Brain and Liver Angiotensinogen Messenger RNA in Genetic Hypertensive and Normotensive Rats

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The brain's renin-angiotensin system is integrally involved in the regulation of blood pressure and fluid/mineral metabolism. Enhanced activity of the angiotensin system in the brain has been implicated as a possible source of the hypertension and the elevated salt appetite of the spontaneously hypertensive rat, as compared with the Wistar-Kyoto rat. This study tested whether these inbred strains of hypertensive and normotensive rats differ in central or peripheral expression of the gene coding for angiotensinogen, the prohormone for the angiotensin peptides. Angiotensinogen messenger RNA was measured in the brain by in situ hybridization and in the liver by Northern blot analysis, using a synthetic oligonucleotide. There was a 28% greater expression of the angiotensinogen gene in the region of the anteroventral hypothalamus, preoptic area, and medial septum of the hypertensive strain. There were no differences between strains in liver angiotensinogen gene expression. These results are consistent with the possibility that enhanced elaboration of the angiotensin prohormone in the brain contributes, in part, to the hypertension or the elevated salt appetite of the spontaneously hypertensive rat. (Hypertension 1991;17:485-491)

The inbred spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rat strains express distinct cardiovascular and behavioral phenotypes that make them useful models for the study of hypertension. Although derived from the same progenitor strain, the SHR are hypertensive and salt-avid, whereas the WKY rats are normotensive and exhibit a substantially lower appetite for saline solutions. The brain's renin-angiotensin system is integrally involved in the regulation of blood pressure and aspects of fluid/mineral metabolism, including salt appetite. Moreover, in comparison with WKY rats, SHR have increased brain angiotensinogen, angiotensin II (Ang II), and Ang II binding. This heightened action of the angiotensin system in the brain has been implicated as a possible source of the hypertension and the elevated salt appetite of the SHR. We hypothesized that if differences in the brain's renin-angiotensin system were responsible for the differences in cardiovascular or behavioral phenotypes between these genetically distinct strains, then this might be manifested, in part, as a difference between strains in the expression of the gene coding for angiotensinogen, the Ang II prohormone.

Several laboratories have demonstrated the presence of angiotensinogen messenger RNA (mRNA) in the brain. Low but measurable levels of angiotensinogen mRNA are spread diffusely throughout the brain, including the medial septum, bed nucleus of the stria terminalis, preoptic area, the paraventricular, ventromedial, and arcuate nuclei of the hypothalamus, the inferior olive, and the nucleus of the solitary tract. It is interesting that although Ang II has been shown to be localized in neurons, the prohormone, angiotensinogen, and the angiotensinogen mRNA appear to be found predominantly, if not exclusively, in glial cells.

The present study was designed to begin to identify potential relations between brain angiotensinogen gene expression and variability in behavioral or cardiovascular processes that are regulated by Ang II. Comparisons between SHR and WKY rats of angiotensinogen gene expression were made in the central...
nervous system by low resolution in situ hybridization histochemistry and in the liver by Northern analysis to determine whether differences between these strains in angiotensinogen gene expression were consistent with the strain differences in phenotypic blood pressure and salt appetite. In the brain, the anteroven tral hypothalamic area was examined because this area is rich in angiotensinogen mRNA and because of considerable evidence of the importance of the anteroven tral third ventricular (AV3V) region in both cardiovascular and fluid/electrolyte regulation. Hepatic angiotensinogen mRNA was measured because the liver is the primary source of peripheral angiotensinogen.

**Methods**

Subjects for this study were adult (approximately 20 weeks old) male SHR (n=11) and WKY rats (n=11), obtained from Taconic Farms, Germantown, N.Y., at approximately 7 weeks of age. The rats from which brain tissue was taken (six of each strain) had served as control subjects in another experiment, and it was during this other procedure that measurements of salt appetite were obtained. Liver tissue was obtained from half of these subjects (three of each strain). Additional liver tissue was obtained from separate groups (n=5) of SHR and WKY rats. Blood pressure, but not salt appetite measurements were made on these additional subjects.

Except during salt appetite measurements, subjects were housed in groups of three in polycarbonate cages with wood shavings in the bottom and a wire grid top, with access to standard rat chow and water ad libitum. Temperature and humidity were controlled, and lighting was maintained on a 12-hour photocycle.

The procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committees of Columbia University, Health Sciences Division, and the New York State Psychiatric Institute.

**Blood Pressure Measurement**

Subjects were removed from the home cage and placed in a Plexiglas cylinder to prevent locomotion and turning. The cylinder was maintained at a temperature of approximately 30°C. A unit containing an inflatable cuff and photosensor (IITC, Inc., Woodland Hills, Calif.) for tail occlusion and detection of arterial pulse waves was placed on the subject's tail. The arterial pulse waves were amplified on a Grass Model 7 polygraph (Grass Instrument Co., Quincy, Mass.) and displayed on chart paper. When the animal was quiet, the cuff was inflated and then deflated at a constant rate. The cuff pressure at which the arterial pulse returned after tail occlusion was recorded as the systolic blood pressure for a trial, and the median pressure of at least five artifact-free trials was used as the blood pressure measure for a subject.

**Salt Intake Measurement**

Subjects were weighed and housed individually in suspended wire cages, with ad libitum access to (TD 81263, Teklad, Madison, Wis.) sodium deficient chow (approximately 0.01% sodium). Low sodium chow was used so all variability in sodium consumption could be measured through saline intake. Fluids were presented in inverted 100-ml graduated cylinders with rubber stoppers and stainless steel sipper tubes. Two cylinders, one containing 0.9% NaCl and the other containing demineralized water, were attached to the front of each cage. Fluid levels were recorded daily, for 4 days, within a 2-hour period in the middle of the light phase of the photocycle. Daily fluid consumption was calculated as the difference between consecutive measures. At the end of the saline consumption test, the subjects were weighed and mean body weight was calculated as the average of the beginning and end weights. Fluid intake was normalized for body weight. This salt appetite measurement constitutes the only prior treatment of the subjects from which brain tissue was obtained. The subjects were returned to standard housing conditions for at least 2 weeks before being killed for the angiotensinogen mRNA measures.

**In Situ Hybridization**

Subjects were killed by decapitation. The brains and livers were frozen in powdered dry ice, then stored at −70°C until sectioning. Coronal 16-µm brain sections were cut on a cryostat at −20°C, mounted on RNase-free, gelatin-subbed slides and kept frozen at −70°C until fixation. Sections were collected beginning approximately 0.5 mm rostral to the middle of the anterior commissure and extending approximately 0.5 mm caudal to the anterior commissure. Approximately 20 sections for each subject, representing the rostrocaudal extent of this region, were hybridized for this study.

The angiotensinogen probe was a synthetic oligonucleotide (30-mer; 5' dTGCCTACTCAGCATCT-TGTACAAGCTGGAA) corresponding to amino acids 106-115 of the rat prohormone. The probe was radiolabeled at the 3' end with adenine monophosphate residues from [32P]dATP by terminal deoxynucleotidyl transferase. The reaction mixture was composed of 100 mM potassium cacodylate buffer (pH 7.2), 25 mM cobalt chloride, 0.2 mM dithiothreitol, 70 µCi of [α-32P]dATP (ICN; specific activity, approximately 3,000 Ci/mmol), 0.2 µg oligonucleotide, and 12 units of terminal deoxynucleotidyl transferase in a total volume of 10 µl. The reaction mixture was incubated for 2 hours at 37°C. The labeled oligonucleotide was purified on a NENsorb 20 cartridge (New England Nuclear, NEN Du Pont, Wilmington, Del.), according to the manufacturer's directions. A computerized literature search indicated that the 30-mer oligonucleotide sequence chosen for the angiotensinogen mRNA probe should not cross-hybridize with the mRNA for any other published nucleic acid sequence. To further test the specificity of labeling, test hybridizations were performed in the presence or absence of a 30-fold molar excess of unlabeled angiotensinogen probe. The excess unlabeled...
beled probe resulted in an approximately 90% reduction in hybridization in brain, under the conditions described below. The theoretical melting temperature for this 30-mer oligonucleotide, under the conditions used in this study is 61.5°C. The actual melting temperature was evaluated by in situ hybridization in duplicate brain sections using the procedures described below, except that wash temperatures varied from 45° to 85°C (in 5° steps). The empirically determined melting temperature for this oligonucleotide was 62.7°C, indicating that the actual melting temperature for this oligonucleotide closely approximated the theoretical.

Sectioned tissue was fixed for 2 minutes in ice-cold 2% paraformaldehyde, 0.1 M sodium phosphate buffer (pH 7.2), and then washed in ice-cold 0.5x SSC buffer for 2 minutes (1xSSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The sections were dried under an airstream and then hybridized (10×Denhardt’s, 4×SSC, 50% formamide, 100 μg/ml sheared and denatured salmon sperm DNA, and 1×10⁶ cpm/ml phosphorus-32–labeled angiotensinogen probe) overnight at 37°C in a humidified environment. The sections were washed first in 2x SSC at room temperature for 25 minutes, and then 2 hours in 1xSSC at 45°C. Film autoradiograms were produced by apposing the hybridized sections to x-ray film (Kodak XAR-5) in x-ray cassettes with intensifying screens (Cronex Lightning Plus, DuPont, Wilmington, Del.) at −70°C. The film autoradiograms were developed after 3–4 days. Optical densities of the autoradiographic images were quantitated using the Drexel DUMAS imaging system (Philadelphia, Pa.). For each section, the labeled area encompassing the medial septum, medial and lateral preoptic area, anteroventral hypothalamus, and ventral pallidum was outlined and quantitated. Because the angiotensinogen mRNA probe was labeled with phosphorus-32, which produces low resolution autoradiograms, no attempt was made to evaluate hybridization in discrete nuclei. A similar study is currently in progress, using high resolution in situ hybridization techniques, which will provide a detailed anatomical analysis of the SHR-WKY differences in angiotensinogen gene expression.

Each repetition of the hybridization procedure, and resulting autoradiogram, contained at least one SHR-WKY comparison. However, because batches of the probe were labeled in different reactions, tissue was exposed to film for varied lengths of time, and different assays were run at different times, some interassay variability was assumed. To control for potential interassay variability in the data analysis, the optical density measures within assays were normalized using z
transformation. The \( z \) transformation produces a sample with mean equaling 0 and standard deviation equaling 1. Thus, the mean differences between assays were eliminated while the quantitative distribution of observations about the mean, within assays, was preserved. The average optical density \( z \) score was calculated for each subject (within assays) and then a test of strain differences in optical density \( z \) scores (across assays) was made by unpaired \( t \) test.

**Northern Blot Analysis**

Northern analysis of angiotensinogen mRNA was performed on approximately 100 mg liver from each subject. Liver RNA was extracted using the Acid Guanidinium Thiocyanate-Phenol-Chloroform procedure. Tissue was cut from frozen samples and homogenized in 1 ml denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) in a glass-Teflon homogenizer. The homogenate was transferred to a test tube with 0.1 ml 2 M sodium acetate (pH 4), 1 ml water-saturated phenol, and 0.2 ml chloroform-isooamyl alcohol (49:1), added sequentially. After 15 minutes on ice, samples were centrifuged at 10,000g for 20 minutes at 4°C. The aqueous phase (0.5 ml) was removed to another tube with 1 ml isopropanol at -20°C overnight. The next day the samples were centrifuged at 13,000 rpm for 10 minutes at 4°C, the pellet was rinsed in 80% ethanol, dried, then dissolved in 300 \( \mu \)l 0.1% sodium dodecy sulfate by boiling the tubes for 2 minutes. The RNA content was measured by spectrophotometry assuming that an absorption at 260 nm of 24 is equal to an RNA content of 1 mg/ml.

Replicate aliquots of the samples (2–3 per subject), each containing 40 \( \mu \)g RNA, were electropho-
Results

Blood pressure measures on five subjects of each strain and saline consumption measures on six subjects of each strain confirmed the differences between strains in cardiovascular and behavioral phenotypes (Figure 1). The WKY rats were within the normal range for systolic blood pressure (117 ± 7 mm Hg), whereas the SHR were hypertensive (166 ± 10 mm Hg; t(8) = 4.51, p < 0.002). As illustrated in Figure 1B, the SHR also had significantly elevated saline consumption (11.8 ml/100 g/day), relative to WKY rats (3.4 ml/100 g/day; t(10) = 4.49, p < 0.001).

Quantitation of the optical density of the in situ hybridization autoradiograms revealed a 28% greater level of angiotensinogen mRNA in the anteroveral hypothalamic and medial septal area of SHR, relative to WKY rats (t(10) = 7.33, p < 0.001). However, there were no differences between strains in liver angiotensinogen gene expression (t(14) = 0.047, NS). Figure 2 illustrates brain and liver angiotensinogen gene expression in SHR as a percentage of WKY as compared with WKY rats. This finding is consistent with the hypothesis that differences in function of the central nervous system renin-angiotensin system contribute to the differences between these strains in phenotypic blood pressure or salt appetite.

Discussion

Enhanced basal activity of the central nervous system renin-angiotensin system has been implicated as a partial mediator of the hypertension and elevated salt appetite of the SHR. For instance, disruption of endogenous central Ang II action via receptor blockade or converting enzyme inhibition causes a marked reduction of blood pressure27 and salt consumption14 in SHR, with no effect in WKY rats. The present study has revealed substantially greater angiotensinogen gene expression in the anterovernal hypothalamic and contiguous forebrain areas of SHR as compared with WKY rats. This finding is consistent with the hypothesis that differences in function of the central nervous system renin-angiotensin system contribute to the differences between these strains in phenotypic blood pressure or salt appetite. More extensive investigations using high resolution in situ hybridization techniques will determine whether these strains differ in angiotensinogen gene expression in brain areas other than those reported herein. Our results for liver angiotensinogen gene expression are similar to those of others,28 who report that SHR and WKY rats do not differ in hepatic angiotensinogen mRNA. Because the liver is the primary source of peripheral angiotensinogen, the absence of strain differences in liver angiotensinogen gene expression suggests that if the renin-angiotensin system contributes to the hypertension or increased salt appetite of SHR, then it is likely to occur via brain mechanisms rather than increased production of the angiotensin prohormone in the periphery.

SHR and WKY rats have been selectively bred for their blood pressure phenotypes, and it is tempting to believe that any physiological or behavioral differences between SHR and WKY rats are in some way related to their phenotypic differences in blood pressure. However, in the course of inbreeding a strain for generations, it is not only possible but probable that many unrelated, nonselected characteristics will become genetically "fixed" in the individual strains. Genetic analysis has demonstrated that several traits, including salt appetite,29,30 sodium metabolism,31 motoric activity,32 and adrenal catecholamine stress response,33 once believed to be related to hypertension in SHR, are genetically independent of the blood pressure phenotype in this strain.

There is reason to expect that for portions of the brain area investigated in the present study, the strain differences in angiotensinogen gene expression...
might also be independent of the blood pressure differences between SHR and WKY rats. Although lesion of the AV3V region attenuates blood pressure in many forms of experimental hypertension, destruction of this area appears not to alter the course of hypertension in SHR. Thus, if the AV3V tissue is not necessary for the expression of hypertension in SHR, then it follows that the increased angiotensinogen gene expression found in this area is not a necessary prerequisite for the hypertensive phenotype. Of course this does not mean that brain areas that lie outside of the AV3V region and express elevated angiotensinogen mRNA are unrelated to the hypertensive phenotype in SHR. The possibility remains that strain differences in angiotensinogen gene expression in AV3V, and other brain regions, are related to strain differences in the behavioral salt appetite phenotype.

To determine whether the strain differences in central nervous system angiotensinogen gene expression are related to strain differences in either blood pressure or salt appetite, genetic linkage should be tested by way of a cosegregation analysis. It seems reasonable to hypothesize that angiotensinogen gene expression in certain circumscribed brain regions will be genetically linked to the blood pressure phenotype, whereas angiotensinogen gene expression in other brain regions will be coupled to the salt appetite phenotype. Experiments are currently underway to assess the coupling of brain angiotensinogen gene expression to both blood pressure and salt appetite in F2 descendants of SHR x WKY crosses.

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References


**KEY WORDS** • hybridization • genetics • blood pressure • salt appetite • hypothalamus • spontaneously hypertensive rats • Wistar-Kyoto rats
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