Kidney International, Vol. 43 (1993), pp. 1229–1235

Intrarenal localization of angiotensin II type 1 receptor mRNA in the rat

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Intrarenal localization of angiotensin II type 1 receptor mRNA in the rat. We examined intrarenal localization of angiotensin II type 1 receptor (AT1) mRNA in kidneys of normal adult male Munich Wistar rats using the methods of reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization. For RT-PCR, we used a rat AT1 subtype A (AT1A)-specific oligonucleotide primer pair. To semi-quantitatively assess the expression level of AT1 mRNA among several regions of kidney, AT1 cDNA was coamplified with β-actin cDNA. When compared to the level in the adrenal gland (expressed as 100%), the level of AT1 mRNA was markedly higher in glomeruli (273 ± 69%), followed in intensity by the renal papilla (151 ± 57%), renal cortex (139 ± 19%), and renal medulla (114 ± 35%). *In situ* hybridization studies, using a 479 bp nucleotide fragment from AT1A-coding exon as a probe, also revealed a glomerular preponderant pattern of AT1 mRNA localization. Thus, within the glomerulus, AT1 mRNA localized in mesangial areas, predominantly at the vascular pole. In the vascular components of the juxtaglomerular apparatus (JGA), namely the terminal portion of the afferent arteriole (that is, immunohistochemically renin-positive site) and extraglomerular mesangial cells, the latter showed AT1 mRNA localization in the non-manipulated kidney, while AT1 mRNA was undetectable in the arteriole outside the JGA. The kidneys of rats treated with an angiotensin I converting enzyme inhibitor (ACEI) showed extension of the AT1 mRNA localization on the afferent arteriole toward the interlobular artery. We speculate that the observed pattern of AT1 mRNA localizing in the glomerular vascular pole and JGA may account for the unique physiological and pathophysiological actions of angiotensin II (Ang II), that is: (1) the renal hemodynamic effect of Ang II involves simultaneous increases in afferent and efferent arteriolar resistances and a reduction in ultrafiltration coefficient; (2) experimental glomerular sclerosis, sensitive to pharmacological inhibition of angiotensin II, is commonly prominent at the vascular pole; and (3) a negative feedback relationship exists between angiotensin II and renin, presumably via the AT1 on the JGA.

With the introduction of novel nonpeptide angiotensin II (Ang II) receptor (Ang IIR) antagonists [1, 2], several types of Ang IIR have been identified. These subtypes of Ang IIR show organ specific distribution [3, 4] with correlation between specific types and function in some organs [1]. Ang IIR is highly expressed in the kidney, adrenal gland, brain, liver and vasculature [2, 3], and Ang II has varying effects via its interactions with specific Ang IIR. Ang II maintains circulatory homeostasis [5, 6], acts as a neurotransmitter in the central nervous system [7] and stimulates hypertrophy and proliferation of cells [8–15].

Received for publication September 21, 1992
and in revised form January 11, 1993
Accepted for publication January 14, 1993

The cloning of Ang IIR type 1 cDNA (AT1) [16, 17] has allowed further study of this receptor's distribution. Subtypes of AT1 have been identified in the rat (1A and 1B) [18], but not in humans.

Although Ang IIR has already been recognized in mesangial and vascular smooth muscle cells, the localization of the specific AT1 mRNA in kidney has not been fully elucidated. In this study, the expression pattern of AT1 mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization.

Methods

Animals

Male adult Munich Wistar rats (total $N = 12$), body weight 250 to 300 g, were studied. *In situ* hybridization studies were performed in rats receiving a normal rat chow diet and tap water *ad libitum*, and in rats treated with enalapril maleate, 200 mg/l drinking water (Merck Sharp and Dohme Research Lab., Rahway, New Jersey, USA) for two weeks ($N = 6$). Rats were anesthetized with sodium pentobarbital (Abbott Lab., South Chicago, Illinois, USA) for tissue harvesting for RT-PCR or *in situ* hybridization.

Glomerular isolation for RT-PCR and mesangial culture for in situ *hybridization*

Kidneys were removed under anesthesia, and renal cortex was dissected free from the remainder of the kidney, cut into small pieces and then passed through serial sieves to isolate glomeruli. The glomerular preparations had less than 5% tubular contamination [19]. Primary cultures of mesangial cells were obtained from outgrowths of isolated rat renal glomeruli as described [20]. Briefly, the glomeruli were cultured in medium which consisted of RPMI with glutamine supplemented with 16% FBS, penicillin, streptomycin and amphotericin B. After mesangial cells had reached confluence they were subcultured, and after three subcultures they were used for study. They represented an apparently uniform cell population as evaluated by the following criteria, that is: (a) morphology, (b) histochemically positive for actomyosin with fibrillar appearance, but (c) not staining for factor VIII.

Reverse transcription-polymerase chain reaction (RT-PCR)

To compare relative amounts of AT1 mRNA in different regions of the kidney, we extracted RNA from glomeruli

isolated by differential sieving (see above), papilla, renal cortex, renal medulla and adrenal gland. Total RNA from tissues from three rats (six kidneys) was extracted and pooled from each part as previously described [21]. It was further purified with 4 M LiCl and finally resuspended in DEPC-treated water. Three micrograms total RNA were reverse-transcribed to first strand cDNA by 100 U Moloney murine leukemia virus (Mo-MLV) transcriptase (BRL, Gaithersburg, Missouri, USA) with 50 ng random hexamer, 10 mM DTT, 2 mM dNTP in buffer containing 50 mM Tris-HCl, 75 mM KCl and 3 mM $MgCl_2$ at 37°C for 60 minutes, followed by RNase digestion [22]. PCR was performed by coamplification with two different primers [23–25], one for rat AT1A and another for rat β-actin. AT1 gene, primer 1 (antisense) was 5′-GGGAGCGTCGAATTCCGAGACTCAT-AATGA-3′, and primer 2 (sense) was 5′-ACCCTCTACAG-CATCATCTTTGTGGTGGGGA-3′. Thus, the cDNA amplification product was predicted to be 479 bp in length. RT-PCR with rat cytoplasmic β-actin served as an invariant control. The primers for β-actin [26] were defined by the following genomic base sequence: primer 1′ (antisense), 5′-ACCTTCAACAC-CCCAGCCATGTACG-3′; primer 2′ (sense), 5′-CTGATCCA-CATCTGCTGGAAGGTGG-3′. β-actin primers spanned two introns and resulted in a 703 bp cDNA amplification product. All the primers were checked for absence of fortuitous homology to any sequence listed in a gene sequence data bank (IntelliGenetics, Inc., Mountain View, California, USA). After preparation of the first strand cDNA, one-tenth of the reaction solution was mixed with PCR reagents. After all the PCR reactions, 100 μl contained the following components: 2.5 U Taq polymerase (Promega, Madison, Wisconsin, USA), 20 pmoles each of AT1A primers, 6 pmoles each of β-actin primers, 0.2 mM dNTP, 10 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$ [22, 27]. Reaction tubes were overlaid with 70 μl of mineral oil to prevent evaporation. PCR was performed in a programmable thermal controller (MJ Research, Inc., Watertown, Massachusetts, USA) which was programmed as follows: incubation at 92°C for five minutes (initial melt), followed by 25 cycles, 92°C for one minute (denature), 65°C for 40 seconds (anneal) and 72°C for 1.5 minutes (extend). PCR was completed by a final extension at 72°C for 10 minutes. For analysis of the PCR products, each half of the reaction solution was extracted by chloroform, dried and size-fractionated on 1.5% agarose gels, and stained with ethidium bromide. For Southern blot analysis of PCR products, gels were denatured, transferred, and blotted onto Zeta-bind nylon membranes. To test for specific PCR amplification products, fragments of 359 bp AT1 and 703 bp β-actin were used for hybridization probe. The sequence of the probe was located in the middle of the amplified fragment and did not include the region of primers. Areas of both AT1 and β-actin bands coamplified from each sample were measured by densitometry and the ratio of AT1 to β-actin calculated. RT-PCR was performed four times on RNA from each region of the kidney, then results were averaged. Results are given as mean ± 1 SE.

Prehybridization

To prevent RNA degradation, all reagents and solutions were treated by 0.1% diethylpyrocarbonate, DEPC (Fluka, Ronkonkoma, New York, USA). All procedures, unless otherwise specified, were performed at room temperature. After anesthesia, rats were perfused *in vivo* via the left ventricle with 20 ml PBS followed by 20 ml of 4% paraformaldehyde [28]. Tissues were harvested from six rats (3 normal and 3 ACEI treated) and placed overnight in 4% paraformaldehyde at 4°C, processed routinely, embedded in paraffin, and stored at 4°C until beginning of hybridization studies. Five micrometer sections were then cut and placed on polylysine (Sigma, St. Louis, Missouri, USA) treated glass slides and prepared in a concentration of 50 μg/ml in 10 mM Tris-HCl, pH 8.0 [29]. Slides, on which paraffin-embedded sections were placed, were dewaxed in xylene, then hydrated serially in 100% to 30% ethanol. Slides were refixed in 4% paraformaldehyde for 20 minutes, PBS washed, and then treated with 20 μg/ml proteinase K in 50 mM Tris-HCl, pH 7.4 and 5 mM EDTA for eight minutes [28]. Subsequently, slides were treated with 0.1 M triethanolamine for 10 minutes, followed by addition of 0.4% (vol/vol) acetic anhydride for 10 minutes. After rinsing in PBS, slides were successively dehydrated in 30% to 100% ethanol.

Hybridization and posthybridization

5×10^5 cpm/slide of ^{35}S-labeled cRNA for AT1 and control ^{35}S-labeled probes (see below) were used. Approximate volume per slide of hybridization mixture was 50 μl. Hybridization buffer contained 50% deionized formamide, 10% dextran sulfate, 8 mM DTT, 0.2 mg/ml tRNA and 1 × salts (300 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 0.02% polyvinylpyrolidone, 0.02% Ficoll and 0.02% BSA). Slides were incubated overnight at 50°C in a sealed humidified container, into which 1 × salts and 50% formamide were placed to maintain moisture. Subsequently, slides were washed in 50% formamide, 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), 20 mM DTT at 50°C for 60 minutes, followed by TEN buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, and 500 mM NaCl) at 37°C, and then treated with 50 μg/ml RNase at 37°C for 30 minutes [30]. Slides were washed in 2 × SSC, followed by 0.1 × SSC at 50°C, dehydrated in 30% to 100% ethanol with 0.3 M ammonium acetate, and air-dried. They were dipped into photographic emulsion (Polysciences Inc., Warrington, Pennsylvania, USA), diluted 1:1 in 2% glycerol solution, left on ice for 15 minutes, dried overnight in a dark box, and exposed at 4°C for seven weeks [31]. The slides were developed with D-19 developer and counterstained with 0.05% toluidine blue.

Probe for in situ *hybridization*

We used a 472 bp fragment from cDNA of rat angiotensin II receptor type 1A (AT1A), vascular type [32]. This fragment has the least homology with the rat Ang IIR subtype 1B (AT1B) gene. The fragment was obtained after double-digestion by *EcoR* I and *Sac* I from whole cDNA of rat AT1A and was located within the exon of the protein coding region. This 472 bp-length DNA was then inserted into PGEM4Z vector (Promega) for *in vitro* synthesis of cRNA. After linearization of the plasmid containing AT1A by pertinent restriction enzymes, we transcribed sense and antisense cRNA of AT1A using RNA polymerases with labeled ^{35}S-UTP (Du Pont, Boston, Massachusetts, USA) in the reaction mixture. Sense cRNA was used as a negative control.

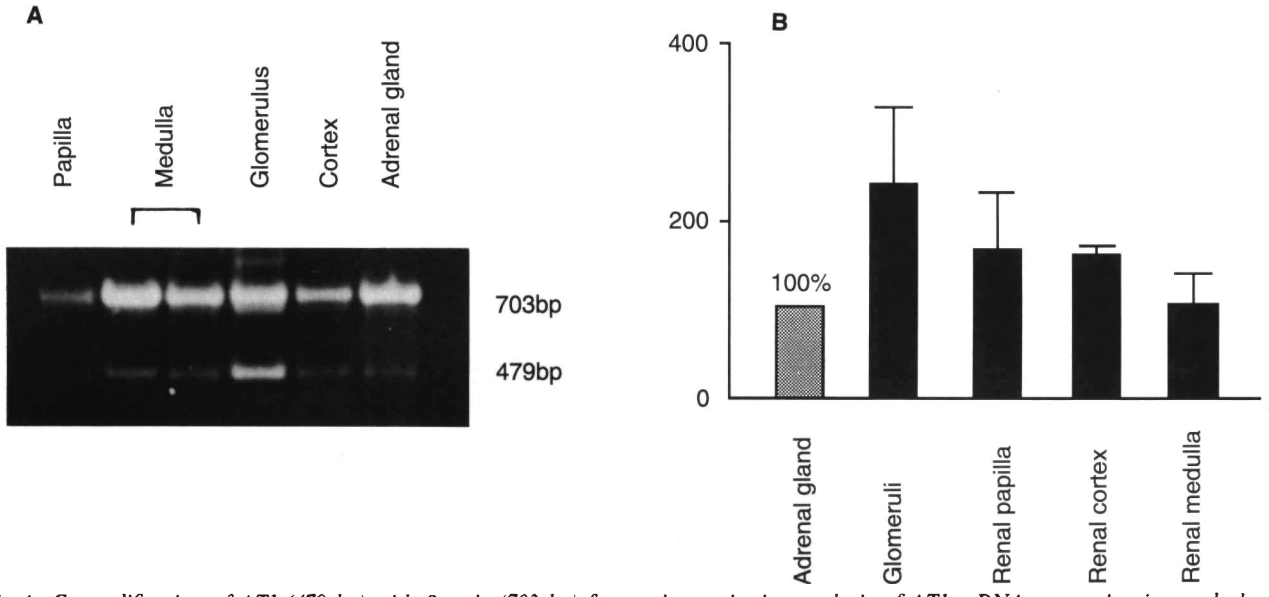


Fig. 1. *A. Coamplification of AT1 (479 bp) with β-actin (703 bp) for semi-quantitative analysis of AT1 mRNA expression in renal glomeruli, papilla, cortex, medulla and adrenal gland.* Glomeruli showed the most intense band for AT1 mRNA. **B.** Varied degrees of AT1 expression in different regions of the kidney. Following Southern analysis, the relative ratio of expression of AT1 mRNA to β-actin mRNA was measured by densitometry. AT1 was expressed most abundantly in glomeruli ($P < 0.05$ vs. adrenal gland). Data are shown as mean ± 1 SE.

Immunohistochemistry

Five micrometer sections of paraffin-embedded tissues were stained by an indirect immunoperoxidase technique (avidin-biotin-peroxidase complex method) [33]. After removal of paraffin by xylene, the tissue sections were incubated with 0.3% hydrogen peroxide for 30 minutes to inactivate endogenous peroxidase and to decrease background. Washing in PBS was followed by covering slides with goat serum (Sigma). Immunoreaction was done by primary rabbit anti-rat renin antibody at dilution 1:1000 in PBS, 1% BSA, and 0.1% sodium azide at room temperature overnight [34]. After PBS washing, slides were incubated with goat biotinylated anti-rabbit antibody (Sigma) at 1:30 dilution in 50 mM Tris-HCl, pH 7.6. Then slides were incubated with avidin conjugated with hydrogen peroxide at room temperature for 30 minutes, followed in sequence by PBS washing and addition of 3,3′-diaminobenzidine (Sigma) as a chromogenic substrate. Counterstaining was done with toluidine blue or Meyer's hematoxylin.

Results

Semi-quantitative RT-PCR of AT1 mRNA

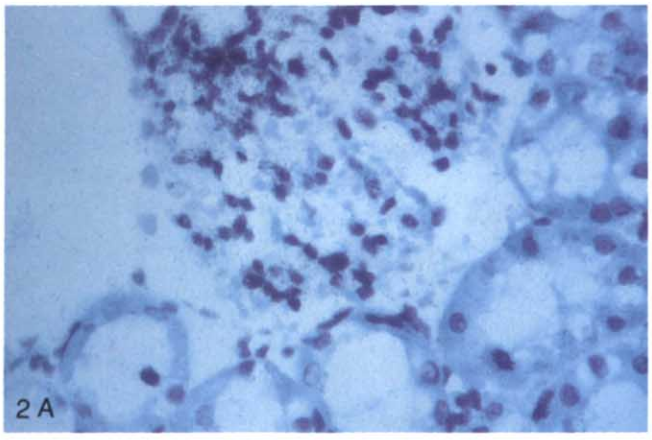
RT-PCR was performed to study relative amount of AT1 mRNA using total RNA derived from adrenal gland, isolated glomeruli, papilla, renal cortex and renal medulla. Coamplification of β-actin and AT1 was performed in the exponential phase to avoid the "tube effect" of PCR [35]. As illustrated in Figure 1A, AT1 mRNA expression was most prominent in glomeruli among the regions of the kidney studied. After assessment by densitometry, amounts of amplified AT1 PCR product were normalized by β-actin product to evaluate the relative amount of AT1 mRNA (Fig. 1B), and expressed as a relative ratio to the adrenal gland, set as 100%. Glomeruli showed the highest level of mRNA (273 ± 69%, $P < 0.05$ vs. adrenal gland) with lesser amounts, in decreasing order, in papilla (151 ± 57%), renal cortex (139 ± 19%), renal medulla (114 ± 35%) and adrenal gland (100%). Controls performed without reverse transcriptase, using procedures identical to those for RT-PCR, verified the absence of contamination by genomic DNA.

Localization of AT1 in kidney

In situ hybridization with AT1A antisense cRNA was performed to localize the mRNA of this receptor. Within the kidney, the signal localized primarily to the glomeruli (Fig. 2, bright field). Proximal tubules, but not distal, showed occasional weak but not definite mRNA signal (Fig. 2, dark field). Within glomeruli, the signal localized especially in mesangial areas. The density of the signal was more intense toward the vascular pole. Glomerular epithelial and endothelial cells showed no appreciable signal.

Interlobular arteries and large vessels did not show the signal. Sections of arterioles distant from the vascular pole also did not express AT1 mRNA. However, the vascular component of the juxtaglomerular apparatus (JGA), namely the extraglomerular mesangial cells (Fig. 3), located between the afferent and efferent arterioles, distinctively expressed AT1, whereas vascular smooth muscle cells of the terminal afferent arteriole showed only very weak signal in the normal rats. The macula densa area did not show any message for AT1 mRNA.

After manipulation of renin-angiotensin system (RAS) by oral administration of enalapril for two weeks, the expression of the AT1 gene was extended more along the afferent arteriole, and the signal was stronger in the early portion of the efferent arteriole (Fig. 4, bright and dark field). The specificity of hybridization was supported by the findings in the adrenal gland. In the adrenal gland, the signal was distributed densely within the cortex, forming a stripe, with less signal in the medulla (data not shown).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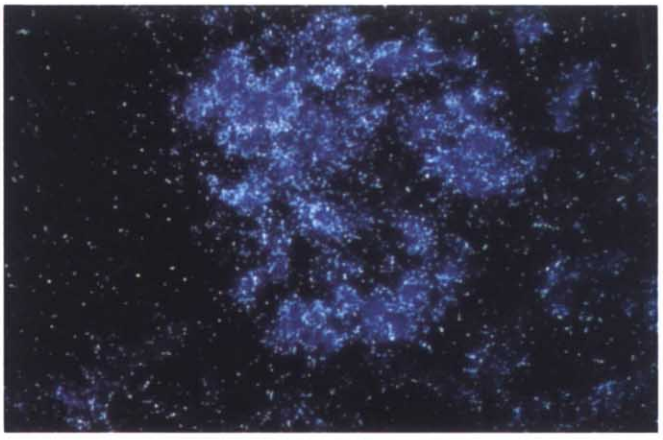

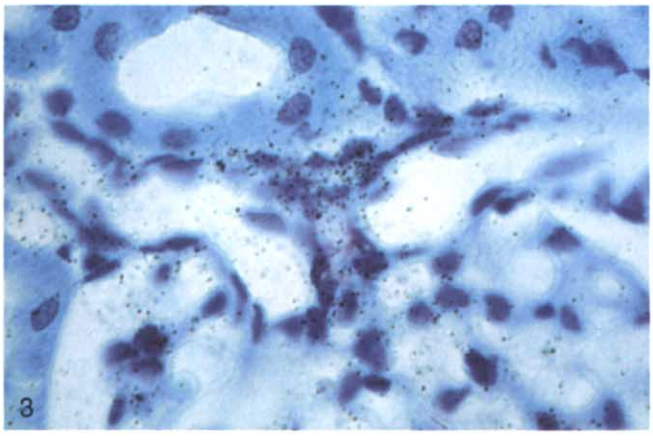
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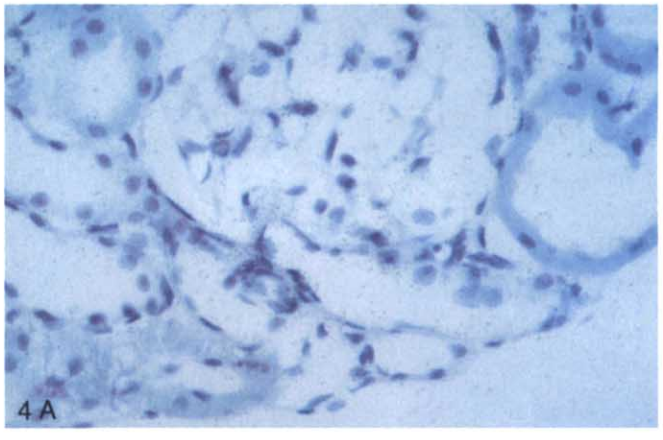
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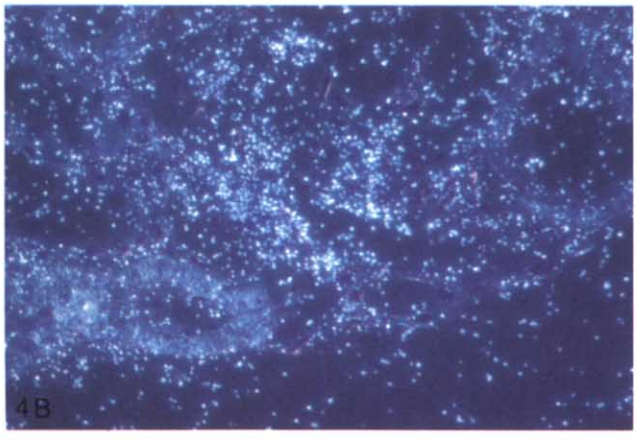
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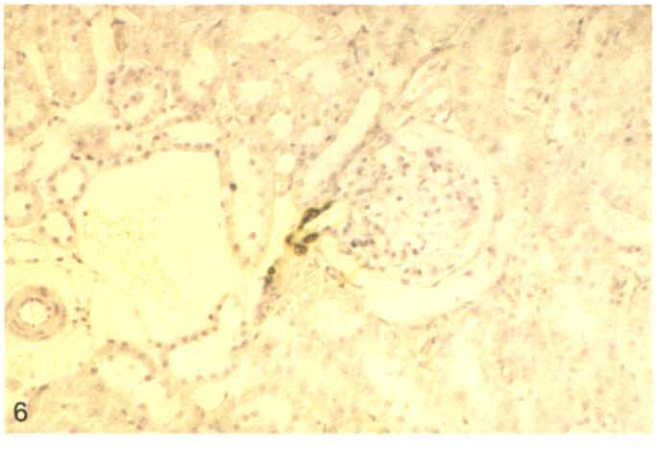
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In vitro, in situ hybridization of cultured rat mesangial cells

The localization of AT1 mRNA was verified in cultured rat mesangial cells. After the third passage from primary culture, mesangial cells showed distinctive expression of AT1 mRNA distributed around the nuclei, as shown in Figure 5.

Immunohistochemistry of renin in juxtaglomerular apparatus

To verify whether the regions of renin production are correlated with the localization of AT1, we performed immunohistochemical staining on adjacent serial sections of the tissues which were used for *in situ* hybridization. In the normal rat, as many previous studies have shown, renin positive cells showed restrictive localization in the terminal afferent arteriole at the vascular pole, and renin was rarely detected in the efferent arteriole. As shown in Figure 6, after enalapril treatment, renin positive cells extended toward the interlobular artery from the glomerular end of the afferent arteriole (one component of JGA), and were rarely detected in efferent arteriole and extraglomerular mesangial cells. Comparison between Figures 4 and 6 illustrates renin-positive cells with AT1 mRNA in the terminal end of the afferent arteriole, although AT1 mRNA positive extraglomerular mesangial cells stained only weakly for renin.

Discussion

This study demonstrates localization of AT1 mRNA in the adult rat kidney by RT-PCR and *in situ* hybridization. To evaluate the message of AT1 semi-quantitatively, we performed RT-PCR using total RNA from several regions of the kidney. The RT-PCR method is a powerful tool to analyze small amounts of mRNA. However, the varied yield of amplification [35], the so-called "tube effect," necessitates coamplification of AT1 with a relatively invariant mRNA, such as β-actin or with an internal standard for semi-quantitative comparisons, as recommended [27].

Further, such a coamplification must be done in the exponential phase, and contamination of genomic DNA in samples must be ruled out. With these caveats, this semi-quantitative technique allows comparison of relative amounts of mRNA.

Previously, high homology between the two subtypes of AT1 (1A and 1B) was identified. However, the distribution of these subtypes varied in different organs. Of special interest, a dominant gene expression of the 1A type was seen in the adult kidney [12]. Therefore, we used a terminal end of the AT1A protein coding region as a probe, which was less homologous to AT1B. The specificity of this probe was evaluated by comparing the results of RT-PCR to those of *in situ* hybridization. Thus, our RT-PCR study, using a specific primer pair for AT1A, revealed the greatest amount of AT1A mRNA in glomeruli. This was paralleled by the findings of our *in situ* hybridization study, confirming the specificity of the probe for AT1A.

Our *in situ* hybridization study showed AT1 mRNA localized specifically within the kidney and the adrenal cortex [36, 37]. Ang II has actions on both sodium and bicarbonate reabsorption in proximal tubules [38, 39], indicating the presence of tubular Ang II receptor, as suggested by previous studies of the site of Ang II in proximal tubules [40–42]. However, in our study, the very weak AT1 signal detected in proximal tubules, due to the limited resolution of the *in situ* hybridization technique itself to distinguish real signal from background, could not be definitively interpreted.

Glomeruli showed the greatest amount of AT1 mRNA expression in the mesangial area. Further, by *in vitro in situ* hybridization, cultured mesangial cells expressed AT1 mRNA. Thus, these *in vivo* and *in vitro* studies demonstrate that mesangial cells are potential effecter sites for Ang II in physiological and pathophysiological settings.

In addition, this study investigated the potential relationship of AT1 and the JGA. The JGA is composed of myoepithelial cells (also called granular cells) and extraglomerular mesangial cells. The latter are identified on the basis of their anatomical localization [43, 44]. The existence of an intrinsic renin angiotensin system in the JGA has been suggested by several immunohistochemical studies showing co-localization of renin and Ang II [34, 45, 46]. Our data show that extraglomerular mesangial cells actually expressed more abundant AT1 mRNA than the granular cells in normal rats. Immunostaining in normal rats on adjacent sections for renin showed positive cells in only the part of the afferent arterioles immediately adjacent to the glomerular vascular poles.

Renin production by the JGA is regulated by several factors. Of note, Ang II has negative feedback on renin gene expression. Conversely, inhibition of Ang II by ACEI has been reported to enhance the expression of renin mRNA [47, 48]. This upregulation of renin was accompanied by a change in the pattern of renin-positive cells, with recruitment of renin gene expression from JGA to the proximal afferent arteriole.

When RAS was manipulated by ACEI [49, 50], we found that AT1 expression was expanded in close association with enhanced renin expression. Thus, the extension of the AT1 mRNA positive region along the afferent arteriole toward the interlobular artery, as well as definite expression of AT1 in the efferent arteriole, was seen in association with extension of renin positive cells to these areas (Fig. 6). RT-PCR (data not shown) revealed that ACEI manipulation enhanced the AT1 expression level numerically (but not statistically) at the whole kidney level, as other investigators have suggested [32]. The

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Fig. 2. *Distribution pattern of AT1 mRNA in cortex of the normal adult rat kidney.* AT1 was expressed mainly in mesangial area. The intensity of the message was higher at the vascular pole (bright and dark field, ×145).

Fig. 3. *AT1 mRNA in normal adult rats expressed predominantly in extraglomerular mesangial cells, defined anatomically to be localized between the afferent and the efferent arteriole.* However, in both of the arterioles at the vascular pole, AT1 message was undetectable (bright field, ×293).

Fig. 4. *AT1 mRNA expression in rats treated with ACEI extended along the afferent arteriole (arrow) from the glomerular vascular pole.* AT1 also was expressed in the efferent arteriole (bright and dark field, ×293).

Fig. 5. *AT1 mRNA expressed in cultured rat mesangial cells* (×293).

Fig. 6. *Study of the JGA by immunohistochemical staining for renin, showing renin-positive cells at the afferent arteriole at the glomerular vascular pole.* Manipulation of RAS by ACEI treatment, induced extension of renin-positive cells (arrow) from the vascular pole to the interlobular artery (×122).

enhanced AT1 mRNA expression in the afferent arterioles shown by our *in situ* hybridization study represents only a fraction of the mRNA of the whole kidney; therefore, such a selective change did not result in marked differences by RT-PCR. The similar pattern of AT1 and renin expression suggests that renin producing JGA cells express AT1 and, further, that AT1 on JGA may be involved in the negative feedback regulation, which is known to exist between Ang II and the renin level.

In addition to JGA cells, AT1 was expressed on mesangial cells and extraglomerular mesangial cells. These patterns of localization are reminiscent of the functions of Ang II, that is, glomerular sclerosis and mesangial matrix accumulation sensitive to Ang II are more prominent in the vascular pole, Ang II results in increased afferent (R_A) and efferent arteriolar (R_E) resistances, and Ang II decreases ultrafiltration coefficient (K_f). It was previously postulated that these simultaneous constrictive responses in R_A, R_E, and K_f represent contraction of both intra- and extraglomerular mesangial cells, which juxtapose the glomerular capillary network and arterioles at the vascular pole.

This anatomical arrangement of mesangial cells together with AT1 receptor localization on them, may provide a highly efficient measure to induce concurrent narrowing of the arterioles and reduction in filtering surface area. Further, the presence of AT1 receptor on JGA and modulation of its expression in parallel with renin supports local regulation of the RAS.

Acknowledgments

This work was supported by National Institute of Health grants DK-44757 and DK-42131. Dr. Agnes Fogo is a recipient of a Clinician-Scientist Award from the American Heart Association. The authors thank Dr. Yasuhisa Matsui, Tohoku University, Sendai, Japan for his valuable input in the early stages of these experiments.

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