AT<sub>2</sub> Receptor Activation Induces Natriuresis and Lowers Blood Pressure


**Rationale:** Compound 21 (C-21) is a highly selective nonpeptide AT<sub>1</sub> receptor (AT<sub>1</sub>R) agonist.

**Objective:** To test the hypothesis that renal proximal tubule AT<sub>2</sub>Rs induce natriuresis and lower blood pressure in Sprague-Dawley rats and mice.

**Methods and Results:** In rats, AT<sub>1</sub>R activation with intravenous C-21 increased urinary sodium excretion by 10-fold (P<0.0001); this natriuresis was abolished by direct renal interstitial infusion of specific AT<sub>1</sub>R antagonist PD-123319. C-21 increased fractional excretion of Na<sup>+</sup> (P<0.05) and lithium (P<0.01) without altering renal hemodynamic function. AT<sub>2</sub>R activation increased renal proximal tubule cell apical membrane AT<sub>2</sub>R protein (P<0.001) without changing total AT<sub>2</sub>R expression and internalized/inactivated Na<sup>+</sup>/H<sup>+</sup> exchanger-3 and Na<sup>+</sup>/K<sup>+</sup>ATPase. C-21-induced natriuresis was accompanied by an increase in renal interstitial cGMP (P<0.01); C-21-induced increases in urinary sodium excretion and renal interstitial cGMP were abolished by renal interstitial nitric oxide synthase inhibitor L-N<sup>6</sup>-nitroarginine methyl ester or bradykinin B<sub>2</sub> receptor antagonist icatiban. Renal AT<sub>2</sub>R activation with C-21 prevented Na<sup>+</sup> retention and lowered blood pressure in the angiotensin II infusion model of experimental hypertension.

**Conclusions:** AT<sub>2</sub>R activation initiates its translocation to the renal proximal tubule cell apical membrane and the internalization of Na<sup>+</sup>/H<sup>+</sup> exchanger-3 and Na<sup>+</sup>/K<sup>+</sup>ATPase, inducing natriuresis in a bradykinin-nitric oxide-cGMP-dependent manner. Intrarenal AT<sub>2</sub>R activation prevents Na<sup>+</sup> retention and lowers blood pressure in angiotensin II-dependent hypertension. AT<sub>2</sub>R activation holds promise as a renal proximal tubule natriuretic/diuretic target for the treatment of fluid-retaining states and hypertension. (*Circ. Res.* 2014;115:388-399.)

**Key Words:** blood pressure • kidney tubules, proximal • natriuresis • sodium

Composed of multiple enzymes, peptide hormones, and receptors, the renin–angiotensin system (RAS) is a major regulatory element in the control of cardiovascular and renal function. Angiotensin II (Ang II), the pivotal peptide hormone of the RAS, directly binds to and activates 2 G protein–coupled receptors, the type 1 (AT<sub>1</sub>R) and type 2 (AT<sub>2</sub>R) angiotensin receptors that generally oppose each other. Activation of AT<sub>1</sub>Rs induces cellular dedifferentiation and growth, vasoconstriction, antinatriuresis, aldosterone secretion, and sympathetic activation that ultimately lead to hypertension. In contrast, AT<sub>2</sub>R activation induces cellular differentiation and growth inhibition, vasodilation, and natriuresis and potentially lowers blood pressure (BP). Because AT<sub>1</sub>R expression in cardiovascular and renal tissues is ubiquitously present and quantitatively greater than that of AT<sub>2</sub>Rs, AT<sub>1</sub>R actions generally predominate in vivo. However, AT<sub>2</sub>R actions can be demonstrated in vivo when the RAS is activated or AT<sub>1</sub>Rs are blocked, and several actions of AT<sub>1</sub>R blockade have been attributed, at least in part, to AT<sub>2</sub>R activation.

The RAS is thought to be a fundamental driving force contributing to the development of hypertension in experimental animals and humans. According to the Guyton hypothesis, the capacity of the kidneys to excrete sodium (Na<sup>+</sup>) via the pressure-natriuresis mechanism is central in the regulation of BP. That is, to sustain a hypertensive process, the increase in renal perfusion pressure cannot be offset by an increase in Na<sup>+</sup> excretion. Recent receptor cross-transplantation and selective renal tubule receptor knockout studies have validated this concept by demonstrating that renal proximal tubule (RPT) AT<sub>1</sub>Rs are required to sustain a hypertensive response to exogenous Ang II. Importantly, Li and Zhuo have recently demonstrated that RPT-dominant transfer of AT<sub>1</sub>Rs (short-term knock-in) induces increased BP responses to both extracellular and intracellular Ang II in AT<sub>1</sub>R-deficient mice. These observations underscore the importance of RPT...
AT$_2$Rs in the control of BP through their effects to increase Na$^+$ reabsorption. AT$_2$Rs are expressed in the adult kidney primarily in the RPT. We have recently demonstrated that RPT AT$_2$Rs inhibit renal Na$^+$ reabsorption and that, rather than Ang II, des-aspartyl$^1$-Ang II (Ang III) is the predominant endogenous agonist for this response. The natriuretic actions of intrarenal Ang III were demonstrable, however, only when systemic AT$_1$Rs were blocked, unless Ang III metabolism was also abrogated with an aminopeptidase N inhibitor.

The present study was designed to explore the mechanisms of renal Na$^+$ transport in response to both systemic and intrarenal AT$_2$R activation with the highly selective nonpeptide AT$_2$R agonist compound 21 (C-21) in vivo in the rat and mouse. We hypothesized that C-21 (Vicore Pharma), a highly selective, nonpeptide AT$_2$R agonist for this response, was added to secure the catheters and prevent interstitial pressure loss using sterile technique. On the day of experimentation, the rats were anesthetized with inactin (100 mg/kg body weight) via intraperitoneal injection, and the pumps were implanted in the interscapular region using sterile technique.

**Methods**

Please see the Online Data Supplement for detailed methods (total renal cortical cell membrane and Western blot analysis, RPT cell [RPTC] apical membrane isolation and Western blot analysis, in vivo kidney perfusion and fixation, confocal immunofluorescence microscopy, immunoelectron microscopy, and specific experimental protocols).

**Animal Preparation**

All experimental protocols were approved by the Animal Care and Use Committee at the University of Virginia and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The rat experiments were conducted on 12-week-old female (Harlan; protocols 1–5, 7) and male Sprague-Dawley (SD) rats (protocol 6). The mouse experiments were conducted on 12-week-old female wild-type (WT) C57BL/6 (Harlan) and AT$_2$R-null mice (whole-body knockout) on a C57BL/6 background generously provided by Dr Tadashi Inagami of the Vanderbilt University Department of Biochemistry (protocol 8). All animals were housed in a vivarium under controlled conditions (temperature, 21±1°C; humidity, 60±10%; light, 8:00–20:00) and fed a normal Na$^+$ diet (0.3% Na$^+$; Harlan).

**Standard Protocol for Rat In Vivo Studies**

For studies that involved systemic AT$_1$R blockade (protocols 1 and 6), a 24-hour osmotic mini-pump (Alzet Model 2001D) infusing candesartan (0.01 mg/kg per minute) was inserted 24 hours before experimentation. The rats were placed under short-term anesthesia with ketamine (100 mg/mL) and xylazine (20 mg/mL) via intraperitoneal injection, and the pumps were implanted in the interscapular region using sterile technique.

**Renal Cortical Interstitial Infusion**

An open-bore microinfusion catheter (PE-10) was inserted in the renal capsule into the cortex of the left kidney to ensure renal interstitial access to the kidney would not spill over to the opposite kidney confounding the results and the ureter of the remaining left kidney was cannulated (PE-10) to collect urine for the quantification of urine Na$^+$ excretion ($U_{Na}^V$).

**RI Fluid Microdialysis Technique**

RI cGMP was collected using microdialysis probes as described previously (Cayman Chemical). Direct cannulation of the right internal jugular vein using PE-10 tubing provided intravenous access through which 2% BSA made in 5% dextrose in water (protocols 1, 3–5, 7), 2% BSA made in 5% dextrose in water with inulin and lithium chloride (protocol 2), 2% BSA made in 0.9% saline (protocol 6), or C-21 made in either solution was infused at 50 μL/min. Direct cannulation of the right carotid artery with PE-50 tubing provided arterial access for monitoring mean arterial pressure (MAP). After a midline laparotomy, the right kidney was excised (so that substances infused directly into the kidney would not spill over to the opposite kidney confounding the results) and the ureter of the remaining left kidney was cannulated (PE-10) to collect urine for the quantification of urine Na$^+$ excretion ($U_{Na}^V$).

**Pharmacological Agents**

C-21 (Vicore Pharma), a highly selective, nonpeptide AT$_2$R agonist ($K_i=0.4$ mol/L) was used to activate systemic and renal AT$_2$Rs. C-21 demonstrates 25000-fold selectivity at AT$_2$Rs compared with AT$_1$Rs. Candesartan (0.01 mg/kg per minute; AstraZeneca), a specific, potent, insurmountable inhibitor of AT$_1$Rs ($I_{C50}>1×10^{-5}$ mol/L and 2.9×10$^{-3}$ mol/L for AT$_2$Rs and AT$_1$Rs, respectively), was used for systemic AT$_1$R blockade. PD-123319 (PD; 10 μg/kg per minute;

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Effects of Systemic C-21 Infusion+Intrarenal Infusion of AT₁R Antagonist PD±Systemic AT₁R Blockade on UᵦₑV and MAP in Volume-Expanded Female SD Rats

UᵦₑV (Figure 1A) was unchanged in response to vehicle infusion throughout the experiment (P=NS). In response to systemic C-21 infusion of 100, 200, and 300 ng/kg per minute, UᵦₑV increased immediately from 0.24±0.06 μmol/min in a dose-dependent manner to 1.12±0.20 (P<0.001), 1.51±0.25 (P<0.001), and 2.04±0.21 μmol/min (P<0.0001), respectively (overall ANOVA F=18.9; P<0.0001 versus vehicle infusion). The C-21–induced natriuresis was abolished by concurrent intrarenal administration of PD (10 μg/kg per minute) at all C-21 infusion rates. UᵦₑV was not enhanced by systemic pretreatment with AT₁R antagonist candesartan at any C-21 infusion rate tested (P=NS). MAP (Figure 1B) was reduced by systemic infusion of candesartan (P<0.0001) but was otherwise unchanged by C-21 and PD.

Effects of Systemic C-21 Infusion+Intrarenal Infusion of AT₁R Antagonist PD, NO Synthase Inhibitor L-NAME, or Bradykinin B₂ Receptor Antagonist Icatibant on RI cGMP, UᵦₑV, and MAP in the Absence of Systemic AT₁R Blockade in Volume-Expanded Female SD Rats

RI cGMP (Figure 3A) was unchanged in response to systemic vehicle infusion (P=NS). In response to systemic C-21 infusion, RI cGMP increased immediately from 4.92±0.83 pmol/mL to 13.0±2.0 (P<0.01), 13.0±2.4 (P<0.01), and 17.2±3.4 pmol/mL (P<0.01) at 100, 200, and 300 ng/kg per minute C-21 infusion, respectively (overall ANOVA F=10.6; P<0.0001). The C-21–induced increase in RI cGMP was abolished with concurrent intrarenal infusion of PD (10 μg/kg per minute), NO synthase inhibitor L-NAME (100 ng/kg per minute), or

Statistical Analysis

Data are presented as mean±1SE. Statistical significance was determined using 1-way ANOVA followed by multiple comparisons testing with the Student–Newman–Keuls test with 95% confidence. The level of significance was set at P<0.05.

Results

Effects of Systemic C-21 Infusion±Systemic AT₁R Blockade on UNaV and MAP in Volume-Expanded Female SD Rats

UNaV (Figure 2A) was unchanged in response to vehicle infusion (F=7.8; P<0.01). MAP (Figure 2B), renal blood flow (Figure 2C), and glomerular filtration rate (Figure 2D) were unchanged by vehicle or C-21 infusion (P=NS). FE Na (Figure 2E) increased from 35.3±3.1% to 57.3±5.3% (P<0.01), 53.8±5.5% (P<0.01), and 52.6±6.8% (P<0.05) in response to C-21 infusion of 100, 200, and 300 ng/kg per minute, respectively (overall ANOVA F=11.8; P<0.005). FE Li (Figure 2F) also increased in parallel with FE Na from 35.3±3.1% to 57.3±5.3% (P<0.01), 53.8±5.5% (P<0.01), and 52.6±6.8% (P<0.05) in response to C-21 infusion of 100, 200, and 300 ng/kg per minute, respectively (overall ANOVA F=16.1; P<0.001).
bradykinin B₂ receptor antagonist icatibant (100 ng/kg per minute). UNaV (Figure 3B) was unchanged in response to vehicle infusion (P=NS). Systemic C-21 infusion induced an increase in UNaV from 0.26±0.05 μmol/min to 1.20±0.29 (P<0.05), 1.35±0.27 (P<0.01), and 1.42±0.22 μmol/min (P<0.01) at 100, 200, and 300 ng/kg per minute, respectively (overall ANOVA F=7.0; P<0.0005). Intrarenal administration of PD, L-NAME, or icatibant abolished C-21–induced natriuresis. MAP (Figure 3C) did not change in response to administration of any pharmacological agent.

**Effects of Systemic C-21 Infusion on Total Cortical and Apical Plasma Membrane AT₂R Density in the Absence of Systemic AT₁R Blockade in Volume-Expanded Female SD Rats**

To determine whether AT₂R activation induces receptor recruitment to the apical plasma membranes of RPTCs, we used confocal immunofluorescence microscopy, immunoelectron microscopy, and Western blot analysis. Figure 4A to 4L demonstrates the subcellular distribution of AT₂Rs as determined by confocal immunofluorescence microscopy from a representative set of rat RPTCs in response to systemic vehicle (Figure 4A–4F) and systemic C-21 (Figure 4G–4L) infusion (100 ng/kg per minute). Figure 4A and 4B, respectively, depicts the RPTC distribution of adaptor protein-2 (blue) marking the subapical membrane and phalloidin (red) marking the apical brush border. Figure 4C demonstrates the cellular distribution of AT₂R protein using antibody (Chemicon) proven to be specific for AT₂Rs by immunoblotting AT₂R-null mouse adrenal glands (Figure 4P) that normally have a high degree of AT₂R expression. Figure 4D demonstrates the merged adaptor protein-2, phalloidin, and AT₂R image. As demonstrated in merged Figure 4D (vehicle infusion) and Figure 4J (C-21 infusion), C-21 administration induced a color shift in immunofluorescence from red to orange, indicating increased AT₂R density in the apical plasma membrane in response to C-21. This color shift can be more easily visualized in Figure 4E and 4K, which are high-power images of the areas depicted in the squares from Figure 4D and 4J. Figure 4F and 4L depicts the AT₂R immunofluorescence staining only in the apical
Effects of Systemic C-21 Infusion on RPTC NHE-3 Apical Plasma Membrane Retraction and Cellular Internalization in the Absence of Systemic AT\(_2\)R Blockade in Volume-Expanded Female SD Rats

To determine whether AT\(_2\)Rs induce natriuresis by internalizing/inhibiting Na\(^+\) apical transporter NHE-3, we also performed immunofluorescence microscopy, immunoelectron microscopy, and Western blot analysis. Figure 6A to 6D demonstrates the subcellular distribution of NHE-3 as determined by confocal immunofluorescence microscopy from a representative set of rat RPTCs in response to systemic vehicle (Figure 6A–6E) and C-21 (Figure 6F–6J) infusion (100 ng/kg per minute). Figure 6A and 6F shows autofluorescence (blue) of the RPTC. Figure 6B and 6G shows NHE-3 (green) expressed in the apical brush border membranes of RPTCs. Figure 6C and 6H demonstrates subapical membranes visualized by adaptor protein-2 staining (red). In merged Figure 6D, there is no visible translocation of NHE-3 from apical to subapical membranes. In contrast, in response to C-21 infusion (Figure 6I), there is visible translocation from apical to subapical membranes, as demonstrated by the extensive yellow transformation. This C-21–induced color shift is more easily visualized in the high-power magnifications (Figure 6E and 6J) taken from the squares of Figure 6D and 6I, respectively. Figure 6K demonstrates the significant quantitative translocation of NHE-3 to subapical membranes in response to C-21 (n=4; P<0.01). Western blot analysis of NHE-3 total cortical distribution is shown in Figure 6L, where there was no change in response to C-21 (100, 200, and 300 ng/kg per minute). Furthermore, Figure 6M shows that systemic C-21 infusion significantly increased total cortical membrane phospho-NHE-3 (Ser 522) protein levels (P<0.001), an established indicator of NHE-3 retraction/internalization. Figure 7 depicts high-powered electron photomicrographs of immunogold-labeled NHE-3 in the apical brush border microvilli and apical plasma membrane base/subapical regions of RPTCs after systemic vehicle (Figure 7A and 7B) and C-21 (Figure 7C and 7D) infusion (100 ng/kg per minute), respectively. Figure 7E demonstrates that C-21 infusion did not affect the NHE-3 density in the apical membrane of RPTCs but dramatically increased the distribution of NHE-3 in the apical plasma membrane/subapical membrane region (P<0.01; Figure 7F). Collectively, these studies demonstrate the ability of C-21 to induce the retraction of NHE-3 from the apical to subapical region of RPTCs.

Effects of Systemic C-21 Infusion on Total Cortical Membrane Phospho-ERK1/2, ERK1/2, Phospho-Src (Tyr 416), Src, Phospho-αNKA (Ser 23), and αNKA Protein Expression in the Absence of Systemic AT\(_2\)R Blockade in Volume-Expanded Female SD Rats

To determine whether AT\(_2\)R activation can internalize/inhibit NKA and activate the Src/extracellular-signal–regulated kinase (ERK) signaling pathway, we performed Western blot analysis of renal cortical membranes. Western blot analyses of total cortical membrane phospho-ERK1/2 and ERK1/2 are shown in Online Figure IIA and IIB, respectively. C-21 treatment (100, 200, and 300 ng/kg per minute) significantly increased phospho-ERK1/2 protein (P<0.01) without changing total cortical ERK1/2 protein expression. Online Figure IIC and IID depicts Western blot analysis of total cortical
membrane phospho-Src (Tyr 416) and Src, respectively. C-21 infusion also significantly increased phospho-Src protein (P<0.01), without changing total cortical Src protein expression. Online Figure IIE and IIF depicts Western blot analysis of total cortical membrane phospho-αNKA (Ser 23) and total αNKA respectively. Although there was no change in total cortical membrane αNKA protein (Online Figure IIF), C-21 infusion significantly decreased phospho-αNKA protein expression (Online Figure IIE; P<0.05), an established indicator of αNKA retraction/internalization. Collectively, these studies suggest that along with NHE-3, NKA is internalized/inactivated during C-21 infusion.

Effects of Systemic C-21 Infusion±Intrarenal Infusion of AT2 Antagonist PD±Systemic AT1R Blockade on UNaV and MAP in Na+-Loaded Female and Male SD Rats

In female rats (Online Figure IIIA), cumulative systemic C-21 infusion (100, 200, and 300 ng/kg per minute) increased UNaV from 1.49±0.17 to 8.07±0.71 μmol/min (overall ANOVA F=36.3; P<0.0001). This response was abolished with concurrent intrarenal infusion of AT1R antagonist PD (10 μg/kg per minute). Systemic pretreatment with AT1R antagonist candesartan increased UNaV to 10.7±0.70 μmol/min, a value significantly higher than with C-21 alone (P<0.02). In male rats (Online Figure IIIA), systemic C-21 infusion increased UNaV from 0.61±0.13 to 6.24±1.08 μmol/min (P<0.01); this response was also abolished by intrarenal PD. In the presence of systemic candesartan pretreatment, there was no difference in UNaV versus C-21 alone (P=NS). In contrast to female volume-expanded rats, UNaV in female Na+-loaded rats was enhanced by systemic pretreatment with candesartan (P<0.05). There was no significant sex difference in the natriuretic response to C-21 alone or C-21+PD (P=NS). However, the natriuretic response to C-21 in the presence of candesartan was significantly greater in female than male rats (overall ANOVA F=5.6; P<0.005). As shown in Online Figure IIIB, MAP was...
reduced ($P<0.0001$) by systemic candesartan administration but was otherwise unchanged in response to administration of pharmacological agents ($P=NS$).

**Effects of Intrarenal C-21 Infusion±Intrarenal Infusion of AT$_2$R Antagonist PD on $U_{\text{Na}}V$ and MAP in the Absence of Systemic AT$_2$R Blockade in Volume-Expanded Female SD Rats**

$U_{\text{Na}}V$ (Online Figure IVA) was unchanged as a result of intrarenal vehicle administration ($P=NS$). In response to intrarenal C-21 infusion, $U_{\text{Na}}V$ increased in a dose-dependent manner from 0.14±0.02 μmol/min to 0.50±0.11 ($P<0.05$), 0.68±0.13 ($P<0.01$), and 0.85±0.11 μmol/min ($P<0.01$) at 20, 40, and 80 ng/kg per minute, respectively (overall ANOVA $F=15.2$; $P<0.0001$). The natriuresis in response to intrarenal C-21 administration was abolished with concomitant intrarenal administration of PD (10 μg/kg per minute). MAP (Online Figure IVB) did not change in response to administration of any pharmacological agent (Online Figure IV).

**Effects of Chronic Intrarenal C-21 Infusion on Mean Systolic BP and 24-Hour $U_{\text{Na}}V$ in Ang II–Dependent Hypertension in Female SD Rats**

As shown in Figure 8A, systemic Ang II infusion (200 ng/kg per minute) increased systolic BP from 126±5 to 188±20 mm Hg during a 7-day period (ANOVA $F=48$; $P<0.0001$). Concurrent intrarenal administration of C-21 (60 ng/kg per minute) markedly inhibited the pressor effect of systemic Ang II infusion ($F=12$; $P<0.0001$). As shown in Figure 8B, consecutive 24-hour $U_{\text{Na}}V$ was reduced from 0.95±0.04 to 0.34±0.08 μmol/min ($P<0.0001$) on day 1 of systemic Ang II infusion. Ang II–induced antinatriuresis was inhibited by intrarenal administration of C-21 ($F=23.3$; $P<0.0001$) during the entire 7-day period of infusion.

**Discussion**

The present study demonstrates, for the first time to our knowledge, that both systemic and direct intrarenal administration of highly selective AT$_2$R agonist C-21 can induce a sustained increase in renal Na$^+$ excretion in normal animals by activating RPTC AT$_2$R. We show that systemic AT$_2$R...
activation induces natriuresis both acutely in rats and chronically in mice, responses that were reversible with intrarenal administration of specific AT2R antagonist PD and genetic deletion of AT2Rs, respectively. The importance of these findings is underscored by the absence of a requirement for concurrent AT1R blockade to unmask AT2R-mediated natriuresis. This is a novel finding, in that past studies cardiovascular and renal responses to AT2R activation have been observed only when the RAS is activated or AT1Rs are concurrently blocked.2–4 Our results support the concept that potent, highly selective nonpeptide AT2R agonist administration may contribute to the future therapeutic management of fluid-retaining disorders and possibly hypertension. This study also demonstrates that chronic renal AT2R activation prevents Na+ retention and lowers BP in an experimental model of Ang II–dependent hypertension. Intrarenal administration of C-21 not only abrogated the initial Ang II–induced antinatriuresis but also augmented Na+ excretion chronically in this model. Thus, intrarenal AT2R activation improved the pressure-natriuresis relationship in this model. Additional factors, other than natriuresis, may also contribute to BP reduction in this model. Future studies will include determination of the effect of sex and the route of C-21 administration on BP reduction in this model.

Previous studies have demonstrated the importance of the bradykinin, NO, and cGMP signaling cascade in the actions of AT2Rs in multiple cells and tissues, including the kidney.2–4,22–24 This signaling pathway can operate either by bradykinin B2 receptor activation or directly via NO and cGMP production without involving bradykinin.24 We explored the mechanisms of AT2R-induced natriuresis in the present study. The increases in renal Na+ excretion with AT2R activation were accompanied by increases in renal cGMP. The C-21–induced increases in both cGMP and Na+ excretion were abolished with intrarenal administration of either bradykinin B2 receptor antagonist icatibant or NO synthase inhibitor L-NAME, demonstrating dependence of the AT2R-induced natriuretic responses on the bradykinin, NO, and cGMP signaling cascade. Similar dependence of AT2R-induced natriuresis on cGMP signaling has been demonstrated for the major endogenous renal AT2R
agonist, Ang III. Our previous studies identified a putative new signaling pathway by which cGMP released into the RI compartment may facilitate natriuresis, that is, by binding to the extracellular domain of αNKA inducing phosphorylation of downstream signaling molecules Src and ERK1/2. Here, we show that C-21 induces Src and ERK phosphorylation and internalization/inactivation of αNKA. This finding suggests, but does not prove, that αNKA internalization/inactivation may be induced by increased extracellular renal cGMP production secondary to renal AT2R activation. On the basis of these results, we hypothesize that extracellular renal cGMP is the major driving force for AT2R-mediated natriuresis by internalizing and inactivating the major RPT Na+ transporters. Additional studies will be required to determine definitively whether this signaling pathway mediates AT2R- and cGMP-induced natriuresis.

Because AT, Rs are encoded by a gene residing on the X-chromosome, sex differences in AT, R actions have recently been explored. Studies to date have shown that female rats have enhanced AT2R-mediated renal vasodilator and tubuloglomerular feedback responses compared with males. However, this difference does not translate to renal Na+ excretion, which was identical in male and female rats. In the present study, AT2R activation with C-21 induced natriuresis to a similar extent in male and female rats, and natriuresis could be attributed to reduced RPTC reabsorption as reflected by increased FENa and FELi without alteration in renal hemodynamic function. Lithium clearance studies have been shown to accurately identify RPT events with a maximum 4% error rate in Na+-replete animals. However, we did observe a significant augmentation of AT2R-induced natriuresis with concurrent AT1R blockade in female but not male rats. The reasons for this difference are not clear, and it is possible that differences in renal blood flow and glomerulotubular feedback may have occurred, which were not monitored during our experiments using candesartan. In addition to these studies, reports of C-21 induction of natriuresis have appeared in the literature. Kemp et al in 2011 first demonstrated that systemic administration of C-21 (0.3 μg/kg per minute) induced natriuresis (12-fold) that was blocked with intrarenal PD infusion in male uninephrectomized SD rats. In addition, Ali and Hussain reported that intravenous infusion of C-21 (5.0 μg/kg per minute, a 16-fold higher dose) increased UNaV, FENa, and FELi in obese Zucker rats and that this response was neutralized by systemic coadministration of PD.

![High-powered electron photomicrographs (x30000) of the apical brush border and apical membrane base/subapical regions of renal proximal tubule cells (RPTCs) from kidneys of volume-expanded female Sprague-Dawley rats after vehicle (A and B) and systemic compound 21 (C-21; 100 ng/kg per minute) treatment (C and D). Black dots represent immunogold (10 nm particles) labeling of Na+–H+ exchanger-3 (NHE-3) after each experimental infusion. Bar at the bottom of C, 0.5 μm. The electron micrographs confirm increased apical membrane base/subapical NHE-3 distribution after C-21 administration. E and F. The quantification of RPTC apical membrane and apical membrane base/subapical membrane NHE-3 staining, respectively, after vehicle and C-21 treatment. Data represent means±1SE from 8 different electron micrographs of RPTCs for each condition. **P<0.01 compared with control treatment. CON indicates control.](http://circres.ahajournals.org/)}
demonstrated by a combination of Western blot analysis and confocal and immunoelectron microscopy. This finding is consistent with previous results from our laboratory showing that natriuretic responses to endogenous renal AT2R agonist Ang III are also accompanied by translocation of AT2R from intracellular sites along microtubules to the apical plasma membranes of RPTCs.31 In addition, dopamine D1 receptor activation with fenoldopam induces natriuresis in an AT2R-dependent manner and translocates AT2Rs to the apical plasma membranes of RPTCs via cAMP and protein phosphatase 2A signaling mechanisms.32 Interestingly, hypertensive 12-week-old spontaneously hypertensive rats fail to recruit AT2Rs or mount natriuretic responses to Ang III, but both receptor translocation and natriuresis can be restored by inhibition of aminopeptidase N, increasing Ang III formation.20,31 Combined with our previous findings, the present results suggest that AT2R recruitment to the apical plasma membranes of RPTCs may be a critical common mechanism initiating and supporting sustained natriuretic responses to dopamine, Ang III, and C-21.

The RPT reabsorbs 67% of Na+ filtered into the nephron.33 NHE-3 is the principal apical Na+ transporter in the RPT, and flow-modulated NHE-3 activity is the mechanism for glomerulotubular balance.34 NHE-3 is expressed along the microvilli of the RPTC brush border but can also be detected in subapical, intracellular, and vesicular compartments, consistent with the regulation of its activity by membrane trafficking.35,36 Direct and indirect binding of NHE-3 to ezrin is required for its intracellular trafficking, and NHE regulatory factor-1 links NHE-3 to ezrin and the cytoskeleton, which are in turn regulated by RhoGTPase.37,38

In the present study, we hypothesized that AT2R activation with C-21 would internalize/inactivate not only NKA (discussed above) but also NHE-3 in the RPT. We demonstrated trafficking of NHE-3 from the tips to the bases of the apical microvilli and also into the subapical membranes of RPTCs in response to C-21. Retraction/internalization of NHE-3 is a marker of reduced NHE-3 activity.39 For example, acutely increased BP induces trafficking of RPTC NHE-3 from the tips to the bases of the apical microvilli (without internalization into the subapical domain), resulting in pressure-natriuresis.40 Activation of RPTC dopamine D1 receptors also inhibits proximal Na+ reabsorption by decreasing NHE-3 activity and protein abundance in the apical plasma membrane without changing total cellular NHE-3 expression.41 As discussed above, C-21 also induced internalization of αNKA, the major Na+ transporter at the RPTC basolateral membrane. Thus, AT2R activation internalizes and inactivates the 2 major transporters governing Na+ reabsorption in the RPT. Exploration of the detailed signaling mechanisms mediating transporter internalization and inactivation will be performed in future studies.

In summary, AT2R activation induces natriuresis by recruiting the receptor to the apical plasma membranes of RPTCs, stimulating a bradykinin–NO–cGMP–Src–ERK signaling cascade and internalizing NHE-3 and NKA. Renal AT2R activation prevents Na+ retention and lowers BP by improving the pressure-natriuresis relationship in Ang II–dependent hypertension. Because no clinically effective diuretic/natriuretic agents acting in the RPT are currently available, systemic AT2R activation potentially represents a unique opportunity for treatment of volume-overload/edema-forming states and hypertension in humans.
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Disclosures
None.

References
What Is Known?

- Angiotensin II (Ang II) acts via one of 2 major receptors, type 1 (AT1R) and type 2 (AT2R).
- AT2Rs generally oppose the actions of Ang II via AT1Rs.
- Instead of Ang II, renal des-aspartyl1-Ang II activates AT2Rs, inducing natriuresis and opposing the usual antinatriuretic action of Ang II via AT1Rs.

What New Information Does This Article Contribute?

- In the renal proximal tubule, compound 21 (C-21), a nonpeptide angiotensin AT2 receptor (AT2R) agonist, administered systemically induces natriuresis by increasing renal production of bradykinin, nitric oxide, and cGMP.
- Renal AT2R activation is accompanied by the recruitment of AT2Rs to the apical plasma membranes of proximal tubule cells and by internalization and inactivation of the major proximal tubule apical and basolateral membrane sodium (Na+) transporters.
- C-21 administered directly into the kidney ameliorates Ang II–induced antinatriuresis and hypertension.

Ang II acts via 2 major receptors, type 1 (AT1R) and type 2 (AT2R). Most of Ang II actions are mediated via AT1Rs, including vasoconstriction, antinatriuresis, aldosterone secretion, and sympathetic activation that increase blood pressure and lead to hypertension. However, AT2R activation can dilate blood vessels and induce natriuresis by increasing renal production of bradykinin, NO, and cGMP. The endogenous ligand activating AT2Rs in the kidney seems to be des-aspartyl1-Ang II instead of Ang II.

We report that specific activation of renal AT2Rs with systemic nonpeptide agonist C-21 induces natriuresis in a cGMP-dependent manner by recruiting AT2Rs from intracellular sites to the apical plasma membranes of proximal tubule cells and by inhibiting the major Na+ transporters, NHE-3 and NKA, in this nephron segment. We found that C-21 could ameliorate Ang II–dependent hypertension by renal activation of AT2Rs.

These findings suggest that proximal tubule AT2Rs could be a therapeutic target for hypertension and edema-forming states when the renin–angiotensin system is activated. Currently, no clinically effective diuretic/natriuretic agents acting at the proximal tubule are available, and therefore C-21 or similar agents could provide a complimentary nephron-specific site of diuresis/natriuresis for future clinical use.

Novelty and Significance

AT2 Receptor Activation Induces Natriuresis and Lowers Blood Pressure

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Detailed Methods

Acute Blood Pressure (BP) Measurements

Mean arterial pressure (MAP) was measured by the direct intra-carotid method with the use of a digital BP analyzer (Micromed, Inc). MAPs were recorded every 5 min and averaged for all periods. Experiments were initiated at the same time each day to prevent any diurnal variation in BP.

Measurement of Glomerular Filtration Rate (GFR), Fractional Excretion of Sodium (FE\textsubscript{Na}), and Fractional Excretion of Lithium (FE\textsubscript{Li})

Urinary and plasma Na\textsuperscript{+} and Li\textsuperscript{+} concentrations were measured using a flame photometer (Instrumentation Laboratory-943). GFR was measured by inulin clearance (1% inulin infusion at 50\(\mu\text{L/min}\)) and reported as mL/min/g kidney weight. Tubular Na\textsuperscript{+} reabsorption was determined by calculating the FE\textsubscript{Na} and renal proximal tubule (RPT) Na\textsuperscript{+} reabsorption was estimated by calculating the FE\textsubscript{Li} (0.1% LiCl infusion at 50 \(\mu\text{L/min}\); maximum error of 4%), as published previously (1,2).

Renal Blood Flow (RBF) Measurement

Following acute uninephrectomy, a flow probe was secured around the left renal artery and connected to a dual-channel flowmeter (Transonic Systems Inc.) to measure RBF.
Total Cortical Cell Membrane Preparation and Western Blot Analysis

Slices of kidney cortex (~100 mg per kidney) were homogenized in detergent free lysis buffer with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) and then centrifuged at 3100 rpm for 10 min at 4°C to remove cellular debris. The supernatant was removed and total protein was quantified using a bicinchoninic acid (BCA) assay (Pierce). Sodium dodecylsulfate (SDS) samples were prepared, separated by SDS-PAGE (10% Tris-HCl polyacrylamide gels; 40 µg of protein loaded per lane), and transferred onto a nitrocellulose membrane by electroblotting. Membranes were blocked in 5% milk in TBS with 0.1% Tween-20 (TBST) for 4h and then incubated overnight at 4°C with the following primary antibodies in 5% milk TBST: AT₂R (Chemicon; 1:200 or Alomone Labs; 1:500), phospho-NHE-3 [Ser 522] (Thermo Scientific; 1:500), NHE-3 (Millipore; 1:500), phospho-Erk 1/2 (Cell Signaling; 1:1000), Erk 1/2 (Cell Signaling; 1:1000), phospho-Src [Tyr 416] (Cell Signaling; 1:1000), Src (Cell Signaling; 1:1000), phospho-αNKA [Ser 23] (Abcam; 1:1000), or αNKA (Thermo Scientific; 1:1000). Membranes were subsequently incubated with their respective HRP-conjugated secondary antibodies in 5% milk TBST (GE Healthcare; 1:2500) for 2h. Signals were detected using chemiluminescence and band densities were measured with Image J software (NIH). The blots were then rinsed in several washes of TBS and incubated with the primary antibody GAPDH (Ambion Life Technologies; 1:2500) in Licor blocking buffer (Licor Biosciences) for 1h. Membranes were subsequently incubated with infrared secondary antibody (anti-mouse IRDye 680; 1:15,000) in Licor blocking buffer for 1h. Immunoreactivity was performed using the Odyssey Infrared Imaging System. All blots were normalized to GAPDH expression.
Renal Proximal Tubule Cell (RPTC) Apical Membrane Isolation and Western Blot Analysis

After the kidney cortex slices were homogenized, 1 mg of total protein was resuspended in 10 mL of detergent free lysis buffer and incubated with 20 μg of biotinylated *Lotus tetragonolobus* agglutinin lectin (Vector Laboratories) on a 360° rocker for 2h at room temperature as previously published (3-5). A 50% vol/vol slurry (20 μL) of Ultralink Neutravidin beads (Pierce) was then added and incubated on a 360° rocker for 30 min at room temperature. 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 100 μL of 70°C 2 X sample buffer for 5 min and underwent Western blot analysis as previously described. The membranes were incubated with the following primary antibodies in 5% milk TBST: AT2R (Chemicon; 1:200 or Alomone Labs; 1:500) followed by their respective HRP-conjugated secondary antibodies as mentioned previously. Membranes were subsequently incubated with GAPDH as mentioned previously for normalization.

In Vivo Kidney Perfusion and Fixation Procedure

Following the first systemic Compound 21 (C-21) IV dose (100 ng/kg/min), the rat heart left ventricular cavity was cannulated and the rat was perfused with 40 mL of 4% sucrose in PBS++ followed by 40 mL of 4% paraformaldehyde (PFA) in PBS++. Kidneys were harvested and cut into 1 mm thick sections using a McIlwaine Tissue Chopper before being placed in 4% PFA for 2h at room temperature. The slices were rinsed in
several changes of PBS++, immersed in 100 mM Tris-HCl for 30 min, and then rinsed again in PBS++ before being stored in 30% sucrose in PBS++ overnight at 4°C. The kidney slices were embedded in Tissue Tek OCT Compound in Cryomold vinyl specimen molds. Cryostat thin sections (5-8 μm) were placed on Probe On Plus positively charged microscope slides through the University of Virginia Research Histology Core. The kidney slices were immediately stained.

Confocal Immunofluorescence Microscopy

After the kidney sections had been spotted onto slides and washed with TBS, they were permeabilized with 0.2% Triton-X in TBS for 10 min. The sections were washed in TBS with 0.02% Tween-20 (TBST) and then blocked in 1% milk TBST for 1h at room temperature. The kidney sections were incubated with the following primary antibodies: AT2R (Chemicon; 1:100) and AP2 (Santa Cruz; 1:100) in 1% milk TBST overnight at 4°C. After washing with TBST, ALEXA 647 conjugated donkey anti-rabbit, ALEXA 488 conjugated donkey anti-mouse secondary antibodies (Invitrogen; 1:500), and Texas-Red phalloidin (Invitrogen; 1:200) were added in 1% milk TBST for 1h at room temperature. In a separate set of slides, the kidney sections were incubated with the following primary antibodies: NHE-3 (Millipore; 1:2000) and AP2 (1:200) and processed as stated previously, minus Texas-Red phalloidin treatment. Following secondary antibody incubation, the slices were washed in TBST, Fluoromount G (Southern Biotech) was applied, and the specimens were covered with a glass coverslip. Stained tubules were photographed under epifluorescence illumination using an automated Olympus IX81 spinning disk confocal microscope using a 60 X plan apo
water immersion objective with a numeric aperture of 1.2. The microscope was controlled using Slidebook 5.5 software (3i, Denver CO) and 5 micron thick z-stack images were captured using a Hamamatsu EMCCD camera at 0.25 micron intervals and deconvolved using the autoquant spinning disk deconvolution module. Calculations of immunoreactive AT$_2$R within the proximal tubule brush border were conducted using F-actin staining with Alexa 594 phalloidin to create the mask and measuring AT$_2$R fluorescence within that region. Calculation of the immunoreactive NHE-3 fluorescence intensity at the subapical region of the RPTC was performed by creating a mask using the AP2 fluorescence intensity and measuring the NHE-3 fluorescence within that region. Quantifications were performed on RPTCs in kidney sections from control (N=4) and C-21 treated (N=4) rats (2 sections per rat and 20 RPTCs per section were analyzed).

**Immuno-electron Microscopy**

On-section labelling immunofluorescence and immuno-electron microscopy was performed using the same procedure as for confocal microscopy, with minor changes. Another AT$_2$R antibody that has been verified for use for immuno-electron microscopy was used (Abcam; 1:100) and the secondary antibodies were substituted with fluorescently labelled Alexa 594 conjugated FluoroNanogold goat anti-rabbit IgG (Invitrogen; 1:20) for AT$_2$R and Alexa 594 conjugated FluoroNanogold goat anti-mouse (Invitrogen; 1:20) for NHE-3. After visual confirmation of staining by fluorescence microscopy, the nanogold particles were silver enhanced according to the manufacturer’s suggested protocol (Silver Enhancement Kit, Invitrogen). Briefly, slices
were washed twice in water to remove chloride ions, incubated in freshly mixed
component A and B, and allowed to develop for 25 min. The slices were rinsed in water
to stop the reaction and then post fixed in 2% glutaraldehyde (EM grade, Electron
Microscopy Sciences) in 0.1 mol/L PBS overnight at 4°C. The slices were then
delivered to the Advanced Microscopy Facility at the University of Virginia for further
processing for transmission electron microscopy. All of the subsequent processing was
carried out at 24°C unless otherwise noted. Slides with attached sections were washed
in distilled water (4x5 min), postfixed for 1h in 1% osmium-tetroxide, dehydrated through
a graded ethanol series followed by transition into 100% acetone, and infiltrated with
epoxy resin (EPON 812, EMS). Although the resin on the slides was still liquid, an
embedding capsule (BEEM, EMS) filled with epoxy resin was inverted directly over each
section and the entire unit (slide with section and capsule) polymerized for 48h at 60°C.
To separate capsules with underlying embedded tissue sections from the slides, each
slide was immersed in boiling water, followed by immersion in liquid nitrogen, and
returned to boiling water a second time. Ultrathin sections (70–80 nm) were prepared
with a Diatome diamond knife (Diatome, EMS) on a Leica Ultracut UCT ultramicrotome,
collected on 200 mesh copper grids (EMS), and contrast stained using a double-lead
procedure (Daddow) as follows: 5 min in lead citrate; 15 min in uranyl acetate (3.0% in
50.0% acetone); and a final 5 min in lead citrate. Thin sections were examined in a
JEOL 1230 transmission electron microscope (Japan Electron Optics Limited, Tokyo,
Japan), and digital images of RPTCs were acquired with an SIA 12-C slow-scan 16.8
megapixel camera (Scientific Instruments and Applications). Immunogold-labeling of
apical plasma membrane AT$_2$Rs and apical/subapical membrane NHE-3 was quantified
from 6-8 different electron micrographs of RPTCs for each experimental condition.
Quantitative counting of immunogold particles was performed by first calibrating
the images using FIJI software, selecting the area to be measured using the drawing tool,
using the find maxima feature, using single point and light background checkboxes
selected, and then adjusting the noise tolerance. Measurements were quantitated as
immunogold particles per µm².

**Telemetric BP Probe and Mini-Pump Implantation for Mice In Vivo Studies**

Mice were placed under short term anesthesia via intraperitoneal (IP) injection of
0.2 mL of a Ketamine and Xylazine mixture (1.9 mL 5% dextrose in water [D₅W], 0.425
mL Ketamine, 0.175 mL Xylazine) for the implantation of the DSI telemetry probe
(TA11PA-C10) and 7d osmotic mini-pump (Alzet Model 1007D) infusing vehicle D₅W or
C-21 (300 ng/kg/min). Using sterile technique, a small incision was made along the left
side of the trachea and the catheter of the telemetry probe was inserted directly into the
carotid artery per manufacturer’s suggested protocol. A small pocket was created along
the left rib cage for the telemetry probe. The mouse was turned over and a small
incision was made in the interscapular region for the implantation of the subcutaneous
(SC) osmotic mini-pump. MAP was measured daily for 7d at the same time each day to
prevent any diurnal variation in BP measurements. In a separate group of mice, 24h
urine Na⁺ excretion (UₙaV) was measured at baseline and on days 1, 3, and 7 of the
study.
Telemetric BP Probe, Systemic Mini-Pump Implantation, and Intrarenal Mini-Pump Implantation for Rat In Vivo Studies

Rats were placed under short term anesthesia via IP injection of ketamine (100 mg/mL) and xylazine (20 mg/mL) for the implantation of the DSI telemetry probe (PA-C40), 7d SC osmotic mini-pump (Alzet Model 2001) infusing angiotensin II (Ang II; 200 ng/kg/min) systemically, and 7d intrarenal osmotic mini-pump (Alzet Model 1007D) infusing D5W or C-21 (60 ng/kg/min). Using sterile technique, a midline abdominal incision was made and the catheter of the telemetry probe was implanted directly into the descending aorta per manufacturer’s suggested protocol. Once the catheter was secure, the telemetry probe was anchored to the abdominal wall with suture, the right kidney was removed (so that substances infused directly into the left kidney would not spill over to the opposite kidney confounding the results), and the incision was closed. There was a 1 wk equilibration period before measurements were taken. After the equilibration period, the rat was placed under short term anesthesia as stated previously and a flank incision was made to expose the left kidney. A PE-10 catheter was inserted into the renal cortex of the remaining kidney for intrarenal infusions and secured with mesh and Vetbond tissue adhesive (3M Animal Care Products). The other end of the tubing was tunneled towards the scapular region. Through a small incision in the scapular region, a SC pocket was made with forceps and the intrarenal PE-10 tubing was connected to an osmotic mini-pump (Alzet, model 1007D) to infuse D5W or C-21. A second osmotic mini-pump (Alzet, Model 2001) was inserted into the SC pocket in the subscapular region in order to infuse Ang II systemically. Mean systolic blood pressure (SBP) was measured daily for 7d at the same time each day to prevent any diurnal
variation in BP measurements. In a separate group of rats, 24h \( U_{Na}V \) was measured daily.

**Rationale for Using Male and Female Rats and Female Mice**

Male and female rats were employed to determine sex differences, if any, in natriuretic responses to C-21. Female mice were used to match the sex of the rats that were employed in the majority of rat studies.

**Specific Protocols:**

(1) Effects of Systemic C-21 Infusion ± Intrarenal Infusion of AT\(_2\)R Antagonist PD ± Systemic AT\(_1\)R Blockade on \( U_{Na}V \) and MAP in Volume-Expanded Female Sprague Dawley (SD) Rats

All rats were volume-expanded and received IV infusions of 2% bovine serum albumin (BSA) made in D\(_5\)W. Following a 1h equilibration period, the following groups of rats were studied: (1) **Control (N=8)**: rats received IV infusions of 2% BSA in D\(_5\)W and renal interstitial (RI) infusions of D\(_5\)W throughout the study. (2) **Systemic C-21 (N=8)**: rats received 3 cumulative IV infusions of C-21 (100, 200, and 300 ng/kg/min; each dose for 30 min) and RI infusions of D\(_5\)W during the experimental periods following a 30 min control period in which 2% BSA in D\(_5\)W was infused in the IV and D\(_5\)W into the RI space. (3) **Systemic C-21 + Intrarenal PD (N=10)**: rats received 3 cumulative IV infusions of C-21 and RI infusion of PD (10 μg/kg/min) during the experimental periods following a 30 min control period in which 2% BSA in D\(_5\)W was infused in the IV and D\(_5\)W into the RI space. (4) **Systemic C-21 + Systemic Candesartan (CAND; N=7)**: rats
followed the same procedure as group 2, but in the presence of 24h AT₁R blockade with CAND. 
\( U_{Na}V \) and MAP were measured for each period.

(2) Effects of Systemic C-21 Infusion on \( U_{Na}V, \) MAP, RBF, GFR, \( F_{E_{Na}}, \) and \( F_{E_{Li}} \) in the Absence of Systemic AT₁R Blockade in Volume-Expanded Female SD Rats

All rats were volume-expanded and received IV infusions of 2% BSA made in \( D_5W \) with inulin and LiCl. Following a 1h equilibration period, the following groups of rats were studied: (1) Control (N=10): rats received IV infusions of 2% BSA in \( D_5W \) and RI infusions of \( D_5W \) throughout the study. (2) Systemic C-21 (N=10): rats received 3 cumulative IV infusions of C-21 (100, 200, and 300 ng/kg/min; each dose for 30 min) and RI infusions of \( D_5W \) during the experimental periods following a 30 min control period in which 2% BSA in \( D_5W \) was infused in the IV and \( D_5W \) into the RI space. \( U_{Na}V, \) MAP, RBF, GFR, \( F_{E_{Na}}, \) and \( F_{E_{Li}} \) were calculated for each period.

(3) Effects of Systemic C-21 Infusion ± Intrarenal Infusion of AT₂R Antagonist PD, Nitric Oxide Synthase (NOS) Inhibitor L-NAME, or Bradykinin (BK) B₂ Receptor Antagonist Icatibant on RI cGMP, \( U_{Na}V, \) and MAP in the Absence of Systemic AT₁R Blockade in Volume-Expanded Female SD Rats

All rats were volume-expanded and received IV infusions of 2% BSA made in \( D_5W \). Following a 1h equilibration period, the following groups of rats were studied: (1) Control (N=8): rats received IV infusions of 2% BSA in \( D_5W \) and RI infusions of \( D_5W \) throughout the study. (2) Systemic C-21 (N=8): rats received 3 cumulative IV infusions of C-21 (100, 200, and 300 ng/kg/min; each dose for 30 min) and RI infusions of \( D_5W \)
during the experimental periods following a 30 min control period in which 2% BSA in D₅W was infused in the IV and D₅W into the RI space. (3) Systemic C-21 + Intrarenal PD (N=8): rats received 3 cumulative IV infusions of C-21 and RI infusion of PD (10 µg/kg/min) during the experimental periods following a 30 min control period in which 2% BSA in D₅W was infused in the IV and D₅W into the RI space. (4) Systemic C-21 + Intrarenal L-NAME (N=8): rats followed the same procedure as Group 3 with RI infusion of L-NAME (100 ng/kg/min). (5) Systemic C-21 + Intrarenal Icatibant (N=8): rats followed the same procedure as Group 3 with RI infusion of icatibant (100 ng/kg/min). RiCGMP levels, UNaV and MAP were quantified for each period.

(4) Effects of Systemic C-21 Infusion on Apical Plasma Membrane AT₂R and Total Cortical Membrane AT₂R, Phospho-NHE-3 (Ser 522), NHE-3, Phospho-Erk 1/2, Erk 1/2, Phospho-Src (Tyr 416), Src, Phospho-αNKA (Ser 23), and αNKA Protein Expression in the Absence of AT₁R blockade in Volume-Expanded Female SD Rats

All rats were volume-expanded and received IV infusions of 2% BSA made in D₅W. Following a 1h equilibration period, the following groups of rats were studied: (1) Control (N=8): rats received IV infusions of 2% BSA in D₅W and RI infusions of D₅W throughout the study. (2) Systemic C-21(N=8): rats received 3 cumulative IV infusions of C-21 (100, 200, and 300 ng/kg/min; each dose for 30 min) and RI infusions of D₅W during the experimental periods following a 30 min control period in which 2% BSA in D₅W was infused in the IV and D₅W into the RI space. Kidneys were harvested at the end of the study for Western blot analysis.
(5) Effects of Systemic C-21 Infusion on RPTC AT$_2$R Translocation and NHE-3 Retraction and Cellular Internalization in the Absence of Systemic AT$_1$R Blockade in Volume-Expanded Female SD Rats

All rats were volume-expanded and received IV infusions of 2% BSA made in D$_5$W. Following a 1h equilibration period, the following groups of rats were studied: (1) Control (N=54): rats received IV infusions of 2% BSA in D$_5$W and RI infusions of D$_5$W for both 30 min periods. (2) Systemic C-21 (N=54): rats received 30 min systemic IV infusion of C-21 (100 ng/kg/min) and RI infusion of D$_5$W during the experimental period following a 30 min control period in which 2% BSA in D$_5$W was infused in the IV and D$_5$W into the RI space. At the end of the experimental period, the rat was perfused and processed for immunofluorescence and immuno-electron microscopy.

(6) Effects of Systemic C-21 Infusion ± Intrarenal Infusion of AT$_2$R Antagonist PD ± Systemic AT$_1$R Blockade on U$_{NaV}$ and MAP in Na$^+$-Loaded Female and Male SD Rats

All rats were Na$^+$-loaded and received IV infusions of 2% BSA made in 0.9% saline. Following a 1h equilibration period, the following groups of rats were studied: (1) Female Systemic C-21 (N=10): rats received 3 cumulative IV infusions of C-21 (100, 200, and 300 ng/kg/min; each dose for 30 min) and RI infusions of D$_5$W during the experimental periods following a 30 min control period in which 2% BSA in 0.9% saline was infused in the IV and D$_5$W into the RI space. (2) Female Systemic C-21 + Intrarenal PD (N=6): rats received 3 cumulative IV infusions of C-21 and RI infusion of
PD (10 µg/kg/min) during the experimental periods following a 30 min control period in which 2% BSA in 0.9% saline was infused in the IV and D₅W into the RI space. (3) Female Systemic C-21 + Systemic CAND (N=6): rats followed the same procedure as Group 1, but in the presence of 24h systemic AT₁R blockade with CAND. (4) Male Systemic C-21 (N=8): rats followed the same procedure as Group 1. (5) Male Systemic C-21 + Intrarenal PD (N=6): rats followed the same procedure as Group 2. (6) Male Systemic C-21 + Systemic CAND (N=8): rats followed the same procedure as Group 3. \(U_{\text{NaV}}\) and MAP were reported for the control and final infusion periods.

(7) Effects of Intrarenal C-21 infusion ± Intrarenal Infusion of AT₂R Antagonist PD on \(U_{\text{NaV}}\) and MAP in the Absence of Systemic AT₁R Blockade in Volume-Expanded Female SD Rats

All rats were volume-expanded and received IV infusions of 2% BSA made in D₅W throughout the study. Following a 1h equilibration period, the following groups of rats were studied: (1) Control (N=8): rats received RI infusion of D₅W throughout the study. (2) C-21 (N=8): rats received RI infusion of C-21 (20, 40, and 60 ng/kg/min; each dose for 30 min) following a 30 min RI infusion of D₅W. (3) C-21 + Intrarenal PD (N=8): rats received RI co-infusion of C-21 + PD (10 µg/kg/min) following a 30 min RI infusion of D₅W. \(U_{\text{NaV}}\) and MAP were measured for each period.

(8) Effects of Chronic Systemic C-21 infusion on 24h \(U_{\text{NaV}}\) and MAP in Female Wild Type (WT; C57BL/6) and AT₂R-Null Mice
Four groups of mice were studied: (1) WT D$_5$W (N=8): mice received continuous systemic infusions of D$_5$W for 7d. (2) WT C-21 (N=8): mice received continuous systemic infusions of C-21 (300 ng/kg/min) for 7d. (3) AT$_2$R-Null D$_5$W (N=8): mice followed the same protocol as Group 1. (4) AT$_2$R-Null C-21 (N=8): mice followed the same protocol as Group 2. MAP was measured daily by telemetry. For the effects of systemic C-21 infusion on 24h $U_{Na}V$, a separate group of mice were studied in the identical 4 groups of 8 mice each as indicated previously. Mice were housed continuously in metabolic cages for 8d. 24h $U_{Na}V$ was measured at baseline and days 1, 3, and 7 thereafter.

(9) Effects of Chronic Intrarenal C-21 Infusion on SBP and 24h $U_{Na}V$ in Ang II-Dependent Hypertension in Female SD Rats

Two groups of rats were studied: (1) Ang II + Intrarenal D$_5$W (N=8): rats received continuous systemic infusion of Ang II (200 ng/kg/min) +RI D$_5$W infusion for 7d. (2) Ang II + Intrarenal C-21 (N=6): rats received continuous systemic infusion of Ang II +RI C-21 (60 ng/kg/min) infusion for 7d. Mean SBPs were measured daily by telemetry. For the effects of systemic C-21 infusion on 24h $U_{Na}V$, a separate group of rats were studied in the identical 2 groups (Ang II + Intrarenal D$_5$W; N=6 and Ang II + Intrarenal C-21; N=7) as indicated previously. Rats were housed continuously in metabolic cages for 8d for urine collections.

Supplemental References


Online Figure 1: Western blot analysis of renal AT$_2$R protein expression (Alomone Labs antibody) in response to systemic vehicle (□) or C-21 (■) infusion (100, 200, and 300 ng/kg/min) in volume-expanded female SD rats in the absence of systemic AT$_1$R blockade. **Panel A.** Total cortical membrane AT$_2$R protein expression normalized to GAPDH. **Panel B.** Renal proximal tubule cell (RPTC) apical membrane AT$_2$R protein expression normalized to GAPDH. **Panel C.** Mouse adrenal whole cell AT$_2$R protein expression from female wild-type (WT; C57BL/6) and AT$_2$R-null mice verifying antibody specificity. Data represent mean ± 1 SE. **P<0.01 compared to control.**
Online Figure II: Western blot analysis of phosphoERK 1/2 and total cortical membrane ERK 1/2 (Panels A and B), phosphoSrc (Tyr 416) and total cortical membrane Src (Panels C and D), and phosphoαNa⁺/K⁺ATPase (NKA) and total cortical αNKA (Panels E and F) in response to systemic vehicle (□) and C-21 (■) infusion (100, 200, and 300 ng/kg/min) in volume-expanded female SD rats in the absence of systemic AT₁R blockade. All blots are normalized to GAPDH. Data represent mean ± 1 SE. *P<0.05 and **P<0.01 compared to control.
Online Figure III: Panel A. Urine Na⁺ excretion ($U_{Na^+}$) in Na⁺-loaded female and male SD rats in response to the following conditions: (□) female systemic C-21 infusion (100, 200, and 300 ng/kg/min), (■) female systemic C-21 infusion + renal interstitial (RI) infusion of PD (10 µg/kg/min), (▲) female systemic C-21 infusion in the presence of concurrent systemic AT₁R blockade, (●) male systemic C-21 infusion, (▲) male systemic C-21 infusion + RI infusion of PD, and (●) male systemic C-21 infusion in the presence of concurrent systemic AT₁R blockade. Results are reported as µmol/min. Panel B. Mean arterial pressure (MAP) in response to the conditions in Panel A. Results are reported as mm Hg. Data represent mean ± 1 SE. **P<0.01 and
****P<0.0001 from respective control period.  xP<0.05 from female systemic C-21 period.  +P<0.0001 from corresponding periods.

ONLINE FIGURE IV

Online Figure IV: Panel A. Urine Na⁺ excretion (U_{Na}V) in volume-expanded female SD rats in response to the following conditions: (□) time control, (■) renal interstitial (RI) C-21 infusion (20, 40, and 60 ng/kg/min), and (▲) RI co-infusion of C-21 infusion + PD (10 µg/kg/min). Results are reported as µmol/min and depict the control and final C-21 infusion period. Panel B. Mean arterial pressure (MAP) in response to the conditions in Panel A. Results are reported as mm Hg. Data represent mean ± 1 SE. *P<0.05 and **P<0.01 from respective control period.
Online Figure V: 24h urine Na⁺ excretion (U_{NaV}) in female mice with the following systemic treatments: (○) wild-type (WT; C57BL/6) with systemic D₃W infusion, (●) WT with systemic C-21 (300 ng/kg/min) infusion, (□) AT₂R-null with systemic D₃W infusion, and (■) AT₂R-null with systemic C-21 infusion. Results are reported as µmol/min and measured at day 0, 1, 3, and 7 of treatment. Data represent mean ± 1 SE. *P<0.05 and ***P<0.001 from corresponding AT₂R-null with systemic C-21 time period.
Online Figure VI: Mean arterial pressure (MAP) in female mice with the following systemic treatments: (〇) wild-type (WT; C57BL/6) with systemic D₅W infusion, (●) WT with systemic C-21 (300 ng/kg/min) infusion, (□) AT₂R-null with systemic D₅W infusion, and (■) AT₂R-null with systemic C-21 infusion. Results are reported as mm Hg and measured daily for 7d. Data represent mean ± 1 SE.