Intrarenal Angiotensin III Is the Predominant Agonist for Proximal Tubule Angiotensin Type 2 Receptors

Brandon A. Kemp, John F. Bell, Daniele M. Rottkamp, Nancy L. Howell, Weijian Shao, L. Gabriel Navar, Shetal H. Padia, Robert M. Carey

Abstract—In angiotensin type 1 receptor–blocked rats, renal interstitial (RI) administration of des-aspartyl\(^1\)-angiotensin II (Ang III) but not angiotensin II induces natriuresis via activation of angiotensin type 2 receptors. In the present study, renal function was documented during systemic angiotensin type 1 receptor blockade with candesartan in Sprague-Dawley rats receiving unilateral RI infusion of Ang III. Ang III increased urine sodium excretion, fractional sodium, and lithium excretion. RI coinfusion of specific angiotensin type 2 receptor antagonist PD-123319 abolished Ang III–induced natriuresis. The natriuretic response observed with RI Ang III was not reproducible with RI angiotensin (1-7) alone or together with angiotensin-converting enzyme inhibition. Similarly, neither RI angiotensin II alone or in the presence of aminopeptidase A inhibitor increased urine sodium excretion. In the absence of systemic angiotensin type 1 receptor blockade, Ang III alone did not increase urine sodium excretion, but natriuresis was enabled by the coinfusion of aminopeptidase N inhibitor and subsequently blocked by PD-123319. In angiotensin type 1 receptor–blocked rats, RI administration of aminopeptidase N inhibitor alone also induced natriuresis that was abolished by PD-123319. Ang III–induced natriuresis was accompanied by increased RI cGMP levels and was abolished by inhibition of soluble guanylyl cyclase. RI and renal tissue Ang III levels increased in response to Ang III infusion and were augmented by aminopeptidase N inhibition. These data demonstrate that endogenous intrarenal Ang III but not angiotensin II or angiotensin (1-7)–induced natriuresis via activation of angiotensin type 2 receptors in the proximal tubule via a cGMP–dependent mechanism and suggest aminopeptidase N inhibition as a potential therapeutic target in hypertension. (Hypertension. 2012;60:387-395.)

Key Words: angiotensin ■ sodium ■ natriuresis ■ kidney ■ receptor ■ cGMP

The basal state of renal sodium (Na\(^+\)) excretion (\(U_{NaV}\)) and its effect on blood pressure are regulated by the intrarenal renin-angiotensin system.\(^{1,2}\) Renin-angiotensin system actions are mediated largely by angiotensin II (Ang II) acting via 2 major receptors, angiotensin type 1 (AT\(_1\),R) and angiotensin type 2 (AT\(_2\),R). The activation of AT\(_1\)Rs by Ang II induces vasoconstriction and increases renal tubule Na\(^+\) reabsorption, sympathetic nervous system activation, and secretion of aldosterone, vasopressin, and endothelin.\(^{1,2}\) Collectively, these actions lead to the elevation of systemic blood pressure. Conversely, the activation of AT\(_2\)Rs results in vasodilation and natriuresis.\(^{3,5}\) Angiotensin (1-7) (Ang(1-7)) is another product of the renin-angiotensin system for which its role in the kidney has not fully been established. In vitro studies in rat renal proximal tubule cells demonstrate that Ang(1-7) inhibits Na\(^+\)/K\(^+\)-ATPase activity, suggesting that Ang(1-7) might induce natriuresis in vivo possibly mediated via AT\(_2\)Rs, the Ang(1-7) mas receptor, or both.\(^5\) However, whether Ang(1-7) reduces tubular Na\(^+\) transport and induces natriuresis in vivo remains uncertain.\(^5\)

Ang II, the principal effector peptide of the renin-angiotensin system, is metabolized within the kidney to des-aspartyl\(^1\)-Ang II (angiotensin III; Ang III) by aminopeptidase A (APA).\(^6\) Intrarenal Ang III has been demonstrated recently to be a preferred AT\(_2\),R agonist in the induction of natriuresis.\(^7-9\) The natriuretic effect of exogenous renal interstitial (RI) Ang III infusion is markedly augmented by coinfusion with the selective aminopeptidase N (APN) inhibitor PC-18, which blocks the degradation of Ang III.\(^8\) In contrast, RI Ang II infusion does not increase \(U_{NaV}\) unless APN is concomitantly inhibited, and this natriuretic response is abolished when Ang II metabolism to Ang III is blocked with the APA inhibitor EC-33.\(^9\)

Previous studies have localized the precursors of angiotensin peptide synthesis, including angiotensinogen, renin, and angiotensin-converting enzyme (ACE) mRNA, to the renal proximal tubule.\(^1\) Renal proximal tubule cells, which are responsible for reabsorption of \(\approx60\%\) of filtered Na\(^+\) in the nephron, express a high level of AT\(_2\)Rs, as verified by Western blot analysis and immunohistochemistry.\(^10,11\)

A major cell signaling pathway of AT\(_2\)Rs is the activation of the NO/soluble guanylyl cyclase/cGMP cascade, but this pathway is involved in the AT\(_2\),R natriuretic response is...
unknown. In the present study, we examined renal functional responses, RI fluid (RIF), and tissue Ang II and Ang III levels to direct RI Ang III infusion to determine whether Ang III induces natriuresis by acting at AT_{1}Rs in renal proximal tubule cells by a soluble guanylyl cyclase–dependent mechanism. We also determined the natriuretic effects of the following: (1) RI Ang III infusion in the presence or absence of the specific AT_{1}R antagonist PD-123319 (PD); (2) RI Ang(1-7) infusion in the presence or absence of ACE inhibition with enalaprilat (to inhibit Ang[1-7] metabolism); and (3) RI Ang II infusion in the presence of the APA inhibitor EC-33 used to block Ang II degradation and shunt the octapeptide to Ang(1-7) in rats with concurrent systemic AT_{1}R blockade. In addition, we studied the RI confuision of Ang III and APN inhibitor PC-18 in both the presence and absence of systemic AT_{1}R blockade to determine the influence of AT_{1}Rs on Ang III–induced natriuresis. In addition, we explored RI infusion of PC-18 alone in the presence of systemic AT_{1}R blockade to determine whether endogenous Ang III can induce natriuresis. The results of these studies demonstrate that endogenous intrarenal Ang III but not Ang II or Ang(1-7) induces natriuresis via activation of AT_{1}Rs in a soluble guanylyl cyclase–dependent manner, likely in the renal proximal tubule.

**Methods**

**Animal Preparation**

The experiments were conducted on 12-week-old female Sprague-Dawley rats except that the experiments in protocol 8 were conducted on 10-week-old male Sprague-Dawley rats. Please see the online-only Data Supplement for the following methods: renal cortical interstitial infusion, RIF microdialysis, mean arterial pressure (MAP) measurements, pharmacological agents, measurement of glomerular filtration rate (GFR) and fractional excretion of sodium (FE_{Na}), and lithium (FE_{Li}), measurement of Ang peptide levels in RIF and tissue, and AT_{1}R Western blot analysis. Except where described otherwise, all of the protocols were performed on rats subjected to 24-hour systemic AT_{1}R blockade with candesartan (CAND).

**Protocols**

**Effects of Unilateral RI Infusion of Ang III on U_{Na}V, MAP, GFR, FE_{Na}, and FE_{Li} in the Presence of Systemic AT_{1}R Blockade**

The left (experimental) kidney received RI infusion of Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg per minute; each dose for 30 minutes) after a 30-minute control period with RI vehicle (V; 5% dextrose in water) infusion. The right (control) kidney received RI V infusion for all of the periods. U_{Na}V, MAP, GFR, FE_{Na}, and FE_{Li} were quantified separately for the experimental and control kidneys for each infusion period.

**Effects of Unilateral RI Infusion of Ang III±AT_{1}R Antagonist PD on U_{Na}V and MAP in the Presence of Systemic AT_{1}R Blockade**

The left (experimental) kidney received RI infusion of Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg per minute; each dose for 30 minutes)±PD (10 μg/kg per minute) or systemic AT_{1}R blockade before the study. The left (experimental) kidney received RI infusion of Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg per minute)±PC-18 (25 μg/min) or Ang III+PC-18+PD (10 μg/kg per minute) throughout the 4 experimental periods after a 30-minute control period before RI infusion. The right (control) kidney received RI V infusion for all of the periods. U_{Na}V and MAP were quantified separately for the experimental and control kidneys for each infusion period.

**Effects of Unilateral RI Infusion of Ang III±APN Inhibitor PC-18 or Ang III+PC-18+AT_{1}R Antagonist PD on U_{Na}V and MAP in the Presence of Systemic AT_{1}R Blockade**

In this series, the rats were not subjected to 24-hour systemic AT_{1}R blockade before the study. The left (experimental) kidney received RI infusion of Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg per minute)±PC-18 (25 μg/min) or Ang III+PC-18+PD (10 μg/kg per minute) throughout the 4 experimental periods after a 30-minute control period with RI V infusion. The right (control) kidney received RI V infusion for all of the periods. U_{Na}V and MAP were quantified separately for the experimental and control kidneys for each infusion period.

**Effects of Unilateral RI Infusion of Ang III and Ang III+PC-18±PD or ±Soluble Guanylyl Cyclase Inhibitor 1-H-(1,2,4) Oxadiazolo-[4,3-c]Quinoxalin-1-One on RIF cGMP, U_{Na}V, and MAP in the Presence of Systemic AT_{1}R Blockade**

The left (experimental) kidney received RI infusion of Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg per minute; each dose for 30 minutes), Ang III+PC-18 (25 μg/min) or Ang III+PC-18+PD (10 μg/kg per minute) throughout the 4 experimental periods after a 30-minute control period with RI V infusion. The right (control) kidney received RI V infusion for all of the periods. RIF cGMP, U_{Na}V, and MAP were quantified separately for the experimental and control kidneys for each infusion period.

**Effects of Systematic AT_{1}R Blockade and Unilateral RI Infusion of Ang II or Ang III or Ang III+PC-18 on Renal Tissue and RIF Levels of Ang II and Ang III**

Male rats (n=11) anesthetized with Inactin underwent acute unilateral nephrectomy. After a 1-hour control period, the rats received a systemic infusion of compound 21 at 100, 200, and 300 ng/kg per minute, each dose for 30 minutes either in the presence or absence of RI infusion of PD (10 μg/kg per minute) or systemic AT_{1}R blockade before the study. U_{Na}V and MAP were quantified for the control and final infusion periods.

**Effects of Systematic AT_{1}R Blockade on Uninephrectomized Male Sprague-Dawley Rats**

The left (experimental) kidney received RI infusion of Ang II (3.5, 7.0, 14.0, and 28.0 nmol/kg per minute; each dose for 30 minutes)±enalaprilat (10 μg/kg per minute) or A-779 (3.5, 7.0, 14.0, and 28.0 nmol/kg per minute; each dose for 30 minutes) throughout the 4 experimental periods after a 30-minute control period with RI V infusion. The right (control) kidney received RI V infusion for all of the periods. U_{Na}V and MAP were quantified separately for the experimental and control kidneys for each infusion period.
or Ang III+PC-18 (25 μg/min) after a 30-minute control period with RI V infusion. Renal tissue and RIF Ang II and III levels were separated using high-performance liquid chromatography and quantified separately by radioimmunoassay, as described previously.12

**Statistical Analysis**

Data are presented as mean±1 SE. Statistical significance was determined by 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 Unilateral RI Infusion of Ang III on UNaV, MAP, GFR, FENa, and FELi in the Presence of Systemic AT1R Blockade**

As shown in Figure 1A, cumulative RI delivery of Ang III in the presence of systemic AT1R blockade with CAND induced statistically significant natriuresis at all of the infusion rates (F=34.0; P<0.0001). UNaV increased from a control of 0.07±0.01 μmol/min to 0.11±0.01 (P<0.01), 0.17±0.02 (P<0.01), 0.18±0.01 (P<0.01), and 0.13±0.02 μmol/min (P<0.05) at 3.5, 7.0, 14.0, and 28.0 nmol/kg per minute of Ang III, respectively. RI Ang III infusion had no significant effect on MAP (Figure 1B), as monitored by a direct carotid artery cannula, or GFR (Figure 1C), as measured by inulin clearance. The rise in UNaV was accompanied by a significant increase in both FENa (F=14.6; P<0.0001) and FELi (F=14.9; P<0.0001), FENa (Figure 1D) increased from a control of 0.26±0.04% to 0.57±0.06% (P=0.01), 0.87±0.26% (P<0.0001), and 0.70±0.10% (P=0.0003) at Ang III infusion rates of 7, 14, and 28 nmol/kg per minute, respectively. FELi (Figure 1E) increased in a dose-dependent fashion from a control of 24.4±2.7% to 36.9±1.9% (P=0.007), 47.4±4.0% (P=0.0002), and 53.3±4.6% (P<0.001) at 7, 14, and 28 nmol/kg per minute of Ang III, respectively. There were no changes in UNaV, MAP, FENa, or FELi in the V-infused control kidneys. Because most of the experiments used systemically blocked AT1Rs for 24 hours, we measured total whole-cell AT1-R protein expression in cortical kidney samples by Western

**Figure 1.** A, Urine Na+ excretion (UNaV) in response to renal interstitial (RI) infusion of vehicle (--; n=6) and angiotensin III (Ang III; ●; n=6) in the presence of systemic angiotensin type 1 receptor (AT1, R) blockade with candesartan. Results are reported as micromoles per minute. B, Mean arterial pressure in response to conditions in A. Results are reported as millimeters of mercury. C, Glomerular filtration rate (GFR) in response to conditions in A. Results are reported as milliliters per minute per gram of kidney weight. D, Fractional excretion of Na+ (FENa) in response to conditions in A. Results are reported as a percentage. Data represent mean±1 SE. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 from own control.
Effects of Unilateral RI Infusion of Ang III±AT_{2}R Antagonist PD on U_{Na}V and MAP in the Presence of Systemic AT_{1}R Blockade

As depicted in Figure S1A (online-only Data Supplement), cumulative RI administration of Ang III in the presence of systemic CAND induced a rise in U_{Na}V (F=23.5; P<0.001). U_{Na}V increased from a control value of 0.05 µmol/min to a peak of 0.14±0.01 µmol/min (P<0.0001) at an Ang III infusion rate of 14 nmol/kg per minute. The increase in U_{Na}V in response to Ang III was abolished by coinfusion of the heptapeptide with AT_{2}R antagonist PD. There was no significant change in MAP (Figure S1B) in any of these experiments or in U_{Na}V in the V-infused control kidneys (Figure S1A).

Effects of Unilateral RI Infusion of Ang (1-7)±ACE Inhibitor Enalaprilate or Ang(1-7) Antagonist A779 on U_{Na}V and MAP in the Presence of Systemic AT_{1}R Blockade

In contrast to the aforementioned results with Ang III, RI infusion of equimolar doses of Ang(1-7) (Figure S1A) did not increase U_{Na}V at any of the administered infusion rates. Similarly, RI coinfusion of Ang(1-7) and ACE inhibitor enalaprilate (to reduce the catabolism of infused Ang[1-7]) failed to increase U_{Na}V. There was also no significant change in MAP (Figure S1B) in any of these experiments or in U_{Na}V in the V-infused control kidneys (Figure S1A). We also demonstrated that Ang(1-7) does not engender natriuresis even in the absence of AT_{1}R blockade with CAND and that Ang(1-7) antagonist A-779 alone has no effect U_{Na}V (data not shown).

Effects of Unilateral RI Infusion of Ang II±APA Antagonist EC-33 on U_{Na}V and MAP in the Presence of Systemic AT_{1}R Blockade

In contrast to Ang III, RI infusion of Ang II (Figure S1A) failed to increase U_{Na}V. To determine whether an increase in endogenous Ang(1-7) is capable of increasing U_{Na}V, we administered APA inhibitor EC-33 interstitially to shunt endogenous Ang II to Ang(1-7). However, this maneuver also failed to increase U_{Na}V (Figure S1A). Similarly, there were no significant changes in MAP (Figure S1B) in any of these experiments or in U_{Na}V in the V-infused control kidneys (Figure S1A).

Effects of Unilateral RI Infusion of Ang III±APN Inhibitor PC-18 or Ang III+PC-18+AT_{2}R Antagonist PD on U_{Na}V and MAP in the Absence of Systemic AT_{1}R Blockade

After demonstrating that RI Ang III infusion induces significant natriuresis in the presence of systemically blocked AT_{1}Rs, we designed a set of experiments to determine whether Ang III–induced natriuresis depends on reduced AT_{1}R activation. As shown in Figure 2A, in the absence of systemic CAND infusion, Ang III alone failed to engender a natriuretic response. However, coinfusion with the APN inhibitor PC-18 enabled Ang III to induce a significant natriuresis (F=23.7; P<0.0001). Coinfusion of Ang III+PC-18+PD abolished the natriuresis induced by Ang III at 3.5, 7.0, and 14.0 nmol/kg per minute in the presence of APN inhibitor PC-18. During these experiments, no significant changes in MAP (Figure 2B) or U_{Na}V (Figure 2A) in the V-infused control kidneys were observed. Because PD decreased U_{Na}V to a value below V-infused control kidneys in these experiments, we also provide a control that PD alone does not reduce U_{Na}V (data not shown).
Figure 3 compares natriuretic and MAP responses to RI coinfusion of Ang III+PC-18 in the presence and absence of systemic AT1R blockade. As shown in Figure 3A, Ang III+PC-18 induced a robust, dose-dependent natriuresis in the presence of CAND (F=30.0; P<0.0001). UNaV increased from control values of 0.08±0.02 µmol/min in a stepwise fashion to a peak of 0.32±0.08 µmol/min (P<0.0001) at the highest infusion rate of Ang III (28 nmol/kg per minute). In the absence of systemic CAND, RI infusion of Ang III+PC-18 induced a comparable natriuresis to that with CAND at 3.5 and 7.0 nmol/kg per minute (P<0.05 from control values; P value not significant from CAND). However, in the rats with uninhibited AT1Rs, Ang III+PC-18 was unable to sustain the natriuresis at the higher Ang III infusion rates and for the duration of the study. The overall natriuretic response to Ang III+PC-18 was greater in the presence than in the absence of systemic AT1R blockade (F=6.7; P<0.0001). As shown in Figure 3B, MAP was significantly lower in the CAND-treated rats than in those without CAND (F=28.4; P<0.0001), but MAP remained unaltered during the RI Ang III+PC-18 infusions (P value not significant).

Effects of Unilateral RI Infusion of Ang III+PC-18±PD on UNaV and MAP in the Presence of Systemic AT1R Blockade
To determine whether endogenous Ang III is capable of inducing natriuresis, we infused PC-18 alone interstitially in AT1R-blocked rats. As shown in Figure 4A, PC-18 induced a sustained natriuresis (F=14.7; P<0.0001) with UNaV rising from 0.05±0.004 µmol/min to a peak of 0.09±0.008 µmol/min (P<0.05) after 60 minutes of infusion. The natriuretic response to RI PC-18 infusion was abolished by the coadministration of AT1R antagonist PD. During these experiments, no significant change in MAP (Figure 4B) or UNaV (Figure 4A) in the V-infused control kidneys was observed.

Effects of Unilateral RI Infusion of Ang III and Ang III+PC-18±PD or ±ODQ on RIF cGMP, UNaV, and MAP in the Presence of Systemic AT1R Blockade
As shown in Figure 5A, RI administration of Ang III increased RIF cGMP concentrations (F=8.49; P<0.0001). PC-18 did not significantly augment the cGMP response to Ang III. The increased cGMP response to Ang III+PC-18 was abolished with both PD and ODQ. Ang III alone and in the presence of PC-18 increased natriuresis (Figure 5B; F=6.5, P<0.0001; F=12.55, P<0.0001, respectively). Natriuretic responses to Ang III+PC-18 were numerically greater than with Ang III alone, although this did not achieve statistical significance (F=1.90; P=0.11). Ang III–induced natriuresis was abolished by both the AT1R blocker PD and by the guanylyl cyclase inhibitor ODQ. There was no significant difference in MAP in response to any of the interstitially infused agents (Figure 5C).

Effects of Systemic AT2R Agonist Compound 21 Alone With or Without Systemic AT1R Blockade and Combined With RI Infusion of PD on UNaV and MAP in Uninephrectomized Male Rats
Compound 21 increased UNaV from 0.46±0.08 to 6.22±1.33 µmol/min (P<0.01). This response was augmented by systemic AT1R blockade (P<0.05) and blocked by intrarenal PD (P<0.05; Figure S2). MAP did not change significantly after compound 21 or PD (data not shown).
Measurement of RIF and Renal Tissue Ang II and III Levels in Response to Systemic AT1R Blockade and RI Infusion of Ang II or Ang III Alone and +PC-18

As demonstrated in Figure 6, RIF Ang II (Figure 6A) and Ang III (Figure 6B) levels in the basal state were 76±12.0 and 77.4±12.1 fmol/mL, respectively (ratio, 1.0; Figure 6C). Systemic AT1R blockade with CAND did not significantly change RIF Ang II or Ang III levels. RI Ang II infusion in the presence of systemic CAND increased RI Ang II levels by 8-fold (P<0.001) compared with those during CAND alone. Although numerically increased, Ang III levels were not significantly different from those with systemic CAND alone. As expected, RI administration of Ang III, although not increasing RIF Ang II levels, increased Ang III levels from those with CAND alone (P<0.01). RI coadministration of Ang III+PC-18 further increased RIF Ang III levels to 789.7±115.9 fmol/mL (P<0.01) but unexpectedly increased Ang II levels significantly.

Baseline renal tissue levels of Ang II and Ang III (Figure S3A and S3B) were 313.0±60.9 and 77.8±18.1 fmol/g of kidney weight, respectively. Systemic AT1R blockade did not alter the levels of either peptide but significantly increased the ratio of Ang III:Ang II (Figure S3C). Ang II infusion increased both Ang II (P<0.01) and Ang III (P<0.05) and decreased the Ang III:II ratio (P<0.05). Ang III infusion increased levels of Ang III (P<0.0001) and also unexpectedly Ang II (P<0.01) but increased the ratio of Ang III:II. Ang III infusion in the presence of PC-18 also unexpectedly increased the levels of both peptides (each P<0.01) to approximately the same extent. Because Ang III increased the tissue concentrations of both peptides, we performed additional experiments in vitro in which Ang III was added to the assay system and Ang II was quantified. There was no significant cross-reactivity of Ang II with Ang III that could account for the increased renal Ang II in response to Ang III or Ang III+PC-18 administration (data not shown).

Discussion

The present study demonstrates the ability of Ang III to induce natriuresis via activation of soluble guanylyl cyclase by AT2Rs specifically localized in the renal proximal tubule cell. RI Ang III–induced natriuresis was abolished by coinfusion with the highly selective AT2R antagonist PD, specific for AT2Rs in the low doses used in this study. In the absence of changes in GFR, FENa increased during Ang III infusions, indicating that the observed natriuretic effects of Ang III are attributable to reduced tubular transport and not to systemic or intrarenal hemodynamic changes. In Na+-replete experimental animals, FENa increases in a dose-dependent fashion, strongly suggesting (but not unequivocally proving) that Ang III acts predominantly in the proximal tubule, where AT2Rs are highly expressed. Ang III–induced natriuresis was accompanied by increased RIF cGMP levels and was abolished by the soluble guanylyl cyclase inhibitor ODQ, strongly suggesting that Ang III–induced natriuresis is mediated by renal production of cGMP.

Our study also provides data showing that, at equimolar concentrations, neither intrarenal Ang II nor Ang(1-7) engenders natriuresis in the AT1R blocked rat. We also demonstrated no effect of the Ang(1-7) antagonist A-779, excluding an effect of endogenous Ang(1-7) on Na+ excretion. Previous studies from our laboratory demonstrated that Ang II was only capable of inducing natriuresis in the presence of an APN inhibitor to decrease Ang III metabolism. In the present study, Ang II, at
an equivalent molar infusion rate as Ang III, either in the presence or absence of APA inhibitor to retard Ang II degradation, was ineffective in increasing UNaV. In these studies, Ang(1-7) also, at the equivalent molar concentration, failed to induce natriuresis either when infused alone or in the presence of an ACE inhibitor to reduce its degradation. It is certainly possible that alternative pathways, such as neprilysin, can metabolize Ang(1-7), but ACE has been identified as a major degrading enzyme for this peptide. Also, APA inhibition, which would be expected to increase Ang II, which would then be available for conversion to Ang(1-7) via ACE-2 and/or alternative pathways, did not induce natriuresis. Taken together, these results support the concept that Ang III is the predominant agonist of AT2R-mediated natriuresis.

Previous studies from our laboratory have indicated that Ang III–induced natriuresis requires the concurrent blockade of systemic AT1Rs. Results from the present study confirm this finding. However, in the present study we demonstrated that, when APN is inhibited to reduce Ang III metabolism, the heptapeptide is capable of inducing natriuresis in the presence of unblocked systemic AT1Rs. Thus, in a pure physiological sense, intrarenal Ang III would not be expected to play a significant role in the control of Na+ excretion when AT1Rs are intact, because the antinatriuretic actions of Ang II via AT1Rs, which are widely expressed in the kidney, would overwhelm the ability of AT1Rs to engender natriuresis. AT1R-induced natriuresis would only apply when AT1Rs are blocked or, as shown by our data, when APN is blocked in the absence of AT1R inhibition. We also demonstrated that the aforementioned natriuretic response is specifically related to renal AT2R activation. In this regard, it is interesting to compare the natriuretic responses to Ang III with and without systemic AT1R blockade. In rats with AT1R blockade, natriuretic responses to Ang III and the APN inhibitor were sustained in a dose-dependent manner, whereas in those without AT1R blockade, an initial natriuretic response was followed by return to baseline levels in spite of increasing peptide infusion rates. These data suggest that AT1R blockade enhances both the magnitude and duration of the natriuretic response to Ang III, albeit in the short-term.

In light of these results, we hypothesized that endogenous Ang III is capable of inducing natriuresis in the normal rat. In AT1R-blocked rats, we found that intrarenal administration of the APN inhibitor PC-18 alone induced a significant and sustained natriuresis that was abolished by the AT1R antagonist PD. These results indicate that, in addition to exogenous Ang III, endogenous renal Ang III induces natriuresis via AT1R activation. The magnitude of the natriuretic response to PC-18 was relatively small compared with that attributed to exogenous Ang III in the presence of PC-18, indicating that renal heptapeptide biosynthesis may be a rate-limiting step in Ang III–induced natriuresis.

The present study is limited by the exclusive use of pharmacological agents as heuristic tools to dissect the relative contributions of Ang peptides to the natriuretic response. In the future, it will be important to use approaches targeting, for example, APN at the molecular level. However, our study does demonstrate that renal Ang II and III levels are approximately equal in the basal state, are appropriate for their respective peptide infusions, and that PC-18 effectively inhibits renal APN. The increase in renal Ang II during Ang III infusion may be because of product inhibition of APA, but this will require further study.
We used female rats for these studies. Hilliard et al.17,18 have shown recently that AT2R activation caused a similar increase in Na+/H+ excretion via an action at the renal tubule in both male and female Sprague-Dawley rats. Identified sex differences were limited to renal hemodynamic (vasodilatory) function that did not influence Na+/H+ excretion in their studies. In addition, Hakam and Hussain19 have shown that AT2R activation directly inhibits renal tubule sodium-potassium ATPase activity both in male obese Zucker rats and in male Sprague-Dawley rats.20 Furthermore, Sabuhi et al.21 demonstrated that AT2R activation induces natriuresis via a renal tubule mechanism in male Sprague-Dawley rats. Thus, there is a large body of evidence in the literature that renal tubule AT2R activation induces natriuresis in both male and female rats. However, we do show here that compound 21, a highly selective AT2R agonist,13 induces natriuresis in male rats and that this response is augmented by concomitant AT1R blockade and blocked with intrarenal PD.

In addition to the kidney, 2 other cardiovascular/hormonal systems have been linked to Ang III as a preferred agonist for AT2Rs, the coronary vascular bed and adrenal zona glomerulosa. In the coronary microcirculation, Ang III, rather than Ang II, is the preferred ligand to induce AT2R-mediated vasodilation.22 Ang III is also the preferred agonist for AT2R-mediated aldosterone secretion from the adrenal cortex.23 Together with the results of the present study, the evidence suggests that, at least in certain tissues, Ang III may be a better molecular fit within the AT2R binding pocket than Ang II. The precise molecular conformation that renders Ang III a better fit awaits future investigation.

**Perspectives**

In the present study in the normal Na+-replete rat, we demonstrated that endogenous intrarenal Ang III increases UNaV and that this response is mediated by AT2Rs by a cGMP-dependent mechanism in the proximal tubule. We showed that Ang III is the predominant angiotensin peptide agonist at renal tubule AT2Rs, because neither Ang II nor Ang(1-7) altered UNaV in our experimental model. Our recent published studies have shown that spontaneously hypertensive rats have defective AT2R-mediated natriuresis, which can be restored by intrarenal APN.
inhibition.24 On the basis of these findings, we hypothesize that, in hypertension, renal APN activity is increased, leading to accelerated Ang III metabolism reducing AT2R-mediated Na+ excretion. We further hypothesize that inhibition of APN activity could be beneficial in the treatment of hypertension. These hypotheses will require validation in future studies.

Acknowledgments
We gratefully acknowledge the provision of PC-18 by Drs Bernard P. Roques and Maria-Claude Fournie-Zaluski of Pharmleads SAS, Paris, France.

Sources of Funding
These studies were supported by grants R01-HL-087998 and R01-HL-095796 from the National Heart, Lung, and Blood Institute to R.M.C. Analytical studies performed at Tulane University were also supported by a Center of Biomedical Research Excellence grant (P20-RR-017659) from the Institutional Development Award Program of the National Institute for General Medical Sciences.

Disclosures
None.

References

What Is New?
- Ang III, but not Ang II or Ang(1-7), induces natriuresis during AT1R blockade.
- This effect is mediated by activation of AT2Rs in the renal proximal tubule.
- In the absence of AT1R blockade, natriuretic responses to Ang II require concurrent inhibition of APN.
- APN inhibition alone can induce natriuresis.

What Is Relevant?
- Endogenous intrarenal Ang III, but not Ang II or Ang(1-7), induces natriuresis by activation of AT2Rs in the renal proximal tubule.
- This action of AT2Rs is mediated by renal formation of cGMP.
- A major initiating event in hypertension is thought to be increased renal proximal tubule reabsorption of sodium.

Novelty and Significance

Renal AT1R activation increases proximal tubule sodium reabsorption, and this response is counteracted by renal AT2R activation triggered by endogenous intrarenal Ang III.

Ang II activation of AT2Rs is markedly augmented by blocking APN.

These studies suggest that APN inhibition would be a novel therapeutic target in hypertension.

Summary

This study demonstrates that endogenous intrarenal Ang III, but not Ang II or Ang(1-7), induces natriuresis via activation of AT2Rs in the proximal tubule via a cGMP-dependent mechanism and suggests APN inhibition as a potential therapeutic target in hypertension.

Intrarenal Angiotensin III Is the Predominant Agonist for Proximal Tubule Angiotensin Type 2 Receptors

Brandon A. Kemp, John F. Bell, Daniele M. Rottkamp, Nancy L. Howell, Weijian Shao, L. Gabriel Navar, Shetal H. Padia and Robert M. Carey

*Hypertension*. 2012;60:387-395; originally published online June 11, 2012; doi: 10.1161/HYPERTENSIONAHA.112.191403

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2012 American Heart Association, Inc. All rights reserved.

Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://hyper.ahajournals.org/content/60/2/387

Data Supplement (unedited) at:

http://hyper.ahajournals.org/content/suppl/2012/06/11/HYPERTENSIONAHA.112.191403.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Hypertension* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Hypertension* is online at:

http://hyper.ahajournals.org//subscriptions/
ONLINE DATA SUPPLEMENT

INTRARENAL ANGIOTENSIN III IS THE PREDOMINANT AGONIST FOR PROXIMAL TUBULE AT$_2$ RECEPTORS

Brandon A. Kemp$^1$
John F. Bell$^1$
Daniele M. Rottkamp$^2$
Nancy L. Howell$^1$
Weijian Shao$^3$
L. Gabriel Navar$^3$
Shetal H. Padia$^4$
Robert M. Carey$^1$

Running title: Angiotensin III in natriuresis

From the Division of Endocrinology and Metabolism, Department of Medicine, University of Virginia School of Medicine, Charlottesville, VA$^1$; the Division of Endocrinology and Metabolism, University of California San Francisco, San Francisco, CA$^2$; and Department of Physiology, Tulane University School of Medicine, New Orleans, LA$^3$.

Correspondence: Dr. Robert M. Carey, P.O. Box 801414, University of Virginia Health System, Charlottesville, VA 22908-1414; telephone: 434-924-5510; fax: 434-924-1284; e-mail: rmc4c@virginia.edu.
DETAILED METHODS

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. All experiments were conducted on 12-week-old female Sprague–Dawley rats (Harlan; N=102) 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 (0.28% Na⁺), except for the experiments with Compound 21, which were conducted in 10-week-old male Sprague-Dawley rats(N=11).

For all studies (except for Protocols 5 and 8), a 24-hr osmotic mini-pump (Alzet Model 2001D) infusing candesartan (CAND; 0.01 mg/kg/min) was inserted 24-hr prior to experimentation in order to block systemic AT₁Rs. While the rats were under short-term anesthesia with ketamine (100 mg/mL) and xylazine (20 mg/mL) via an intra-peritoneal (IP) injection, the pumps were implanted in the interscapular region using sterile technique.

On the day of experimentation, the rats were anesthetized with pentobarbital (50 mg/mL) given 5 mg/100 g body weight via 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 vehicle (V) 5% dextrose in water (D₅W) or V with inulin and lithium (Li⁺) chloride (Protocol 1) was infused at 20 µL/min. Direct cannulation of the right carotid artery with PE-50 tubing provided arterial access for monitoring blood pressure (BP). Following a midline laparotomy, both ureters were cannulated (PE-10) to collect urine for the quantification of UNaV.

Renal Cortical Interstitial Infusion

An open bore micro-infusion catheter (PE-10) was inserted under the renal capsule into the cortex of each kidney to ensure the RI infusion of pharmacological agent or V at 2.5 µL/min with a syringe pump (Harvard; model 55-222) as reported previously (1). When more than one agent was simultaneously infused, separate interstitial catheters were used. Vetbond tissue adhesive (3M Animal Care Products) was added to secure the catheter(s) and prevent interstitial pressure loss in the kidney.

Renal Interstitial Fluid (RIF) Microdialysis Technique:

RIF Ang II, Ang III and cGMP levels were measured using microdialysis probes that were constructed and utilized as described previously in our laboratory (1).

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.

**Pharmacological Agents**

Candesartan (CAND), a specific, potent, insurmountable inhibitor of AT$_1$Rs ($IC_{50}$ >1x10$^{-5}$ mol/L and 2.9x10$^{-8}$ mol/L for AT$_2$Rs and AT$_1$Rs, respectively), was used for systemic AT$_1$R blockade. Angiotensin II (Ang II; Bachem; $K_i$=10.5x10$^{-9}$ mol/L and 2.2x10$^{-9}$ mol/L for AT$_1$Rs and AT$_2$Rs, respectively), [des-Asp$^1$]-Ang II (Ang III; Bachem; $K_i$=10.5x10$^{-9}$ mol/L and 2.2x10$^{-9}$ mol/L for AT$_1$Rs and AT$_2$Rs, respectively), angiotensin (1-7) (Ang [1-7]; Bachem) and Ang (1-7) antagonist A-779 (Bachem) were employed for these studies. PC-18 (2-amino-4-methylsulfonyl-butane-thiol); 25 µg/min; $K_i$=8.0 nmol/L) was used interstitially to inhibit APN enzymatic activity (2,3) and EC-33 (3-amino-4-thio-butyl-sulfonate; 25 µg/min; $K_i$=0.29 µmol/L) was used interstitially to inhibit APA enzymatic activity (4) and were generous gifts from Drs Fournie-Zaluski and Roques. Enalaprilate (Sigma; 10 µg/kg/min) was used interstitially to inhibit ACE activity. PD-123319 (PD; Parke-Davis; 10 µg/kg/min) a specific AT$_2$R antagonist ($IC_{50}$=2x10$^{-8}$ mol/L and > 1x10$^{-4}$ mol/L for AT$_2$R and AT$_1$Rs respectively) was employed interstitially to block AT$_2$Rs. Compound 21 (C21), a highly selective AT$_2$R agonist was employed to activate the AT$_2$Rs specifically in male rats (5,6).

**Western Blot Analysis**

Total whole cell AT$_2$R protein expression levels were evaluated between CAND-treated and untreated control kidneys. Slices of kidney cortex (approx. 100 mg per kidney) were homogenized in Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific) with Halt protease inhibitor cocktail (Thermo Scientific) and spun at 900 x g for 10 min in order to pellet insoluble material. The supernatant was removed and the total protein was quantified using a bicinchonic acid (BCA) assay (Pierce). Sodium dodecylsulfate (SDS) samples were prepared, separated by SDS-PAGE (10% polyacrylamide gels; Biorad; 20 µg of protein loaded per lane), and transferred onto a nitrocellulose membrane by electroblotting. Membranes were blocked for 1-hr in Odyssey blocking buffer (Licor Biosciences) and then incubated overnight at 4°C with polyclonal anti-AT$_2$R (1:100; H-143 Santa Cruz) and monoclonal anti-β-actin (1:2000; Sigma) antibodies made up in Odyssey blocking buffer. Subsequently, the membranes were incubated with infrared secondary antibodies (anti-mouse IRDye 680 nm and anti-rabbit IRDye 800 nm; both 1:15,000 dilutions, Licor Biosciences). Immunoreactivity and quantitative assessment of band densities was performed using the Odyssey Infrared Imaging System (Licor Biosciences). The results were reported as a ratio of AT$_2$R/β-actin protein expression levels in which β-actin served as the loading control for the samples.

**Measurement of Glomerular Filtration Rate (GFR), Fractional Excretion of Sodium (FE$_{Na}$), and Fractional Excretion of Lithium (FE$_{Li}$)**
Urinary and plasma Na\(^+\) and Li\(^+\) concentrations were measured using a flame photometer (Instrumentation Laboratory-943). GFR was measured by inulin clearance and reported as mL/min/gram kidney weight. Tubular Na\(^+\) reabsorption was determined by calculating the FE\(_{Na}\) and renal proximal tubule Na\(^+\) reabsorption was estimated using the (FE\(_{Li}\) (maximum error of 4%), as published previously (7).

Measurement of RI and kidney Ang II and Ang III Levels

Separation of Ang II from Ang III by HPLC

Before injecting samples to HPLC, the retention time and collection interval for Ang II and Ang III were determined after preparation of mobile phase and confirmed daily by injecting 100 pmol of Ang II and Ang III calibrators and determining their elution pattern by monitoring the effluent at 230 nm. To prevent carryover of the calibrators after their injection, we washed the column and the injector valve with 150 and 10 ml, respectively, of wash solution (90% acetonitrile / 0.13% HFBA). Lyophilized renal interstitial fluid (RIF) and kidney samples were resuspended by vortex-mixing after addition of 300-µl equilibration solvent which included 65% mobile phase A (0.13% HFBA) and 35% mobile phase B (80% acetonitrile / 0.13% HFBA). Before HPLC, insoluble matter in the resuspension was sedimented by centrifugation. A 200-µl aliquot of supernate was chromatographed by isocratic elution at 1 ml/min on a 25x0.46-cm, 5-µm Vydac C18 reverse-phase HPLC column (Separations Group). According to the retention time from high concentration of Ang II and Ang III calibrators, collection time was determined. Ang II eluted at 17 min, and Ang III had an elution peak at 19 min. During separation of samples, the peaks of Ang II and Ang III could not be observed due to the low amount of angiotensin peptides. Sample fractions were collected from 16 to 18 min for Ang II and from 18 to 20 min for Ang III, evaporated to dryness, reconstituted in assay diluent, and measured directly by radioimmunoassay (8).

Measurement of Ang II and Ang III by Radioimmunoassay

The reconstituted RIF and kidney sample fractions were incubated with rabbit anti–Ang II antisera (Peninsula Laboratories Inc) and \(^{125}\)I-radiolabeled Ang II (Perkin Elmer Life and Analytical Sciences) for 48 h at 4°C. Bound and free Ang peptides were separated by dextran-coated charcoal, and the supernatants were counted on a computer-linked gamma counter for 3 min. Immunoreactivities of the antibody for Ang II and Ang III were virtually identical. The sensitivity of the Ang II and Ang III assay was 1.48 fmol. For the Ang II and Ang III assays, the specific binding was 66.4%, and nonspecific binding was 0.6%. The intra-assay(CV) was 2.6% and inter-assay(CV) was 4.2%. 
REFERENCES


FIGURE S1: Panel A. Urine Na⁺ excretion (Uₙₐᵥ) in response to renal interstitial (RI) infusion of vehicle ( ), (N=27), Ang III ( ), (N=8), Ang III + PD-123319 ( ), (N=6), Ang 1-7 ( ), (N=8), Ang 1-7 + ACE inhibitor Enalaprilate ( ), (N=7), Ang II ( ), (N=6), and Ang II + APA inhibitor EC-33 ( ), (N=6) in the presence of systemic AT₁R blockade with candesartan. Results are reported as µmol/min. Panel B. Mean arterial pressure (MAP) in response to conditions in Panel A. Results are reported as mm Hg. Data represent mean ± 1 SE. *P<0.05, **P<0.01, and ***P<0.001 from own control.
FIGURE S2: \( U_{Na}V \) in male Sprague-Dawley rats (N=15) in response to systemic administration of selective AT\(_2\)R agonist Compound 21 (white bars), Compound 21 in rats pre-treated with systemic AT\(_1\)R blockade with candesartan (black bars) or Compound 21 in the presence of renal interstitial infusion of AT\(_2\)R antagonist PD-123319 (gray bars). ** \( P<0.01 \) from respective control; *** \( P<0.001 \) from respective control; + \( P<0.05 \) from Compound 21 alone.
**FIGURE S3:** Angiotensin peptide levels in whole kidney. **Panel A.** Ang II peptide levels following the renal interstitial (RI) infusion of vehicle in the absence of systemic AT$_1$R blockade and the RI infusion of vehicle, Ang II, Ang III, and Ang III + APN inhibitor PC-18 in the presence of systemic AT$_1$R blockade with candesartan. N=6 for all conditions and results are reported as fmol/gram kidney weight. **Panel B.** Ang III peptide levels measurements in response to conditions in Panel A. **Panel C.** Ratios of Ang III to Ang II peptide levels in response to conditions in Panel A. Data represent mean ± 1 SE.