Differential Regulation of Renal Angiotensin Subtype \( \text{AT}_{1A} \) and \( \text{AT}_2 \) Receptor Protein in Rats With Angiotensin-Dependent Hypertension

Zhi-Qin Wang, Lesley J. Millatt, Nicholas T. Heiderstadt, Helmy M. Siragy, Roger A. Johns, Robert M. Carey

Abstract—This study was designed to investigate distribution and regulation of the renal \( \text{AT}_{1A} \) and \( \text{AT}_2 \) subtype receptors in rats with either systemic angiotensin II (Ang II)–induced hypertension or acute phase renal hypertension (2-kidney, 1-clip [2K1C] or 2-kidney, 1-figure-of-8-wrap [2K1W]). In normal rat kidneys, positive immunostaining for the \( \text{AT}_{1A} \) receptor was observed in the intrarenal vasculature, glomeruli, proximal and distal tubules, and collecting ducts. The \( \text{AT}_2 \) receptor was localized mainly to the glomeruli. The \( \text{AT}_{1A} \) but not \( \text{AT}_2 \) receptor protein expression was significantly reduced in rats with 10-day systemic Ang II–induced hypertension. In both 7-day 2K1C and 3-day 2K1W rats, the \( \text{AT}_{1A} \) receptor was significantly reduced in ischemic and contralateral kidneys compared with sham-operated control rats. Reduction in \( \text{AT}_2 \) receptor expression was observed only in the ischemic kidneys in 2K1C and 2K1W renal hypertensive rats. These results demonstrate that the \( \text{AT}_{1A} \) receptor is widely distributed in the glomerulus and all other nephron segments of the rat kidney. Renal \( \text{AT}_{1A} \) but not \( \text{AT}_2 \) receptor protein is downregulated in rats with Ang II–induced hypertension. In renal hypertensive rats, the \( \text{AT}_{1A} \) receptor is bilaterally downregulated and the \( \text{AT}_2 \) receptor is downregulated only in the ischemic kidney. (Hypertension. 1999;33:96-101.)

Key Words: receptors, angiotensin II ■ immunohistochemistry ■ kidney ■ hypertension, renal

The kidney is an important target organ for angiotensin II (Ang II), which plays a critical role in the regulation of blood flow, glomerular filtration, tubular sodium reabsorption, and renin secretion. The 2 subtypes of the \( \text{AT}_1 \) receptor (\( \text{AT}_{1A} \) and \( \text{AT}_{1B} \)) are encoded by separate genes on different chromosomes. Their cDNAs have 91% identity in their nucleotide sequences within the coding regions but exhibit only 60% homology in the 5'- and 3'-untranslated regions. Both \( \text{AT}_{1A} \) and \( \text{AT}_{1B} \) receptor mRNAs are present in the kidney, with the \( \text{AT}_{1A} \) receptor as the predominant isoform, and their mRNA expression is differentially regulated. However, the intrarenal localization of the \( \text{AT}_1 \) receptor subtype protein remains unknown largely because of the lack of specific ligand for different \( \text{AT}_1 \) receptor isoforms. The \( \text{AT}_1 \) receptor is expressed in the adult kidney at a much lower level and is localized mainly to the glomerular mesangial cells. The majority of studies suggest that the renal actions of Ang II are mediated primarily by the \( \text{AT}_1 \) receptor. However, activation of renal \( \text{AT}_2 \) receptors recently has been reported to regulate pressure-natriuresis, to cause vasodilation in the preglomerular afferent arteriole, and to stimulate renal nitric oxide production. Both 2-kidney, 1-clip (2K1C) (Goldblatt) and 2-kidney, 1-figure-of-8-wrap (2K1W) (Grollman) renal hypertension are renin dependent. Differential regulation of renal \( \text{AT}_1 \) subtype receptor mRNA in renal hypertension has been reported in 2K1C rats. However, regulation of the \( \text{AT}_2 \) receptor expression in renal hypertension is unknown. The present study is designed to investigate the distribution and regulation of renin subtype \( \text{AT}_{1A} \) and \( \text{AT}_2 \) receptor protein in Ang II–dependent hypertension in rats induced by systemic Ang II infusion and rats in the acute phase of renal hypertension (either 7-day 2K1C or 3-day 2K1W hypertension).

Methods

All animal procedures were conducted with the approval of the Animal Research Committee of the University of Virginia School of Medicine.

Characteristics of Polyclonal Antisera

The peptide (\( ^{225} \text{AYEQKKNKPRNDD}^{237} \)), a part of the third cytoplasmic domain of the rat \( \text{AT}_{1A} \) receptor, was cross-linked to thyroglobulin and used to generate a specific anti–\( \text{AT}_{1A} \) receptor polyclonal antiserum in the rabbit. The antiserum recognized a specific protein with an estimated molecular mass of 60 kd, consistent with the predicted molecular weight of the glycosylated form of the \( \text{AT}_{1A} \) receptor. The specificity of the antiserum was further supported by a single band of 60 kd detected from the \( \text{AT}_{1A} \) receptor–rich rat liver but not from the \( \text{AT}_{1B} \) receptor–rich adult (12-week old) rat liver.
Rogers, Ariz) by immunoblot analysis, performed as described in the following section (Figure 1, left); a significant decrease in the enzyme-linked immunosorbent assay response (10 ng of the peptide used as antigen target) after preadsorption challenge of the antiserum with the peptide at 120 μg/mL diluted antiserum, performed as described6 (Figure 1, right). The polyclonal antiserum to the AT1 receptor was raised in the rabbit against a synthetic peptide sequence (MKDNFSFAATSRNITSS) derived from the rat AT1 receptor, and its specificity has been documented.4,16,17

**Animal Preparations**

Female Sprague-Dawley rats (200 to 220 g body weight, Hilltop Laboratory Animals; Scottsdale, Pa) were randomly allocated to the following groups1: 2K1C hypertension and a sham-operated control group; 2K1W hypertension and a sham-operated control group; and Ang II–induced hypertension and a vehicle-infused control group.3 After rats were anesthetized with pentobarbital sodium (50 mg/kg body wt intraperitoneally), the left kidneys were exposed by a midline laparotomy in the first 2 groups. In the first group, the left renal artery was clipped by placement of a solid U-shaped silver clip. The effective clipping of the left renal artery was confirmed by a 70% to 75% reduction in renal cortical blood flow as determined by a laser Doppler probe (superficial probe, type C, Advance Co, Ltd) coupled to a laser Doppler flowmeter (ALF 21D Dual Channel Flowmeter, Advance Co, Ltd). In the second group, the left kidney was tautly wrapped in a figure-of-8 pattern around the kidney to compress the poles and body with 2.0 silk thread (Ethicon Inc). All rats in the sham control groups received identical surgical treatment as described above.

**Figure 1.** Characteristics of polyclonal antiserum against rat AT1A subtype receptor. Left, Representative immunoblot of AT1A receptor protein from liver (lane 1) and pituitary gland (lane 2) of adult (12-week-old) rat (40 μg of total protein per lane). Right, Summary of the enzyme-linked immunosorbent assay response after preadsorption challenge of AT1A receptor antiserum with peptide immunogen. n=3, *P<0.01 vs antiserum.

**Figure 2.** Light photomicrographs of frozen sections of rat kidneys immunostained for AT1A receptor expression. In normal rat kidney, positive immunoreactivity was observed in glomeruli, proximal and distal tubules, and collecting ducts (A, B). Macula densa cells were strongly stained (A, arrow). Positive immunostaining was also detected in intrarenal vasculature (A), including afferent arteriole (C, arrow) and interlobar artery (D). No immunoreactive signal was observed in consecutive sections incubated with preabsorbed antiserum (E). Systemic Ang II infusion apparently decreased the intensity of immunostaining (F). AT1A receptor immunoreactivity also was notably decreased in clipped (G) and unclipped (H) kidneys of 2K1C and in wrapped (I) and unwrapped (J) kidneys of 2K1W rats. Magnification ×250 except C, ×500.
apart from clip placement or renal wrap. In the third group, Ang II (25 ng/min in lactated Ringer’s solution) or vehicle was infused into the jugular vein through mini-osmotic pumps (model 2002, Alza). After 3 days (for 2K1W and sham-operated control rats), 7 days (for 2K1C and sham-operated control rats), or 10 days (for Ang II–infused or vehicle-infused rats), systolic arterial blood pressure was measured by a tail-cuff photosensor (Rat Tail Monometer-Tachometer system, Natsume model KN-210, Peninsula Laboratories). The kidneys were quickly perfused and then harvested for immunohistochemical studies (n = 3/group) and Western blot analysis (n = 7/group) from rats deeply anesthetized with pentobarbital sodium (80 mg/kg body wt intraperitoneally).

Light Microscopic Immunohistochemistry
Indirect immunoperoxidase immunohistochemistry was performed as previously described.4,16 Frozen sections (10 to 12 μm) were incubated with one of the following, diluted 1:500 in 1.5% normal goat serum and 0.5% nonfat dry milk in phosphate-buffered saline: AT1A (7.7 protein mg/mL) or AT2 (3.1 mg protein/mL) receptor primary antiserum; AT1A or AT2 receptor primary antiserum preabsorbed against its respective synthetic peptide antigen. For preabsorption, antiserum was incubated overnight at 4°C with its respective peptide immunogen at 120 μg/mL (for AT1A) or 50 μg/mL (for AT2) diluted antiserum. Immunoreactive signal was detected with an avidin-biotin immunoperoxidase reaction (Vectastain ABC kit, Vector Laboratory) and visualized by exposure to diaminobenzidine for 40 seconds.

Western Blot Analysis
Western blot analysis was performed as previously described.4,16 The nitrocellulose membrane was incubated with the AT1A or AT2 receptor antibody (both 1:1000 dilution in Tris-buffered saline with 5% nonfat dry milk and 0.1% Tween 20) in random order. Blots were then incubated with peroxidase-conjugated donkey anti-rabbit secondary antibody (1:5000 dilution, Amersham, Arlington Heights, Ill.). The immunoreactivity was visualized with an enhanced chemiluminescence Western blotting detection kit (Amersham). Membranes were stripped between incubations with different antibodies in a Tris-buffered solution containing 2% sodium dodecyl sulfate and 100 mmol/L β-mercaptoethanol at 50°C.

Quantitative assessment of band densities was performed by scanning densitometry (ImageQuant, Molecular Dynamics). Statistical analysis was performed with a Macintosh StatView program (Abacus Concepts). All data were expressed as mean ± SEM. Comparisons were made with either the unpaired t test or 1-way ANOVA followed by Fisher protected least significant difference test for multiple comparisons when appropriate. A value of P < 0.05 was considered statistically significant.

Results
The 2K1C and 2K1W animals exhibited significantly increased systolic blood pressure compared with their respective sham-operated controls (166 ± 6 vs 95 ± 4 mm Hg in 2K1C, P < 0.01; 148 ± 2 vs 104 ± 3 mm Hg in 2K1W, P < 0.01). Ang II–infused rats also had elevated blood pressure compared with vehicle-infused controls (159 ± 9 vs 100 ± 7 mm Hg, P < 0.01).

Intrarenal Distribution of AT1A and AT2 Subtype Receptors
In the frozen sections of normal rat kidney, intense immunoreactivity for the AT1A receptor was observed in glomeruli, proximal and distal tubules, and collecting ducts (Figure 2, A and B). The macula densa cells were strongly stained (Figure 2, A). Positive immunostaining also was observed in the intrarenal vasculature (Figure 2, A), including juxtaglomerular cells (Figure 2, C) and interlobular artery (Figure 2, D).
No specific staining was observed in consecutive sections incubated with preabsorbed antiserum (Figure 2, E). Notably, less staining than normal control rats was observed in the kidney of the Ang II–infused rat (Figure 2, F) and both ischemic and contralateral kidneys of the 2K1C (Figure 2, G and H) and 2K1W (Figure 2, I and J) rats, respectively.

The immunoreactive signal for the AT\(_2\) receptor was present mainly in the glomeruli and also lightly in the proximal tubules of the normal rat kidney (Figure 3, A). No specific staining was observed in consecutive sections incubated with preabsorbed antiserum (Figure 3, B). The AT\(_2\) receptor staining was absent in the clipped kidney of the 2K1C rat (Figure 3, C) and apparently decreased in both the glomeruli and tubules in the wrapped kidney of the 2K1W rat (Figure 3, D). However, both the glomeruli and tubular epithelium were positively stained in the contralateral (nonischemic) kidneys of the 2K1C (Figure 3, E) and 2K1W rats (Figure 3, F). No apparent change from the normal rat in the renal AT\(_2\) receptor staining was observed in the Ang II–infused rat (Figure 3, G).

**Western Blot Analysis of Renal AT\(_{1A}\) and AT\(_{2}\) Subtype Receptors**

In both 2K1C and 2K1W renal hypertensive rats, the AT\(_{1A}\) receptor protein was significantly reduced in the ischemic and contralateral kidneys compared with sham-operated control rats (n=7/group, P<0.01) and 2K1W (n=7/group, P<0.05) rats (Figure 5). Renal AT\(_{1A}\) receptor protein expression was significantly decreased in rats with Ang II–induced hypertension compared with vehicle-infused controls (n=7/group, P<0.05, Figure 4). No significant difference in renal AT\(_2\) receptor protein was observed between Ang II–infused and vehicle-infused rats (n=7/group, P>0.05, Figure 5).

**Discussion**

Because all known AT\(_1\) receptor ligands bind with equivalent affinities to AT\(_{1A}\) and AT\(_{1B}\) receptor subtypes, the distribution of the AT\(_1\) subtype receptor in the kidney has been difficult to evaluate with ligand binding and autoradiographic techniques. Moreover, previous efforts to localize the renal AT\(_1\) receptor protein failed to distinguish AT\(_{1A}\) from AT\(_{1B}\) receptor subtypes because the anti-peptide antibodies used were raised against shared amino acid sequences between AT1A and AT\(_{1B}\) receptor subtypes. In this study, AT\(_{1A}\) subtype receptor protein was localized to the glomeruli and all other nephron segments of rat kidney by immunohistochemistry with the use of a specific anti–AT\(_{1A}\) receptor antibody. Our results are in good agreement with the expression pattern of renal AT\(_{1A}\) receptor mRNA in that the AT\(_{1A}\) receptor subtype is the major (>80%) isoform of AT\(_1\) receptor mRNA detected in all nephron segments except the glomerulus, where AT\(_{1A}\) and AT\(_{1B}\) mRNA are expressed in similar proportions. Renal AT\(_{1A}\) receptor expression in the afferent and efferent arterio-
lar smooth muscle and in the glomerular mesangial cells was recently demonstrated indirectly by immunohistochemical localization of β-galactosidase in the heterozygous AT1A receptor of mutant mice, generated by a targeted replacement of the AT1A receptor loci by the lacZ gene. However, whether there is any tubular localization of the AT1A receptor was not reported. Ang II influences single-nephron glomerular filtration rate by modifying glomerular vascular tone, inducing contraction of mesangial cells, and reducing glomerular ultrafiltration coefficient. Ang II regulates sodium and bicarbonate transport in proximal tubules; modulates sodium, chloride, and bicarbonate transport in distal tubules; and modifies H1/ATPase activity and amiloride-sensitive sodium transport in the cortical collecting ducts. Our findings provide structural evidence for the possible role of the AT1A receptor in these Ang II–mediated renal effects.

Renal hypertension produced by unilateral renal artery clipping or renal compression is associated with increased renin release and Ang II production from the ischemic kidney (also unpublished data). The current findings provide direct evidence for the bilateral downregulation of the renal AT1A subtype receptor protein in rats with acute-phase 2K1C and 2K1W renal hypertension. Renal AT1A receptor mRNA expression is decreased after systemic administration of a nonpressor dose of Ang II and in the clipped kidney 2 days after unilateral renal artery clipping. These results are in agreement with the notion that increased Ang II exerts a negative regulatory effect on the renal expression of the AT1A receptor.

This study demonstrated differential regulation of the renal AT2 receptor in renal hypertension. The AT2 receptor was downregulated only in the ischemic kidneys of 2K1C and 2K1W hypertensive rats, whereas renal AT2 receptor expression was not significantly affected in Ang II–induced hypertensive rats. These results suggest that increased Ang II itself may not be directly responsible for this downregulation. Hypoperfusion and/or other local changes secondary to reduced blood supply to the clipped/wrapped kidney are possible causes of suppressed AT2 receptor expression in the ischemic kidneys of renal hypertensive rats. A lack of inducible AT2 receptor expression in cultured rat glomerular mesangial cells from stroke-prone spontaneously hypertensive rats and aortic vascular smooth muscle cells from spontaneously hypertensive rats has been described. The AT2 receptor not only causes vasodilation in the preglomerular afferent arteriole but also exerts an antiproliferative effect and induces apoptosis. Recently, accelerated renal interstitial fibrosis and collagen deposition has been observed in adult AT2 receptor null mutant mice during unilateral ureteral obstruction. Thus, decreased AT2 receptor expression in the ischemic kidney may aggravate renal ischemia and promote cell proliferation/fibrosis, possibly augmenting kidney damage in renal hypertension.
In summary, we localized the AT_{1A} subtype receptor protein to glomeruli, proximal and distal tubules (including macula densa), and collecting ducts, as well as the intrarenal vasculature. Renal AT_{1A} but not AT_{2} receptor protein was downregulated in Ang II–induced hypertension. Expression of AT_{1A} and AT_{2} receptor protein in the kidney was differentially regulated in renal vascular hypertensive rats: The AT_{1A} receptor was bilaterally downregulated, whereas the AT_{2} receptor was downregulated only in the ischemic kidney. Differential regulation of angiotensin receptor subtypes may play a role in the pathophysiology of renal hypertension.

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References


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