Chapter VI: Expression of Angiotensin II Receptors in Human Kidney

Summary

All studies analyzing localization of angiotensin II (Ang II) receptors in the human kidney have been performed on the protein level using 125I-Ang II as a probe. In this study, cellular localization of Ang II type 1 (AT1-R) and type 2 (AT2-R) receptor mRNAs in the adult human renal cortex were examined for the first time using in situ hybridization and its expression pattern determined by RNase protection assay was compared with those in other human tissues. In the human renal cortex obtained from tumor-free portions in renal cell carcinoma, AT1-R mRNA levels were about 8- to 10-fold higher than AT2-R mRNA levels. Human liver and aorta predominantly expressed AT1-R mRNA, while human right atrium contained both AT1-R and AT2-R mRNAs. Ligand binding assays revealed that total Ang II receptor numbers in the human renal cortex was 16.0±3.3 fmol/mg protein, similar to that in liver (17.7±5.8) but significantly higher than in right atrium (11.6±3.2) and aorta (5.6±2.7). Relative distribution ratios of AT1-R and AT2-R numbers in the renal cortex and right atrium were 82/17 and 56/42 %, respectively. In situ hybridization study indicated that strongest AT1-R mRNA signals were located in interlobular arteries and tubulointerstitial fibrous regions surrounding interlobular arteries and glomeruli, followed in decreasing order by glomeruli and cortical tubules. Expression of AT2-R mRNA was highly localized in interlobular arteries. Cells present in

tubulointerstitial regions were positive for vimentin and collagen type1, indicating that the majority of the cells present in the regions are fibroblasts. Presence of strong AT1-R mRNA signals in the tubulointerstitial fibrous regions surrounding arteries and glomeruli and the expression of AT2-R mRNA in the interlobular artery were the first evidence, suggesting a pharmacologic framework for the differential effects of Ang II receptor subtypes-mediated renal function in the adult human kidney.

Introduction

Angiotensin II (Ang II) has multiple effects on renal function, including modulation of renal blood flow, glomerular filtration rate, tubular epithelial transport, renin release and cellular growth [101,102]. At present AngII receptors are separated into two major subtypes, designated as type 1 receptor (AT1-R) and type 2 receptor (AT2-R) and most of the effects of Ang II in the renal and cardiovascular systems are mediated by Ang II receptor type 1 (AT1-R) [101-103].

Autoradiography coupled with competitive binding studies has been used to characterize the distribution of Ang II receptor subtypes in the kidney. Using these techniques, the distribution of AT1-R and AT2-R subtypes within the kidney was shown to be species dependent. For example, in the rat and rabbit kidneys Ang II receptors were essentially of the AT1-R [104-108], while both AT1-R and AT2-R were present within the kidney of the opossum and of primates including humans [106-111]. The distribution of renal Ang II receptors has been most extensively studied in the rat where the AT1-R binding sites are predominantly located in the glomeruli, renal tubules and renal vasculature[112,113]. These observations in the rat kidney were further confirmed at mRNA levels using reverse transcriptase-polymerase chain reactions and in situ hybridization [114-116] ; large AT1-R mRNA signals were detected in the glomerulus, proximal tubules, cortical blood vessels and collecting ducts. Smaller AT1-R mRNA signals were present in medullary thick ascending limb, medullary collecting duct. Within the rat glomerulus, AT1-R mRNA was localized in mesangial areas, predominantly at the vascular pole and on the terminal portion of the afferent arteriole, whereas the study using polyclonal antisera for the rat AT1-R indicated the presence

of AT1-R protein in both afferent and efferent arterioles [117]. Using mutant mice with a targeted replacement of the AT1-R loci by the lacZ, we also found that the strong lacZ staining was detected in both afferent and efferent arterioles [118], which agreed with the immunocytochemical observation [117].

Analyses using Northern blot [119] or in situ hybridization [115] indicated that AT2-R mRNA was not detected in the rat or mouse kidneys. However, autoradiography revealed that in the rabbit the fibrous sheath around the kidney contained AT2-R binding sites [120] and in the rhesus monkey AT2-R binding sites were present on the juxtaglomerular apparatus and vasculature in the renal cortex [106]. With respect to localization of Ang II receptor subtypes in the adult human kidney, all studies were performed on the protein level by the autoradiography using [125I]-Ang II as a ligand[108,110,111] and distribution patterns at mRNA levels have not yet been analyzed. Grone et al. [110] and Goldfarb et al. [111] demonstrated that AT1-R binding sites were predominantly present in the glomeruli, while AT2-R is the major subtype in large cortical blood vessels. In contrast, Sechi et al. reported that AT1-R is present primarily in both glomeruli and cortical blood vessels and AT2-R protein is not expressed in the human kidney [108]. In this study, we examined for the first time the cellular localization of AT1-R and AT2-R in the adult human kidney at mRNA levels using in situ hybridization and its expression pattern determined by RNase protection assay was compared with distribution patterns in other human tissues such as atrium, liver or aorta. RNase protection assays and in situ hybridization established that both AT1-R and AT2-R mRNAs were present in the human kidneys. Strong AT1-R mRNA signals were localized in interlobular arteries and tubulointerstitial fibrous regions surrounding arteries and glomeruli, and weaker signals were detected in glomeruli and proximal tubules. AT2-R mRNA signals were highly localized in

interlobular arteries. AT1-R expression levels in the kidneys were similar to that in liver and higher than in atrium or aorta. AT2-R was expressed in the human kidney and right atrium, but not in liver or aorta.

Materials and Methods

Human tissues

The human kidneys were obtained during operation for surgical removal due to renal cell carcinoma (six patients) and tumor-free cortical parts were excised. Sex and age of patients are listed in Table 1 (age 60±3.3, range:49-71, n=6). No patients were pretreated with the ACE inhibitors. Patients with complications of hypertension, diabetes mellitus or hyperlipidemia were not included in this study. Sechi et al. reported that the presence of tumor in the kidney or the type of anesthesia did not significantly influence the binding studies [108]. Right atrium, renal cortex, liver and ascending aorta were excised at autopsy (about 2 hr after death) from patients with brain death who had no history of cardiac disease or pulmonary and renal diseases (age 54±5.2, range:28-64, n=4).

Preparation of plasma membranes

Tissue samples (100-200g) were immediately placed in iced, oxygenated Tyrode's solution, and then homogenized by a Polytron at 4°C in 2 volumes of buffer A comprising of ice-cold 0.32 M sucrose, 0.5 mmol/L EDTA and 25 mmol/L Tris, pH7.5 including protease inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride, 10 mg/L bacitracin, 4μg/mL leupeptin, 4 μg/mL pepstatin, 40 U/mL trasylol, and antipain, phosphoramidon, amastatin and bestatin, each at 1ug/mL) [121]. The homogenates were centrifuged at 500g for 10 min at 4 °C, and the supernatant fractions were recentrifuged at 48,000 g for 30 min at 4 °C. The pellets were suspended in a solution containing 0.6 mol/L KCl and 30 mmol/L histidine at pH 7.0 (including protease inhibitors mentioned above) to solubilize actin and myosin filaments and

were recentrifuged at 48,000 g for 30 min at 4°C. The pellets obtained after the final centrifugation were resuspended in binding buffer containing protease inhibitors mentioned above and immediately used for the binding assay. All steps were performed at 4°C. Thus, in the present study the tissue samples and membrane preparations were not subject to the frozen storage process, since we had found that during the frozen step of tissue samples AT1-R and AT2-R numbers were decreased by 30±2.7% and 8.2±1.4% (n=4), respectively.

Binding assay and analysis of data

The binding assay for 125I-Ang II was performed as previously described [121,122]. Briefly, the membrane fractions (500 to 600μg of protein) were incubated with different concentrations (eight to ten points in 0.05 to 5 nmol/L) of 125I-[Sar1, Ile8] Ang II for the saturation experiment (0.2 nmol/L 125I-[Sar1, Ile8] Ang II for the competition experiment) in a total assay volume of 300μl for 90 min at 21°C. The assay buffer contained 50 mmol/L Tris (pH 7.6), 100 mmol/L NaCl, 10 mmol/L MgCl2, 1mmol/L EGTA, 0.25mg/ml BSA and a variety of protease inhibitors used for membrane preparation. The degradation rate of [Sar1 Ile8] AngII after 90 min incubation at 21°C was determined by reversed-phase high performance liquid chromatography. The results showed that under the incubation conditions used as much as 97.1±2.4% (n=3) of the radioligand remained intact. Specific 125I-[Sar1, Ile8] Ang II binding was determined from the difference between counts in the absence and presence of 3μmol/L unlabeled Ang II. All assays were conducted in duplicate. Analysis of the binding data was performed as described previously [123]. The Kd and Bmax values were estimated by Rosenthal analysis of the saturation data. The ability of antagonists to inhibit specific Ang II binding was estimated by IC50 values, which are the molar concentrations of

unlabeled drugs necessary to displace 50% of specific binding (estimated by log probit analysis). Values for Ki were calculated from the equation Ki=IC50 /(1+L/Kd), where L is the concentration of 125I-[Sar1, Ile8] Ang II. The distribution ratio of AT1-R/AT2-R was calculated from nonlinear least-squares regression analysis, using the GraphPad InPlot computer program (Graph Pad, San Diego, CA) [123]. Total Ang II receptor numbers were determined from Scatchard analysis, and AT1-R and AT2-R numbers were calculated from total Ang II receptor numbers on the basis of distribution ratio determined with inhibition by CGP42112A.

RNase protection analyses

RNase protection assays were performed with 40μg of total RNA as described [124,125]. RNA was isolated by guanidium isothiocyanate-cesium chloride centrifugation followed by digestion with DNase (Takara Shuzo, Kyoto, Japan) in the presence of RNase inhibitor (Takara Shuzo) to remove contaminating genomic DNA. PCR amplification of this RNA using PCR primers designed from the coding region of the human AT1-R gene did not produce any bands, indicating that there was no genomic DNA contamination in RNA samples. A RNA probe for the AT1-R (270 bp) was produced from Dra I-digested EcoRI and Pst I cDNA fragment of human AT1-R (nt -280 to +1140 relative to the ATG codon). An AT2-R RNA probe (100 bp) was generated from Ava II-digested EcoRI and Kpn I fragment of human AT2-R cDNA (nt -189 to +425 relative to the ATG codon). Both cDNA clones were kindly provided by Dr. R. Takayanagi, Kyushu University. The probe was hybridized with total RNA at450C and digested with RNase T1 and A, resulting in a 210 bp protected signal for the AT1-R mRNA and a 90 bp protected signal for the AT2-R mRNA. For quantitative analyses, the

densities of AT1-R and AT2-R mRNA signals were measured by laser densitometry and normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels quantified by Northern blotting [126,127].

In situ hybridization

All procedures were performed as previously [117]. Briefly, frozen tissue samples were cut into 15μm cryostat sections. Same AT1-R and AT2-R RNA probes as used in RNase protection assays were used. Antisense and sense probes were made by in vitro transcription in the presence of digoxigenin-labeled dUTP using T3 or T7 RNA polymerase (Toyobo, Osaka Japan).

Fluorescent immunocytochemistry

Cells present in fibrous regions were characterized by fluorescent immunocytochemistry. Tissue sections were cut on a cryostat at -20°C, fixed in acetone. Monoclonal antibodies against vimentin and collagen type 1 (Sigma Immunochemicals) were tested for the detection of fibroblasts. Immunocytochemistry was performed by Biotin/Avidin system (Elite ABC kit, Vector Lab. Burlingame, CA) using diaminobenzidine tetrahydrochloride as a substrate.

Reagents and statistical methods

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated below. The AT1-R antagonist losartan was provided by DuPont Merck Pharmaceutical (Wilmington, DE). The AT2-R antagonist CGP42112A was purchased from

NEOSYSTEM (Strasbourg, France). Results are expressed as means±SE. Analysis of variance and the Fisher's PLSD test were used for multigroup comparisons, with $p<0.05$ considered significant.

Results

AT1-R and AT2-R mRNA were expressed in human kidneys and hearts, while in liver and aorta only AT1-R mRNA was detected. All kidney samples obtained from tumor-free cortical portions had similar AT1-R and AT2-R mRNA distributions (Fig.17, lanes #1 to #6) : AT1-R mRNA was abundant in all RNA samples, whereas AT2-R mRNA expression was relatively low in the same samples. When AT1-R mRNA levels determined by a densitometer were compared with AT2-R mRNA levels in each patient (lanes #1 to #6), the relative expression level of AT1-R was about 8.2-fold higher than that in AT2-R. Similar distribution patterns were observed in the renal and right atrial samples obtained at autopsy (Fig.17, lanes #7 and #8). The expression level of AT1-R in right atrium was about 1.8-fold higher than that of AT2-R. The presence of both AT1-R and AT2-R in the human right atrium was consistent with the previous reports [128,129]. We also examined AT1-R and AT2-R mRNA levels in human liver and aorta (Fig.17, lanes #9 and #10). The AT1-R mRNA was dominant in these tissues, whereas AT2-R mRNA was not detected. Similar expression patterns of mRNA distribution in the kidney, right atrium, liver and aorta were also observed in other autopsy samples (n=4, data not shown). When AT1-R mRNA levels in the aorta determined by a densitometer were arbitrarily normalized to 1 (Fig.17, lanes #10), the relative expression levels in liver and kidney were 9.6±0.4 and 8.4±0.5, respectively. No specific signals for AT1-R and AT2-R mRNAs were observed in tRNA.

AT1-R and AT2-R binding sites were present in human kidneys and hearts, while only AT1-R binding sites were detected in liver and aorta. Experiments using [125I]-Sar1 Ile8-Ang II as a ligand revealed that membrane fractions from the renal cortex had high affinity binding

sites with a Ki of 0.89±0.15 nmol/L (Figs.18A and 18B). Competition binding experiments yielded complex curves suggesting the presence of two classes of binding sites (Fig.18C), and nonlinear least-squares regression analysis indicated the existence of two populations of Ang II binding sites, one with high (Ki=7.1±0.2 nmol/L) and the other with low (Ki=0.11±0.02 μmol/L) affinity for losartan. When CGP42112A was used for competitive inhibition, Ki values of high and low affinity sites were 0.35±0.06 nmol/L and 0.63±0.33 μmol/L, respectively. AT2-R numbers determined by the experiments using losartan and CGP42112A as competitors to specific [125I]Sar, Ile-Ang II binding (0.2 nmol/L) were about 3 fmol/mg protein (Table 2), which was a low-capacity site compared with AT1-R numbers. However, as shown in the saturation curve in Fig.18A, the specific binding at 0.2 nmol/L of [125I]Sar, Ile-Ang II was 85 to 95 % of total Ang II binding, suggesting that the specific Ang II binding is sufficient to determine the distribution of a low-capacity site. The relative ratio of high- and low- affinity sites for CGP42112A was calculated as 17.3±1.8 (AT2-R) / 82.4±2.3 % (AT1-R) (Table 2). The ratio of AT1-R/AT2-R on the basis of inhibition by losartan was calculated as 84.1±2.2 / 15.2±2.1 %, in good agreement with the ratio calculated from inhibition by CGP42112A.

To further confirm the accuracy of the assay method used in this study, we determined AT2-R numbers by Scatchard analysis using AT2-R selective ligand [125I]-CGP42112A and compared them with AT2-R numbers calculated from inhibition by CGP42112A. AT2-R numbers determined using [125I]-CGP42112A were 2.9±0.3 fmol/mg protein, which was very similar to AT2-R numbers calculated from inhibition by CGP42112A (2.7±0.3, Table 2) and the inter-assay variation in AT2-R numbers determined with two different methods was less than 7 % (6.7±0.42 %). These findings established the accuracy of this approach and

proved the validity of the data in Table 2 and Figs. 17 to 19 calculated from inhibition by CGP42112A.

We next examined the distribution patterns of Ang II receptors in right atrium, liver and aorta obtained at autopsy from four patients. Nonlinear least-squares regression analysis indicated the presence of two classes of binding sites in right atria with Ki values of 0.34±0.06 nmol/L and 0.58±0.25 μmol/L for CGP42112A, and the relative ratio of AT1-R/AT2-R was calculated to be about 56±2.4/42±5.5 % on the basis of inhibition by CGP42112A (Fig.19A). On the other hand, both human liver and aorta exclusively expressed AT1-R with similar affinities (Fig.19B and 19C).

AT1-R and AT2-R numbers were calculated from total Ang II receptor numbers on the basis of subtype distribution ratio by competitive inhibition using CGP42112A (Fig.20). Total Ang II receptor numbers (fmol/mg protein) in the renal cortex samples removed during operation (16.0±2.5) were very similar to those in the kidney samples obtained at autopsy (14.6±4.1), indicating that the length of time expired before hearts were removed from autopsy samples had no significant influence on measurement of Ang II receptor numbers. In membrane fractions from autopsy samples, there was no significant difference between kidney (13.6±3.5) and liver (17.7±5.8) but the receptor numbers in right atrium (11.2±3.7) and aorta (5.6±1.8) were lower ($p<0.05$) than that in the kidney, in good agreement with distribution patterns in the mRNA levels.

Cellular distribution of AT1-R and AT2-R mRNAs in the human renal cortex was examined using in situ hybridization. As shown in Figs. 21A and 21D, strong signals for AT1-R mRNA (shown by red color) were localized in interlobular arteries and perivascular fibrous regions (indicated by yellow arrow) and tubulointerstitial regions around the glomeruli

(indicated by blue arrow). Moderate densities of signals were observed in the glomeruli and proximal tubules. Previous studies at protein levels using 125I-Ang II demonstrated that AT1-R binding sites were chiefly detected in the interlobular arteries and glomeruli in the human renal cortex, and that lower densities of diffuse binding were observed in the tubulointerstitial regions without obvious fibrosis [110,111]. Taken together, these findings suggested that the pathological changes associated with the progression of tubulointerstitial fibrosis are at least partially involved in the expression of AT1-R in this region.

The intensity of AT2-R mRNA signal was much lower than AT1-R mRNA signals and primarily localized in interlobular arteries, and there were no detectable signals in tubulointerstitial regions and proximal tubules (Figs. 21B and 21E). The sense probe for AT2-R mRNA did not yield such specific signals in these regions (Fig. 21C and 21F). The expression of AT2-R protein in the glomeruli was reported by Goldfarb et al. [111], whereas Grone et al. [110] and Sechi et al.[108] did not detect AT2-R protein in this region. Our present assay using in situ hybridization more sensitive than autoradiography revealed no or undetectable level of AT2-R mRNA expression in the glomeruli, while distribution pattern of AT1-R and AT2-R mRNAs in the interlobular arteries of human kidneys agreed with the previous studies at protein level [110,111]. Similar expression pattern of Ang II receptor subtypes was also observed in the kidney samples from different patients.

The tubulointerstitial fibrous regions in the renal cortex contained abundant collagen fibers as shown in blue by Azan staining (Fig.22A). To identify the presence of fibroblasts in this region, we stained the sections using monoclonal antibodies against vimentin and collagen type 1. Cells present in tubulointerstitial regions were positive for vimentin (Fig.22B) and collagen type 1 (Fig.22D), demonstrating that the majority of cells present in tubulointerstitial

fibrous regions are fibroblasts and surrounded by collagenous matrix protein.

Discussion

The results of this study demonstrated that in the cortex of the human kidney the strongest AT1-R mRNA signals are located in interlobular arteries and tubulointerstitial fibrous regions surrounding glomeruli and interlobular arteries, followed in decreasing order by glomeruli and tubules. Although the AT2-R mRNA was also expressed in the interlobular arteries, its expression level was very low and the AT1-R signal is the predominant subtype in this region. Although Grone et al. [110] and Goldfarb et al. [111] reported in autoradiography using 125I-Ang II that densities of AT1-R binding in the tubulointerstitial regions of the human kidney were weaker than in the glomeruli, the extent of interstitial fibrosis in their studies [110,111] was much lesser than that in this study. Whereas the renal cortex from tumor-free portions of renal cell carcinoma was obtained from the patients with normal renal function, the presence of remarkable fibrosis around the interlobular artery or glomeruli suggested that the kidneys examined in this study were in the pathological state and not the normal kidneys. Considering that the immunocytochemical study indicated that fibroblast was the major cell type present in the tubulointerstitial fibrous region with accumulation of collagenous matrix protein, the pathological changes associated with the progression of tubulointerstitial fibrosis might be at least partially involved in the expression of AT1-R in this region. Thus, it is likely that AT1-R in the normal kidney is mainly localized in glomeruli and interlobular arteries, while in the pathological state with tubulointerstitial fibrosis AT1-R is re-expressed by fibroblasts present in the fibrous regions. AT1-R was reported to be strongly expressed in the rat [117] or mouse [118] in the macula densa, while this study indicated that its presence in the adult human kidneys was relatively low. Further studies are required to define whether such low expression

of AT1-R was due to species-specific difference or pathological changes associated with tubulointerstitial fibrosis of the kidney.

With respect to the localization of AT2-R binding site, Grone et al. [110] and Goldfarb et al.[111] showed its presence of binding sites in the interlobular arteries, while Sechi et al. reported that there was no detection of AT2-R binding sites in the human renal cortex [108] . Our present result clearly demonstrated that substantial amounts of AT2-R are present in the adult human kidney at mRNA and protein levels and established that in the adult human renal cortex AT2-R gene is predominantly expressed in the interlobular arteries. In human glomeruli, Grone et al. [110] and Sechi et al [108] reported that there is no expression of AT2-R protein, whereas Goldfarb et al. [111] showed the presence of a small population of AT2-R binding sites. In the rhesus monkey renal cortex, an AT1-R site was localized to the glomerular tuft itself, while an AT2-R site was localized to the cells of the juxtaglomerular apparatus [106] . Moreover, in membrane binding studies using rat mesangial cells, while AT1-R was the major subtype, a small AT2-R population was also identified [130] . In this study using the sensitive assay to detect AT2-R mRNA levels, we could not observe a significant expression of AT2-R mRNA in the glomeruli. Although the lack of AT2-R message in the glomeruli may be partly result from the pathological changes associated with the progression of tubulointerstitial fibrosis, it was suggested that AT2-R is mainly present in the cortical vascular system rather than in mesangial cells.

Our present study revealed that large cortical blood vessels had the strong signals for AT1-R mRNA and the weaker AT2-R mRNA signals. Strong AT1-R mRNA signals were accumulated in the adventitia of blood vessels, outer or central portion of media and perivascular fibrous regions, while the signal density in the inner portion of the media or

endothelial cells was not prominent. AT2-R binding sites appeared to be present in the outer and central layers of the media. Human renal arterial smooth muscle cells grown in culture have been reported to express only AT1-R ; however, binding studies using membranes freshly prepared from the whole human renal artery demonstrated both AT1-R and AT2-R binding sites [131] . Thus, it is likely that low levels of AT2-R are localized in the smooth muscle layer of in situ renal vessels, while AT1-R is localized chiefly to both adventitia and smooth muscle layer. This differential localization of AT1-R and AT2-R in cortical blood vessels may be important in explaining subtle differences in vascular reactivity, depending upon whether Ang II is delivered intravascularly or adluminally via the interstitium.

In summary, we demonstrated that both AT1-R and AT2-R mRNAs are expressed in the adult human renal cortex. The expression level of AT1-R in the human kidney was comparable with that in the liver and higher than the level in atrium or aorta. AT2-R mRNA and protein were present in the kidney and atrium but not in liver and aorta. In situ hybridization study indicated the predominant expression of the AT1-R mRNA in the interlobular artery and tubulointerstitial fibrous regions surrounding interlobular artery and glomeruli, followed in decreasing order by glomeruli and cortical tubules. The presence of strong AT1-R mRNA signal in the tubulointerstitial fibrous regions around the glomerulus or artery is the first report, which may imply that the pathological changes associated with the progression of tubulointerstitial fibrosis might be at least partially involved in the expression of AT1-R in this region. The AT2-R mRNA was present in the interlobular artery in a very low expression level. These findings provide a pharmacologic framework for the differential effects of Ang II receptor subtypes-mediated renal function in the human kidney.

Table 2 Patient characteristics

Patient No.	Sex	Age years	Creatinine mg/dl	Blood pressure mm Hg	AT1-R fmol/mg protein	AT2-R fmol/mg protein
1	male	66	1.1	130/74	12.2	2.1
2	male	71	0.9	104/68	14.4	1.8
3	male	54	1.0	116/62	10.4	2.8
4	male	63	0.9	144/82	13.8	3.3
5	male	58	1.2	154/84	12.8	2.4
6	male	49	1.0	148/78	15.6	3.5
Mean ± SE		60 ± 3.3	1.0 ± 0.5	133 ± 8/75 ± 3	13.2 ± 0.7	2.7 ± 0.3

Renal cortex was obtained from tumor-free portions of renal cell carcinoma. B_{max} values were determined by Scatchard plots derived from the specific [^{125}I]Sar^{1}Ile^{8} Ang II binding as described in Materials and Methods.

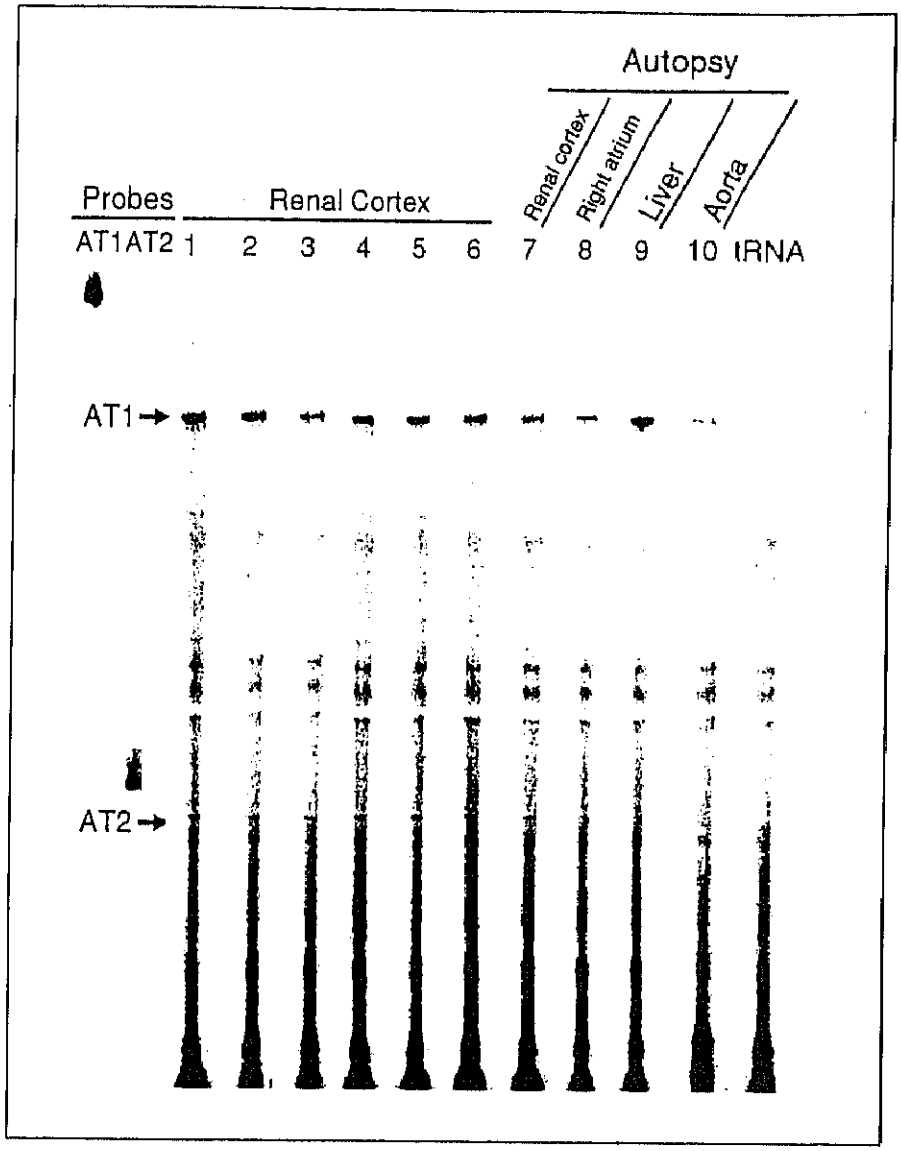

Fig. 17. RNase protection analyses of AT1-R and AT2-R mRNA distributions in human kidney, right atrium, liver, and aorta. Total RNA (20 μg) treated with DNase in the presence of RNase inhibitor was simultaneously hybridized with RNA probes for AT1-R and AT2-R, then digested with RNase. Renal cortex was obtained from tumor-free portions of renal cell carcinomas (lanes 1–6). Renal cortex (lane 7), right atrium (lane 8), liver (lane 9), and aorta (lane 10) samples were obtained at autopsy from the same patient with no history of renal or heart diseases. AT2-R mRNA signals were detectable in all kidney and right atrium samples, whereas liver and aorta contained only AT1-R mRNA. Profiles of patients with renal cell carcinoma (lanes 1–6) are described in table 1, and the lane numbers in the figure correspond to the patient numbers in table 1. tRNA was used as a negative control. AT1 and AT2 indicate the positions of protected mRNA signals. Exposure time was 48 h.

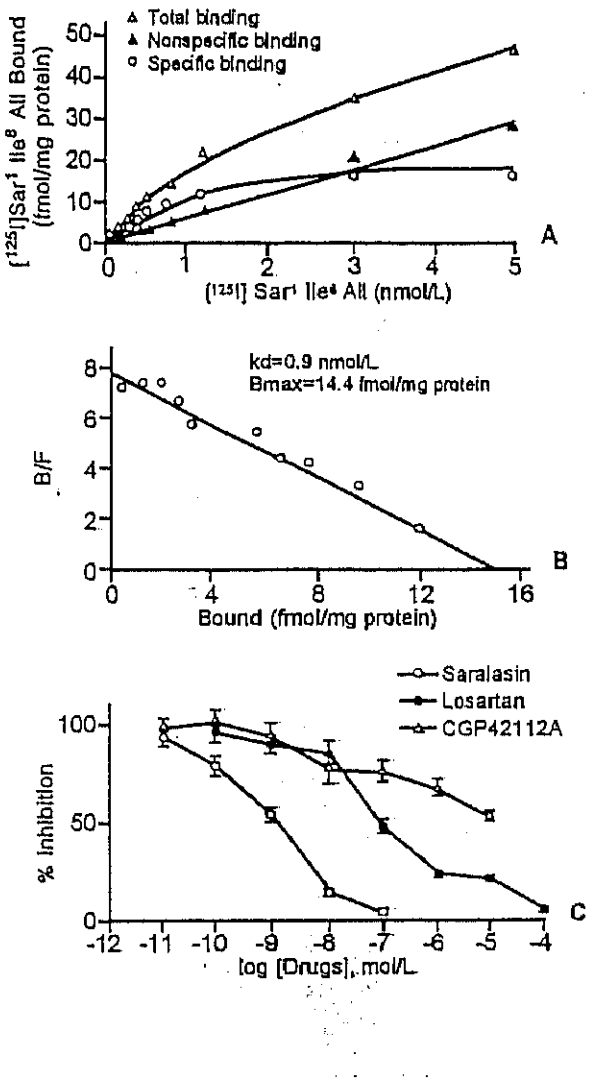

Fig. 18. Inhibition studies of [^{125}I]Sar^{1}Ile^8 Ang II binding to human renal cortex. **A** Saturation binding of [^{125}I]Sar^{1}Ile^8 Ang II to membranes from human renal cortex. Membrane fractions were incubated for 90 min at 21 °C with various concentrations (ten assay points in 0.05–5 nmol/l) of [^{125}I]Sar^{1}Ile^8 Ang II. Specific [^{125}I]Sar^{1}Ile^8 Ang II binding was determined experimentally as the difference between total and nonspecific binding in parallel assays in the absence and presence of 3 µmol Ang II. **B** Scatchard plots derived from the specific [^{125}I]Sar^{1}Ile^8 Ang II binding data. Each point in **A** and **B** represents the average of duplicate determinations using membranes of renal cortex obtained from patient 1 (table 1). **C** Membrane fractions of renal cortex were incubated with 0.2 nmol/l [^{125}I]Sar^{1}Ile^8 Ang II for 90 min at 21 °C in the presence of varying concentrations of CGP4112A, saralasin, or losartan. [^{125}I]Sar^{1}Ile^8 Ang II binding in the presence of 3 µmol/l Ang II was subtracted from each value, and results were expressed as percentages of specific [^{125}I]Sar^{1}Ile^8 Ang II bound in the absence of any drugs. Each point represents the mean ± SE from six samples (patients 1–6) with duplicate determinations in each experiment. Data analyses in the Scatchard plots and competitive inhibition experiments were as described in Materials and Methods.

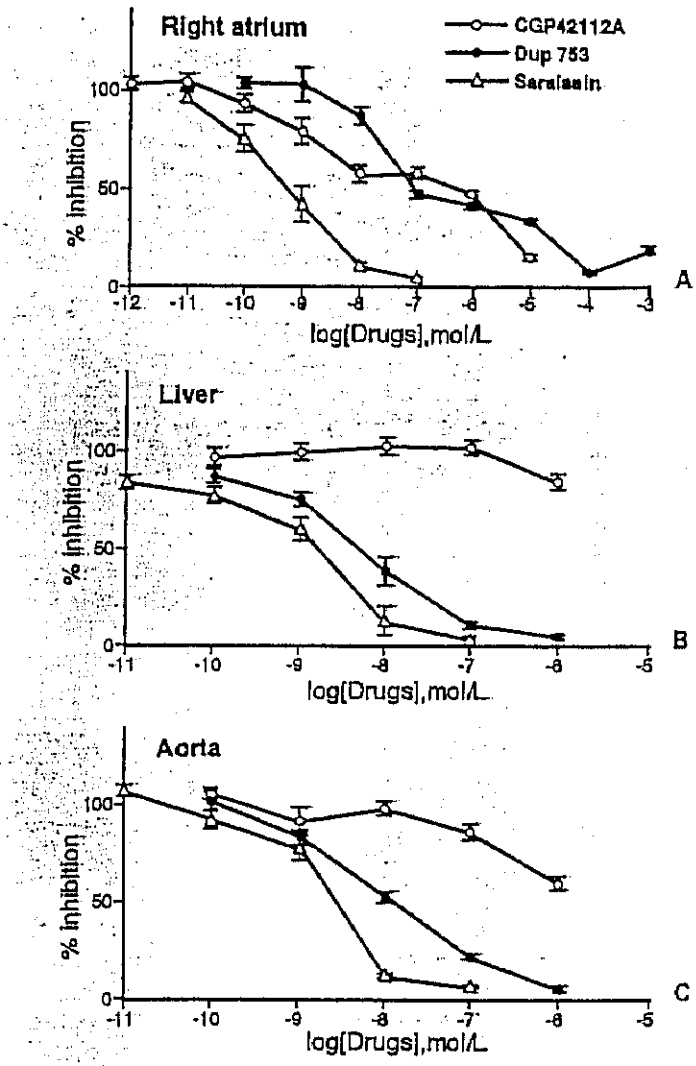

Fig. 19. Competitive inhibition studies of [^{125}I]Sar^{1}Ile^{8} Ang II binding to membrane fractions from atrium (**A**), liver (**B**) and aorta (**C**). Membranes from right atrium, liver, and aorta obtained at autopsy from the 4 patients with no history of renal or heart diseases were incubated with 0.2 nmol/l [^{125}I]Sar^{1}Ile^{8} Ang II in the presence of varying concentrations of CGP42112A, saralasin, or losartan. [^{125}I]Sar^{1}Ile^{8} Ang II binding in the presence of 3 µmol/l Ang II was subtracted from each value, and results are expressed as percentages of specific [^{125}I]Sar^{1}Ile^{8} Ang II bound in the absence of competitors. Each point represents the mean ± SE from four samples with duplicate determinations in each experiment. Data analyses in the Scatchard plots and competitive inhibition experiments were as described in Materials and Methods.

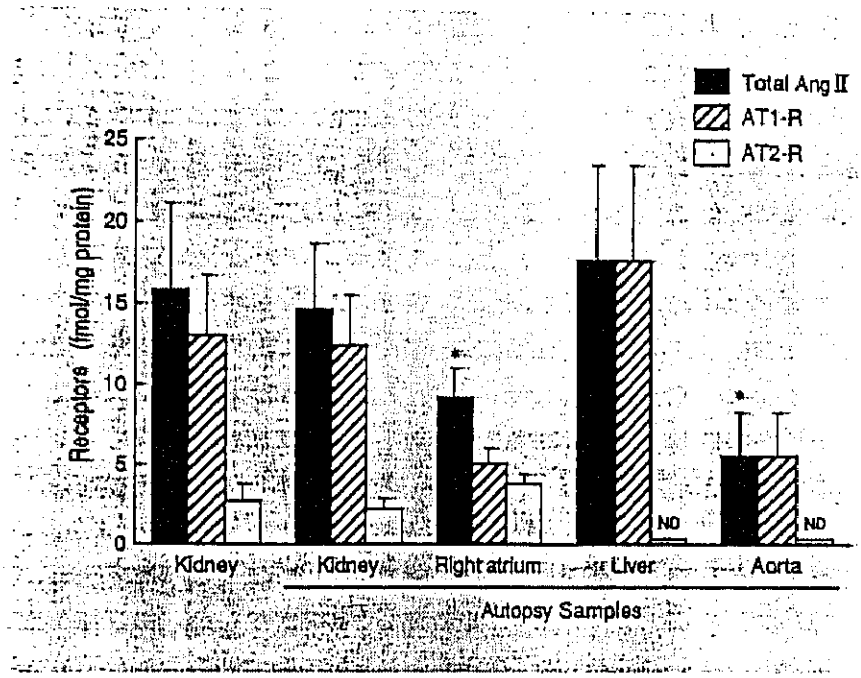

Fig. 20. Distribution of AT1-R and AT2-R in human tissues. Total Ang II receptor densities were determined by Scatchard plots, and AT1-R and AT2-R densities were calculated by nonlinear least-squares regression analysis on the basis of inhibition by CGP42112A. Renal cortex samples were obtained from tumor-free portions of renal cell carcinoma (n = 6, table 1) or from autopsy samples (n = 4). Right atrium, liver, and aorta were obtained at autopsy from 4 patients with no history of renal or heart diseases. Specific [^{125}I]Sar^{1}Ile^{8} Ang II binding was determined experimentally as the difference between total and nonspecific binding in parallel assays in the absence and presence of 3 μmol/l Ang II. The values indicated are mean ± SE. * $p < 0.05$ vs. the receptor numbers in kidney samples obtained at autopsy.

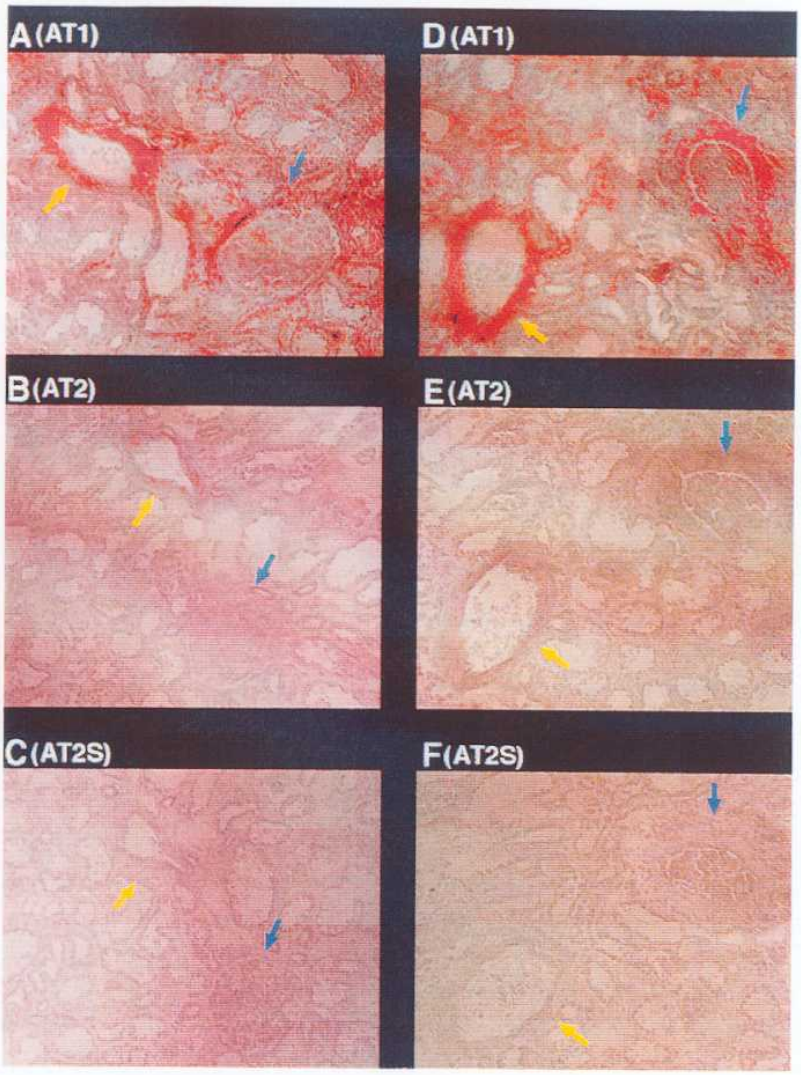

Fig. 21. In situ hybridization of human renal cortex. Renal cortex samples were examined by in situ hybridization using specific antisense cRNA probes for AT1-R (**A, D**) and AT2-R (**B, E**) mRNAs and sense cRNA probes for AT2-R mRNA (**C, F**). Adjacent serial sections shown in **A–C** were from patient 1, and those shown in **D–F** were from patient 2. Strong signals for AT1-R mRNA (shown by red color) were localized in interlobular arteries and perivascular fibrous regions (indicated by yellow arrow) and tubulointerstitial regions surrounding the glomeruli (indicated by blue arrow). Moderate densities of signals were observed in the glomeruli, and weaker signals were present in proximal tubules. The AT2-R mRNA signal (shown by red color in **B** and **E**) was localized in interlobular arteries, and there were no significant detectable signals in tubulointerstitial regions and proximal tubules. No AT2-R mRNA signals were observed when sense cRNA probes for AT2-R mRNA were used (**C, F**).

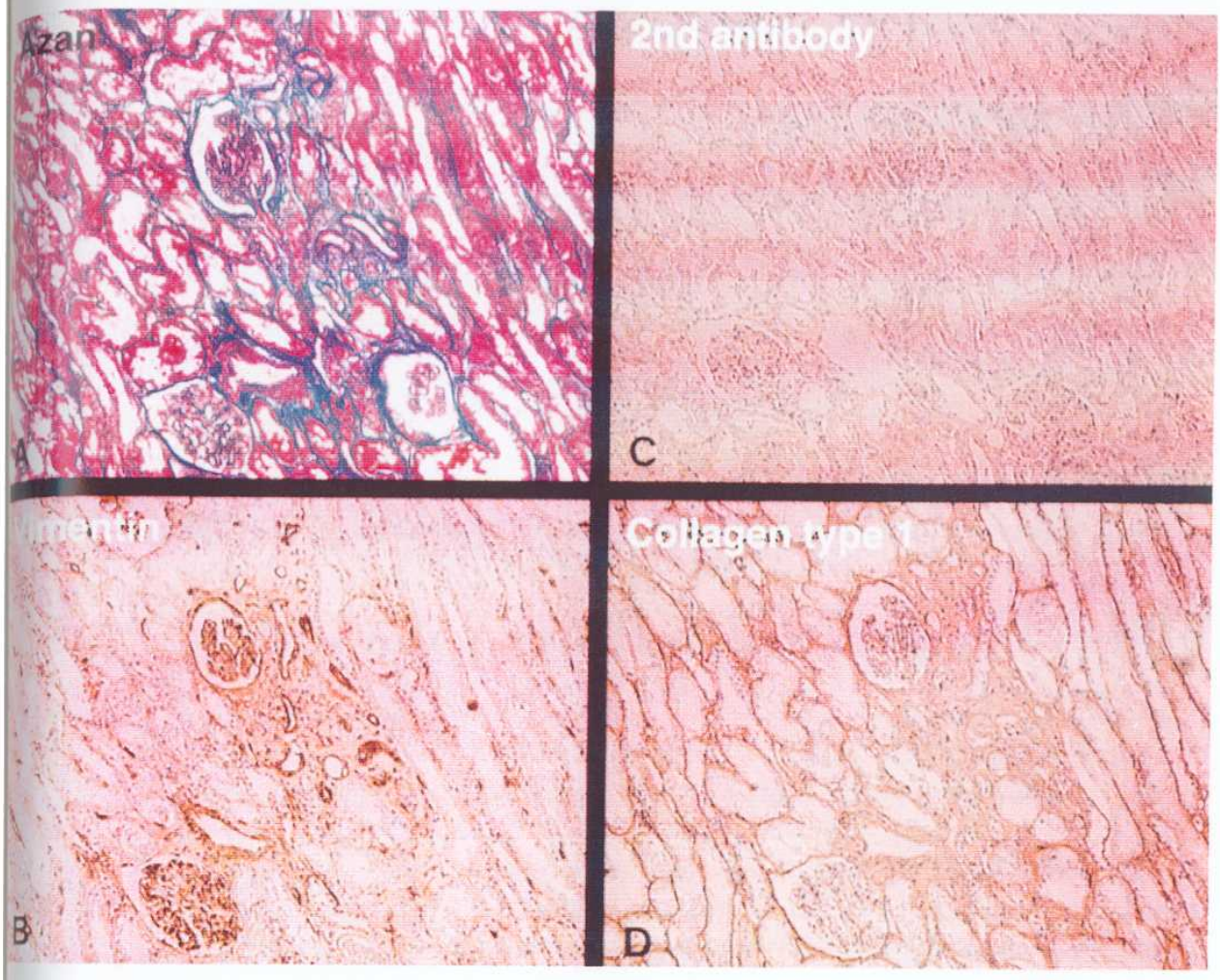

Fig. 22. Immunocytochemical analysis of human renal cortex. Adjacent serial sections from patient 1 were stained with azan or analyzed by fluorescence immunocytochemistry using the biotin-avidin-immunoperoxidase system. In azan-stained sections, collagen fibers were indicated by blue color (**A**). Adjacent sections were stained with monoclonal antibodies against vimentin (**B**) and collagen type 1 (**D**) or second antibody (**C**). Cells present in tubulointerstitial fibrous regions were positive for vimentin and collagen type 1 and negative for second antibody.