Identification of Renin and Angiotensinogen Messenger RNA Sequences in Mouse and Rat Brains

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SUMMARY Components of the renin angiotensin system have been demonstrated in mouse and rat brains. However, local synthesis of renin has not been documented. In this study, we employed mouse submandibular gland renin complementary DNA (pDD-lD2) and rat liver angiotensinogen complementary DNA (pRang3) to examine whether renin and angiotensinogen RNA sequences exist in mouse and rat brains. Angiotensinogen messenger RNA sequences were readily demonstrable in whole rat and mouse brain using Northern blot hybridization analysis. Using large quantities (>100 μg) of brain total RNA and the sensitive complementary RNA probe, we were able to detect low levels of renin RNA sequences in the brains of both species. The relatively low concentration of brain renin messenger RNA and high concentration of angiotensinogen messenger RNA raises several interesting questions about the distribution of these two proteins and their relative contribution to the activity of the brain renin-angiotensin system. In summary, our data demonstrate the expression of both renin and angiotensinogen genes in mouse and rat brains and provide definitive evidence for an independent endogenous brain renin angiotensin system. (Hypertension 8: 544-548, 1986)

KEY WORDS • brain renin • brain angiotensinogen • complementary DNA • hybridization • pSP64 riboprobe

FIFTEEN years after the initial reports of renin-like activity in the brain,1,2 the issue of endogenous renin production in the brain remains unsettled. With the use of immunocytochemical and immunochemical techniques several studies demonstrated immunoreactive renin in mouse, rat, and human brains.3-7 On the other hand, Husain et al.8 have been unable to demonstrate immunoreactive renin in dog brain but instead have identified a neutral protease that is capable of producing angiotensin I from angiotensinogen. The problems associated with biochemical and immunological studies appear to be related to conditions of assay, to contamination with plasma, and to the animal species studied. Unfortunately, none of the previous reports have clearly demonstrated local synthesis of renin. Hence, the concept of an independent endogenous brain renin system remains to be proved.

In the past few years, the advent of recombinant DNA technology has enabled molecular cloning of mouse submandibular gland (SMG) and human renal renin complementary (c) DNAs, as well as rat and human liver angiotensinogen cDNAs.9-12 These provide the possibility of examining whether angiotensinogen and renin RNA sequences exist in the brain. Using a rat angiotensinogen cDNA as a hybridization probe, Ohkubo et al.13 demonstrated reactive RNA sequences in whole rat brain. Campbell et al.14 reported two forms of immunoprecipitable angiotensinogen precursors from cell-free translational products of rat brain poly(A)+ RNA. To our knowledge, the issue of angiotensinogen expression in mouse brain has not been examined, nor have brain renin RNA sequences been reported in any animal species despite the availability of renin cDNA. In the present study, we employed mouse SMG renin cDNA and rat liver angiotensinogen cDNA to examine the presence of renin and angiotensinogen RNA sequences in whole mouse and rat brains.
Materials and Methods
Adult male outbred Swiss mice (Crl:CD-1[ICR]BR) and adult male Wistar-Kyoto rats from Charles River Breeding Laboratories (North Wilmington, MA, USA) were studied. They were housed and handled according to the guidelines of the Brigham and Women’s Hospital. Animals were killed by decapitation; various organs (brain, kidney, liver) were removed within 3 minutes and immediately snap-frozen in liquid nitrogen and stored at −80°C until used.

Isolation of Total RNA
Various tissues were homogenized in 10 ml of 4 M guanidine thioctanate, 0.5% sodium-N-lauryl sarcosine, 25 mM sodium citrate, 0.1 M β-mercaptoethanol, and 2 M CsCl.15, 16 The homogenate was applied to 5 ml of autoclaved 5.7 M CsCl/25 mM sodium acetate, pH 5.5, and the RNA pellet by centrifugation at 35,000 rpm for 16 hours at 20°C in a 70 Ti fixed angle rotor (Beckman Instruments, Palo Alto, CA, USA). The RNA, resuspended in 0.2 M sodium acetate (pH 5.5) by rocking at 4°C for 1 hour, was precipitated by ethanol. The precipitated RNA, collected by centrifugation, was dissolved in water and quantitated by absorbance at 260 nm, aliquoted for future use, and stored at −70°C.

Gel Electrophoresis and Hybridization Studies
Aliquots of RNA, lyophilized and denatured with glyoxal at 50°C for 60 minutes, was size-separated by electrophoresis through 1.5% agarose gels in 0.01 M NaPO₄, pH 7.0.17 In studies of brain renin messenger (m) RNA, up to 300 µg total RNA were run on the agarose gel. Denaturing mixture consisted of 1.2 M glyoxal, 50% (vol/vol) dimethyl sulfoxide and 0.02 M NaPO₄, pH 7.0; 24 µl was used for RNA amounts less than 100 µg, and 36 µl was used for larger amounts. A Hae III digest of ϕX174RF was denatured and run with the RNA as molecular weight standard. Gels were transblotted by capillary action with 10 × SSC (0.15 M NaCl plus 0.015 M sodium citrate) for 20 hours onto a nylon filter paper (Gene Screen, New England Nuclear, Boston, MA, USA). The filter with bound RNA was baked at 80°C in a vacuum oven for 2 hours. When nick-translated cDNA was to be used, the filter was prehybridized at 42°C for 3 to 4 hours in a buffer consisting of 5 × SSC, 50% formamide, 5 × Denhardt’s solution, 25 µg/ml yeast transfer (t) RNA, and 25 µg/ml salmon sperm DNA in 0.2% sodium dodecyl sulfate. The blots were then hybridized overnight in the same buffer to which was added 32P-labeled renin cDNA (pDD-1D2)4 or angiotensinogen cDNA (pRang3).19 Following hybridization, the ribo-probe-hybridized blots were washed at 35°C with 0.5 × SSC with 0.1% sodium dodecyl sulfate at 56°C. In other experiments, 32P-labeled complementary RNA for renin or angiotensinogen mRNA was generated by transcription of a pSP64-1D2 or pSP65-Rang3 fusion plasmid (ribo-probe).20 respectively (see below). Prehybridization buffer was as described above but had 1 mg/ml of yeast tRNA, and prehybridization and hybridization were performed at 56°C. Following hybridization, the ribo-probe-hybridized blots were washed three to five times (20 minutes each) in 0.1 × SSC containing 0.1% sodium dodecyl sulfate at 65°C. Blots were dried and subsequently exposed to x-ray film (Kodak XAR, Eastman Kodak, Rochester, NY, USA) at −70°C in the presence of an image intensifying screen. Increasing amounts of total RNA from each organ were employed for hybridization, and the autoradiographs were scanned with a Chromoscan 3 Microdensitometer (Joyce, Loebl & Co., Gateshead, England). Regression lines were calculated from integral values, and the relative signals of the transcripts for each organ were estimated from the slope of the regression line. Comparisons of mRNA levels from the different tissues were only made using densitometric analysis within a single autoradiogram, thus eliminating the need for an external RNA standard. If multiple exposures were necessary, the data were corrected for exposure time and, if necessary, radioisotopic decay.

Hybridized probe could be removed from the bound RNA by boiling the filter at 100°C for 5 minutes. The boiled filter was exposed overnight to ensure that no residual radioactivity remained. The filter with bound RNA could then be used for hybridization to a different probe to determine coexpression in the same tissue.

Renin and Angiotensinogen Complementary DNAs
Angiotensinogen cDNA (pRang3) is a partial-length rat liver angiotensinogen cDNA cloned by Lynch et al.19 into the BamHI site of pUC9. Nucleotide sequence analysis revealed that this cDNA was homologous to nucleotides 650 to 1140 of rat angiotensinogen cDNA sequence as reported by Ohkubo et al.20 Renin cDNA (pDD-1D2) is a full-length mouse SMG renin cDNA cloned by Field et al.4 into the Pst I site of pBR322. This cDNA readily hybridizes with mouse kidney renin mRNA.

Labeling of Complementary DNA
The renin cDNA insert (1D2), prepared by Pst I digestion of pDD-1D2 followed by electrophoresis in agarose, was recovered by electroelution and purified by phenol/chloroform extraction followed by chromatography on NACS column (BRL, Bethesda, MD, USA). The angiotensinogen cDNA insert, similarly isolated from pRang3 by Bam H1 digestion and electrophoresis in polyacrylamide gel, was labeled by nick-translation with 150 µCi [α32P]d-ctydidine 5’-triphosphate (CTP; 1000–1500 Ci/mmole; New England Nuclear) as described.18 The labeled cDNA was purified by Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ, USA) gel filtration chromatography.

Complementary RNA
High specific activity synthesis of complementary RNA probe was accomplished as described.20 Briefly, the 1D2 Pst fragment was cloned into the Pst I site of pSP64, and the orientation was determined by restric-
tion mapping. The plasmid containing the proper orientation (pSP64-1D2) was linearized with Bam H1 and transcribed using SP6 RNA polymerase (Promega Biotech, Madison, WI, USA) in the presence of \(\alpha^{32}\)P-CTP, (New England Nuclear). Generation of full-length transcripts was demonstrated by agarose gel electrophoresis followed by autoradiography. Similarly, the pRang3 Bam fragment was cloned into the Bam H1 site of pSP65 and linearized with Xba I. Incorporation was routinely \(2 \times 10^6\) cpm/\(\mu\)g DNA template.

**Results**

Nick-translated pDD-1D2 hybridized with renin RNA sequences in mouse and rat kidneys. Renin RNA sequences could not be detected when less than 100 \(\mu\)g total RNA of mouse or rat brain was analyzed. When larger quantities (\(\geq 100 \mu\)g) of total RNA were used, however, positive hybridization signals with nick-translated pDD-1D2 could be detected at low levels for both species (Figure 1). The signals became more evident when highly sensitive cRNA probe was used (see Figure 1). Renin mRNAs in the brain and kidney of both rat and mouse were indistinguishable by size according to their migration in agarose gels. The estimated size (1550 bases) is in agreement with the mouse SMG renin mRNA size as calculated by nucleotide sequence of the full-length cDNA.\(^9\) The relative ratio of renin mRNA in brain and kidney was 1:100 for the mouse (data not shown) and 1:25 for the rat (Figure 2).

In similar experiments, the presence of angiotensinogen mRNAs in the brain kidney and liver of both species was demonstrated (see Figure 1). The size of the angiotensinogen mRNA in the above rat and mouse tissues was the same (approximately 1660 bases), consistent with previously reported size of rat liver angiotensin mRNA. The relative ratio of angiotensinogen mRNA in the liver, brain, and kidney was 100:10:1 for the rat (see Figure 2) and 100:30:30 for the mouse (data not shown).

**Discussion**

The potential biological importance of an endogenous brain renin-angiotensin system has been extensively reviewed.\(^{21,22}\) Despite much biochemical and immunological data on the presence of various components of the renin-angiotensin system in the brain of different animal species, the production of endogenous renin in the brain is still unproved. The aim of this study was to employ renin and angiotensinogen cDNAs to examine the expressions of these two proteins in mouse and rat brains.

We demonstrated the presence of very low concen-

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**Figure 1.** Northern blot analyses of rat brain and kidney total RNA hybridized to angiotensinogen cRNA, pSP65-Rang3 (A), and to renin cRNA, pSP64-1D2 (B). Northern blot analyses of mouse brain and kidney total RNA hybridized to nick-translated \(\alpha^{32}\)P-labeled cDNA for angiotensinogen, pRang3 (C), and renin, pDD-1D2 (D). Autoradiograms were exposed for 21 (A and B), 96 (C), and 168 (D) hours. The sensitive riboprobe permits more ready identification of brain renin mRNA as compared with nick-translated renin cDNA (data not shown). The specific activity of the probes was 1 to 2 \(\times 10^6\) cpm/\(\mu\)g DNA or RNA template.
trations of renin RNA sequences in mouse and rat brain. The application of a highly sensitive cRNA generated by pSP64-1D2 fusion plasmid and the use of large quantities of total brain RNA enabled us to detect their presence. To our knowledge, this is the first application of the cRNA approach for the detection of low quantities of renin mRNA in any tissue. Our results also confirmed a recent report on the accumulation of angiotensinogen mRNA in rat brain. In addition, we demonstrated that angiotensinogen mRNA could be detected in mouse brain. The size of brain renin RNA sequence was indistinguishable from the kidney renin RNA sequence. Similarly, brain angiotensinogen RNA sequence was the same size as its liver counterpart, suggesting a high degree of homology. An additional novel finding is the presence of angiotensinogen RNA sequences in rat and mouse kidneys. Although Ohkubo et al. published the finding of angiotensinogen mRNA in rat kidney, to our knowledge this finding in the mouse has not been published before.

Despite the limitations in sensitivity when the cDNA was used to study RNA from a different animal species, we were able to estimate the quantities of brain renin and angiotensinogen RNA sequences relative to their homologous kidney or liver RNA sequences. According to our results, rat brain renin mRNA was approximately 4% of rat kidney renin mRNA level and mouse brain renin mRNA concentration was approximately 1% of that of mouse kidney. In comparison, angiotensinogen RNA sequences accumulated in relatively higher concentrations in both mouse and rat brains, representing 30% and 10% of their respective liver angiotensinogen mRNA concentrations.

A clear limitation in our study is the use of whole brains. Our results cannot address whether renin mRNA accumulates in high concentrations in selective brain regions. Lynch et al. observed that rat midbrain, brainstem, cerebellum, and diencephalon contained higher concentrations of angiotensinogen RNA sequences than did hippocampus, cortex striatum, or pituitary. Previous biochemical and immunohistochemical studies indicated that there are marked differences in renin and angiotensinogen distribution in the brain. In mouse and humans, immunoreactive renin appears to be widely distributed in the brain. In contrast, in the rat, immunoreactive renin accumulates selectively in different regions. There is an apparent discordance in the distribution of rat brain renin and angiotensinogen. The low concentration of renin RNA sequences and the relatively higher concentration of angiotensinogen RNA sequences raise several important questions. Are renin and angiotensinogen coexpressed in the same region and in the same cells? What is the meaning of the lower concentration of renin mRNA as compared with angiotensinogen mRNA? Which plays the more important role in the regulation of angiotensin production? Is angiotensin formed intracellularly or extracellularly? Finally, the discrepancies in quantity and perhaps distribution of angiotensinogen and renin expressions raise the possibility that nonrenin angiotensinogenases may be involved in the production of angiotensin in selective brain regions.

In summary, our data indicate the expression of both renin and angiotensinogen genes in mouse and rat brains. The relative amount of brain renin mRNA was quite low. In contrast, brain angiotensinogen mRNA was relatively abundant. The regional and cellular localization of renin and angiotensinogen mRNA levels and their regulation in the brains of various species should provide insight into the biology of the brain renin angiotensin system.

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