AT₂ Receptor Activation Prevents Sodium Retention and Reduces Blood Pressure in Angiotensin II–Dependent Hypertension


Rationale: Compound 21 (C-21) is a highly selective nonpeptide angiotensin AT₂ receptor (AT₂R) agonist.

Objective: To test the hypothesis that chronic AT₂R activation with C-21 induces natriuresis via an action at the renal proximal tubule (RPT) and lowers blood pressure (BP) in experimental angiotensin II (Ang II)–dependent hypertension.

Methods and Results: In rats, Ang II infusion increased both sodium (Na⁺) retention and BP on day 1, and BP remained elevated throughout the 7-day infusion period. Either intrarenal or systemic administration of C-21 prevented Ang II–mediated Na⁺ retention on day 1, induced continuously negative cumulative Na⁺ balance compared with Ang II alone, and reduced BP chronically. The effects of C-21 are likely to be mediated by action on the RPT as acute systemic C-21–induced natriuresis was additive to that induced by chlorothiazide and amiloride. At 24 hours of Ang II infusion, AT₂R activation with C-21, both intrarenally and systemically, translocated AT₂Rs from intracellular sites to the apical plasma membranes of RPT cells without altering the total cellular pool of AT₂Rs and internalized/inactivated major RPT Na⁺ transporters Na⁺-H⁺-exchanger-3 and Na⁺/K⁺-ATPase. C-21 lowered BP to a similar degree whether administered before or subsequent to the establishment of Ang II–dependent hypertension.

Conclusions: Chronic AT₂R activation initiates and sustains receptor translocation to RPT apical plasma membranes, internalizes/inactivates Na⁺-H⁺-exchanger-3 and Na⁺/K⁺-ATPase, prevents Na⁺ retention resulting in negative cumulative Na⁺ balance, and lowers BP in experimental Ang II–induced hypertension. Acting uniquely at the RPT, C-21 is a promising candidate for the treatment of hypertension and Na⁺-retaining states in humans. (Circ Res. 2016;119:532-543. DOI: 10.1161/CIRCRESAHA.116.308384.)

Key Words: blood pressure ▪ hypertension ▪ kidney ▪ natriuresis ▪ sodium channels

The renin–angiotensin system is a complex hormonal system composed of multiple enzymes, peptides, and receptors controlling sodium (Na⁺) excretion and blood pressure (BP). Angiotensin II (Ang II), its major effector peptide, acts at 2 major angiotensin receptors, the type-1 (AT₁R) and type-2 (AT₂R) receptors. The vast majority of Ang II biological actions are mediated by AT₁Rs, including vasoconstriction, antinatriuresis, cellular differentiation and growth, aldosterone secretion, and sympathetic nervous system activation, each of which can increase BP. In contrast, AT₂Rs generally induce the opposite effects, including vasodilation, natriuresis, cellular differentiation, and growth inhibition. Because AT₁Rs are expressed at a higher level than AT₂Rs in most adult tissues, including vasculature and the kidney, AT₁R actions usually predominate in vivo, especially under baseline unstimulated conditions. However, on renin–angiotensin system stimulation or during AT₁R blockade, when unblocked AT₂Rs are exposed to high levels of angiotensin, AT₂R-mediated vasodilation and natriuresis can readily be demonstrated.

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According to the Guyton hypothesis, the kidneys play a critical role in the control of BP by regulating Na⁺ excretion. Under normal physiological conditions, a primary increase in renal Na⁺ retention expands extracellular fluid volume and increases BP. However, the increase in BP in turn is offset by pressure-induced natriuresis, returning BP to or toward its original baseline level. The development of hypertension requires an abnormality in pressure natriuresis, wherein the increase in BP is accompanied by a natriuretic response that is insufficient to lower BP to normal.

The renal proximal tubule (RPT) is a critical nephron segment in the development of hypertension induced by Ang II.
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>AP2</td>
<td>adaptor protein 2</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AT₁R</td>
<td>angiotensin type-1 receptor</td>
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<td>AT₂R</td>
<td>angiotensin type-2 receptor</td>
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<tr>
<td>BP</td>
<td>blood pressure</td>
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<tr>
<td>C-21</td>
<td>compound 21</td>
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<tr>
<td>cAMP</td>
<td>adenosine cyclic 3’,5’-monophosphate</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
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<tr>
<td>NHE-3</td>
<td>sodium-hydrogen exchanger-3</td>
</tr>
<tr>
<td>NKA</td>
<td>sodium/potassium ATPase</td>
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<tr>
<td>PD</td>
<td>PD-123319</td>
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<tr>
<td>Phospho-NHE-3</td>
<td>phosphorylated sodium-hydrogen exchanger-3</td>
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<tr>
<td>RPT</td>
<td>renal proximal tubule</td>
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<td>RPTC</td>
<td>renal proximal tubule cell</td>
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<tr>
<td>SBP</td>
<td>systolic blood pressure</td>
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<tr>
<td>Src</td>
<td>Src family kinase</td>
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<td>UₙV</td>
<td>urinary sodium excretion</td>
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Gurley et al. have demonstrated in cross-transplantation studies in renal tubule-selective AT₁R knockout mice that AT₁Rs must be present in the RPT to sustain a hypertensive response to continuous systemic Ang II infusion. Li and Zhuo recently have confirmed the critical importance of RPT AT₁Rs to control BP through their actions to increase Na⁺ reabsorption by demonstrating in AT₁R-null mice that transfer of AT₁Rs into RPT cells (RPTC) enables a hypertensive response to Ang II. AT₂Rs are expressed in the adult kidney RPT. Recently, we demonstrated that acute systemic administration of the highly selective nonpeptide AT₂R agonist compound 21 (C-21) induces natriuresis by activating and recruiting AT₂Rs to the apical plasma membranes of RPTCs in vivo. C-21-induced AT₂R activation evoked a bradykinin-NO-cGMP signaling cascade that stimulated downstream signaling mediators Src kinase and extracellular signal-related kinase (ERK), internalizing/inactivating major RPT Na⁺ transporters Na⁺/H⁺ exchanger-3 (NHE-3) and Na⁺/K⁺-ATPase (NKA) resulting in natriuresis. Preliminary data indicated that AT₂R activation might prevent Na⁺ retention and lower BP by improving the pressure–natriuresis relationship in Ang II–dependent hypertension.

The present study was designed to explore in depth the therapeutic potential of AT₂R activation in the Ang II infusion model of experimental hypertension in the rat. We hypothesized that AT₂R activation would increase Na⁺ excretion and prevent or reverse hypertension chronically in this model via an action in the RPT. Here, we show that both systemic and intrarenal AT₂R activation with C-21 reduces BP and augments cumulative Na⁺ excretion chronically in a model of Ang II–dependent hypertension. Our results suggest that long-term AT₂R activation is a potential new approach in the treatment of Na⁺/fluid-retaining states and hypertension in humans.

**Methods**

Please see the Online Data Supplement for detailed methods (telemetric BP probe and systemic and intrarenal mini-pump implantation for chronic systemic and intrarenal C-21 infusion studies; respectively; standard protocol for acute in vivo studies; renal cortical interstitial infusion and BP measurements for acute in vivo studies; total renal cortical homogenate preparation and Western blot analysis; RPTC apical membrane isolation and Western blot analysis; in vivo kidney perfusion and fixation procedure; confocal immunofluorescence 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 experiments were conducted on 12-week-old female Sprague-Dawley rats (Harlan; n=182). Female animals were used to maximize the renal expression of AT₂Rs. 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).

**Urine Na⁺ and Plasma Potassium Measurements**

Urinary Na⁺ and plasma potassium concentrations were measured using a flame photometer (Instrumentation Laboratory-943) and reported as μmol/min and mmol/L, respectively. Cumulative Na⁺ balance was measured as the amount of Na⁺ consumed minus urinary sodium excretion (UₙV) and reported as mEq.

**Pharmacological Agents**

C-21 (60 ng/kg/min for chronic intrarenal studies, 300 ng/kg/min for chronic systemic studies, and 100, 200, and 300 ng/kg/min for acute systemic studies; Vicore Pharma), a highly selective, nonpeptide AT₂R agonist (Kᵢ=0.4 mol/L) was used to activate intrarenal and systemic AT₂Rs. C-21 demonstrates 25000-fold selectivity at AT₂Rs compared with AT₁Rs. C-21 was administered systemically at doses that are selective for AT₂Rs in the rat. The intrarenal dose of C-21 was chosen as 20% of the systemic dose based on renal blood flow. Ang II (200 ng/kg/min; Bachem) was used to induce Ang II–dependent hypertension. PD-123319 (PD; 10 µg/kg/min; Parke-Davis), a specific AT₂R antagonist (IC₅₀=2×10⁻⁸ mol/L and >1×10⁻⁴ mol/L for AT₂R and AT₁Rs, respectively), was used to block intrarenal AT₂Rs. Amiloride (0.8 µg/kg/min; Torcix) was used to inhibit intrarenal epithelial sodium channel activity. Chlorothiazide (0.1 µg/kg/min; Sigma) was used to inhibit intrarenal sodium-chloride channel activity. Amiloride and chlorothiazide doses were administered on the basis of dose-ranging studies with target incremental natriuretic responses of 1 to 2 µmol/min.

**Statistical Analysis**

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

**Results**

**Effects of Chronic Intrarenal and Systemic C-21 Infusion on Systolic BP, Diastolic BP, Activity Levels, Cardiac Output, and Total Peripheral Vascular Resistance in Ang II–Dependent Hypertension**

We first determined whether direct intrarenal administration of C-21 would lower BP in the Ang II infusion model. As demonstrated in Figure 1A, systemic Ang II infusion (200 ng/kg/min) increased systolic BP (SBP) from 126±3 to 156±4 mmHg on day 1 (F=35.6; P<0.0001) and to 187±2 mmHg at the end of the 7-day infusion period (F=23.2; P<0.0001). As shown in Online Figure 1A, Ang II induced a parallel increase in diastolic BP from 91±1 to 122±3 mmHg (F=85.6;
P<0.0001) on day 1 and to 134±7 mmHg (F=7.7; P<0.0001) on day 7. Concurrent intrarenal administration of AT,R antagonist PD for 7 days did not significantly change the pressor response to Ang II (Figure 1A; Online Figure 1A), although PD did cause a nonsignificant increase in SBP. Chronic intrarenal administration of the nonpeptide AT2R agonist C-21 abolished the systolic pressor response to systemic Ang II on day 1 (F=7.03; P<0.01) and throughout the 7-day infusion period (F=4.54; P<0.001) with a parallel reduction in diastolic BP (Online Figure IA).

Next, we determined whether systemic C-21 infusion would induce a similar depressor action as intrarenal infusion. Systemic C-21 again abolished the increase in SBP in response to Ang II on infusion day 1 (F=18.54; P<0.001) and throughout the 7-day infusion period (F=7.33; P<0.001) with a parallel reduction of diastolic BP (Online Figure IA). There was no significant difference in hypotensive responses to intrarenal versus systemic C-21 administration. Activity levels (Online Figure IB) were not significantly changed by Ang II, C-21, or PD. Similarly, cardiac output and total peripheral vascular resistance measurements (Online Figure II) were not significantly influenced by any of the experimental agents.

Effects of Chronic Intrarenal and Systemic C-21 infusion on 24-Hour UNaV and Cumulative Na+ Balance in Ang II–Dependent Hypertension

As shown in Figure 1B, systemic Ang II administration reduced 24-hour UNaV from 0.92±0.04 to 0.33±0.06 μmol/min (F=61.89; P<0.0001) on day 1. Subsequently, there was no significant difference in UNaV responses to Ang II or vehicle control. Although the antinatriuretic effects of systemic Ang II in the presence or absence of PD were similar during the first 24 hours, intrarenal PD administration enhanced the antinatriuretic response to systemic Ang II (F=6.76; P<0.001) during the entire 6-day collection period. Intrarenal administration of C-21 abolished the antinatriuretic response to systemic Ang II on day 1 (F=5.05; P<0.05) and reduced Ang II–mediated Na+ retention during the entire 6-day period (F=4.71; P<0.01).

Cumulative Na+ balance is depicted in Online Figure III. In response to systemic Ang II infusion, cumulative Na+ balance increased from 0 to 0.32±0.36 mEq on day 1 after which Na+ balance returned to control levels for the remaining 5 days of study. Concurrent administration of intrarenal PD induced a similar degree of positive Na+ balance on day 1 but resulted in a more positive Na+ balance for the entire study period (F=2.5; P<0.05) than Ang II alone. Intrarenal C-21 abolished the Ang II–induced positive Na+ balance on day 1 (F=10.78; P<0.01) and resulted in a sustained negative Na+ balance state for the entire study compared with that induced by Ang II alone (F=3.13; P<0.05).

Effects of Chronic Systemic C-21 Infusion on Plasma Renin Activity, Aldosterone, Potassium, and Creatinine in Ang II–Dependent Hypertension

Plasma renin activity (Online Figure IVA), potassium (Online Figure IVB), and creatinine (Online Figure IVD)
were not significantly changed by Ang II or systemic C-21 administration although there was a nonsignificant decrease in creatinine with Ang II that was not present when C-21 was coadministered with Ang II. Plasma aldosterone (Online Figure IVC), as expected, was markedly increased by Ang II alone either in the absence or presence of systemic C-21.

**Effects of Acute Systemic C-21 Infusion±Intrarenal Infusion of Epithelial Sodium Channel Inhibitor Amiloride or Sodium-Chloride Channel Inhibitor Chlorothiazide on U_{NaV} and Mean Arterial Pressure in Volume-Expanded Rats**

We then determined whether the natriuretic actions of C-21, which occur largely in the RPT, would be additive to those of diuretics acting in the distal tubule and cortical collecting duct. In acute experiments in anesthetized rats, systemic C-21 administration induced a dose-dependent natriuretic response (F=19.3; P<0.001; Figure 2A). Epithelial sodium channel inhibitor amiloride administered intrarenally induced a similar dose-dependent natriuretic response (F=7.6; P<0.001). Combination of systemic C-21 and intrarenal amiloride induced a greater natriuretic response compared with either systemic C-21 (F=5.6; P<0.01) or intrarenal amiloride (F=7.1; P<0.001) alone. The U_{NaV} response to the combination of systemic C-21 and intrarenal amiloride was additive. Similarly, intrarenal chlorothiazide induced a dose-dependent natriuretic response (F=6.1; P<0.001). The combination of systemic C-21 and intrarenal chlorothiazide also induced a greater increase in U_{NaV} than either C-21 (F=7.9; P<0.001) or chlorothiazide (F=4.0; P<0.01) alone.

Again, the natriuretic response to C-21 and chlorothiazide was additive. As shown in Figure 2B, there was no significant change in mean arterial pressure from control with any of these agents in these acute experiments.

**Effects of Systemic or Intrarenal C-21 Infusion on 24-Hour Total Cortical Homogenate and RPTC Apical Plasma Membrane \( \text{AT}_{2}\)R Density in Ang II-Induced Hypertension**

To determine whether \( \text{AT}_{2}\)R activation with C-21 in the presence of Ang II still induces receptor translocation to the apical plasma membranes of RPTCs, we used confocal immunofluorescence microscopy and Western blot analysis. Figure 3A depicts the subcellular distribution of \( \text{AT}_{2}\)Rs as determined by confocal immunofluorescence microscopy in a representative set of rat RPTCs in response to systemic vehicle (control), systemic Ang II infusion, systemic Ang II+intrarenal C-21, and systemic Ang II+intrarenal C-21. The RPTC apical plasma membrane marker phalloidin (red), subapical membrane marker adaptor protein-2 (AP2; blue), and the \( \text{AT}_{2}\)R (green) are depicted sequentially left-to-right for all 4 conditions. As shown in the merged, enlarged merged, and enlarged merged panels and as green fluorescence in the enlarged \( \text{AT}_{2}\)R panel (P<0.001). Similar to systemic C-21 infusion, intrarenal administration
of C-21 increased the RPTC apical plasma membrane AT$_2$R fluorescence intensity ($P<0.01$).

As shown in Figure 4A, there was no significant difference among control, systemic Ang II, or systemic Ang II+systemic C-21 in total cortical homogenate AT$_2$R protein by Western blot analysis, although there was a nonsignificant trend to reduced levels in Ang II–infused animals. To complement the immunofluorescence studies on AT$_2$R recruitment, we isolated RPTC apical plasma membranes using the lectin pull-down method. Western blot analysis clearly demonstrated AT$_2$R recruitment to the RPTC apical plasma membranes in response to systemic C-21 ($P<0.01$; Figure 4B). There was a nonsignificant decrease in RPTC apical plasma membrane AT$_2$Rs in response to Ang II alone. Similarly, intrarenal C-21 increased apical membrane expression of AT$_2$R protein without changing total AT$_2$R levels as determined in cortical homogenates (Figure 4C and 4D; $P<0.01$). Ang II reduced total cortical AT$_2$R protein levels ($P<0.05$) in these experiments.

Effects of Systemic or Intrarenal C-21 Infusion on 24-Hour Total Cortical Homogenate and RPTC Apical Plasma Membrane NHE-3 and Phospho-NHE-3 in Ang II–Induced Hypertension

The next experiments were aimed at determining whether chronic C-21–induced natriuresis is related to internalization
and inactivation of the major RPTC apical membrane Na⁺ transporter NHE-3. Figure 5 demonstrates the subcellular distribution for NHE-3 in response to systemic or intrarenal C-21 in Ang II–infused rats. Representative confocal micrographs are shown in Figure 5A and quantification of the relative fluorescence intensity in Figure 5B. Ang II alone did not alter total NHE-3 fluorescence intensity or distribution in RPTCs. However, systemic C-21+Ang II infusion increased the retraction of NHE-3 to the RPTC subapical membranes (P<0.01; yellow color in the subapical layer marked by AP2). Similar to systemic C-21 infusion, intrarenal C-21 increased NHE-3 translocation into the subapical membranes of RPTCs (P<0.01).

Western blot analysis of NHE-3 is shown in Figure 6. Although Ang II or Ang II+systemic or intrarenal C-21 did not alter total NHE-3 protein with 24-hour total cortical homogenate and RPTC basolateral membrane NKA during Ang II–induced hypertension

Effects of Systemic or Intrarenal C-21 Infusion on 24-Hour Total Cortical Homogenate and RPTC Basolateral Membrane NKA During Ang II–Induced Hypertension

We next determined whether C-21 also induces internalization and inactivation of αNKA. Systemic Ang II infusion reduced basolateral intracellular fluorescence intensity of αNKA, and concurrent systemic C-21 infusion reversed this effect (P<0.0001; Online Figure VIIA and VIIIB). However, there was no significant effect on total αNKA protein with systemic Ang II alone or combined with systemic C-21 (Online Figure VIIIA). Similar results were demonstrated when C-21 was administered directly into the kidney instead of systemically (Online Figures VIIIA, VIIIB, and VIIIB). Phosphorylated NKA [Ser 23] (pSer⁵²⁵-NKA), an established marker for NKA internalization, was not significantly increased (pSer⁵²⁵-NKA; Online Figure IX) in response to Ang II administration but was markedly reduced in RPTCs in the

Figure 4. Western blot analysis of total cortical homogenate (A and C) and renal proximal tubule cell (RPTC) apical membrane (B and D) AT²R protein expression after 1 day of control ([]), systemic ANG II (sANG II; [●]), sANG II+systemic C-21 (sC-21; [■]), and sANG II+intrarenal (IR) C-21 (●) treatments.

All blots are normalized to GAPDH. Data represent mean±1 SE. **P<0.01 from control. ***P<0.001 from sANG II. &P<0.05 from control.
presence of systemic C-21 (P<0.0001). A similar reduction in pSer23-NKA in RPTCs was observed when systemic Ang II was combined with direct intrarenal administration of C-21 (P<0.0001).

**Effects of Systemic C-21 Infusion on Total Cortical Homogenate ERK 1/2, Phospho-ERK 1/2, Src, and Phospho-Src in Ang II–Induced Hypertension**

To determine whether AT₉R activation stimulates the Src/ERK signaling pathway, we performed Western blot analysis of total renal cortical homogenate. Western blot analyses of phospho-Src [Tyr 416] (pTyr⁴¹⁶,Src), Src, phospho-ERK 1/2 [Thr 202/Tyr 204] (pThr/Tyr²⁰²/²⁰⁴,ERK 1/2), and total ERK 1/2 are shown in Online Figure X. C-21 infusion did not significantly alter Src or ERK levels during these experiments.

**Effects of Chronic Systemic AT₉R Treatment on SBP After Establishment of Ang II–Induced Hypertension**

As shown in Online Figure XI, systemic administration of C-21, initiated on day 3 after the establishment of Ang II–induced hypertension, reduced both SBP (F=3.5; P<0.01) and diastolic BP (F=3.21; P<0.01) to baseline control levels by day 7.

**Discussion**

Our previous study in normal Sprague-Dawley rats and genetically engineered mice documented that acute systemic AT₉R activation with C-21 induces natriuresis by translocating AT₉Rs to the apical plasma membranes of RPTCs and internalizing/inhibiting major RPTC transporters NHE-3.
and NKA. This study builds on these findings by demonstrating in an Ang II infusion model of experimental hypertension that chronic AT2R activation with C-21 prevents initial renal Na+ retention and lowers BP during a 7-day period. In addition, C-21 was an effective natriuretic and antihypertensive agent whether administered systemically or directly into the kidney in this experimental model. We also demonstrated that continuous C-21 administration, both systemically and intrarenally, induced sustained negative cumulative Na+ balance accompanied by AT2R recruitment from intracellular sites to the apical plasma membranes of RPTCs and internalization/inactivation of major RPT Na+ transporters NHE-3 and NKA. Importantly, C-21–induced natriuresis was related to inhibition of Na+ transport in the RPT because it was additive to that observed with diuretics acting at either the distal tubule or the cortical collecting duct. We further demonstrated that C-21 was equally effective in lowering BP whether administered before or after Ang II–dependent hypertension had been established. Taken together, these results strongly support a role for AT2R agonists as natriuretic/diuretic agents that improve the pressure–natriuresis relationship and are potential candidates for the treatment of hypertension and disorders of Na+ and fluid retention in humans.

In the Ang II infusion model of experimental hypertension, major Na+ retention occurred within the first 24 hours of initiating the infusion, as previously reported, and Na+ excretion returned to approximately normal levels after 24 hours and beyond. Although the major effect of C-21 was to prevent Na+ retention at 24 hours, a continuing effect of C-21 to promote negative cumulative Na+ balance was observed throughout the study. Our data are highly consistent with a primary RPT action of C-21. Because we did not perform formal renal function studies, we cannot rule out a small renal hemodynamic effect as contributing to the natriuresis and reduced BP level. However, the action of C-21 was purely tubular in our previous acute study. Similarly, we cannot exclude a systemic vascular effect of C-21 as contributing, at least in part, to the chronic reduction in BP in these animals. However, the fact that C-21 prevented Ang II–induced hypertension in equivalent fashion whether the C-21 was administered systemically or intrarenally argues strongly for the primacy of the kidneys in causing the reduction in BP.

Importantly, concurrent use of AT1R antagonist was not required to uncover the chronic natriuretic and hypotensive actions of AT2R agonist administration in this study. Past studies indicated that cardiovascular and renal responses to AT2R activation are only observed when the renin–angiotensin system is activated, as is the case in this study, or when AT1Rs are concurrently blocked. Indeed, the hypotensive action of chronic C-21 was somewhat unexpected because most studies have failed to demonstrate BP-lowering effects in the absence of AT1R blockade, albeit in spontaneously hypertensive rats or stroke-prone spontaneously hypertensive rats. However, further studies on the effects of the combination of AT1R antagonist and AT2R agonist administration are clearly indicated to clarify the
therapeutic utility of AT$_2$R agonist/AT$_1$R antagonist combination in this model.

In this study, AT$_2$R activation in the presence of Ang II–induced translocation of AT$_2$Rs from intracellular sites to the apical plasma membranes of RPTCs 24 hours after initiation of C-21 infusion without any alteration in total cellular AT$_2$R expression, as validated by both confocal immunofluorescence studies and Western blot analysis of isolated RPTC apical membranes and total renal cortical cells. This is consistent with our previous demonstration that systemic C-21 administration acutely (at 30–90 minutes) recruits AT$_2$Rs to the apical plasma membranes of RPTCs.$^{16}$ Our previous studies have shown that natriuretic responses to endogenous renal AT$_2$R agonist Ang III are also accompanied by translocation of AT$_2$Rs along microtubules to the

Figure 7. Confocal micrographs (×600) of renal proximal tubule cell (RPTC) thin sections (5–8 μm) pSer$_{552}$-NHE-3 protein in response to 1 day of chronic systemic infusion of 5% dextrose in water (D W; control), systemic infusion of ANG II (sANG II; 200 ng/kg/min), sANG II-systemic infusion of C-21 (sC-21; 300 ng/kg/min), and sANG II+intrarenal (IR) infusion of C-21 (60 ng/kg/min). A, The first row of images represents control treatment, the second row represents sANG II treatment, the third row represents sANG II+sC-21 treatment, and the fourth row represents sANG II+IR C-21 treatment from a representative set of RPTCs. The first column depicts confocal autofluorescence. The second column depicts subapical membrane staining with adaptor protein-2 (AP2). The third column depicts pSer$_{552}$-NHE-3 staining. The fourth column depicts a merged image of confocal autofluorescence and pSer$_{552}$-NHE-3. The fifth column depicts an enlarged image of the square section in the merged image. The scale bars in the first and fifth columns represent 10 and 2 μm, respectively. B, The quantification of RPTC subapical pSer$_{552}$-NHE-3 fluorescence intensity performed on 20 independent measurements of RPTCs from a representative rat after control (□), sANG II (■), sANG II+sC-21 (▲), and sANG II+IR C-21 (■) treatments. Data represent mean±1 SE. ***P<0.001 from control. ++++P<0.0001 from sANG II. &P<0.05 from control.
epithelial cells, their recruitment and continuous apical plasma membrane expression may stabilize and reinforce the natriuretic response.

Of the Na+ filtered into the nephron, 67% is reabsorbed isotonically in the RPT. The principal apical membrane Na+ transporter in the RPT is NHE-3, which also participates in supporting flow-dependent glomerulotubular balance. NHE-3–null mice are hypovolemic and hypotensive, exhibit metabolic acidosis, reduced reabsorption of Na+, HCO3−, and fluid and increased mortality when subjected to low-Na+ intake. In light of the major physiological role of NHE-3 in the RPT, relatively small changes in its activity could have significant pathophysiological consequences. Indeed, NHE-3 is among the most highly regulated transport proteins of cell membranes, modulated by multiple physiological and pathological conditions. 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.

Acute regulation of NHE-3 occurs via changes in phosphorylation, membrane trafficking, and/or membrane localization acting on the existing cellular NHE-3 pool. The cytoplasmic loop of NHE-3 contains several phosphorylation sites that are targeted by different kinases. The cAMP-dependent protein kinase A phosphorylates NHE-3 in its carboxy terminus. Phosphorylation of serines 552 and 605 is required for maximum inhibition of NHE-3 by cAMP because mutation of these serines individually reduces the inhibitory effect on NHE-3 promoted by cAMP.

Retraction/internalization of NHE-3 is a marker of reduced NHE-3 activity. In our previous studies, we showed that acute systemic C-21 administration (30–90 minutes) retracts NHE-3 from the tips to the bases of apical microvilli and into the subapical membranes of RPTCs as a mechanism to reduce RPTC Na+ transport. In the present study, we demonstrated chronic (24 hours) C-21 administration induced retraction/internalization of NHE-3 by confocal fluorescence microscopy and Western blot analysis. We further demonstrated increased total cortical homogenate pSer552-NHE-3 and pSer605-NHE-3 levels, as established indicators of NHE-3 retraction/internalization.

In addition to NHE-3, we demonstrated a parallel internalization and inactivation of NKA, the major Na+ transporter across the basolateral membranes of RPTCs. We showed that Ang II markedly reduced intracellular NKA fluorescence intensity, whereas C-21 reversed this response without change in the total cellular pool of NKA. In the rat RPT, the α-1 subunit of NKA is phosphorylated at serine 23 by the action of protein kinase C. NKA phosphorylation at this site plays a major role both in the regulation of NKA enzyme activity and subcellular distribution, as demonstrated by mutation of this phosphorylation site. In this study, we demonstrated that C-21–induced NKA dephosphorylation at serine 23, indicating retraction and inactivation of NKA.

However, we found no evidence of chronic activation of Src or ERK signaling molecules downstream of AT1Rs. This contrasts with C-21–induced Src and ERK activation after acute systemic administration of C-21 in our previous study that is likely explained by the early response of these pathways to AT1R activation and cGMP, as demonstrated previously.

The results of this study encourage the evaluation of acute and chronic AT1R activation in the control of Na+ excretion and BP, with and without concurrent AT1R blockade, in the TGR(mRen2)27 rat, a model of human primary hypertension in which the endogenous tissue renin–angiotensin system is activated, as well as a volume-expanded hypertensive model (DOCA [11-deoxycorticosterone acetate]/salt hypertension). Future investigations will explore the potential role for AT1R agonists as natriuretic/diuretic agents in hypertension and disorders of Na+ and fluid retention in humans.

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Disclosures
None.

References


Novelty and Significance

What Is Known?

- Angiotensin II (Ang II) activates 2 major receptors, type-1 (AT₁R) and type-2 (AT₂R).
- AT₂R activation generally opposes the actions of Ang II via AT₁Rs.
- Within the kidney proximal tubule, acute AT₂R activation with nonpeptide agonist compound 21 (C-21) recruits AT₂Rs to apical plasma membranes and increases sodium (Na⁺) excretion in a bradykinin–nitric oxide–cyclic guanosine 3',5'-monophosphate (cyclic GMP)–dependent manner in normal Sprague-Dawley rats.

What New Information Does This Article Contribute?

- Chronic AT₂R activation (systemically or intrarenally) with C-21 increased Na⁺ excretion and normalized blood pressure (BP) in the Ang II–infusion model of experimental hypertension.
- Chronic AT₂R activation translocated AT₂Rs to apical plasma membranes, internalized and inactivated sodium transporters sodium-hydrogen exchanger-3 (NHE-3) and Na⁺/K⁺-ATPase (NKA) and functionally reduced sodium transport in renal proximal tubule cells.
- C-21 normalized BP whether administered previously or subsequent to the establishment of Ang II–dependent hypertension, and the natriuresis engendered by C-21 was additive to that of diuretics acting at the distal tubule (chlorothiazide) and cortical collecting duct (amiloride), indicating a primary RPT effect.

These findings indicate that AT₂R activation can lower BP chronically under conditions when the renin–angiotensin system is stimulated; thus, AT₂Rs are predicted to be legitimate therapeutic targets for hypertension in humans. Currently, no effective diuretic/natriuretic agent is available that acts in the RPT. AT₂R activation, therefore, may provide a complimentary nephron-specific site for diuresis/natriuresis in humans.
AT2 Receptor Activation Prevents Sodium Retention and Reduces Blood Pressure in Angiotensin II–Dependent Hypertension

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SUPPLEMENTAL MATERIAL

Standard Protocol for Acute *In Vivo* Studies

On the day of experimentation, the rats were anesthetized with inactin (100 mg/kg body weight) via intraperitoneal (IP) injection, and a tracheostomy was performed using polyethylene tubing (PE-240) to assist respiration. Direct cannulation of the right internal jugular vein using PE-10 tubing provided intravenous access through which 2% BSA made in D\textsubscript{5}W or C-21 (100, 200, and 300 ng/kg/min) made in 2% BSA 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 $U_{NaV}$.

Blood Pressure (BP) Measurements for Acute *In Vivo* Studies

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.

Renal Cortical Interstitial Infusion for Acute *In Vivo* Studies

An open-bore micro-infusion catheter (PE-10) was inserted under the renal capsule into the cortex of the left kidney to ensure intrarenal infusion of vehicle D\textsubscript{5}W or
pharmacological agent at 2.5 μL/min with a syringe pump (Harvard; model 55-222) as reported previously (1-5). Vetbond tissue adhesive was added to secure the catheter and prevent interstitial pressure loss in the kidney.

**Telemetric BP Probe, Systemic Mini-Pump Implantation, and Intrarenal Mini-Pump Implantation for Chronic Intrarenal Compound 21 (C-21) In Vivo Studies**

Rats were placed under short term anesthesia via IP injection of ketamine (100 mg/mL) and xylazine (20 mg/mL). Using sterile technique, a midline abdominal incision was made and the catheter of the DSI telemetry probe (PA-C40) was implanted directly into the descending aorta per manufacturer’s suggested protocol. The telemetry probe was anchored to the abdominal wall with suture and the right kidney was removed (so that substances later infused directly into the left kidney would not spill over to the opposite kidney confounding the results). After a 1 week equilibration period, the rat was placed under short term anesthesia as stated above 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 and connected to an osmotic mini-pump (Alzet, model 1007D) to infuse 5% dextrose in water (D₅W), C-21 (60 ng/kg/min), or PD-123319 (PD; 10 ng/kg/min). A second osmotic mini-pump (Alzet, Model 2001) was inserted into the subcutaneous (SC) pocket in the subscapular region in order to infuse D₅W or Ang II (200 ng/kg/min) systemically. Mean systolic blood pressure (SBP) mean diastolic blood pressure (DBP), and total activity were measured daily for 7 days at the same time each
day to prevent any diurnal variation in BP measurements. In a separate group of rats, the telemetry probe was not implanted and 24h urine Na$^+$ excretion ($U_{NaV}$) and cumulative Na$^+$ balance were measured daily for 6 days. In a third and fourth set of rats, kidneys were removed after 1 day of treatment for Western blot analysis or perfused for confocal microscopy respectively.

**Telemetric BP Probe and Systemic Mini-Pump Implantation for Chronic Systemic C-21 In Vivo Studies**

Rats were placed under short term anesthesia for the implantation of the DSI telemetry probe as described above. The right kidney was also removed so that the rats could be compared to the chronic intrarenally infused rats. After a 1 week equilibration, 7 day SC osmotic mini-pumps for Ang II (200 ng/kg/min) and C-21 (300 ng/kg/min) infusions were implanted in the subscapular region. Mean SBP, DBP, total activity, 24h $U_{NaV}$, cumulative Na$^+$ balance, and kidneys for Western blot analysis and confocal microscopy were measured and collected as described above.

**Telemetric BP Probe and Systemic Mini-Pump Implantation for Chronic Systemic C-21 Treatment Following Ang II-Induced Hypertension**

The procedure was as described for “Chronic Systemic C-21 In Vivo Studies” with the following modification. After the 1 week equilibration, a 7 day SC osmotic mini-pump infusing Ang II was placed. After SBP was measured on the third day, the rats were placed under short term anesthesia. Half of the rats received a 7 day SC osmotic
mini-pump infusing D$_5$W while the other half received a SC osmotic mini-pump infusing C-21. Mean SBP was then measured daily for the remaining 4 days.

**Total Cortical Homogenate Preparation and Western Blot Analysis for Chronic In Vivo Studies**

After 1 day of treatment, rats were anesthetized and the left kidney was removed. Slices of kidney cortex (~100 mg) were homogenized in Mammalian Protein Extraction Reagent (MPER; Thermo Scientific) 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: AT$_2$R (Chemicon; 1:200), NHE-3 (a generous gift of Dr. Peter Aronson Yale School of Medicine; 1:10), pSer$^{552}$-NHE-3 (Thermo Scientific; 1:500), pSer$^{605}$-NHE-3 (Thermo Scientific; 1:500), αNKA (Millipore; 1:1000), and or ERK 1/2 (Cell Signaling; 1:1000) pThr/Tyr$^{202/204}$-ERK 1/2 (Cell Signaling; 1:1000), Src (Cell Signaling; 1:1000), and pTyr$^{416}$-Src (Cell Signaling; 1:1000). Membranes were subsequently incubated with their respective HRP-conjugated secondary antibodies in 5% milk TBST (GE Healthcare; 1:2500) for 2h at 4°C. Signals were detected using chemiluminescense 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). Membranes were subsequently incubated with infrared secondary antibody (anti-mouse IRDye 680; 1:15,000) in LI-COR blocking buffer for 1h. Immunoreactivity was determined 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 for Chronic *In Vivo* Studies**

After 1 day of treatment, rats were anesthetized and the left kidney was removed. Slices of kidney cortex (~100 mg) were homogenized in detergent free lysis buffer with Halt protease and phosphatase inhibitor cocktail and then centrifuged at 3100 rpm for 10 min at 4ºC to remove cellular debris. Following a BCA assay to determine total protein, 1 mg of protein was resuspended in 10 mL of detergent free lysis buffer and incubated with 20 μg of biotinylated Lotus tetragonolobus agglutinin lectin (LTA; Vector Laboratories) on a 360º rocker for 2h at room temperature as previously published (1). A 50% vol/vol slurry (20 μL) of Ultralink Neutravidin beads (Pierce) was then added and incubated on a 360º rocker for 30 min at room temperature. The beads were then pelleted and thoroughly washed using a microcentrifuge spin cup filter. The LTA affinity–attached membranes were eluted by incubating the beads in the spin cup filter with 125 μL of 70ºC 2X sample buffer for 10 min and Western blot analysis was performed as previously described (1). The membranes were incubated with AT₂R primary antibody (1:200) and or NHE-3 (1:10) in 5% milk TBST followed by the respective HRP-conjugated secondary antibody as mentioned above. Membranes were
subsequently incubated with GAPDH antibodies as described previously for normalization.

**Kidney Perfusion and Fixation Procedure for Chronic In Vivo Studies**

After 1 day of treatment, 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 Mcllwaine 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 4ºC. 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 by the University of Virginia Research Histology Core and stored at -80ºC until stained.

**Confocal Immunofluorescence Microscopy for Chronic In Vivo Studies**

After the kidney sections had been spotted onto slides and washed with TBS, they were permeabilized with 0.2% Triton-X in TBS or 1% SDS in TBS for 10 min. The sections were washed in TBS with 0.02% Tween-20 (TBST) and then blocked in 1% milk TBST or LI-COR blocking buffer for 1h at room temperature. The kidney sections were incubated with primary antibodies in 1% milk TBST or LI-COR blocking buffer overnight at 4ºC. After washing with TBST, ALEXA 647 conjugated donkey anti-rabbit, ALEXA 488 conjugated donkey anti-mouse secondary antibodies (Invitrogen; 1:500),
and or Texas-Red phalloidin (Invitrogen; 1:200) were added in 1% milk TBST or LI-COR blocking buffer for 1h at room temperature. The following antibody pairs were used in 1% milk TBST after permeabilization with 0.2% Triton-X detergent: (1) AT$_2$R (1:100), AP2 (Santa Cruz; 1:100), and phalloidin (Invitrogen; 1:200). (2) NHE-3 (Millipore; 1:2000) and AP2 (1:100). The following primary antibody pairs were used in LI-COR blocking buffer after permeabilization with 0.2% Triton-X detergent: (1) pSer$^{552}$-NHE-3 (1:100) and AP2 (1:100). (2) pSer$^{605}$-NHE-3 (1:100) and AP2 (1:100). The following antibody pair was used in 1% milk TBST after permeabilization with 1% SDS detergent: αNKA (1:100) and AP2 (1:100). The following antibody pair was used in LI-COR blocking buffer after permeabilization with 1% SDS detergent: pSer$^{23}$-NKA (Abcam; 1:100) and AP2 (1:100). 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 5 water immersion objective with a numeric aperature 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. Calculation of immunoreactive AT$_2$R within the RPTC brush border was 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. The immunoreactive pSer\textsuperscript{552}-NHE-3 and pSer\textsuperscript{605}-NHE-3 fluorescence intensity were measured at the subapical region of the RPTC. The immunoreactive αNKA fluorescence intensity was measured in the intracellular region of the RPTC, 4 μm from the basolateral membrane. 10 boxes were measured per RPTC. The immunoreactive pSer\textsuperscript{23}-NKA fluorescence intensity was calculated as total intercellular fluorescence intensity within the RPTC. For each antibody, quantifications were performed on 20 independent measurements of RPTCs from a rat and averaged.

**MRI Measurements**

Magnetic Resonance Imaging (MRI) was performed in the UVA molecular imaging core (UVAMIC) facility using the state-of-art 7T Bruker-Siemens scanner (ClinScan) for imaging rodents. Cardiac gated MRI was performed using the 7T ClinScan MRI. After attaching ECG leads to the arms of the rats, they were placed, under 1.5-2% isoflurane, in a prone position inside the radiofrequency coil. The heart rate, respiration, and core body temperature were continuously monitored using a Small Animal Monitoring and Gating system (Small Animal Instruments Inc., Stony Brook, NY). The left ventricular ejection function (LVEF) measurements were performed using ECG-triggered cine black blood pulse sequence with a slice thickness of 1.5 mm and an in-plane resolution of 100 x 100 μm\textsuperscript{2} (7,8). During each session, the entire LV was imaged with 7 contiguous short-axis slices. The black blood cine images were imported to a workstation and analyzed with SEGMENT (Medviso, AB, Sweden, open source) software. Specifically, the end-diastolic (ED) and end-systolic (ES) frames were
identified and thereafter the endocardial and epicardial contours were drawn on these frames for all the slices. Using the software, the ED volume (EDV), ESV, stroke volume (EDV-ESV), ejection fraction (EF%=(EDV-ESV)/EDV) and cardiac output (CO= heart rate x stroke volume) were measured. Following the imaging studies, the rats were anesthetized and MAP measurements were taken for 30 minutes according to the methods explained previously in “BP Measurements for Acute In Vivo Studies” Vascular resistance was computed by dividing the measured MAP by the CO.

ELISA Assay Kits

A creatine colorimetric assay kit (Cayman Chemicals), an aldosterone ELISA kit (Cayman Chemicals), and renin assay kit (Sigma) were used to measure plasma levels after 1 day of chronic systemic infusion of 5% dextrose in water (D5W; control), systemic infusion of ANG II (sANG II; 200 ng/kg/min), and sANG II + systemic infusion of C-21 (sC-21).

Specific Protocols:

(1) Effects of Chronic Systemic AT2R Stimulation, Chronic Intrarenal AT2R Stimulation, and Chronic Intrarenal AT2R Inhibition on SBP, DBP, Total Activity, 24h UNaV, and Cumulative Na+ Balance in Ang II-Dependent Hypertension

Five groups of rats were studied: (1) Control (N=10): rats received continuous systemic and intrarenal (IR) infusions of D5W for 7 days. (2) Systemic Ang II (sANG II; N=8): rats received continuous sANG II (200 ng/kg/min) and IR infusions of D5W for 7 days. (3) sANG II + Systemic C-21 (sC-21; N=6): rats received continuous sANG II +
sC-21 (300 ng/kg/min) infusions for 7 days. (4) sANG II + IR C-21 (N=9): rats received continuous sANG II and IR C-21 (60 ng/kg/min) infusions for 7 days. (5) sANG II + IR PD (N=6): rats received continuous sANG II and RI PD (10 μg/kg/min) infusions for 7 days. Mean SBP, DBP, and total activity were recorded daily for ~30 min between 1-3 pm by telemetry. For the effects of chronic systemic AT\(_2\)R stimulation, chronic intrarenal AT\(_2\)R stimulation, and chronic intrarenal AT\(_2\)R inhibition in the presence of sANG II on 24h U\(_{Na}\)V and cumulative Na\(^+\) balance measurements, a separate group of rats were studied in the identical 5 groups (Control (N=6), sANG II (N=7), sANG II + sC-21 (N=6), sANG II + IR C-21 (N=9), and sANG II + IR PD (N=6) as indicated previously. Rats were housed continuously in metabolic cages for 6 days for urine collections.

(2) Effects of Chronic Systemic C-21 infusion in the Presence of sANG II on Cardiac Output and Vascular Resistance, Plasma Aldosterone, Plasma Renin Activity, Plasma Potassium, and Serum Creatinine Measurements

Three groups of rats were studied: (1) Control (N=3): rats received continuous systemic and intrarenal (IR) infusions of D\(_5\)W for 1 day. (2) sANG II (N=3): rats received continuous sANG II (200 ng/kg/min) + IR infusions of D\(_5\)W for 1 day. (3) sANG II + sC-21 (N=3): rats received continuous sANG II + sC-21 (300 ng/kg/min) infusions for 1 day. In a separate set of rats (N=6 for each condition) plasma was collected after 1 day of treatment for creatinine, aldosterone, renin activity and potassium measurements.
Effects of Chronic Systemic and Chronic Intrarenal C-21 Infusion in the Presence of sANG II on Apical Plasma Membrane AT$_2$R and or NHE-3 and Total Cortical Homogenate AT$_2$R, NHE-3, pSer$^{552}$-NHE-3, pSer$^{605}$-NHE-3, αNKA, and or pSer$^{23}$-NKA, ERK 1/2, pThr/Tyr$^{202/204}$-ERK 1/2, Src, and pTyr$^{416}$-Src Protein Expression

Four groups of rats were studied: (1) Control (N=6): rats received continuous systemic and intrarenal (IR) infusions of D$_5$W for 1 day. (2) sANG II (N=6): rats received continuous sANG II (200 ng/kg/min) + IR infusions of D$_5$W for 1 day. (3) sANG II + sC-21 (N=6): rats received continuous sANG II + sC-21 (300 ng/kg/min) infusions for 1 day. (4) sANG II + IR C-21 (N=6): rats received continuous sANG II + IR C-21 (60 ng/kg/min) infusions for 1 day. After 1 day of experimental infusion, the kidneys were harvested for Western blot analysis. In a separate set of animals (N=4), the rat was perfused and processed for immunofluorescence.

Effects of Acute Systemic C-21 Infusion ± Intrarenal Infusion of E$_{Na}C$ inhibitor Amiloride ± Intrarenal Infusion of NCC inhibitor Chlorothiazide on U$_{Na}V$ and MAP in Volume Expanded 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=8): rats received IV infusions of 2% BSA and intrarenal (IR) infusions of D$_5$W throughout the study. (2) sC-21 (N=8): rats received 3 cumulative IV infusions of C-21 (100, 200, and 300 ng/kg/min; each dose for 30 min) in 2% BSA and IR infusion of D$_5$W during the experimental periods following a 30 min control period in which 2% BSA was
infused in the IV and D5W into the IR space. (3) sC-21 + IR Amiloride (N=6): rats received 3 cumulative IV infusions of C-21 and IR infusion of amiloride (0.8 µg/kg/min) during the experimental periods following a 30 min control period in which 2% BSA was infused in the IV and D5W into the IR space. (4) IR Amiloride (N=9): rats received IV infusions of 2% BSA and IR infusion of amiloride (0.8 µg/kg/min) during the 3 experimental periods following a 30 min control period in which 2% BSA was infused in the IV and D5W into the IR space. (5) sC-21 + IR Chlorothiazide (N=6): rats received 3 cumulative IV infusions of C-21 in BSA and IR infusion of chlorothiazide (0.1 µg/kg/min) during the experimental periods following a 30 min control period in which 2% BSA was infused in the IV and D5W into the IR space. (6) IR Chlorothiazide (N=8): rats received IV infusions of 2% BSA and IR infusion of chlorothiazide (0.1 µg/kg/min) during the 3 experimental periods following a 30 min control period in which 2% BSA was infused in the IV and D5W into the IR space. UNaV and MAP were measured for each period.

(5) Effects of Chronic Systemic AT2R Treatment on SBP and DBP Following Ang II-Induced Hypertension

Two groups of rats were studied: (1) sANG II Treatment Controls (N=4): rats received continuous sANG II (200 ng/kg/min) for 3 days followed by 4 days of sANG II + sD5W infusions. (2) sANG II + sC-21 Treatment (N=5): rats received continuous sANG II infusions for 3 days followed by 4 days of sANG II + sC-21 (300 ng/kg/min) infusions.
REFERENCES


Online Figure I: Panel A. Mean diastolic blood pressure (DBP) in response to chronic systemic infusion of 5% dextrose in water (D5W; control; ), systemic infusion of ANG II (sANG II; 200 ng/kg/min; ), sANG II + systemic infusion of C-21 (sC-21; 300 ng/kg/min; ), sANG II + intrarenal (IR) c-21( ), and sANG + IR PD ( ). Results are reported as mm Hg. Panel B. Total activity measurements in response to
conditions in **Panel A**. Results are reported as counts/min. Data represent mean ± 1 SE. **Panel A.** Day 1: sANG II vs. sANG II + sC-21, F=8.15, P<0.01. sANG II vs. sANG II + IR C-21, F=6.14, P<0.05. **All Days:** sANG II vs. sANG II + sC-21, F=3.09, P<0.01. sANG II vs. sANG II + IR C-21, F=3.30, P<0.01.

**Online Figure II: Panel A.** Cardiac output in response 1 day of chronic systemic infusion of 5% dextrose in water (D$_5$W; control; □) systemic infusion of ANG II (sANG II; 200 ng/kg/min; □), and sANG II + systemic infusion of C-21 (sC-21; 300 ng/kg/min; □).
Results are reported as mL/min. Panel B. Vascular resistance in response to the conditions in Panel A. Results are reported as mm Hg min/mL. Data represent mean ± 1 SE.

Online Figure III: Cumulative Na\(^+\) balance in response to chronic systemic infusion of ANG II (sANG II; 200 ng/kg/min; □), sANG II + systemic infusion of C-21 (sC-21; ▶), sANG II + intrarenal (IR) infusion of C-21 (60 ng/kg/min; ▪), and sANG II + IR infusion of PD (10 μg/kg/min; ▼). Results are reported as mEq. Data represent mean ± 1 SE. Day 1: sANG II vs. sANG II + sC-21, F=16.14, P<0.001. sANG II vs. sANG II + IR C-21,
Online Figure IV: Panel A. Plasma renin activity measurements in response to 1 day of chronic systemic infusion of 5% dextrose in water (D₅W; control; □) systemic infusion of ANG II (sANG II; 200 ng/kg/min; ■), and sANG II + systemic infusion of C-21 (sC-21; 300 ng/kg/min; □). Results are reported as ng/mL. Panel B. Plasma potassium measurements in response to the conditions in Panel A. Results are
reported as mmol/L. Panel C. Plasma aldosterone measurements in response to conditions in Panel A. Results are reported as ng/dL. Panel D. Plasma creatinine measurements in response to conditions in Panel A. Results are reported as mg/dL. Data represent mean ± 1 SE. *P<0.05 and **P≤0.01 from control.

Online Figure V: Confocal micrographs (600X) of renal proximal tubule cell (RPTC) thin sections (5-8 μm) pSer⁶⁰⁵-NHE-3 protein in response to 1 day of chronic systemic infusion of 5% dextrose in water (D₅W; control), systemic infusion of ANG II (sANG II;
200 ng/kg/min), sANG II + systemic infusion of C-21 (sC-21; 300 ng/kg/min), and sANG II + intrarenal (IR) infusion of C-21 (60 ng/kg/min). **Panel A.** The first row of images represents control treatment, the second row represents sANG II treatment, the third row represents sANG II + sC-21 treatment, and the fourth row represents sANG II + IR C-21 treatment from a representative set of RPTCs. The first column depicts confocal autofluorescence. The second column depicts subapical membrane staining with adaptor protein-2 (AP2). The third column depicts pSer<sup>605</sup>-NHE-3 staining. The fourth column depicts a merged image of confocal autofluorescence and pSer<sup>605</sup>-NHE-3. The fifth column depicts an enlarged image of the square section in the merged image. The scale bars in the first and fifth columns represent 10 and 2 μm, respectively. **Panel B.** The quantification of RPTC subapical pSer<sup>605</sup>-NHE-3 fluorescence intensity performed on 20 independent measurements of RPTCs from a representative rat following control ( ■ ), sANG II ( ■ ), sANG II + sC-21 ( ■ ), and sANG II + IR C-21 ( ■ ) treatments. Data represent mean ± 1 SE. ****P<0.0001 from control. +++P<0.0001 from sANG II. ^P<0.05 from control.
Online Figure VI: Western blot analysis of total cortical homogenate pSer$^{605}$-NHE-3 protein expression following 1 day of control ( ), systemic ANG II (sANG II; ), sANG II + systemic C-21 (sC-21; ), and sANG II + intrarenal (IR) C-21 ( ) treatments. All blots are normalized to GAPDH. Data represent mean ± 1 SE. *P<0.05 from control. +P<0.05 from sANG II.
Online Figure VII: Confocal micrographs (600X) of renal proximal tubule cell (RPTC) thin sections (5-8 μm) αNKA protein in response to 1 day of chronic systemic infusion of 5% dextrose in water (D₅W; control), systemic infusion of ANG II (sANG II; 200 ng/kg/min), sANG II + systemic infusion of C-21 (sC-21; 300 ng/kg/min), and sANG II + intrarenal (IR) infusion of C-21 (60 ng/kg/min). Panel A. The first row of images represents control treatment, the second row represents sANG II treatment, the third row represents sANG II + sC-21 treatment, and the fourth row represents sANG II + IR C-21 treatment from a representative set of RPTCs. The first column depicts brush
border membrane staining with phalloidin. The second column depicts subapical membrane staining with adaptor protein-2 (AP2). The third column depicts αNKA staining. The fourth column depicts a merged image. The fifth column depicts an enlarged image of the square section in the merged image. The sixth column depicts the enlarged image with αNKA staining only. The tiny white boxes encompass the intracellular regions that were quantified for αNKA intensity. The scale bars in the first and sixth columns represent 10 and 2 μm, respectively. Panel B. The quantification of RPTC intracellular αNKA fluorescence intensity performed on 20 independent measurements of RPTCs from a representative rat following control ( ), sANG II ( ■ ), sANG II + sC-21 ( ■ ), and sANG II + IR C-21 ( ○ ) treatments. Data represent mean ± 1 SE. **P<0.01 from control. +++P<0.001 and ++++P<0.0001 from sANG II. &&&&P<0.0001 from control.
**Online Figure VIII:** Western blot analysis of total cortical homogenate αNKA protein expression following 1 day of control ( ), systemic ANG II (sANG II; ), sANG II + systemic C-21 (sC-21; ), and sANG II + intrarenal (IR) C-21 ( ) treatments. All blots are normalized to GAPDH. Data represent mean ± 1 SE.

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**Online Figure IX:** Confocal micrographs (600X) of renal proximal tubule cell (RPTC) thin sections (5-8 μm) pSer^{23}-NKA protein in response to 1 day of chronic systemic infusion of 5% dextrose in water (D5W; control), systemic infusion of ANG II (sANG II;
200 ng/kg/min), sANG II + systemic infusion of C-21 (sC-21; 300 ng/kg/min), and sANG II + intrarenal (IR) infusion of C-21 (60 ng/kg/min). **Panel A.** The first row of images represents control treatment, the second row represents sANG II treatment, the third row represents sANG II + sC-21 treatment, and the fourth row represents sANG II + IR C-21 treatment from a representative set of RPTCs. The first column depicts confocal autofluorescence. The second column depicts subapical membrane staining with adaptor protein-2 (AP2). The third column depicts pSer^{23}-NKA staining. The fourth column depicts a merged image. The fifth column depicts an enlarged image of the square section in the merged image. The scale bars in the first and fifth columns represent 10 and 2 μm, respectively. **Panel B.** The quantification of RPTC total intracellular pSer^{23}-NKA fluorescence intensity performed on 20 independent measurements of RPTCs from a representative rat following control (□), sANG II (■), sANG II + systemic C-21 (sC-21; □), and sANG II + intrarenal (IR) C-21 (☐) treatments. Data represent mean ± 1 SE. ****P<0.01 from control. ++++P<0.0001 from sANG II.
**Online Figure X:** Western blot analysis of total cortical homogenate pThr/Tyr\(^{202/204}\)-ERK 1/2 (Panels A and B) and pTyr\(^{416}\)-Src and Src (Panels C and D) in response to 1 day of control ( ), systemic infusion of Ang II (sANG II; 200 ng/kg/min; ), and sANG II + systemic infusion of C-21 (sC-21; 300 ng/kg/min; ). All blots are normalized to GAPDH. Data represent mean ± 1 SE.
Online Figure XI: Panel A. Mean systolic blood pressure (SBP) in response to chronic systemic infusion of ANG II (sANG II; 200 ng/kg/min; □) and chronic systemic C-21 (sC-21; 300 ng/kg/min; ■) treatment following Ang II-induced hypertension. Results are reported as mm Hg. Panel B. Mean diastolic blood pressure (DBP) in response to conditions in Panel A. Results are reported as mm Hg. Data represent mean ± 1 SE.

Panel A. All Days: F=3.5, P<0.01. Panel B. All Days: F=3.21, P<0.01.