Selective Inhibition of the Renal Angiotensin Type 2 Receptor Increases Blood Pressure in Conscious Rats

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Abstract—The angiotensin II type 2 (AT₂) receptor is present in rat kidney; however, its function is not well understood. The purpose of this study was to evaluate the role of the AT₂ receptor in blood pressure (BP) regulation. The effects of selective inhibition of the renal AT₂ receptor with phosphorothioated antisense oligodeoxynucleotide (AS-ODN) were examined in conscious uninephrectomized rats. Oligodeoxynucleotides (AS-ODN or scrambled [S-ODN]) were infused directly into the renal interstitial space by using an osmotic pump at 1 μL/h for 7 days. Texas red–labeled AS-ODN was distributed in renal tubules in the infused but not the contralateral kidney of normal rats. Continuous renal interstitial infusion of the AS-ODN, but not S-ODN, caused a significant (P<0.01) increase in BP 1 to 5 days after the initiation of the infusion. AS-ODN–treated rats experienced an increase in systolic BP from 109±4 to 130±4 mm Hg (n=8, P<0.01), whereas S-ODN–treated (n=8) and vehicle-treated (n=8) rats did not show any significant change in BP. On day 5 of the oligodeoxynucleotide infusion, AS-ODN–treated rats exhibited a greater pressor response to systemic angiotensin II infusion (30 ng/kg per hour) than did S-ODN–treated rats (P<0.01). Renal interstitial fluid cGMP decreased from 11.9±0.8 to 3.6±0.5 pmol/mL (P<0.001), and bradykinin decreased from 0.05±0.05 to 0.18±0.03 ng/mL (P<0.001) in response to AS-ODN, but they were not significantly changed in response to S-ODN. To evaluate the effects of AS-ODN and S-ODN on AT₂ receptor expression, Western Blot analysis was performed on treated kidneys. Kidneys treated with AS-ODN had ≈40% less expression of AT₂ receptor than did kidneys treated with S-ODN or vehicle (P<0.05). These results suggest that AS-ODN directed selectively against the renal AT₂ receptor decreased receptor expression and caused an increase in BP. We conclude that the renal AT₂ receptor plays an important role in the regulation of BP via a bradykinin/cGMP vasodilator signaling cascade. (Hypertension. 2001;37:1285-1291.)

Key Words: blood pressure ■ receptors, angiotensin II ■ hypertension, experimental ■ kidney ■ rats

Angiotensin II (Ang II) is a vasoactive peptide that plays an important role in blood pressure (BP) regulation. Ang II exerts its physiological effects by interacting with 1 of 2 major Ang II receptors, AT₁ or AT₂. The cDNAs that encode the AT₁ and AT₂ receptors have been cloned, and their cell-signaling pathways have been defined. Ang II has equal affinity for AT₁ and AT₂ receptors. The 2 receptors share a sequence homology of ≈34%, and their cDNAs are 91% identical in their nucleotide sequences within their coding regions but only 60% identical in their untranslated regions. Each receptor subtype has distinctive functional properties and cell-signaling mechanisms.

The vast majority of the known functions of Ang II result from its interaction with the AT₁ receptor, which mediates renal afferent and efferent arteriolar vasoconstriction, decreases glomerular filtration rate and renal plasma flow, and stimulates sodium and fluid reabsorption in the proximal tubules as well as cell growth and differentiation. The AT₁ receptor has been localized to the brain, peripheral blood vessels, adrenal gland, heart, and kidney. Relatively little is known about the AT₂ receptor compared with the AT₁ receptor. AT₂ receptor mRNA has been localized to the rat adrenal gland, heart, and brain, and the receptor protein has been identified by immunohistochemistry and Western blots in rat kidney and heart. AT₂ receptor expression is also known to decrease with age; its mRNA is found in fetal and neonatal rat kidney but disappears after the neonatal period and is not detectable in the adult. Immunohistochemical studies, however, have indicated that the receptor is expressed in fully mature rats, albeit at a reduced level.

The function of the AT₂ receptor is not well understood. Recent studies indicate that the AT₂ receptor mediates cell-signaling pathways responsible for the inhibition of cell growth and differentiation. The renal AT₁ receptor may be activated during sodium depletion, suggesting that it may play a role in regulating renal function. The AT₂ receptor has also been reported to control renal afferent arteriolar vasodilation and renal bradykinin (BK) and nitric oxide (NO) production.
The present study uses antisense oligodeoxynucleotides (AS-ODNs) to ascertain the function of the AT_2 receptor in the control of BP and renal function. Because renal tubule epithelial cells, especially proximal tubule cells, can absorb large quantities of oligodeoxynucleotides (ODNs), the kidney is an appropriate target for a site-directed AS-ODN approach. In the present study, AS-ODN targeted toward AT_2 receptor mRNA was delivered directly into the renal interstitial space to inhibit selectively renal AT_2 receptor biosynthesis. We measured renal AT_2 receptor protein expression, BP, and the renal autacoids, BK and cGMP, in response to AS-ODNs, scrambled ODNs (S-ODNs), Ang II, and vehicle.

**Methods**

**ODN Design**
Phosphorothioated antisense ODN, directed against the rat AT_2 receptor mRNA, and its control, S-ODN, were synthesized by using cyanoethylphosphoramidite chemistry. After removal of the protecting groups by hydrolysis with concentrated ammonium hydroxide, the product was purified by reverse-phase high-performance liquid chromatography (RP grade). Both AS-ODN and S-ODN were generated by the Midland Certified Reagent Co. The designated antisense sequence from nucleotides 140 to 158 of the rat AT_2 receptor cDNA (5' AAC TGA AGT TGT CCT TCA 3') did not show any homology with other known mammalian sequences included in the GenBank database (accession No. D16840). A scrambled sequence composed of the same bases but in a different order (5' ATC CTA AGT TGT CAT GCA 3') was used as a control. Both AS-ODN and S-ODN were infused into the rat renal cortex by using osmotic minipumps, which delivered ODN at a rate of 1 μL/h for 7 days (model 2001, Alza).

**Animal Study Design**
The study was performed in 100-g normotensive female Sprague-Dawley rats provided by Harlan Teklad (Madison, Wis.). After the animals recovered from travel for 24 hours, all were fed normal sodium rat chow (0.28% NaCl, Bioserve) and kept in a temperature- and light-regulated facility. All animal procedures were conducted with the approval of the Animal Research Committee of the University of Virginia School of Medicine.

**Group 1 (Distribution of AS-ODN)**
The distribution of AS-ODN was detected by use of a Texas red–labeled ODN. The rats were anesthetized with ketamine and xylazine, and an intravenous line with PE-50 tubing was inserted into the left jugular vein. Lactated Ringer’s solution, and both kidneys were harvested. The tissues were fixed with 2% paraformaldehyde and cryoprotected with 30% sucrose. The frozen sections were examined with a fluorescence microscope and photographed.

**Group 2 (BP Data)**
Rats were placed under general anesthesia with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (5 mg/kg). First, a dialysis catheter was implanted in the cortex of the right kidney. The dialysis catheter was constructed from a dialysis fiber linking 2 sections of PE-10 tubing (Clay Adams). The dialysis fiber and PE-10 tubing were connected by use of Bipax epoxy resin glue (Tra-Con). The dialysis catheter was implanted ~1 to 2 mm from the exterior of the kidney. Lactated Ringer’s solution was pumped continuously through the catheter at a rate of 3 μL/min for 90 minutes. The lactated Ringer’s solution that traveled through the dialysis catheter was then collected for further analysis while the animals were still under anesthesia. After the collection, an indwelling catheter was implanted in the right kidney as described above. Osmotic minipumps were again attached to the tubing and were filled with AS-ODN, S-ODN, or lactated Ringer’s solution at an infusion rate of 1 μL/h. A second surgery was not performed on the second group because baseline readings had already been established. All animals were allowed to recover from surgery for 24 hours.

Seven days later, the rats were once again anesthetized with ketamine and xylazine, and an intravenous line with PE-50 tubing was inserted into the left jugular vein. Lactated Ringer’s solution was pumped at a rate of 10 μL/min into the left jugular vein through the intravenous line. Dialysis catheters were implanted in the left kidney as described above. Lactated Ringer’s solution was concurrently pumped through the renal catheter at a rate of 3 μL/h for 90 minutes. After allowing the animals to reach equilibrium for 15 minutes before flow collections were made, renal interstitial fluid was collected from the dialysis catheters. The intravenous infusion of lactated Ringer’s solution was then replaced by infusion of Ang II (30 pmol/kg per minute), and renal fluid was again collected for 90 minutes. Fifteen minutes after Ang II infusion was complete, all animals were perfused with lactated Ringer’s solution, and the kidneys were frozen in liquid nitrogen and stored at −80°C for Western blot analysis.

**Western Blot Analysis**
The kidneys were dissected, minced, and homogenized with a Polytron (Brinkmann Instruments) in buffer A (10% glycerol, 20 mmol/L Tris-HCl, 100 mmol/L NaCl, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L EDTA, 2 mmol/L EGTA, 10 mmol/L sodium orthovanadate, 10 μg/L leupeptin, and 10 μg/L aprotonin). The homogenate was centrifuged at 30 000g for 30 minutes at 4°C. The pellet was resuspended in buffer B (buffer A with 1% NP-40), stirred for 2 hours at 4°C, and centrifuged again at 30 000g for 30 minutes at 4°C. The supernatant was used in the analysis.

Protein content of the samples was quantified by using the BCA protein assay kit (Pierce). Protein samples (50 μg per rat) were subjected to polyacrylamide gel electrophoresis under denaturing conditions (Ready Gel Cell, 12% Tris-HCl Ready Gel, Bio-Rad). Proteins were transferred onto a nitrocellulose membrane (Trans-
Blot transfer medium, 0.45 μm, Bio-Rad) by electroblotting at 90 V for 1 hour (Ready Gel Cell, Bio-Rad). The nitrocellulose membrane was then soaked in 5% nonfat dry milk in Tween solution (0.05% Tween 20, 10 mmol/L Tris base, and 250 mmol/L NaCl) overnight. Membranes were then washed in Tween solution and incubated with the appropriate receptor antiserum (3.57 mg/mL, 1:1000 dilution in Tween solution with 5% nonfat dry milk). Blots were washed (Tweensolution, 3 times for 10 minutes each) and incubated with peroxidase-conjugated donkey anti-rabbit secondary antibody (1:3000 dilution in Tween solution with 5% nonfat dry milk, Amersham) for 2 hours. Immunoreactivity was visualized with the ECL Western blotting detection kit (Amersham) and Kodak BioMax ML Light-1 film. Quantitative analysis of band densities was performed by scanning densitometry (ImageQuant, Molecular Dynamics).

Western blot analysis was performed by using AT2 receptor and β-actin antibodies. The AT2 receptor antibody was raised against a synthetic peptide sequence (MKDNFSFAATSR-NITSS) derived from the 17 N-terminal amino acids of the predicted AT1 receptor amino acid sequence. This sequence is unique to the AT2 receptor and does not have any specific homology to any other known receptor proteins. The antiserum was IgG affinity-purified before use.20 The selectivity of the antibody was confirmed through immunohistochemical staining and Western blotting analysis of stably transfected and nontransfected COS-7 cells (provided by Dr Tadashi Inagami, Vanderbilt University School of Medicine, Nashville, Tenn). Preadsorption controls were negative. The specificity of this antibody has been demonstrated.9,11,21,22 Western blots were then repeated by using both AT1A receptor antiserum (225 AYEIQKNK-PRNDD237) and β-actin antiserum (monoclonal anti–β-actin clone b–actin clone 1A4, Sigma Chemical Co) in place of AT2 receptor antiserum (both 1:1000 dilution). The AT1 receptor–immunizing peptide was part of the same plasmidic domain of the rat AT1 receptor and was cross-linked to thyroglobulin and used to generate a specific anti–AT1 receptor polyclonal antiserum in the rabbit.21,22 This antiserum recognized a specific protein with an estimated molecular weight of 60 kDa, consistent with the predicted molecular weight of the glycosylated form of the AT1A receptor.23

BP Measurements
Systolic BP was measured in the tail artery in rats under restraint by using an automated sphygmomanometer (model 679, IITC/Life Sciences Instruments). BPs were recorded at 10-minute intervals for 30 minutes each morning during the study period (model 179 Apollo Recorder, Life Sciences Instruments), and values were averaged each day.

Analytical Methods
Renal interstitial fluid BK levels were measured by ELISA assay (Sigma). The sensitivity of this assay is 1 pg/mL. The assay is 100% specific for BK and does not react with any other cyclic nucleotides.

Data Analysis
Statistical analysis for BP measurements was performed by ANOVA, including a repeated-measures term. Multiple comparisons of individual pairs of effect means were conducted by the use of least square means pooled variance. Statistical analysis for renal interstitial fluid BK and cGMP was performed by a Student t test for paired data in which control and S-ODN or AS-ODN were compared in the same animals. All data were expressed as mean±SEM. A value of P<0.05 was considered statistically significant.

Results
Renal Distribution of AS-ODN
A fluorescent signal (Texas red–conjugated AT2 receptor AS-ODN) was detected in the cortex and medulla of the whole kidney 24 hours after renal interstitial injection. Significant uptake of the AS-ODN occurred in both tubular epithelium and intrarenal vasculature, with minimal signal in the glomerulus (Figure 1A). A fluorescent signal was not detected in the contralateral kidney (Figure 1B).

Western Blot Analysis of Renal AT2, AT1, and β-Actin
Western blot analysis demonstrated a single protein band of 44 kDa for the AT2 receptor. Western blot analysis using the AT2 receptor antibody indicated that AT2 receptor expression was reduced in AS-ODN–treated animals compared with S-ODN–treated and vehicle control animals (Figure 2A). Western blots using β-actin as a “housekeeping” protein revealed that the AT2 receptor to β-actin band density ratio was reduced by 41% in AS-ODN–treated animals compared with S-ODN–treated control animals (Figure 2B). Band densities of vehicle-infused kidneys deviated by <8% from one another. Western blot analysis using the AT1 receptor antibody indicated a deviation among AS-ODN, S-ODN, and vehicle kidney band densities of <2% from each other (data not shown).
Effect of AS-ODN on BP

BP was increased for AS-ODN–treated rats compared with S-ODN–treated rats and vehicle control rats. AS-ODN–treated animals exhibited consistently higher BPs than did either S-ODN–treated or vehicle control animals during each of the infusion days. Continuous renal interstitial infusion of the AS-ODN, but not S-ODN, caused a significant ($P<0.01$) increase in BP 1 to 5 days after the initiation of the infusion. AS-ODN–treated rats experienced an increase in systolic BP from $109\pm 6$ to $130\pm 4$ mm Hg ($n=8$, $P<0.01$), whereas S-ODN–treated ($n=8$) and vehicle-treated ($n=8$) rats did not show a significant change in BP (Figure 3A). On day 5 of the ODN infusion, AS-ODN–treated rats exhibited a pressor response to Ang II of $46\pm 18$ mm Hg compared with a response of $20\pm 11$ mm Hg ($P<0.01$) in S-ODN–treated animals and $36\pm 9$ mm Hg ($P<0.05$) in vehicle control animals (Figure 3B).

Effect of AS-ODN on Renal Interstitial BK and cGMP

Renal interstitial fluid analysis indicated significant reductions in both BK (Figure 4A) and cGMP (Figure 4B) levels in AS-ODN–treated animals compared with S-ODN–treated control animals. BK levels decreased in AS-ODN–treated animals from $0.5\pm 0.05$ to $0.18\pm 0.03$ ng/mL, a decrease of $\approx 75\%$, whereas BK levels in S-ODN–treated control animals did not significantly change. cGMP levels decreased in AS-ODN–treated animals from $11.9\pm 0.8$ to $3.6\pm 0.5$ pmol/mL, a decrease of $\approx 70\%$. S-ODN–treated control animals showed a nonsignificant decrease in renal interstitial fluid cGMP.

Discussion

The present study demonstrates for the first time that selective inhibition of the AT$_2$ receptor within the kidney increases BP and engenders pressor hypersensitivity to Ang II. In addition, the present study shows that the AT$_2$ receptor physiologically mediates a vasodilator signaling pathway, including BK and cGMP. Our results suggest that the renal AT$_2$ receptor plays a physiological role in the control of BP.

The present study demonstrated an increase in BP within the first day of AS-ODN infusion. Studies involving the time course of AT$_2$ receptor turnover are unavailable. We deduce that the half-life of the renal AT$_2$ receptor is short (probably in the neighborhood of 2 to 3 days) to allow for a 40% reduction of AT$_2$ receptor density within 5 days. Similar results were obtained with the use of AS-ODN for the $\beta$-adrenergic receptor, in which a major reduction of BP was observed after 1 day of AS-ODN infusion. Previous studies from our laboratory have suggested that Ang II increases renal BK, NO, and cGMP. These vasodilator autacoids were increased in response to exogenous Ang II in animals during normal sodium intake and were inhibited with the AT$_2$ receptor antagonist PD 123319 in animals whose autacoids had been stimulated by endogenous Ang II during dietary sodium restriction. In the present study, we demonstrate $\approx 70\%$ inhibition of both BK and cGMP in sodium-replete animals for the first time in response to AT$_2$ receptor inhibition.

There are some contradictory reports of AT$_2$ receptor action on vascular tone and BP. AT$_2$ receptors may contribute to the vasoconstricting effect of Ang II in mesenteric arteries in young but not adult spontaneously hypertensive rats. The effects of Ang II infusion on renal vascular reactivity are reported to be mediated by AT$_1$, and not by AT$_2$ receptors. Immunization against AT$_1$ but not AT$_2$ receptors decreased BP in young spontaneously hypertensive rats. However,
many other reports support the vasodilator actions of the AT$_2$ receptor.\textsuperscript{27,28,32–41}

Antisense oligonucleotide technology is a highly specific method of demonstrating the physiological action of a protein if an appropriate reduction in expression is observed.\textsuperscript{42–49} In the present study, we were able to achieve an $\approx$40\% reduction in AT$_2$ receptor protein in response to AS-ODN and no change in response to S-ODN. These results are similar to those using AS-ODN technology in other systems.\textsuperscript{42–49}

The function of the AT$_2$ receptor has been examined in mice lacking the AT$_2$ receptor (AT$_2$-null).\textsuperscript{27} A consistent finding has been normal baseline BP but pressor hypersensitivity to Ang II in AT$_2$-null.\textsuperscript{27} Because AT$_2$-null mice have had an absence of the AT$_2$ receptor for life, it is possible that their pressor hypersensitivity to Ang II could have been related to cross talk between AT$_1$ and AT$_2$ receptors, resulting in upregulation of the AT$_1$ receptor.\textsuperscript{50} In the present study, it is highly unlikely that upregulation of the AT$_1$ receptor accounts for the observed changes in BP because BP was elevated in the animals receiving AS-ODN on the first day of its administration. Furthermore, Western blot analysis showed no change in AT$_1A$ receptor protein in response to AS-ODN administration. The present study demonstrates that both acute and chronic reduction of AT$_2$ receptor expression increases BP during the sodium-replete state.

We used direct renal interstitial administration of oligonucleotides in uninephrectomized animals to study the paracrine role of Ang II at the AT$_2$ receptor. We demonstrated in animals with both kidneys intact that the oligonucleotide is distributed in renal tubule cells and does not leak into the opposite uninfused kidney via the circulation. Thus, it is highly likely that the oligonucleotide was confined to the kidney during these experiments. Renal epithelial cells have been shown to respond to phosphorothioated ODN.\textsuperscript{19,42,43} Earlier studies have used antisense oligonucleotide technology to study the inducible isoform of NO synthase, the AT$_1$ receptor, intracellular adhesion molecule-1, and transforming growth factor-$\beta$ in the kidney.\textsuperscript{45–48} We previously were able to determine the effects of dopamine D$_{1A}$ receptor inhibition on renal sodium excretion in the rat by using direct renal interstitial administration of AS-ODN.\textsuperscript{19}

The AT$_2$ receptor is highly expressed in most fetal tissues, but expression regresses early during the postnatal period.\textsuperscript{7} In the newborn kidney, the receptor protein is expressed in cortical glomeruli, tubule elements, and undifferentiated mesenchymal cells.\textsuperscript{9} In the adult, AT$_2$ receptor protein is expressed in low amounts in glomeruli, tubules, vasculature, and interstitial cells.\textsuperscript{9} In the present study, it is uncertain precisely which cells were responsible for the reduction in AT$_2$ receptor protein in response to AS-ODN, although the oligonucleotide was distributed largely in the tubules.
The renin-angiotensin system not only serves as a systemic hormonal system but also may function as a paracrine system within selected tissues.\(^1\)\(^2\) In the kidney, evidence has accumulated indicating that Ang II is synthesized and acts locally in the control of renal function.\(^1\)\(^2\) An important unanswered question has been whether the renal renin-angiotensin system acts to control BP in the absence of involvement of the systemic system. Indeed, Davisson et al.\(^5\) have recently shown that selective transgenic overexpression of angiotensinogen within the mouse kidney leads to severe hypertension. In the present study, we were able to demonstrate that selective intrarenal blockade of the AT2 receptor in a highly specific manner increases BP and conveys pressor hypersensitivity to Ang II. Therefore, we conclude that the physiological action of Ang II at the AT2 receptor within the kidney provides sufficient vasodilation to prevent hypertension in the normal animal. The precise mechanisms whereby selective reduction in renal AT2 receptor expression leads to systemic hypertension will be the subject of future investigation.

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**References**

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