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RAT ANGIOTENSIN II RECEPTOR: cDNA SEQUENCE AND REGULATION OF THE GENE EXPRESSION

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SUMMARY: The nucleotide and amino acid sequences for rat type I angiotensin II receptor were deduced through molecular cloning and sequence analysis of its complementary DNAs. The rat angiotensin II receptor consists of 359 amino acid residues and has a sequence similar to G protein-coupled receptors. The expression of this receptor gene was detected in the adrenal, liver and kidney by Northern blotting. Sodium deprivation positively modulated the expression of the receptor gene in the adrenal. No detectable change was observed in the expression levels of this receptor gene between spontaneously hypertensive rats and Wistar-Kyoto rats in the tissues examined including the adrenal, brain, kidney and liver. Interestingly the expression of this receptor gene was developmentally regulated.

The renin angiotensin system is the major regulatory mechanism for electrolytes and blood pressure control. The genes of this system have all been cloned and well characterized, except those for angiotensin receptors. The mas protein has been reported to be a possible angiotensin II receptor, while controversy regarding this contention exists (1, 2).

We have recently isolated a cDNA clone for bovine angiotensin II (Ang II) receptor by an expression cloning method (Sasaki et al. to be published). The product of this mRNA is a polypeptide consisting of 359 amino acid residues and contains seven putative membrane-spanning domains. The nucleotide sequence of this mRNA is distinctly different from that of mas oncogene.

Rats are the most frequently used species in cardiovascular research including hypertension, heart failure, glomerulonephritis and vascular diseases. Thus, studies on possible roles of AngII receptor in these diseases demand the cloning of rat angiotensin receptors. In this communication, we report the complete amino acid sequence and the characterization of rat angiotensin II receptor. We also report the regulation of this receptor gene analyzed by quantitative polymerase chain reaction method (3).

MATERIALS AND METHODS

cDNA cloning: A rat kidney cDNA library (λgt 10) prepared from 16 week old Spontaneously hypertensive rats (SHR) (4) was screened with a 1.3 kb [α^{32}P] dCTP nick translated *XhoI-SalI* fragment derived from a bovine angiotensin II receptor cDNA clone (Sasaki et al. to be published). The hybridization was in 35% formamide, 6 X SSC (0.90M NaCl, 0.09M Sodium Citrate), 50mM NaH_2PO_4 /Na_2HPO_4 (ph 7.0), and 1% sodium dodecyl sulfate (SDS) at 42°C for 24 hrs. The filters were washed in 2 X SSC containing 1% SDS at 55°C. Sequence

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analysis was done on both strands directly from double stranded plasmids using Sequenase sequence kit (United States Biochemical Corp., Cleveland, OH) and Exo III/Mung bean nuclease deletion kit (Stratagene, La Jolla, CA).

RNA and DNA Blot Hybridization Analysis: Total RNA, poly(A)$^+$RNA, and genomic DNA were isolated as described previously (4). RNA and genomic DNA hybridization analysis were carried out by using a nylon membrane as described previously (4). The 1213-base pair *Kpn*I(86)-*Sac*I(1298) fragment was used as a probe and was ^{32}P-labelled by nick translation.

DNA transfection and Binding assay: The cloned cDNA fragment was inserted into the mammalian expression vector pcDNA I (Invitrogen, San Diego, CA.). 1.0 X 10^6 COS-7 cells in 300μl phosphate buffered saline were mixed with 4.0 μg rat AIIR expression plasmid, and were electroporated in 0.2cm gap cuvettes (150V, 250μF) by using Gene Pulser Apparatus (Bio Rad, La Jolla, CA). Three days after transfection, the cells were subjected to the receptor binding assay and intracellular Ca^{2+} response assay. The receptor binding assay was performed as described previously (5). DuP 753 and Exp 655 were provided from E. I. DuPont de Nemours Company (Wilmington, DE). For $[Ca^{2+}]_i$ transient assays, the transfected cells were suspended in solution A (140mM NaCl, 4mMKCl, 1mM Na_2HPO_4, 1mM Mg Cl_2, 1.25 mM Ca Cl_2, 11 mM Glucose, 5mM HEPES, pH7.4, 0.2% BSA) containing 4mM Fura-2-penta-acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR.) for 1 hr at 23°C. The Fura-2 loaded cells were resuspended in solution A without dye, and the fluorescence was assayed with excitation at 340 nm and 380nm, and emission at 500nm. $[Ca^{2+}]_i$ was estimated according to Tsien et al. (6).

Quantitative polymerase chain reaction: The 1213-base pair *Kpn*I (86)-*Sac*I (1298) fragment was subcloned into pBluescript II SK + (Stratagene), which was designated as pKS. The pKS was cut by *Msc*I and was self ligated. The resultant plasmid, designated as pΔKS, contained the insert which lacked the region between the two *Msc*I sites (446 and 734). The pΔKS was linealized by cutting with *Sac*I and the deletion mutated RNA was synthesized by T_7 RNA polymerase. Total RNA (20μg) and the deletion mutated RNA (ΔAIIR) (0.1pg ~ 10.0pg depending on the tissues) were mixed and were reverse transcribed using random primers as a primer. The resultant single strand cDNA mixture was amplified by polymerase chain reaction using Taq DNA polymerase (Stratagene). Denaturing, annealing, and polymerase reactions were done 30 times at 94°C for 30 seconds, 65°C for 45 seconds and 72°C for 45 seconds, respectively. The employed primers are as follows: downstream antisense primer 2 (5'-GGGAGCGTCGAATTCCGAGACTCATAATGA-3') and upstream sense primer 1 (5'-ACCCTCTACAGCAT CATCTTTGTGGTGGGGA-3'). Amplification of rat AIIR mRNA and ΔAIIR-RNA using these two primers should give a 479-base pair fragment and a 191-base pair fragment, respectively. The trace amount of [α^{32}P] dCTP was included in PCR reaction mixture to assess the molecular ratio of the 479-bp fragment to the 191-bp fragment.

Animals: Spontaneously hypertensive rats, Wistar-Kyoto rats and Sprague-Dawley rats were obtained from Taconic Farm (Germantown, NY). Low sodium diet (NaCl 0.03%) and high salt diet (3.15%) were obtained form Ralston Purina (St. Louis, MO).

RESULTS

From 500,000 clones screened, six clones were isolated which hybridized to the bovine cDNA fragment. All clones were identified to correspond to the same mRNA based on the nucleotide sequence analysis. Fig. 1. shows the nucleotide and the deduced amino acid sequence of the receptor. The nucleotide sequence surrounding the initiation codon agrees with the Kozak consensus sequence. The receptor consists of 359 amino acid residues and shares a significant sequence similarly with other G protein-coupled receptors.

The positions of the putative transmembrane segments III - VII are tentatively assigned on the basis of a hydropathy profile (window = 20) and a sequence comparison with other G protein-coupled receptors. Some notable features of this receptor include the highly hydrophilic aminoterminal region followed by a large hydrophobic region (residue 26-88 which is described as transmembrane segment I + II), a short third cytoplasmic loop which is also seen in endothelin receptors (7,8), and a segment (residue 286 - 318) which shared a degree of homology with the mas protein. There are several potential sites for post-translational modification in the receptor: three consensus sites for N-glycosylation in extracellular domains, four cystein residues for

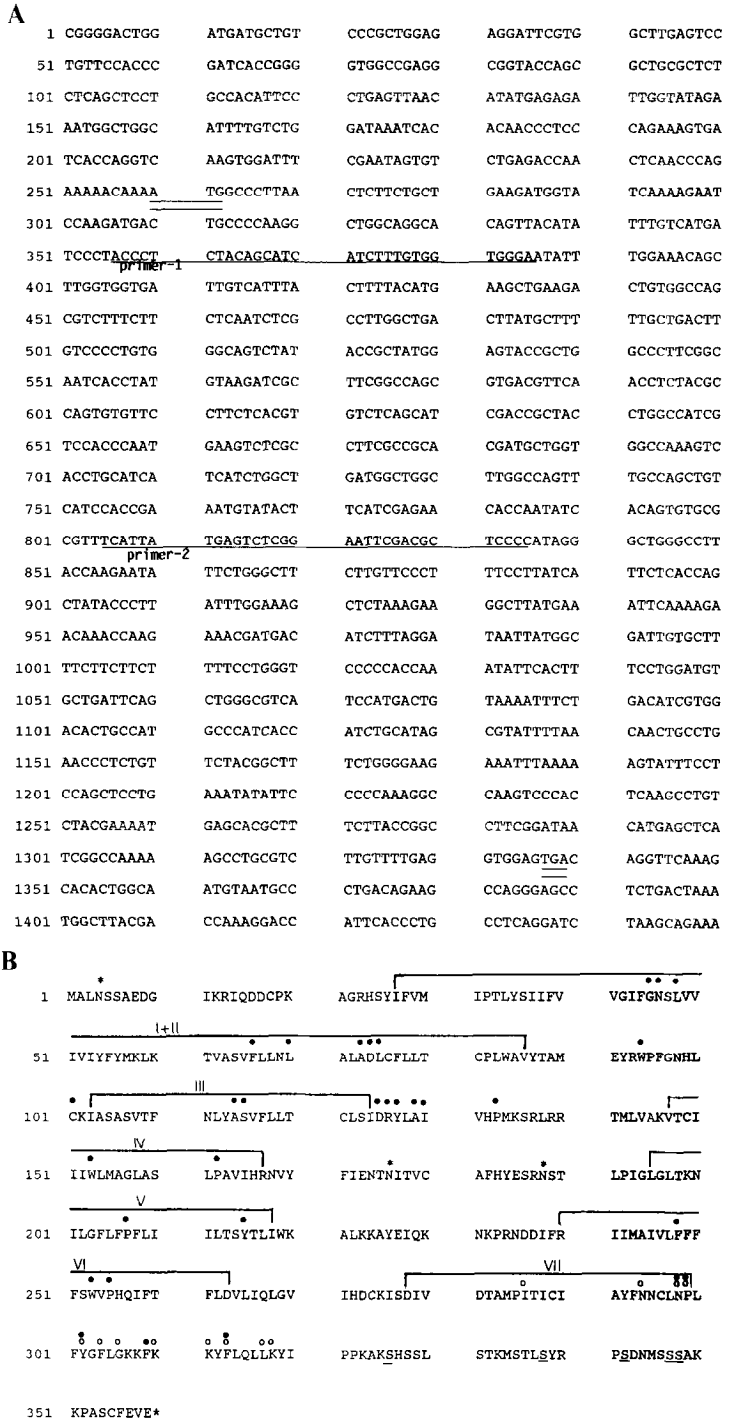


Figure 1.

A. Nucleotide sequence of rat AngII receptor.
The initiation codon and the termination codon are doubly underlined.

B. Deduced amino acid sequence of rat AngII receptor.
Positions of the putative transmembrane segments (I+II)-VII are indicated above the sequence. Asterisks, potential N-glycosylation sites; dots, amino acid residues conserved among G-protein coupled receptors; open dots, identical amino acid residues to human mas protein; possible phosphorylation sites are underlined.

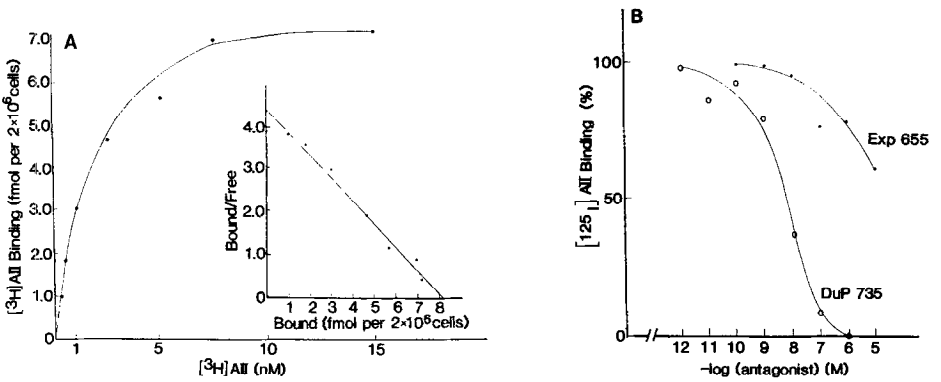


Figure 2.

A. Saturation isotherm.
Inset shows Scatchard plot of [^{3}H]-labelled AngII. Kd was 1.9nM.

B. Displacement of specific binding of [^{125}I]-labelled AngII (0.1nM).

disulfide bridge and several serine residues in the C-terminal cytoplasmic domain for regulatory phosphorylation (9).

Series of pharmacological and biochemical studies suggest that there are at least two types of AngII receptor in mammalian tissues (10, 11, 12). The characterization of the cloned receptor was determined by expressing the cloned cDNA in COS-7 cells. Binding of [^{3}H] Ang II to transfected COS cells was saturable with a dissociation constant (Kd) of 1.9 nM (Fig 2A). No significant binding was detected on the mock-transfected COS cells. Displacement experiment of [^{125}I] AII binding indicated that Dup753 (Type I specific antagonist) is by far a more potent inhibitor than Exp655 (Type II specific antagonist), which suggests that the cloned receptor is a type I receptor (10,11, 12) (Fig. 2B).

The transfected COS-7 cells responded to Ang II with a transient increase in the concentration of intracellular Ca^{2+} (Fig 3).

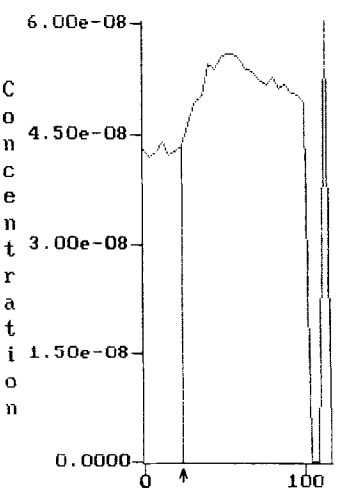
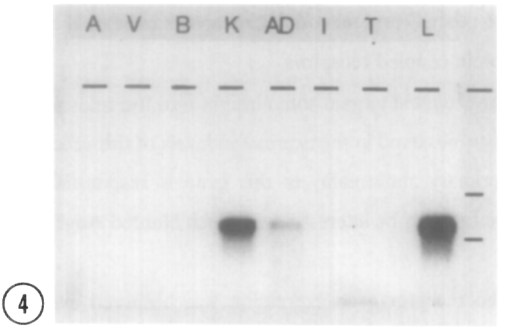


Figure 3. AngII induced intracellular Ca^{2+} changes in the transfected COS-7 cells.
Arrows indicate the time point when AngII (10^{-7}M) was loaded. No increase in $[Ca]_i$ was observed in the mock-transfected COS-7 cells.



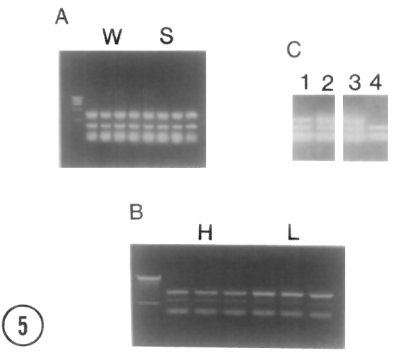


Figure 4. Northern blot analysis.
A,V,B,K,AD,I,T and L indicate atrium, ventricle, brain, kidney, adrenal, intestine, testis and liver. Two micrograms of poly $(A)^+$ RNA from various tissues were electrophoresed. The positions of marker RNAs (4.40kb and 2.37kb) are indicated.

Figure 5. Regulation of rat AngII receptor gene.

A. Assessment of the expression levels of AngII receptor mRNA in SHR and WKY. Panel A shows the typical examples. Twenty micrograms of brain total RNAs from 16 week old SHR (n=4) and age-matched WKY (n=4) were mixed with 0.1pg deletion mutated AngII receptor RNA (ΔAIIR-RNA), and the mixtures were reverse-transcribed, and were amplified by polymerase chain reaction. The upper and the lower bands correspond the the receptor RNA and the ΔAIIR-RNA, respectively. The lowest broad band was originated from degradated RNA and primers.

B. Effect of high (H) and low (L) salt diet on the expression level of AngII receptor mRNA in the adrenals.
Twenty micrograms of adrenal total RNAs were mixed with 3.0pg ΔAIIR-RNA and were reverse transcribed, and were amplified by PCR.

C. Developmental changes in the expression levels of AngII receptor mRNA.
Ten picograms of ΔAIIR-RNA were mixed with twenty micrograms of total RNA from adult kidney (lane 1), one day old kidney (lane 2), adult liver (lane 3) and 1 day-old liver (lane 4), and were reverse transcribed, and amplified by PCR.

The expression of this mRNA was detected in the liver, kidney, adrenal and weakly in the atrium and ventricle of the heart (Fig 4). Use of the polymerase chain reaction method also permitted the detection of the receptor mRNA in all the tissues examined including the aorta, testis, small intestine, lung and brain (data not shown).

The regulation of this receptor gene was analyzed by competitive polymerase chain reaction method (Fig 5). Low sodium diet feeding for a prolonged period (4 weeks) increased the expression level of this mRNA in the adrenal (2.3 fold) (Fig 5B), but not in the kidney, liver and brain. Neither the administration of captopril for 2 weeks, nor the infusion of Ang II for 2 weeks, altered the expression level of this gene in the kidney and liver (data not shown).

The expression levels of this gene in the kidneys, livers, adrenals and brains were assessed in 6 and 16 week old spontaneously hypertensive rats and age-matched Wistar-Kyoto rats. No significant change was observed between the two strains (Fig 5A).

The expression of this gene in the liver of newborn rats was not detected, while that in the kidney of newborn rats was significantly higher (2.5 times) than that in adult rats (Fig 5C).

DISCUSSION

The rat and bovine AngII receptors share a significant sequence homology (91% sequence identity in amino acid residues) and similar receptor properties. The preferential affinity to Dup 753, a typeI-specific

antagonist clearly showed that the cloned receptor is a type I receptor which is intimately involved in blood pressure control (11). And the sequence similarity with other G protein-coupled receptors indicate that this type I AngII receptor belongs to the large family of G protein-coupled receptors.

The modulation of this gene expression was investigated to gain some insight into the relevance of this gene in disease states. Because no detectable change was observed in the expression levels of this receptor gene between SHR and WKY, it is not likely that the primary abnormality in this gene is responsible for the hypertension in SHR. In this sense, Milan hypertensive rats may be interesting, in which blunted AngII receptor response was reported (13).

In the kidney and liver no modulation of the receptor gene expression was observed even by the administration of captopril or by the continuous infusion of AngII. Only in the adrenal, the positive modulation was observed by sodium deprivation. This result is in good agreement with the previous studies (14, 15).

The developmental change of the receptor gene expression is marked. The other components of the renin angiotensin system are also reported to be developmentally regulated (16). The developmental change of the receptor gene expression might support the hypothesis that AngII is not only a vasoactive peptide but also a physiological growth or differentiating factor (17).

In summary, rat type I AngII receptor cDNA was isolated. The clarification of the structure of this receptor will contribute to the understanding not only of the physiological role of the renin angiotensin system, but also of the mechanism of the action of AngII.

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