Conversion of Renal Angiotensin II to Angiotensin III Is Critical for AT₂ Receptor–Mediated Natriuresis In Rats

Shetal H. Padia, Brandon A. Kemp, Nancy L. Howell, Marie-Claude Fournie-Zaluski, Bernard P. Roques, Robert M. Carey

Abstract—In the kidney, angiotensin II (Ang II) is metabolized to angiotensin III (Ang III) by aminopeptidase A (APA). In turn, Ang III is metabolized to angiotensin IV by aminopeptidase N (APN). Renal interstitial (RI) infusion of Ang III, but not Ang II, results in angiotensin type-2 receptor (AT₂R)-mediated natriuresis. This response is augmented by coinfusion of PC-18, a specific inhibitor of APN. The present study addresses the hypotheses that Ang II conversion to Ang III is critical for the natriuretic response. Sprague-Dawley rats received systemic angiotensin type-1 receptor (AT₁R) blockade with candesartan (CAND; 0.01 mg/kg/min) for 24 hours before and during the experiment. After a control period, rats received either RI infusion of Ang II or Ang II+PC-18. The contralateral kidney received a RI infusion of vehicle in all rats. Mean arterial pressure (MAP) was monitored, and urinary sodium excretion rate (Uₙa/V) was calculated separately from experimental and control kidneys for each period. In contrast to Ang II–infused kidneys, Uₙa/V from Ang II+PC-18-infused kidneys increased from a baseline of 0.03±0.01 to 0.09±0.02 μmol/min (P<0.05). MAP was unchanged by either infusion. RI addition of PD-123319, an AT₂R antagonist, inhibited the natriuretic response. Furthermore, RI addition of EC-33, a selective APA inhibitor, abolished the natriuretic response to Ang II+PC-18. These data demonstrate that RI addition of PC-18 to Ang II enables natriuresis mediated by the AT₂R, and that conversion of Ang II to Ang III is critical for this response. (Hypertension. 2008;51[part 2]:460-465.)

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

The intrarenal renin-angiotensin system (RAS) is separate from the peripheral RAS and contributes independently to the regulation of blood pressure and sodium excretion.1–4 All of the precursors of angiotensin peptide synthesis are located within the renal proximal tubule (RPT), including angiotensinogen, renin, and angiotensin converting-enzyme mRNA.5–8 Interstitial fluid concentrations of angiotensin II (Ang II) and angiotensin III (Ang III) are far greater (nannotomolar) than can be explained solely on the basis of equilibration with circulating concentrations (femtomolar).9,10 These findings suggest that important influences are exerted by locally generated angiotensins. Additionally, the 2 major receptor subtypes that mediate actions of RAS, the angiotensin type-1 receptor (AT₁R) and the angiotensin type-2 receptor (AT₂R), are both present in RPT cells, consistent with a primary role for tubular angiotensins as paracrine substances in the control of renal function.11,12

The enzymes responsible for the metabolism of Ang II are also highly expressed in the kidney. Aminopeptidase A (APA), a membrane-bound zinc-dependent aminopeptidase, is distributed at the surface of glomerular endothelial and mesangial cells and podocytes and all along the nephron, with highest expression in the RPT.13,14 As shown in Figure 1, APA preferentially cleaves the N-terminal acidic amino acid (aspartate) from Ang II to generate Ang III, which serves as the rate-limiting step in Ang II metabolism.15 Aminopeptidase N (APN) is also a membrane-bound, zinc-dependent aminopeptidase and is a major constituent of RPT cell brush border membranes.16,17 The metabolism of Ang III to Ang IV is mediated by APN, which preferentially releases neutral amino acids from the N-terminal end of oligopeptides.18

Specific pharmacological inhibitors of APA and APN have been used to elucidate the role of Ang II and Ang III within local tissue RAS. Selective APA inhibitor, EC-33 (3-amino-4-thiobutyl-sulfonic acid, inhibition constant Kᵢ=0.29 μmol/L), blocks the conversion of Ang II to Ang III and increases the half-life of Ang II by 2.6-fold.19 Similarly, the selective APN inhibitor, PC-18 (2-amino-4-methylsulfonyl-butanethiol, Kᵢ=8.0 nmol/L), blocks the conversion of Ang III to Ang IV, and increases the half-life of Ang III by 3.9-fold.20 We have previously reported that renal interstitial (RI) Ang III infusion, but not Ang II infusion, results in natriuresis mediated by the AT₁R when AT₁Rs are blocked systemically.21 This response can be significantly augmented during concomitant infusion of PC-18,22 via accumulation of unmethylated Ang III. In the present set of studies, we sought to determine whether conversion of Ang II to Ang III was critical for AT₁R-mediated natriuresis. We evaluated renal...
sodium excretion rate (UNaV) in response to RI infusion of Ang II with PC-18 to determine whether intrarenal APN inhibition would convert the absence of a natriuretic response to Ang II (without APN inhibition) into a robust natriuretic response because of accumulation of Ang III. Additionally, we determined whether coinhibition of APA would abolish the natriuretic response to Ang II enabled by APN inhibition.

Methods

Animal Preparation
The experiments, which were approved by the University of Virginia Animal Research Committee, were conducted in 12-week-old 250-g female Sprague–Dawley rats (Harlan, Teklad). All of the studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were placed under general anesthesia with pentobarbital (100 mg/mL) and xylazine (20 mg/mL) for candesartan (CAND) (0.01 mg/kg per minute) infusion 24 hours before and during the experiment. The right kidney was infused with vehicle (5% dextrose in water, D5W) directly into the RI space during both control and experimental collection periods (30 minutes each) and served as the control kidney in all studies. In the first set of animals (n=6), Ang II (3.5, 7, 14, and 28 nmol/kg per minute) was infused cumulatively into the RI space of the left (experimental) kidney after a 30 minute control infusion of D5W (2.5 μL/min). In the second set of animals (n=8), Ang II (3.5, 7, 14, and 28 nmol/kg per minute) plus PC-18 (25 μg/min) was infused cumulatively into the RI space of the left (experimental) kidney after a 30-minute control infusion of (D,W) (2.5 μL/min). Both ureters were cannulated individually to collect urine for quantification of UNaV for the control and 4 experimental periods (each 30 minutes) from both the right (control) and left (experimental) kidneys.

Effects of RI Ang II+PC-18+PD in the Presence of Systemic AT1R Blockade
Rats (n=6) were prepared identically to those described above, but instead of Ang II+PC-18 infusion into the left kidney, Ang II+PC-18+PD (10 μg/kg/min) was infused. The right kidney continued to be infused with vehicle into the renal interstitial space during both control and experimental periods. UNaV was determined for control and experimental periods from both the left and right kidneys.

Effects of Unilateral RI Ang II Alone or Ang II+PC-18 on UNaV in Rats on Normal Na+ Intake in the Presence of Systemic AT1R Blockade
Rats were studied on normal Na+ intake with both kidneys intact. Using sterile technique, osmotic minipumps were implanted into the interscapular region with the animals under short-term anesthesia with ketamine (100 mg/mL) and xylazine (20 mg/mL) for candesartan (CAND) (0.01 mg/kg per minute) infusion 24 hours before and during the experiment. The right kidney was infused with vehicle (5% dextrose in water, D,W) directly into the RI space during both control and experimental collection periods (30 minutes each) and served as the control kidney in all studies. In the first set of animals (n=6), Ang II (3.5, 7, 14, and 28 nmol/kg per minute) was infused cumulatively into the RI space of the left (experimental) kidney after a 30 minute control infusion of D5W (2.5 μL/min). In the second set of animals (n=8), Ang II (3.5, 7, 14, and 28 nmol/kg per minute) plus PC-18 (25 μg/min) was infused cumulatively into the RI space of the left (experimental) kidney after a 30-minute control infusion of (D,W) (2.5 μL/min). Both ureters were cannulated individually to collect urine for quantification of UNaV for the control and 4 experimental periods (each 30 minutes) from both the right (control) and left (experimental) kidneys.

Statistical Analysis
Comparisons among vehicle, AT,R blocker (CAND), AT,R blocker (PD), Ang II, PC-18 (APN inhibitor), and EC-33 (APA inhibitor) were estimated by ANOVA, including a repeated-measures term, by using the general linear models procedure of the Statistical Analysis System. Multiple comparisons of individual pairs of effect means were conducted by the use of least-square means pooled variance. Data are expressed as mean±1 SE. Statistical significance was identified at a level of P<0.05.
Results

Effects of Unilateral Renal Interstitial Ang II Alone or Ang II+PC-18 on UNaV in Rats on Normal Sodium Intake in the Presence of Systemic AT1 Receptor Blockade

Figure 2 demonstrates that, in the presence of systemic CAND administration, cumulative RI Ang II infusion does not result in increased UNaV across the duration of the experiment. However, after coinfusion with PC-18, UNaV increased from a baseline of 0.03±0.01 to 0.08±0.03 μmol/min (P<0.05) at 3.5 nmol/kg/min, 0.09±0.02 μmol/min (P<0.05) at 7 nmol/kg/min, 0.07±0.01 μmol/min (P<0.01) at 14 nmol/kg/min, and 0.06±0.01 μmol/min (P<0.05) at 28 nmol/kg/min of Ang II (ANOVA F 8.40; P<0.05). Compared with time control, RI Ang II infusion, addition of EC-33 significantly reduced UNaV across all Ang II infusion rates. Vehicle-infused kidneys did not show a significant change in UNaV across the duration of the experiment.

Effects of Unilateral Renal Interstitial Ang II+PC-18 Versus Ang II+PC-18+PD on UNaV in Rats on Normal Sodium Intake in the Presence of Systemic AT1R Blockade

In the presence of AT1R blockade, the intrarenal addition of PD to Ang II plus PC-18 abolished the natriuretic response (Figure 3). Compared with Ang II plus PC-18 infusion, UNaV was 0.035±0.004 μmol/min at 3.5 nmol/kg/min (P=NS), 0.030±0.006 μmol/min at 7.0 nmol/kg/min (P<0.05), 0.035±0.006 μmol/min at 14 nmol/kg/min (P<0.01), and 0.038±0.012 μmol/min at 28 nmol/kg/min (P=NS) of Ang II infusion concentration. Thus, the addition of PD prevented the natriuretic response to Ang II enabled by PC-18.

Blood Pressure Responses to Renal Interstitial Ang II, Ang II+PC-18, Ang II+PC-18+PD, and Ang II+PC-18+EC-33 Infusions in Rats on Normal Sodium Intake in the Presence of Systemic AT1R Blockade

As shown in Figure 5, compared with time control during which only vehicle was infused, each RI infusion did not alter

![Figure 2](image1.png)

**Figure 2.** Urine Na⁺ excretion (UNaV) rate in response to renal interstitial (RI) infusion of vehicle (D5W) versus Ang II alone versus Ang II+PC-18 in the presence of systemic AT1 receptor blockade with candesartan (n=7 per group). White bars, UNaV responses when only D5W was infused into the renal interstitial space of the control kidney for the duration of the experiment. Grey bars, UNaV responses to 4 cumulative RI Ang II infusions, after a 30-minute precontrol period during which only vehicle was infused. Black bars, UNaV responses to Ang II plus PC-18 (25 μg/min) infusion, after a 30-minute precontrol period during which only vehicle was infused. Data represent mean±1 SE; *P<0.05, **P<0.01, from respective time control and +P<0.05, ++P<0.01 from Ang II alone infused kidney.

![Figure 3](image2.png)

**Figure 3.** UNaV responses to renal interstitial infusion of vehicle (D5W) (white bars), Ang II+PC-18 (black bars), and Ang II+PC-18+PD (patterned bars), each in the presence of systemic AT1R blockade with candesartan (n=7 per group). During the time control period, only D5W was infused into the renal interstitial space in all groups. Data represent mean±1 SE; *P<0.05, **P<0.01, from respective time control and +P<0.05, ++P<0.01 from Ang II+PC-18 infused kidney.

**Effects of Unilateral Renal Interstitial Ang II+PC-18+EC-33 on UNaV in Rats on Normal Sodium Intake in the Presence of Systemic AT1R Blockade**

In the presence of systemic AT1R blockade, the addition of EC-33 to intrarenal infusion of Ang II+PC-18, inhibited the natriuretic response observed with Ang II+PC-18 alone (Figure 4). Compared with time control, RI Ang II+PC-18+EC-33 showed a UNaV of 0.02±0.004 μmol/min (P=NS) at 3.5 nmol/kg/min, 0.04±0.007 μmol/min (P=NS) at 7 nmol/kg/min, 0.04±0.005 μmol/min (P=NS) at 14 nmol/kg/min, and 0.03±0.006 μmol/min (P=NS) at 28 nmol/kg/min of Ang II. Compared with Ang II+PC-18 infusion, addition of EC-33 significantly reduced UNaV across all Ang II infusion concentrations (P<0.05 at 3.5, 7.0, and 28 nmol/kg/min and P<0.01 at 14 nmol/kg/min). Coinhibition of APA with EC-33, therefore, inhibited the natriuretic response to Ang II enabled by PC-18.

![Figure 4](image3.png)

**Figure 4.** UNaV responses to renal interstitial infusion of vehicle (D5W) (white bars), Ang II+PC-18 (black bars), and Ang II+PC-18+EC-33 (striped bars), each in the presence of systemic AT1 receptor blockade with candesartan (n=7 per group). During the time control period, only D5W was infused into the renal interstitial space in all groups. Data represent mean±1 SE; *P<0.05, **P<0.01, from respective time control and +P<0.05, ++P<0.01 from Ang II+PC-18 infused kidney.
mean arterial pressure (MAP) from its respective baseline. MAP responses during RI Ang II alone infusion ranged from 91 mm Hg during control to 85 mm Hg at 28 nmol/kg/min, RI Ang II+PC-18 infusion ranged from 84 to 86 mm Hg, RI Ang II+PC-18+PD ranged from 86 to 87 mm Hg, and RI Ang II+PC-18+EC-33 from 94 mm Hg during control to 88 mm Hg at 28 nmol/kg/min.

Discussion

These studies demonstrate that RI Ang II, an AT1-R ligand, does not induce a natriuresis in the presence of systemic AT1-R blockade. However, a natriuretic response to Ang II, mediated by the AT1-R, is unmasked by the intrarenal addition of PC-18, an APN inhibitor. Because the metabolism of Ang III to Ang IV by APN is inhibited by PC-18, these data suggest that Ang III is a significant mediator of Ang II–induced natriuresis. This conclusion is further supported by the ability of APN inhibition to abolish the natriuretic response to Ang II+PC-18.

Previous studies from our laboratory have shown that in the presence of AT1-R blockade, RI Ang III infusion results in natriuresis that is abolished by concomitant PD infusion, implicating the renal AT1-R in the response.21 Furthermore, addition of RI PC-18 to Ang III infusion causes a 1.8- to 2.8-fold increase in Na+ excretion compared with RI Ang III infusion alone.22 These findings prompted the present set of investigations to determine whether renal conversion of Ang II to Ang III was necessary for the natriuretic response.

In local renin-angiotensin systems (RASs), Ang III has been shown to be a major effector peptide. In the brain, Ang III, and not Ang II, is the agonist responsible for mediating vasoconstriction and BP elevation via the AT1-R.15,23–27 Ang III was also shown to mediate vasopressin release when administered directly into the 3rd ventricle of the brain.15,27 Similarly, in the adrenal RAS, aldosterone production is stimulated by Ang III as well as Ang II.28 In the intrarenal RAS, Ang III has been reported to increase angiotensinogen levels, sodium excretion, transforming growth factor (TGF)-β, fibronectin, and monocyte chemoattractant protein-1 gene expression.21,29,30

The relative affinities of Ang II and Ang III for AT1-Rs and AT2-Rs remain a topic of interest. Some authors argue that Ang III binds mainly to AT2-Rs and exhibits a lower affinity for AT1-Rs compared with that of Ang II.31 In the myometrial cells of the pig uterus, the Ki for Ang III at the AT2-R is 2.2±0.2 nmol/L, and in rat liver membranes, the Ki for Ang III at the AT1-R is 10.5±0.3 nmol/L, corroborating this observation. However, in the brain, Ang II and Ang III have been noted to display similar affinities for AT1-Rs and AT2-Rs,32,33 suggesting that local tissue RAS systems may behave differently. The specific affinities of Ang II and Ang III for AT1-Rs and AT2-Rs have not been studied systematically in the kidney. However, previous studies from our laboratory have demonstrated that RI infusion of Ang III causes a significant natriuresis in the presence of systemic AT1-R blockade, which is abolished by the addition of PD. Thus, within the intrarenal RAS, Ang III is capable of exerting significant actions at AT2-Rs, at least in the presence of AT1 receptor blockade.21

APA is the major enzyme responsible for the metabolism of Ang II to Ang III13,14 and is most highly expressed in the RPT.13,14 APN, the major renal enzyme responsible for the cleavage of Ang III to Ang IV,19 is also expressed on the brush border (apical) membranes of RPT cells and enterocytes.34 The APA inhibitor EC-33 exhibits an inhibitory potency nearly 100-fold greater for APA (Ki=0.29 μmol/L) than for APN (Ki=25 μmol/L),35 whereas the inhibitory potency of PC-18 is 2150- and 125-fold more active on APN (Ki=0.008±0.001 μmol/L) than on APN (Ki=17.2±4.3 μmol/L).36–38 In the systemic circulation, Ang III is metabolized 2 to 4 times more rapidly than Ang II, accounting for a reduced relative efficacy of systemically infused Ang III.28,34 Thus, the infusion of PC-18 directly into the RI compartment in the present study allowed for examination of the effects mediated by Ang III at the local intrarenal tissue RAS, with the advantage of permitting this rapidly degraded peptide to remain available for a longer period of time.

It is possible that PC-18 inhibition of APN, although quite specific, may have permitted accumulation of other intrarenal peptides that are metabolized by APN (eg, kallidin), accounting for the natriuretic response observed to Ang II in the present study. However, the effects of the other substrates of APN would also have been mediated by the renal AT1-R, given that the natriuresis enabled by the addition of PC-18 to Ang II, was abolished by the substitution of PD, a specific AT1-R. Interestingly, the highest Ang II infusion rate (28 nmol/kg/min), when combined with PC-18, resulted in less of an increase in USoV, compared with lower Ang II infusion rates (eg, 7 nmol/kg/min). One possible explanation is that APA becomes “saturated” during the higher concentrations of Ang II infusion, resulting in decreased Ang III formation and thereby decreased natriuresis via the unblocked AT1-R.

Inhibition of APA with EC-33 likely permitted the accumulation of Ang II, which is known to have antinatriuretic effects via the AT1-R.39,41 We believe the inhibition of natriuresis observed during Ang II+PC-18+EC-33 infusion...
was more likely attributable to the decreased availability of Ang III, rather than antinatriuretic effects of Ang II, given that the AT,R was blocked for 24 hours before and during our studies. The adequacy of AT,R blockade was confirmed by the absence of significant MAP increases in response to renal interstitial Ang II infusion and Ang II + PC-18 + EC-33 infusion, when unmetabolized Ang II likely accumulated. Furthermore, the addition of EC-33 to renal interstitial Ang II + PC-18 infusion may have shunted the Ang II degradation pathway toward Ang I-7 formation via ACE-2. Renally infused Ang I-7 has been reported to induce natriuresis in some studies, whereas others argue that renal tubular sodium reabsorption may be stimulated by Ang I-7. In the present study, Ang II + PC-18 + EC-33 infusion, with presumed accumulation of Ang I-7, did not result in significant natriuresis.

The addition of PD, a selective AT2R antagonist, completely eliminated the natriuretic response observed with Ang II + PC-18 back to control levels, indicating that the renal AT2R was largely responsible for the natriuresis. AT2Rs have been implicated in the natriuresis of both normal and diseased rodents. In obese Zucker rats, AT2R blockade induces natriuresis to a greater degree than in lean rats and the natriuresis is abolished with AT1R blocker PD. In streptozotocin-induced diabetes mellitus, the rise in fractional sodium excretion is inhibited by administration of PD in the absence of exogenous hormone, suggesting a tubular action of AT2R blockade. In both disease models, AT2Rs are markedly upregulated in RPT cell basolateral and brush border membranes compared with lean or nondiabetic controls. However, the preferred effector ligand of AT2R-mediated natriuresis has not been established in disease models, and the specific mechanisms of normal Ang II-induced natriuresis via the renal AT2R have not yet been elucidated.

**Perspectives**

The present studies demonstrate that in the presence of systemic AT2R blockade, RI administration of PC-18, a potent inhibitor of the enzyme which converts Ang III to Ang IV, enables a natriuretic response to RI infusion of Ang II alone, and that this response is blocked when Ang II is not able to be converted to Ang III. These studies indicate that Ang III is the major agonist of AT2R-induced natriuresis. Our results suggest that Ang III, the AT2R, APA, and APN are potentially important therapeutic targets for disorders characterized by Na+ and fluid retention, such as hypertension and congestive heart failure.

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None.

**References**


25. Wright JW, Roberts KA, Cook VI, Murray CE, Sardinha MF, Harding JW. Intracerebroventricularly infused [D-Arg1]angiotensin III, is superior
to [D-Asp1]angiotensin II, as a pressor agent in rats. 

25. Wright JW, Harding JW. Important role for angiotensin III and IV in the brain renin-angiotensin system. 

26. Cesari M, Rossi GP, Pessina AC. Biological properties of the angiotensin peptides other than angiotensin II: implications for hypertension and cardiovascular diseases. 


29. Matsubara H. Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. 


31. Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE. Important role for angiotensin III and IV in the development of kidney damage in mesangial cells and renal interstitial fibroblasts. 

32. Cesari M, Rossi GP, Pessina AC. Biological properties of the angiotensin peptides other than angiotensin II: implications for hypertension and cardiovascular diseases. 

33. Wright JW, Harding JW. Important role for angiotensin III and IV in the brain renin-angiotensin system. 

34. Cesari M, Rossi GP, Pessina AC. Biological properties of the angiotensin peptides other than angiotensin II: implications for hypertension and cardiovascular diseases. 

35. Wright JW, Harding JW. Important role for angiotensin III and IV in the brain renin-angiotensin system. 

36. Cesari M, Rossi GP, Pessina AC. Biological properties of the angiotensin peptides other than angiotensin II: implications for hypertension and cardiovascular diseases. 

37. Cesari M, Rossi GP, Pessina AC. Biological properties of the angiotensin peptides other than angiotensin II: implications for hypertension and cardiovascular diseases. 

38. Cesari M, Rossi GP, Pessina AC. Biological properties of the angiotensin peptides other than angiotensin II: implications for hypertension and cardiovascular diseases. 

39. Cesari M, Rossi GP, Pessina AC. Biological properties of the angiotensin peptides other than angiotensin II: implications for hypertension and cardiovascular diseases.
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