Developmental Changes in Expression of Angiotensinogen mRNA in Rat Nephron Segments

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We studied the localization of angiotensinogen mRNA in rat nephron segments and the differences in angiotensinogen mRNA levels between male Sprague-Dawley rats at 6 and 12 wk of age using reverse transcription and polymerase chain reaction (RT-PCR). Each nephron segment of the rat kidney was microdissected. Total RNA was prepared and used in the following RT-PCR assay. The PCR products were size-fractionated by agarose gel electrophoresis, visualized with ethidium bromide staining, and identified by Southern blot analysis. The relative amounts of products were determined by densitometry. Strong bands corresponding to angiotensinogen mRNA were detected from proximal convoluted and straight tubules, and weaker bands were found in glomeruli. The signals in all tissues in 12-wk-old rats were weaker than those in 6-wk-old rats. Since local angiotensinogen is the unique substrate of the tissue renin-angiotensin system and exerts an autocrine-paracrine influence on renal function, the changes in tubular angiotensinogen may be related to physiological and morphological changes in the rat kidney during development. (Hypertens Res 1998; 21: 155-161)

Key Words: angiotensinogen, reverse transcription, polymerase chain reaction, renal tubule, mRNA

Numerous studies supporting the existence of a complete renin-angiotensin system (RAS) within multiple tissues have been reported (1-6). Within renal tissue, evidence of renin, angiotensin-converting enzyme, and angiotensins has been revealed by means of immunohistochemical (7-9) and physiological techniques (10-13). However, these findings cannot exclude the contribution of the circulating RAS.

Recently, through the use of molecular biological techniques, the local synthesis of RAS components in the kidney has been demonstrated. The intrarenal expression of renin, angiotensin-converting enzyme, and angiotensinogen mRNAs has been demonstrated by Northern blot analysis (1-6, 14), and the intrarenal tissue sites of renin and angiotensinogen mRNA synthesis have been localized by in situ hybridization (15-17). Furthermore, studies using reverse transcription and polymerase chain reaction (RT-PCR) have shown the localization of angiotensin II type 1 receptor and angiotensinogen mRNA in microdissected nephron segments (18).

Detecting the localization of components of the RAS is particularly important in understanding the intrarenal RAS. For example, if the renin, angiotensin II type 1 receptor and angiotensinogen genes are expressed within the same cells, we can speculate that intracellular angiotensin II may be formed and secreted and may act at surface angiotensin II receptors, suggesting an autocrine function. Therefore, to elucidate the role and the significance of intrarenal RAS, further information on the detailed localization of components of the RAS is necessary.

Furthermore, the regulation of expression of mRNAs involved in the intrarenal RAS should be investigated to understand its potential roles in the regulation of renal function in health and disease (19). It has been reported that renal angiotensinogen mRNA may be regulated by sodium, androgen, and angiotensin II (19-24). Intrarenal angiotensinogen expression is altered in various disease states, such as heart failure (25), and in spontaneously hypertensive rats (19). However, little is known about the effects of development on renal angiotensinogen mRNA expression, especially in the tubules. Although several studies of changes in renal angiotensinogen mRNA expression with development or aging have been performed, their results are inconsistent (24, 26-28).

Therefore, in this study we examined the localization of angiotensinogen mRNA in microdissected rat nephron segments and investigated differences in angiotensinogen mRNA expression semi-quantitatively in each nephron segment between 6- and 12-wk-old rats with the use of RT-PCR. Similar meth-
Methods

Animals
Male Sprague-Dawley rats obtained from Charles River Laboratories, Inc. (Atsugi, Kanagawa, Japan) were used in this study. 6-week-old rats ($n=4$) weighing 220 to 250 g and 12-week-old rats ($n=4$) weighing 450 to 480 g were fed ad libitum a normal-salt diet and had free access to water.

Renal Tubule Microdissection and RNA Preparation
Animals were anesthetized with pentobarbital. The abdomen of each animal was opened, and the aorta was cannulated with polyethylene tubing below the left kidney. The left kidney was perfused initially with 10 ml of ice-cold dissection solution containing the following (in mmol/l): 135 NaCl, 1 Na$_2$SO$_4$, 1.2 MgSO$_4$, 5 KCl, 2 CaCl$_2$, 5.5 glucose, and 5 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES, pH 7.4). The kidney was then perfused again with 10 ml of the same solution containing 1 mg/ml collagenase (type I, 400 U/mg) and 1 mg/ml bovine serum albumin (BSA). The kidney was removed and decapsulated, and thin coronal sections were cut. The sections were transferred into tubes containing 10 ml of the same collagenase solution, and incubated with 95% O$_2$ and 5% CO$_2$ bubbling for 40 min at 37°C. Then, these sections were transferred to iced petri dishes filled with dissection solution containing 10 mmol/l vanadyl ribonucleotide complex.

Tubule dissection was performed with dissecting forceps (sharpened Dumont No.5, A. Dumont and Fils, Switzerland), under a dissection microscope with dark-field illumination. Tubule segments were identified based on previously described criteria (31). We microdissected the following structures: glomeruli (Glm), proximal convoluted tubule (PCT), cortical collecting duct (CCD), medullary thin ascending limb (MTAL), and medullary thick ascending limb (MTAL). Dissected segments were washed with an ocular micrometer. Each segment (total length 20 to 30 mm, 200 glomeruli) was collected, and transferred using pipettes coated with 0.1% BSA (RNase-free) to clean dissection buffer and washed free of contaminating debris. It took about 1 h for this microdissection. These segments were transferred into individual tubes containing 1 ml of RNAzo1B (Cinna/Biotecx Laboratories, Inc., Houston, TX, USA) and immediately homogenized. Total RNA was precipitated from the extract of each segment with an equal volume of isopropanol in the presence of 25 μg of glycogen. The total RNA was collected by centrifugation, and the resultant pellets were resuspended in 20 μl of diethylpyrocarbonate (DEPC)-treated water, and absorbance was quantified at 260 nm. As control for possible contamination, 10 μl of the final wash buffer was carried through the RNA preparation, reverse transcription, and PCR steps.

Reverse Transcription
Total RNA (9 μl; 0.3 μg) containing 100 pmol/l random hexanucleotide primer was heated to 94°C for 2 min and 37°C for 5 min. RT reaction mixture (11 μl) containing 20 U RNAase inhibitor, 10 mmol/l DTT, 2 mmol/l dNTP, 5× reaction buffer, and 100 U Moloney murine leukemia virus reverse transcriptase was added. The reaction mixture was incubated at 37°C for 60 min and at the end of the incubation period heated to 98°C for 10 min to inactivate the reverse transcriptase activity and to denature RNA-cDNA hybrids. Negative control reactions with all the reagents except for the reverse transcriptase were performed in parallel.

Polymerase Chain Reaction
PCR was performed with rat angiotensinogen specific primers 5'-AGT GCTGAAAGTGGCCAGTGC-3' (antisense, corresponding to nucleic acids 917-937) and 5'-TGTACAAAGATGCTGAAGGCG-3' (sense, corresponding to nucleic acids 323-344). These primers were designed to localize to separate exons and yielded a product of 615 bases. Simultaneously, we performed RT and PCR for the housekeeping gene β-actin in the renal structures as a positive control. The primers for β-actin were defined by the following cDNA base sequences: 5'-GGCCATCTTCTGCTGAACTG-3' (antisense, corresponding to nucleic acids 2457-2476) and 5'-AA GAGGAGCATCTGACCATAC-3' (sense, corresponding to nucleic acids 1509-1528), which spanned an intron and resulted in a 504-bp product. After RT, we divided 20-μl samples into 15 μl for analysis of angiotensinogen and 5 μl for β-actin. The volume was adjusted to 20 μl with sterile water, and parallel PCR reactions were run with each set of primers. To each tube was added 80 μl of a PCR master mix containing 100 picomoles of each primer, 10 μl Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l MgCl$_2$, and 0.001% gelatin), 1 μl of 10 mmol/l dNTP, and 2.5 units of Taq DNA polymerase. The reaction mixture (100 μl) was overlaid with 50 μl of mineral oil, and the tubes were placed in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA), programmed as follows: incubation at 94°C for 3 min (initial melt); then 30 cycles of 94°C for 1 min (melt), 60°C for 1 min (anneal), and 72°C for 3 min (extension). Final incubation was performed at 72°C for 7 min. Then, samples were kept at 4°C until analysis.

Analysis of Products
The identity of PCR products was confirmed by Southern hybridization, after size-fractionation by agarose gel electrophoresis. After electrophoresis and ethidium bromide staining, DNA bands were photographed using an ultraviolet transilluminator (UVP, Inc) and Polaroid type 667 positive-negative film (Polaroid Corp). PCR products were
transferred onto Gene Screen Plus nylon membranes (DuPont-New England Nuclear) as described previously (32). Hybridization was conducted at 65°C for 16 h in 1 mol/l NaCl, 1% SDS, 10% dextran sulfate, 100 mg/ml denatured salmon sperm DNA, and 1 × 10^6 cpm/ml labeled probe; the probe was obtained from the 1.1-Kbp restriction fragment of rat angiotensinogen cDNA (33), using a Random Primed DNA Labeling Kit (Boehringer Mannheim GmbH). Filters were washed twice with 2x SSC at room temperature for 5 min each time, twice with 2x SSC and 1% SDS at 60°C for 30 min, and twice with 0.1x SSC at room temperature. The filters were then subjected to autoradiography at room temperature for 6 h with BAS 2000 imaging plates (Fuji Film Corp).

Relative Quantification of mRNA Level from Autoradiographs
The relative amounts of PCR products were determined by densitometric scanning of autoradiographs, using a BAS 2000 laser image analyzer (Fuji Film Tokyo, Japan). To normalize the variability of each assay, data were expressed as a percentage of the densitometric values obtained in PCT in the same experiment, since PCT consistently gave the largest signal. To confirm whether RT-PCR of angiotensinogen mRNA was performed successfully, we used 1 µg of the same total RNA of whole rat kidney (reference RNA) in every RT-PCR assay. Preliminary experiments were performed to obtain appropriate cycle numbers of PCR and amounts of sample RNA for the semiquantitative analysis. We chose 0.1, 0.3, 0.5, 0.7, 1.0, and 2.0 µg of total RNA of rat whole kidney and 25, 50, and 40 cycles of PCR.

Statistical Analysis
The results are given as means ± SEM. When appropriate, the data were analyzed for significance by Student's t-test for unpaired data. A value of p < .05, was considered to indicate statistical significance.

Results
Preliminary experiments showed that at 30 cycles, PCR products, as reflected by densitometry values, increased in a linear fashion with increasing amounts of total RNA between 0.1 µg and 0.7 µg. However, at other cycles attenuation was observed with a plateau effect above 0.5 µg. Figure 1 shows that the densitometry values of PCR products at 30 cycles, when the 0.7 µg sample was assigned as an arbitrary unit of 100, were 11.4 (0.1 µg), 33.5 (0.3 µg), 53.1 (0.5 µg), and 69.6 (0.7 µg). Therefore, we set the number of amplification cycles in this experiment at 0 cycles and the initial amount of total RNA of each segment at 0.3 µg (0.225 µg was used for PCR reaction of angiotensinogen and 0.075 µg for β-actin).

Figure 2 shows representative photographs of ethidium bromide-stained agarose gels and corresponding Southern blots for the PCR products of angiotensinogen and β-actin mRNA in glomeruli and tubules of 6- and 12-wk-old rats. The expected
size of each PCR product was apparent: angiotensinogen (617 to 615 bp) and *β*-actin (504 bp).

As shown in Fig. 2A, on agarose gel staining a single 615-bp band of the expected size amplified from the angiotensinogen primers was found in PCT and PST, and a very weak band was found in glomeruli. Among renal nephron segments, the strongest signal was consistently found in PCT of 6-wk-old rats, and strong bands were found in PCT of 12-wk-old rats and PST of 6-wk-old rats. A weak, but detectable signal was found in PST of 12-wk-old rats.

As shown in Fig. 2B, Southern hybridization using specific probes confirmed the identity of each of these PCR products. The radioactive intensity of the signal of each segment was virtually the same as the results with ethidium bromide staining: the strongest signal was detected in PCT of 6-wk-old rats; strong signals in PCT of 12-wk-old rats and PST of 6-wk-old rats; a weak signal in PST of 12-wk-old rats; and very weak signals in glomeruli of 6- and 12-wk-old rats. When PCR was carried out in the absence of reverse transcriptase, the bands were not seen, indicating that each band was derived from mRNA and not from contaminating genomic DNA. This observation also confirmed that the sense and antisense primers designed for RT-PCR were located on separate exons. Furthermore, the RT-PCR product from the final wash buffer produced no reaction product, which indicated that the samples were not contaminated by other structures during microdissection and RNA isolation.

Figure 2C shows that the amplification product of *β*-actin was detected from all renal structures at the predicted size (504 bp), indicating that RT-PCR was successful in each nephron segment.

Figure 3 graphically summarizes the relative levels of the angiotensinogen amplification products among the nephron segments. The densitometric value from each segment was normalized by dividing the densitometric value from reference RNA (1 μg of total RNA of whole rat kidney). Data points represent results from four independent experiments and are expressed as percentages of the densitometric values obtained in PCT of 6-wk-old rats in the same experiment. The PCT of 6-wk-old rats consistently gave the largest signal, which was relatively invariant from experiment to experiment (PCT of 6-wk-old rats, arbitrary value 100 ± 2.9%). In proximal convoluted tubules of 12-wk-old rats, the signal was 55.8 ± 27.2%. In proximal straight tubules of 6- and 12-wk-old rats, the signals were 59.1 ± 4.8% and 38.4 ± 15.3%, respectively, and in glomeruli, the values were 7.5 ± 3.1% and 2.8 ± 1.7%, respectively. There were significant decreases in the expression of angiotensinogen mRNA in Glm, PCT, and PST between 6- and 12-wk-old animals (p < .05).

**Discussion**

This study demonstrated the localization of angiotensinogen mRNA in the rat kidney by microdissection and RT-PCR. Angiotensinogen mRNA is expressed in PCT, PST, and glomeruli. Furthermore, we found that the expression of angiotensinogen mRNA in these nephron segments and glomeruli of 12-wk-old rats was significantly decreased as compared with those of 6-wk-old rats.

In the kidney, the immunohistochemical localization of angiotensinogen was first reported by Richoux et al. (7). However, they concluded that kidney angiotensinogen was hepatic in origin. The recent application of molecular biological methodologies have allowed investigators to study endogenous systems, circumventing the problem of contamination by circulatory angiotensinogen. By Northern blot hybridization techniques using angiotensinogen cDNA as a probe, many investigators have demonstrated that the angiotensinogen gene is expressed in many tissues, including the brain, spinal cord, aorta, kidney (cortex and medulla), adrenal gland, atria, spleen, and adipose tissue (1-6, 14). Ingelfinger et al. (16, 17) examined the tissue sites of angiotensinogen synthesis in kidneys of male WKY rats by means of in situ hybridization histochemistry. They demonstrated that angiotensinogen mRNA was primarily localized to the proximal renal tubule, and considerably weaker signals were found in the distal tubular segments and glomerular tufts. Niihura et al. also used in situ hybridization techniques and found the expression of angiotensinogen mRNA within the outer stripe of the outer medulla, indentified as the pars recta (35).

Terada et al. (18) demonstrated that angiotensinogen mRNA was expressed primary in PCT and PST, with small amounts in the glomeruli and vasa recta, using microdissection and RT-PCR. In their
study, RT was performed with mRNA obtained from five glomeruli or 2-mm lengths of renal tubule segments or the arcuate artery by destroying cell membranes with the use of a potent surfactant, Triton X-100, in the presence of a large amount of RNAase inhibitor. In contrast, we performed RT with a fixed amount of total RNA, which was extracted by the acid-guanidium-phenol-chloroform method32 from each segment collected, having a total length of 20 to 30 mm and 200 glomeruli. To evaluate the level of angiotensinogen mRNA semi-quantitatively, they compared the RT-PCR products of each nephron segment per fixed length. On the other hand, we compared RT-PCR products per fixed amount of total RNA extracted from each nephron segment. The comparison using the length of tubules is simple and used in many physiological studies, such as those employing micropерfusion and microdissection techniques (36, 37). During the microdissection step, however, the loss of epithelial cells of tubules and the microscopic destruction of the samples were unavoidable even though we treated the samples carefully. Moreover, it was very difficult to measure the length of tubules, because they often described a complicated course. Thus, the numbers of cells would differ between two tubules of the same length obtained by microdissection. This heterogeneity of cell numbers would cause variabilities in the amount of mRNA. Therefore, the comparison of RT-PCR products per fixed length and the number of the nephron segments could not accurately reflect mRNA expression per functioning cell. Comparison of RT-PCR products per fixed amount of total RNA extracted from the tubules therefore provides a better estimate of the expression of mRNA per cell.

Owing to the extremely high sensitivity of PCR and the limitations of microdissection, we cannot exclude the possibility that contamination by other cell types, such as interstitial cells, adhering to the microdissected segments contributed to the amplified products. However, good concordance between our results and those of in situ hybridization analysis by Ingelfinger et al. (18), strongly suggested that the RT-PCR products were derived principally from the angiotensinogen gene expressed in the specific microdissected structures and not from adherent cells.

Generally, mRNA is quantified by methods such as in situ hybridization, Northern blotting, dot or slot blotting, and S-1 nuclease assays, all of which require large amounts of mRNA. The RT-PCR method is a powerful tool to analyze small amounts of mRNA. Techniques for absolute quantification of RT-PCR, such as, competitive PCR, have been proposed (38). In the present study, we performed RT-PCR using total RNA from nephron segments of the kidney to evaluate the level of angiotensinogen mRNA semi-quantitatively. The quantitative detection of mRNA by RT-PCR was described previously by Makino et al. (39). We chose the appropriate number of PCR cycles during which the amount of product increased exponentially without a saturation effect. Furthermore, we chose the initial amount of total RNA for RT-PCR in the range where there was a linear relationship between the initial amount of RNA and that of the RT-PCR product. Thus, we believe that the semi-quantitative technique used in this study allowed us to compare the relative amounts of mRNA.

Previous studies (14-18, 40, 41) have demonstrated the intrarenal expression of renin, angiotensin-converting enzyme (ACE), and angiotensin II type 1 receptor (AT1) mRNAs. Renin gene expression was found in cells of the juxtaglomerular apparatus. Expression of ACE was found in the proximal tubules in immunocytochemical studies. AT1 mRNA was shown to be localized in glomeruli by in situ hybridization, and the other segments did not show definite mRNA signals. However, by RT-PCR, strong signals were detected in the glomeruli, PCT, PST, CCD, and vascular system, and weak signals were detected in glomeruli of 6- and 12-wk-old rats. Similar results were confirmed in 40-wk-old rats (data not shown). Taken together, these findings support the existence of all components of the RAS in PCT, and suggest that these components are involved in renal functions such as regulation of renal vascular resistance, glomerular filtration, and tubular epithelial transport.

In addition, we investigated differences in levels of expression of angiotensinogen mRNA between 6- and 12-wk-old rats. All the detectable signals in 6-wk-old rats were stronger than those of corresponding segments in 12-wk-old rats. The physiological significance of this growth-dependent change in the level of tubular angiotensinogen gene expression is not clear. Although much work has been done in this area by numerous investigators, angiotensinogen mRNA has not been studied as extensively as other components of the intrarenal RAS. Using Northern blotting, Gomez et al. (14) showed that renin and angiotensinogen mRNA levels were higher in neonatal than in adult SHR rats. By using conventional and electron-microscopic immuno- histochemical techniques, Darby et al. (27) demonstrated that immunoreactive angiotensinogen was more abundant in neonatal than in adult Wistar rats. These results suggested that the intrarenal activity of components of the RAS decreased during development and were consistent with our findings. However, the contrasting observation of androgen-dependent up-regulation of angiotensinogen gene expression in WKY rat kidney was reported by Ellison et al. (24). They also showed very low angiotensinogen mRNA levels in male kidney before puberty (35 d old) and increased levels in adulthood (63 and 91 d old). Furthermore, in studies using slot blot analysis, Jung et al. (28) demonstrated that angiotensinogen and ACE mRNA in male Sprague-Dawley rat kidney remained stable, whereas renin mRNA decreased with age. The discrepant results of these studies might have been due to the different strains and ages of rats used, the different portions of the kidney evaluated (i.e., tubules or whole.
kidney), or the methods used to evaluate the level of angiotensinogen mRNA semi-quantitatively. Furthermore, Heymes et al. (42) showed an age-associated increase in the level of expression of angiotensinogen mRNA in the left ventricle and a decrease in the liver of Wistar rats, suggesting that the regulation of angiotensinogen gene expression might differ among tissues.

In the developing kidney, a series of complex changes associated with cell growth and differentiation might occur. Although numerous factors must be involved in the maturation process, few specific mediators have been identified. The RAS is thought to undergo a series of changes during development, playing an important role in regulating blood pressure in the early stages and influencing renal hemodynamics and functions in the later stages (43-47).

In summary, we demonstrated the localization of angiotensinogen mRNA in the rat kidney by microdissection and RT-PCR and showed that the level of expression of angiotensinogen mRNA in nephron segments of 12-wk-old rats was decreased as compared with that in 6-wk-old rats. Further studies are needed to elucidate the physiological and ontogenetic roles of the intrarenal RAS.

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