playing a role in the maintenance of high blood pressure in SHR.

Methods
Tissue Preparation and Staining
Adult male WKY rats (n = 6) and SHR (n = 6) weighing from 250-350 g were anesthetized with chloral hydrate and physiologically perfused via the left ventricle with buffered picric acid-paraformaldehyde, pH 7.4 (Zamboni's fixative). Brains were removed to the level of the middle medulla and placed in fresh perfusate for 4 hours at room temperature. The tissue was sectioned by vibratome at 50 and 100 μ and incubated in 0.4% Triton X-100 in 0.1 M Tris buffered saline (TBS), pH 7.4, for 30 minutes at room temperature.

Specific antiAll serum (provided by Dr. Detlev Ganten, University of Heidelberg) was generated in rabbits that were immunized and challenged with 5-isoleucine All conjugated to bovine serum albumin (BSA). This antiserum has been titered at 1:90,000 for 50% binding by radioimmunoassay. No significant cross reactivity has been demonstrated with a variety of like and unlike peptides, including arginine vasopressin, bradykinin, thyrotropin-releasing factor, somatostatin, and AI. It is possible that the smaller fragments of the All sequence are being positively stained in this reaction. It has been reported, however, that fragments of the All have the same physiological action on neurons as the intact molecule.

Serial sections were incubated in a 1:1,000 dilution of antiAll serum, preabsorbed with 1 mM BSA, in 0.1 M TBS for 36 hours at 4°C. The tissue was gradually brought to room temperature, washed in 0.1 M TBS, and treated with a 10-fold excess concentration of goat antirabbit immunoglobulin G (Cappel Laboratories, Cochranville, Pennsylvania) and rabbit peroxidase antiperoxidase (Cappel Laboratories) for 1 hour at room temperature, with intermittent washes in TBS. Control sections were incubated with antiAll preabsorbed with excess All, or antiAll preabsorbed with excess arginine vasopressin, or TBS or preimmune rabbit serum substituted for the primary antiserum. The tissue was cytochemically reacted with 0.03% diaminobenzadine in 0.1 M TBS, with 15% absolute ethanol for 6 minutes at room temperature. Sections were mounted and examined with a Leitz Orthoplan light microscope. Areas containing All-like immunoreactive cell bodies and fiber profiles were plotted on parasagittal drawings.

Results
In this study we report the distribution of immunoreactive All-like material in adult SHR and WKY rat brain by the unlabeled antibody enzyme method. Although the most prominent staining was localized to small diameter fibers with densely stained varicosities throughout the brain and brain stem, we observed All immunoreactivity in neuronal soma of selective forebrain areas. Further, we report differences in the fiber and cell body content and distribution in several areas of SHR and WKY rat brain.

Positive All cell bodies were seen in the paraventricular and supraoptic nuclei of the hypothalamus, hippocampus, and cortex. In the hypothalamus, only a few magnocellular neurons of the supraoptic (SON) and paraventricular (PVH) nuclei contained immunoreactive All (fig. 1). Low intensity staining was diffusely localized in these neurons, and occasionally we found immunoreactive fibers originating from positively stained cell bodies. The parvocellular neurons of both nuclei remained unstained. Although there were no demonstrable differences in the staining intensity of SHR and WKY hypothalamic neurons, we observed a significant increase in the number of All containing SON and PVH cell bodies of SHR compared to WKY rats. Staining of hippocampal neurons was confined to the CA3 region. These cells always contained densely stained granules in the nonnuclear soma (fig. 2). Occasionally we found small neurons in the frontal, parietal, and temporal cortex that contained stained granules similar to the CA3 neurons.

Small diameter immunoreactive fiber profiles were demonstrated throughout the brain and brain stem. Most of these All-like immunoreactive fibers had prominently stained varicosities that could be clearly visualized along the length of the profile (fig. 3). With the utilization of 50 and 100 μ coronal and parasagittal sections, we were able to follow the tortuous course of many of these fiber profiles through the surrounding tissue. Positively stained fibers could be traced for...
variable distances, with some fibers coursing for several millimeters through major neuroanatomical subdivisions. Arborizations were occasionally seen along the length of the fiber profiles with branching fibers containing stained varicosities similar to the parent fiber. In many instances, immunoreactive fiber terminals appeared to end in discrete nuclei of the brain and brain stem. A small number of fibers, with densely stained varicosities, followed the course of small and medium sized arteries through the brain parenchyma.

In SHR and WKY rat, moderate concentrations of immunoreactive fibers were found in the arcuate nucleus of the hypothalamus and in structures surrounding the lateral third and fourth ventricles. These ventricular immunoreactive fibers often coursed through the subependymal layer and frequently projected to the ependymal surface of the ventricle (figs. 4 and 5). This staining pattern was most apparent along the ventricular border of the lateral septum and the hypothalamus at the level of and ventral to the paraventricular nu-
BRAIN ANGIOTENSIN IN SHR/Weyhenmeyer and Phillips

Figure 6. Bifurcating All fiber in the posterior thalamic nucleus of the SHR brain. ×1750

Moderate-to-low concentrations of All-like immunoreactive fibers were also localized in the following brain and brain stem areas of SHR and WKY rats in the order of their relative preponderance: stria terminalis and medullaris, basal ganglia, anterior and middle hypothalamus, locus coeruleus, nucleus of the solitary tract, and reticular formation. Other areas contained isolated fibers that were seen in large neuroanatomical subdivisions such as the cortex. Increases in SHR fiber density were most prominent in the medial preoptic area and the stria terminalis, although we noted a higher All fiber content in all areas of SHR brain.

Other differences in the fiber content of SHR and WKY brain were found in the thalamic nuclei: SHR contained varicose fibers in the anteromedial, dorsomedial, and posterior thalamic nuclei (fig. 6). These areas were free of All immunoactivity in WKY rats. Cross sections of SHR and WKY brain are shown in figures 7–10.

Abbreviations for Figures 7–10

AHA Anterior hypothalamic area  MH Medial hypothalamus
AM Anteromedial nucleus of the thalamus  MPO Medial preoptic area
ARH Arcuate nucleus  NPH Prepositus nucleus
BCS Brachium of the superior colliculus  NPT Posterior nucleus of the thalamus
CAP Anterior commissure, posterior part  OT Optic tract
CC Corpus callosum  OVLT Organum vasculosum of the lamina terminalis
CH Hippocampal commissure  POA Lateral preoptic area
CIF Inferior colliculus  PV Paraventricular nucleus of the thalamus
CO Optic chiasm  PVG Periventricular grey
CPO Caudate nucleus/putamen  PVH Paraventricular nucleus of the hypothalamus
CT Cortex  RF Reticular formation
DBB Diagonal band of Broca  RT Reticular nucleus of the thalamus
DBC Decussation of the brachium conjunctivum  SC Suprachiasmatic nucleus
DMH Dorso medial nucleus of the hypothalamus  SCC Splenium of the corpus callosum
DR Dorsal raphe nucleus  SL Lateral septum
FD Dentate gyrus  SM Stria medullaris thalami
FI Fimbria of the hippocampus  SN Substantia nigra
FLM Medial longitudinal fasciculus  SOL Solitary nucleus
FX Fornix  SON Supraoptic nucleus
GCC Genu of the corpus callosum  ST Stria terminalis
HL Lateral habenular nucleus  TOL Lateral olfactory tract
HP Habenulo-interpeduncular tract  TS Nucleus triangularis septi
HPC Hippocampus  V Ventricule
LL Lateral lemniscus  VA Ventral nucleus of the thalamus, anterior part
LT Lateral nucleus of the thalamus  VB Ventral nucleus of the thalamus, basal part
LTP Lateral nucleus of the thalamus, posterior part  VMH Ventromedial nucleus of the hypothalamus
MD Dorso medial nucleus of the thalamus  X Nucleus of the vagus
**FIGURE 7.** Sagittal drawings of WKY and SHR brains showing the distribution of All immunoreactivity in midline structures. Cross-hatched areas represent moderate-to-high density staining in fiber profiles; hatched areas indicate low-to-moderate staining in fiber profiles; and closed circles represent stained cell bodies. The localization of All immunoreactive fiber profiles in the anteromedial nucleus of the thalamus was observed in only the SHR brains.
FIGURE 8. Parasagittal drawings (0.5 mm from the midline) of WKY and SHR brains showing moderate-to-high density staining (cross-hatched) and low-to-moderate density staining (hatched) of AII immunoreactivity in fiber profiles. All fiber profiles in the anteromedial and dorsomedial nuclei of the thalamus were localized in SHR, but not WKY rat brains.
Figure 9. Parasagittal drawings (1.3 mm from the midline) of WKY and SHR brains showing moderate-to-high density staining (cross-hatched) and low-to-moderate density staining (hatched) of immunoreactive All in nerve fibers and terminals. At this level, changes in the distribution of All fiber profiles between the two strains were observed in the dorsomedial, lateral, and posterior nuclei of the thalamus, the hippocampus and hippocampal commissure, the dentate gyrus, and the brachium of the superior colliculus.
FIGURE 10. Parasagittal drawings (2.1 mm from the midline) of WKY and SHR brains showing the distribution of immunoreactive All. Cross-hatched areas represent moderate-to-high density staining in fiber profiles; hatched areas indicate low-to-moderate density staining in fiber profiles; and closed circles represent stained cell bodies. Changes in the distribution of All fiber profiles between the two strains were found in the posterior part of the lateral thalamic nucleus. Occasionally, stained CA3 cell bodies were found in the hippocampus of the WKY rat.
Discussion

The fiber pattern with All-like immunoreactivity confirms the earlier reports of All distribution in rat brain.7 There is a selective distribution of fibers in localized regions of the brain, but the distribution cannot be called widespread and is not found throughout the brain. Areas in which the fibers were absent included the cerebellum and large sections of the midbrain and diencephalon. This gives the impression of a specific and well-localized pattern of fiber distribution emanating from comparatively few cells in the supraoptic and paraventricular nucleus. The main difference between the SHR and WKY controls was the overall increase in fiber density in the SHR, and the presence of fibers in areas where fibers were absent in the controls. These areas included medial preoptic, the stria terminalis, and the thalamic nuclei. The significance of the distribution for any one particular function is not immediately obvious. There is sufficient data to implicate All in thirst, vasopressin release, and sympathetic activation.9,10 Therefore, some of the sites where fibers were found fit with some of the structures known to be involved in these responses. For example, the organum vasculosum of the lamina terminalis (OVLT) has been hypothesized as a receptor site for All in its various responses when given centrally.9,11 and the tractus solitarius is associated with blood pressure control.12 The areas containing angiotensin-like immunoreactive fibers also match the areas that have been shown to contain All receptor binding sites.13-16 These include the OVLT, the septum, hypothalamus, and the colliculi close to the periventricular gray. Thus, it appears that the fibers are more concentrated in SHR where receptors for angiotensin are found. Physiologically, more fibers in receptor areas would have the effect of increasing activation of angiotensin receptors and consequently exaggerating the responses. In fact, that is what one sees with the SHR; they have higher blood pressure, more vasopressin release,8 and, although they do not drink more water, they do drink more sodium if offered sodium.8 Sodium appetite is characteristically elevated when All is injected into the brain.19

Although there are undoubtedly multiple causes for the development and maintenance of hypertension in the SHR, there now appears to be strong evidence that a component of the hypertension is due to the effect of All on brain receptors. The source of the All could be from the periphery or from the brain or both.

Higher circulating levels of plasma renin in SHR would induce vasoconstriction and account for the hypertension very simply. However, measurements of plasma renin levels in the SHR show they are the same as those in the WKY controls, less than 5 ng All ml⁻¹ hr⁻¹ or lower.1,3

We also nephrectomized SHR bilaterally to remove all possible sources of renal renin. The plasma renin levels fell to below 0.5 ± 0.1 ng All ml⁻¹ hr⁻¹, which is negligibly low, and yet the mean arterial pressure, which before nephrectomy had been 178.3 ± 6.2 mmHg, remained 178.0 ± 6.3 mm/kg after nephrectomy. Nephrectomy was carried out 12 hours prior to testing, which was long enough for the plasma renin to have fallen to virtually zero, but short enough to leave the rats in good condition for arterial blood pressure measurement.

To block circulating All in SHR, the All antagonist Sar¹-Ala⁴-All (saralasin) has been infused intravenously to see if plasma All levels were the cause of hypertension.3 The antagonist should have lowered blood pressure. Saralasin infused in cumulative doses of 0.1, 1.0, 10.0, and 100 ng/kg/min for 15 minutes at each dose over a total of 1 hour with increasing doses had no effect on the blood pressure of SHR (n = 6) or WKY (n = 6). Thus, none of the measures for increased plasma renin activity, either direct assay, nephrectomy, or intravenous infusion of saralasin, supported a role for circulating angiotensin to explain hypertension in the SHR.

Another possibility is that SHR are more sensitive to central effects of All than their controls. Hoffman et al.4 found that an intraventricular injection (i.v.t.) of All produces a significantly greater pressor response in SHR than WKY. At 5, 50, and 500 ng All i.v.t. doses, blood pressures were significantly higher in SHR at the p < 0.05 level. All doses were randomly tested and each test separated by 1 hour to allow full recovery. The increase at 5 ng was 24 ± 6 mm Hg for SHR compared to 8 ± 2 mm Hg for WKY, and at 50 ng the increase in SHR was 38 ± 3 mm Hg compared to 18 ± 1 mm Hg in WKY. At the highest dose of 500 ng, the difference was 40 ± 5 mm Hg for SHR compared to 25 ± 1 mm Hg for WKY. Thus, there could be an increased responsiveness in SHR without an increase in total All. However, the combination of an increased amount of angiotensin in the brain in addition to the increased sensitivity could maintain a hypertensive state.

Independently, Ganten et al.20 and Phillips et al.21 showed a reduction of blood pressure in SHR by administering saralasin i.v.t. Together we found that with a 1 or 5 µg dose, blood pressure measured by the tail-cuff method was significantly lowered (p < 0.05).3 This study has since been repeated by others, and although there have been variations in the strain of SHR used, the type of All antagonist and dose, the method of recording blood pressure, and the age of the rats, the findings are the same.21

The central depressor action of saralasin in this model of hypertension implies that there is an elevated or active level of angiotensin in the brain of SHR normally, which contributes to their hypertensive state. Further, since the pressure elevation is independent of renal renin, this angiotensin must be from an extrarenal source. The most likely extrarenal source is the brain isorenin-angiotensin system. Indeed, higher levels of All have been reported in the cerebrospinal fluid of Smirk strain rats5,20 and in the cerebrospinal fluid of essential hypertensive patients.22

Throughout this study we have been aware that the method of immunohistochemistry is limited and the
All immunoreactivity may not be the same as peripheral All. Thus, while the present study is not proof for the involvement of the brain angiotensin system in hypertension, the increased density and distribution of an immunoreactivity to All found in SHR fit with the other fragments of data that point to a role of All in the maintenance of hypertension.

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