Intrarenal Angiotensin III Infusion Induces Natriuresis and Angiotensin Type 2 Receptor Translocation in Wistar-Kyoto but not in Spontaneously Hypertensive Rats


Abstract—In Sprague-Dawley rats, renal angiotensin (Ang) type 2 receptors (AT2Rs) mediate natriuresis in response to renal interstitial (RI) D1-like receptor stimulation or RI Ang III infusion. After D1-like receptor activation, apical membrane (AM) but not total renal proximal tubule cell AT2R expression is increased, suggesting that AM AT2R translocation may be important for natriuresis. The onset of hypertension in spontaneously hypertensive rats (SHRs) is preceded by defects in renal sodium excretion. The present study examines AT2R-mediated natriuresis in response to RI Ang III infusion in Wistar-Kyoto rats (WKYs) and SHRs. WKYs and SHRs received RI Ang III infusion after 24 hours of systemic AT1R blockade with candesartan. In WKYs, urine sodium excretion rate increased from 0.043±0.01 to 0.191±0.06 μmol/min (P<0.05) in response to Ang III infusion, but identical conditions failed to increase the urine sodium excretion rate in SHRs. The increase in the urine sodium excretion rate was blocked by coinfusion of PD-123319, a selective AT2R antagonist. On confocal microscopy images, Ang III–infused WKYs demonstrated greater renal proximal tubule cell AM AT2R fluorescence intensity compared with SHRs (5385±725 versus 919±35; P<0.0001), and Western blot analysis demonstrated increased AM (0.050±0.003 versus 0.038±0.003; P<0.01) but not total cell AT2R expression in WKYs. In SHRs, AM AT2R expression remained unchanged in response to RI Ang III infusion. Thus, RI Ang III infusion elicits natriuresis and renal proximal tubule cell AT2R translocation in WKYs. Identical manipulations fail to induce natriuresis or AT2R translocation in SHRs, suggesting that defects in AT2R-mediated natriuresis and trafficking may be important to the development of hypertension in SHRs.

Key Words: sodium natriuresis angiotensin III AT2 receptor AT1 receptor translocation

The renin-angiotensin system is a coordinated hormonal cascade of crucial importance in cardiovascular and renal function. In recent years, an emphasis has been placed on delineating the role of the intrarenal renin-angiotensin system in the regulation of blood pressure (BP) and sodium (Na+) balance.3–5 The majority of the effects of the intrarenal renin-angiotensin system are mediated by 2 angiotensin receptors, angiotensin (Ang) type I (AT1R) and Ang II type II (AT2R). Renal AT1Rs, as a consequence of their antinatriuretic actions, are required for the development of Ang II–dependent hypertension, because the presence of systemic extrarenal AT1Rs alone is not sufficient to sustain hypertensive responses to Ang II infusion.1 Furthermore, AT1Rs in renal proximal tubule cells (RPTCs), as opposed to other sites along the nephron, are primarily responsible for this response. In Sprague-Dawley rats, renal AT1Rs have been reported to mediate natriuresis in response to renal interstitial (RI) AT1R blockade or Ang III infusion.3 Inhibition of the conversion of Ang II to Ang III in the kidney abolishes natriuresis mediated by renal AT1Rs, indicating that Ang III is the preferred agonist of this response.4 Thus, RPTC AT1Rs and AT2Rs are major determinants of BP and Na+ responses in normal and hypertensive animals.

The intrarenal dopaminergic system also plays an important role in the regulation of Na+ balance. Dopamine, synthesized by the RPTCs, mediates diuresis and natriuresis via D1-like receptor (D1R) activation.7,8 A physiological interaction between the intrarenal renin-angiotensin system and dopaminergic systems has been reported in normal Sprague-Dawley rats. In response to a high-salt diet, RI D1R activation with fenoldapam results in natriuresis and diuresis that is abolished by selective pharmacological inhibition of renal AT1,Rs with PD-123319 (PD).9 Furthermore, fenoldapam-induced natriuresis is accompanied by an increase in apical plasma membrane (AM) but not total RPTC AT1R expression, as quantified by Western blot analysis.9 Thus, D1R-mediated natriuresis depends on functional renal AT1Rs, and one of the mechanisms involves RPTC AT1R translocation. Spontaneously hypertensive rats (SHRs) develop hypertension as they age and are widely used as a model to study the development and maintenance of human primary (essential) hypertension.10 Before the onset of hypertension, SHRs...
demonstrate inappropriately increased RPTC Na\(^+\) reabsorption, which is not accompanied by an increase in glomerular filtration rate or renal blood flow.\(^{11-17}\) These observations suggest a primary defect in the renal tubule function rather than in renal hemodynamics. Over time, however, increased renal perfusion pressure is required by the kidneys to continue to excrete Na\(^+\), and this adaptation is fundamental to the development and maintenance of hypertension.\(^{18,19}\)

Previous studies examining the Na\(^+\) excretory defects in SHRs that ultimately lead to hypertension have focused on alterations in renal dopaminergic or AT\(_1\)R-mediated effects. However, as mentioned previously, natriuresis because of renal D\(_1\)R stimulation and AT\(_1\)R blockade is mediated, at least in part, by renal AT\(_2\)Rs. Thus, the present study was undertaken to determine whether another strain of normal rat, Wistar-Kyoto rats (WKYs), demonstrates AT\(_2\)R-mediated natriuresis in response to RI Ang III infusion, and, if so, whether such natriuresis is demonstrable in SHRs. Because one of the mechanisms of AT\(_2\)R-mediated natriuresis involves AT\(_2\)R translocation from the cytoplasm to the AM of RPTCs, AT\(_2\)R localization and expression after pharmacological stimulation of natriuresis were determined via confocal microscopy and Western blot analysis in both WKYs and SHRs.

Methods

Animal Preparation

The experiments, which were approved by the University of Virginia Animal Care and Use Committee, were conducted in 12-week-old female WKYs and SHRs (Harlan Teklad), in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Rats (N=18) were placed under general anesthesia with pentobarbital (50 mg/mL) given 5 mg/100 g of body weight IP. A tracheostomy was performed, and arterial access was achieved by direct cannulation of the right carotid artery. Renal cortical interstitial infusion catheters were placed, as reported previously.\(^3,4\) When 2 substances were simultaneously infused into the kidney, separate interstitial catheters were used. Intravenous access was obtained via cannulation of the right internal jugular vein. Rats were housed under controlled conditions (temperature: ±1°C; humidity: 60%; light: 8 to 20 hours). Experiments were initiated at the same time each day to prevent any diurnal variation in BP. Mean arterial pressure (MAP) was measured by the direct intracarotid method with the use of a BP analyzer (Micromed Inc). MAP was recorded every 5 minutes and averaged for each of the control and experimental periods.

Pharmacological Agents

Ang III (des-Asp\(^1\))-angiotensin II (Bachem), an AT\(_1\)R and AT\(_2\)R agonist, was used for these studies (Ki 10.5\(^{9}\)mol/L and 2.9\(^{9}\)mol/L for AT\(_1\)Rs and AT\(_2\)Rs, respectively\(^{20}\)). Candesartan (CAND), a specific, potent insurmountable inhibitor of AT\(_1\)R (IC\(_{50}\) >1×10\(^{-5}\)mol/L and 2.9×10\(^{-5}\)mol/L for AT\(_1\)Rs and AT\(_2\)Rs, respectively), was used for systemic AT\(_1\)R blockade, PD (Parke-Davis), a specific AT\(_2\)R antagonist (IC\(_{50}\) 2×10\(^{-8}\)mol/L and >1×10\(^{-4}\)mol/L for AT\(_1\)Rs and AT\(_2\)Rs, respectively), was used interstitially to block the AT\(_2\)R.

Effects on Urine Na\(^+\) Excretion Rate of Unilateral RI Ang III in the Presence of Systemic AT\(_1\)R Blockade With and Without RI AT\(_2\)R Blockade

WKYs and SHRs (n=6 in each group) were studied on normal Na\(^+\) intake with both kidneys intact. There were a total of 2 groups for WKYs and 1 group for SHRs. In all of the rats, the right kidney served as the control kidney and was infused with 5% dextrose in water (D\(_2\)W) directly into the RI space during both control (30 minutes) and experimental collection periods (30 minutes each). In the first group, osmotic minipumps were implanted into the inter-scapular region with the animals under short-term anesthesia with ketamine (100 mg/mL) and xylazine (20 mg/mL) for systemic CAND (0.01 mg/kg per min) infusion 24 hours before and during the experiment. Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg per min) was then infused cumulatively (each concentration for 30 minutes) into the RI space of the left (experimental) kidney after a 30-minute control infusion of D\(_2\)W (2.5 µL/min). In the second group, systemic CAND infusion was achieved in the same manner as in the first group, but Ang III and PD (10 µg/kg per minute) were coinfused into the RI space of the left kidney. In all of the rats, both ureters were cannulated individually to collect urine for quantification of the urine Na\(^+\) excretion rate (U\(_{\text{Na}}\)V) for the control and 4 experimental periods from each kidney.

In Vivo Kidney Perfusion and Fixation Procedure

WKY and SHR kidneys were subject to the following fixation protocol after receiving either vehicle (D\(_2\)W) or RI Ang III infusions. With RI infusions continuing, the rat heart left ventricular cavity was cannulated, and the animal was perfused with 40 mL of 4% sucrose in Dulbecco’s PBS with calcium and magnesium chloride (DBPS\(^{++}\)) followed by perfusion with 40 mL of 4% paraformaldehyde in DBPS\(^{++}\). Sections of cortex were removed and placed in 4% paraformaldehyde for 2 hours at room temperature. Cortical sections were rinsed and immersed in 100 mmol/L of Tris-HCl for 30 minutes before storage in 30% sucrose in DBPS\(^{++}\) overnight at 4°C. Cortical specimens were embedded in Tissue Tek OCT Compound freezing medium, frozen on liquid nitrogen, and stored at −80°C. Cryostat thin sections (8 µm) were placed on Probe On Plus positively charged microscope slides (Fisher Scientific) and a PAP pen was used to draw a hydrophobic ring around the sections for immediate staining.

Immunofluorescence Microscopy

After specimens had been spotted onto slides and washed with trisphosphate- buffered saline (TBS), they were permeabilized with 0.2% Triton X-100 in TBS for 5 minutes. The sections were washed several times with TBS with 0.02% Tween 20 and then blocked in 1% milk in TBS with 0.02% Tween 20 for 1 hour. The kidney sections were incubated with anti-AT\(_2\)R primary antibody at 1:100 dilution in 1% milk overnight at 4°C. ALEXA 647–conjugated donkey antirabbit secondary antibody diluted at 1:500 was then added for 90 minutes at room temperature. To identify RPTCs, the preparation was stained further with Texas-Red phalloidin (1:200), which labels actin-containing structures, including RPTC AMs. Hoechst (10 mg/mL stock) was diluted at 1:2500 and added to identify nuclei. After several TBS with 0.02% Tween 20 washes, fluoromount was applied before covering with a glass coverslip.

Confocal Microscopy and Quantification of Immunofluorescence Signals

Confocal microscopy recordings were performed using a Leica TCS SP inverted confocal scanning laser microscope with excitation at 490 and 647 and detection at 510 and 660 nm. Images were captured using identical capture parameters for each section on an Olympus IX81 Spinning Disk Confocal Microscope using a 60×1.2 NA UIS2 water immersion objective and a Hammamatsu 9100 to 02 EMCCD camera with Slidebook 4.2 software. Images were exported as 16-bit tiffs and analyzed using MacBiophotonics ImageJ version 1.38m and the Sync Measure 3D plug-in written by Joachim Walter. To generate line-intensity plots, the AT2R-stained slide and the corresponding phalloidin-stained slide were opened, and the Sync Measure 3D plug-in was used to draw a line from the tip of the phalloidin stain at the brush border of 1 cell to the phalloidin-stained basolateral membrane at the base of the same cell, and the AT\(_2\)R immunofluorescence intensity plot along this trajectory was measured.
Membrane Preparations and Western Blot Analysis

After the in vivo RI D5W or Ang III infusions, RPTC total membranes and AMs were isolated, as published previously.9 Briefly, RPTCs were obtained after whole body perfusion with 40 mL of 4°C DPBS without Ca ++ or Mg ++ (DPBS+), to halt endosomal movement and slow cellular respiration. Immediately after the perfusion, the kidney cortex was removed and minced into 1-mm³ pieces. The specimens were rinsed in 20 mL of 4°C DPBS+ containing 0.5 mmol of EGTA at 1200 rpm to open the tight junctions between the RPTCs and then rinsed again in 20 mL of 4°C DPBS+ at 1200 rpm to remove the EGTA. The samples were digested in a collagenase solution (2 mg/mL in DPBS+; Boehringer Mannheim Biochemicals) at room temperature for 3.5 hours in a spinner flask that provided gentle agitation. After the digestion, 15 mL of DPBS+ were added to the collagenase mixture, which was then transferred through 2 successive sieves, 212 (USA Standard Sieve Series). The supernatant was collected and spun at 1200 rpm for 10 minutes to isolate RPTCs. Total cell membranes of the RPTCs were subsequently isolated by the method of Nagamatsu et al.10 AMs of RPTCs were isolated by first lysing the cells in detergent-free lysis buffer and performing a biotinylated lectin pull-down assay. After the tissue was homogenized, 1 mg of total protein was incubated with 20 μg of biotinylated Lotus tetragonolobus agglutinin lectin (Vector Laboratories) in 10-mL volume for 2 hours at room temperature. A 50% vol/vol slurry (40 μL) of Ultralink Neutravidin beads (Pierce Laboratory) was then added and incubated for 30 minutes. The beads were then pelleted and thoroughly washed using a microcentrifuge spin cup filter. The Lotus tetragonolobus agglutinin affinity–attached membranes were eluted by incubating the beads in the spin cup filter with 40 μL of 50°C sample buffer and 20 μL loaded per lane on a gel for Western blotting. After preparation, total RPTC membranes and AMs were incubated with rabbit AT2R polyclonal antibody (1:100 dilution; H-143 Santa Cruz) and villin monoclonal antibody (1:2500 dilution; Immunotech), which is enriched in RPTCs.9 A review of the literature suggests that the H-143 AT2R antibody from Santa Cruz is specific to the AT2R and does not cross-react with the AT1R (see product citations for AT1R (H-143) catalog No. sc-9040, Santa Cruz Biotechnology, Inc.). In addition, a database search, eg, Protein Blast, indicates that the epitope recognized by H-143 is unique to the AT2R. Membranes were subsequently incubated with infrared secondary antibodies (antirabbit IRDye 680 nm and antimouse IRDye 800 nm, each at 1:15 000; Licor Biosciences). Immunoreactivity and quantitative assessment of band densities were performed using the Odyssey Infrared Imaging System (Licor Biosciences). Results were reported as a ratio of AT2R:villin expression.

Statistical Analysis

Comparisons among vehicle, Ang III, and Ang III plus PD were estimated by ANOVA, including a repeated-measures term, by using the general linear models procedure of the Statistical Analysis System. Multiple comparisons of individual pairs of effect means were conducted by the use of least-square means pooled variance. Data are expressed as means±1SE. Statistical significance was identified at a level of P<0.05.

Results

Effects of RI Ang III Infusion and Ang III Plus PD Infusion on UNaV and MAP in Uninephrectomized WKYs and SHRs

As demonstrated in Figure 1, in WKYs, RI Ang III infusion (in the presence of systemic AT1R blockade) increased UNaV from a baseline of 0.043±0.006 to 0.11±0.025 μmol/min (P<0.05) during 3.5 nmol/mg per kilogram of Ang III infusion, to 0.23±0.069 μmol/min (P<0.05) during 7.0 nmol/mg per kilogram of Ang III infusion, to 0.18±0.041 μmol/min (P<0.01) during 14 nmol/mg per kilogram of Ang III infusion, and to 0.19±0.060 μmol/min (P<0.05) during 28 nmol/mg per kilogram of Ang III infusion. PD coinfusion abolished the natriuretic responses to RI Ang III (P<0.05 or <0.01 from Ang III infusion). In SHRs, however, identical infusions of Ang III failed to increase UNaV (baseline: 0.033±0.016 to 0.031±0.009 μmol/min after 2 hours of Ang III infusion; P value not significant). Infusion of vehicle (D5W) resulted in no change in UNaV in WKYs or SHRs during control or experimental periods. As illustrated in Figure 2, RI Ang III infusion did not significantly alter MAP from the baseline in WKYs or SHRs. Similarly, coinfusion of PD with Ang III did not influence MAP in WKYs.

Confocal Microscopy Analysis of RPTC AT2R Localization in Response to In Vivo RI Ang III Infusion in the Presence of Systemic AT1R Blockade in WKYs and SHRs

Figure 3 demonstrates high-power (×600) confocal micrographs of WKY (Figure 3A) and SHR (Figure 3B) renal cortex labeled with antibodies to phalloidin (red), to AT2R (green), and to Hoechst nuclear stain (blue) after vehicle,
Ang III infusion did not result in increased apical membrane AT₂R localization in SHRs. Figure 4 quantifies AT₂R fluorescence intensity as a function of its distance from the apical tip of RPTCs. Compared with SHRs, Ang III–infused kidneys from WKYs demonstrated greater RPTC AM fluorescence intensity (5385±725 versus 919±35; P<0.0001, quantified as the average of AT₂R fluorescence intensity 0 to 2 μm from the apical tip) and reduced cytosolic AT₂R fluorescence intensity (1878±311 versus 1044±24; P<0.001, average of AT₂R fluorescence intensity 2 to 6 μm from the apical tip).

**RPTC Total Cell and AM AT₂R Expression in Response to RI Vehicle or Ang III Infusion in WKYs and SHRs**

As demonstrated by Western blot analysis in Figure 5A, RPTC total cell and AM AT₂R protein expression were similar between WKYs and SHRs after 2.5 hours of RI vehicle infusion. However, compared with RI D₅W infusion, RI Ang III infusion in WKYs resulted in a 30% increase in AM (P<0.01) but not total RPTC membrane AT₂R protein expression (Figure 5B). In SHRs, AM AT₂R protein expression failed to increase from baseline in response to RI Ang III infusion (Figure 5C).

**Discussion**

The present studies demonstrate that, in normal WKY rats, RI Ang III infusion, in the presence of systemic AT₁R blockade, results in AT₂R-mediated natriuresis that is accompanied by increased AM but not total RPTC membrane AT₂R expression. Identical Ang III infusions fail to stimulate natriuresis or alter AT₂R localization or expression in SHRs, suggesting that defects in AT₂R function and/or trafficking may be important in the excess Na⁺ retention and ultimate development of hypertension in these animals.

Previous studies have shown that intrarenal Ang III infusion, in the presence of systemic AT₁R blockade, results in AT₂R-mediated natriuresis in Sprague-Dawley rats on normal Na⁺ intake. These results are not specific for the Sprague-Dawley strain, however, because the present study demonstrates similar natriuresis to identical RI Ang III infusions in WKYs. Indeed, in Sprague-Dawley rats, natriuresis to RI Ang III can be augmented by inhibiting the activity of the enzyme.
responsible for metabolizing Ang III (aminopeptidase N) and can be abolished by inhibiting the enzyme responsible for the formation of Ang III (aminopeptidase A). Thus, RI Ang III, and not Ang II, was used to elicit natriuretic responses in WKYS in this study. It is probable, and other authors have also proposed, that Ang III, and not Ang II, is the preferred agonist of AT2Rs. Because the natriuresis in response to Ang III infusion in WKYS was abolished to control levels with PD, the observed natriuresis was likely mediated by renal AT2Rs.

The mechanisms by which renal AT2Rs mediate natriuresis have not been established. However, it is well known that the regulation of cell surface expression of several membrane proteins by different hormones is crucial in the regulation of various aspects of whole body homeostasis. In the kidney, the regulation of the abundance of the water transporter aquaporin-2 at the AM of collecting duct cells by vasopressin determines water excretion and, thus, plays a crucial role in the control of water homeostasis. Likewise, it has been established that the natriuretic D1R translocates to the cell surface in response to D1R activation in cultured kidney cells, kidney section preparations, and isolated proximal tubules. This event requires signaling through cAMP and depends on intact microtubules and vesicular fusion proteins. The physiological significance of RPTC D1R trafficking is not known, but because natriuretic responses to D1R activation are mediated, at least in part, by AT2R activation, the present study investigated the trafficking patterns of renal AT2Rs under natriuretic stimulation.

At baseline, both WKYS and SHRs have been reported to demonstrate similar AT2R mRNA expression levels in the whole kidney and mesenteric vessels. However, to our knowledge, this is the first report that documents similar RPTC AT2R protein expression levels after 24 hours of systemic AT1R blockade between WKYS and SHRs, suggesting that defects in Na+ excretion in response to Ang III in SHRs are not accounted for by decreased expression of natriuretic AT2Rs before infusion. The significance of characterizing RPTC AT2R expression levels is underscored by recent reports suggesting that BP regulation at baseline, and in response to Ang II infusion, is primarily determined by natriuretic responses in the renal proximal and not distal tubules.

In the present study, RPTC AM but not total cell AT2R expression was increased in response to Ang III–induced natriuresis in WKYS. The lack of increase in total RPTC AT2R expression suggests that the trafficking of RPTC AT2Rs may be regulated under conditions that promote

Figure 4. AT2R expression (relative fluorescence units) in thin sections of WKY (black) or SHR (gray) kidneys infused with RI Ang III (in the presence of CAND), as a function of the distance from the tip of the AM of RPTCs. Each data point represents the mean±SE of 9 independent measurements.

Figure 5. Western blot analysis of RPTC total cell and AM AT2R protein expression in response to RI vehicle (A) or Ang III infusion in WKYS (B) and SHRs (C); n=6 per group. A, Total RPTC and AM AT2R expression in vehicle-infused WKYS (□) and SHRs (■). B, Total RPTC and AM AT2R expression in vehicle-infused (□) or Ang III–infused (□) WKYS. C, Total RPTC and AM AT2R expression in vehicle-infused (■) or Ang III–infused (□) SHRs. Data represent means±SE; **P<0.01 vs vehicle-infused WKYS.
natriuresis. Whether AT$_2$R translocation is required for natriuresis is presently unknown, but the absence of natriuresis or increased AM AT$_2$R expression in SHRs strongly suggests that the intracellular trafficking of RPTC AT$_2$Rs may help to determine natriuretic responses. In fact, previous studies have also documented increased AM but not total RPTC AT$_2$R protein expression in response to D$_1$R-induced natriuresis.\(^9\) Given similar baseline RPTC AT$_2$R protein expression levels, the failure of SHRs to increase AM AT$_2$R expression in response to identical natriuretic stimuli indicates that functional abnormalities in RPTC AT$_2$Rs may contribute to the excess Na$^+$ retention that occurs in these animals. Additional studies will investigate the mechanisms of renal AT$_2$R trafficking in normal rodents and determine whether defects in AT$_2$R signaling or trafficking machinery predispose SHRs to defective Na$^+$ excretion.

**Perspectives**

Renal AT$_2$Rs mediate Na$^+$ excretion in normal animals in response to RI Ang III infusion. The natriuresis is accompanied by increased RPTC AM AT$_2$R distribution in WKYs but not in SHRs. In light of the major critical importance of excess Na$^+$ retention in the development of hypertension in SHRs, the present studies provide a potential mechanistic explanation for the pathogenesis of hypertension in SHRs.

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**Disclosures**

None.

**References**


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