

Endogenous Angiotensin Concentrations in Specific Intrarenal Fluid Compartments of the Rat

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Abstract

To examine angiotensin (ANG) concentrations in fluid compartments near known intrarenal ANG receptors, we measured ANG concentrations in glomerular filtrate (GF), star vessel plasma (SVP), and luminal fluid from the early, mid, and late proximal tubule (E, M, and L PT). Samples were collected from euvoletic Munich-Wistar rats by free-flow micropuncture; ANG concentrations were measured by RIA. In one group of rats, concentrations of total immunoreactive ANG (reflecting ANG II and lesser amounts of three fragments) in GF and E, M, and L PT fluid averaged 29–40 nM compared with 32 pM in systemic plasma. In a second group, immunoreactive ANG concentrations in SVP also exceeded systemic levels by a factor of 1,000. In a final group, samples of GF and LPT fluid were purified by HPLC before RIA to measure ANG II and III concentrations specifically: their respective concentrations were 6–8 nM and 14–25 nM. We interpret these results to indicate that substantial amounts of ANG peptides are released into or generated within intrarenal fluid compartments, in which local ANG is likely to effect regulation of renal function independently of systemic ANG. (*J. Clin. Invest.* 1990. 86:1352–1357.) Key words: intrarenal angiotensin II • radioimmunoassay • high performance liquid chromatography • glomerulus • proximal tubule

Introduction

There is considerable evidence that angiotensin II is an important regulator of renal hemodynamics, glomerular function, mesangial contractility, and proximal tubule transport (1–11). However, while it is known that there are receptors for angiotensin in glomeruli, vasa recta, and on the luminal and basolateral sides of proximal tubule cells (12–16), endogenous an-

giotensin concentrations in fluid surrounding these receptors are not known. The present experiments were performed to measure endogenous angiotensin concentrations directly in specific intrarenal fluid compartments, including glomerular filtrate, star vessel plasma, and the lumen of the proximal convoluted tubule. The results show that angiotensins II and III are normally present in these compartments in concentrations much higher than in the systemic circulation, providing strong evidence that local angiotensin effects regulation of renal function independently of systemic angiotensin.

Methods

Studies were performed on 39 male Munich-Wistar rats (Simonson, Gilroy, CA) weighing 180–220 g. 34 were anesthetized by intraperitoneal injection of 100 mg/kg body wt of thiobutabarbital (Byk-Gulden, Konstanz, FRG) and prepared for micropuncture. The remainder were decapitated for collection of blood without prior anesthesia or surgery. In animals prepared for micropuncture, catheters were placed in the right jugular vein for infusion of fluids and in the left carotid artery for blood sampling and continuous monitoring of blood pressure. Fluids were infused according to a modification of the protocol of Ichikawa et al. to simulate euvoletic conditions (17): fluid containing 5% BSA, 140 mM NaCl, and 4 mM KCl was infused at 1 ml/100 g body wt for a total of 45 min. Then an infusion of saline without BSA was begun at 0.15 ml/100 g body wt per h and adjusted as necessary to keep the hematocrit constant. Micropuncture and blood sampling were started 45 min after completion of the BSA infusion.

Experiment 1

Measurement of total immunoreactive angiotensin in glomerular filtrate, proximal tubule fluid, and systemic arterial plasma. In this experiment, total immunoreactive angiotensin concentrations in micropuncture samples and arterial plasma from 17 rats were measured by RIA after peptide extraction as described below. Microsamples were obtained by free-flow micropuncture of surface glomeruli and their corresponding proximal tubule segments, which were identified by injecting small amounts of tinted fluid from a finding pipette (outer tip diameter 2–3 μ m) positioned in Bowman's space. This fluid contained 1 g/liter FD & C Blue No. 1 (H. Kohnstamm & Co., New York) and 115 mM NaCl, 25 mM NaHCO₃, 4 mM KCl, and 14 mM urea. All micropuncture samples were collected for 5–7 min periods in oil-filled pipettes coated with Siliclad (Sigma Chemical Co., St. Louis, MO). Proximal tubule samples were obtained by complete collection of luminal fluid from early, mid, or late proximal tubule segments, where early and late segments were defined as the first and last pairs of surface proximal segments. Mid proximal segments were defined as all intervening segments. In some cases, proximal tubules were punctured at two or three sites beginning with the most downstream site. Results for tubules punctured more than once were identical to those for nephrons punctured once. Samples of glomerular filtrate were obtained by par-

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tial collection of fluid in Bowman's space. Care was taken to avoid suction in all cases. Immediately after collection, individual samples were transferred to a constant bore capillary (containing siliconized mineral oil) for measurement of volume, added to separate 1-ml aliquots of chilled deionized water containing 1.2 mM Na₂EDTA (to inhibit angiotensin-converting enzyme activity) and 1.2 mM 1,10-phenanthroline (to inhibit other proteases), and then stored at -20°C until the time of assay.

Multiple 50- μ l blood samples were obtained from the carotid artery for determination of hematocrit. A single 0.5-ml sample of carotid blood was collected from each rat in a siliconized polyethylene tube containing 10 μ l of 125 mM Na₂EDTA and 10 μ l of 125 mM phenanthroline. This blood was immediately centrifuged at 4°C and the plasma frozen at -20°C until the time of assay. Plasma was also obtained from five decapitated littermates for the measurement of angiotensin concentration in animals not subjected to anesthesia and surgery.

Experiment 2

Measurement of total immunoreactive angiotensin in star vessel plasma. In each of another six rats prepared for micropuncture, blood was collected without suction from three or four randomly selected star vessels for 4-5 min per collection. The pipettes for this experiment were siliconized as described above, rinsed with 125 mM Na₂EDTA and 125 mM phenanthroline, and filled with mineral oil. Plasma was separated by immediate centrifugation, transferred for volume measurement, and promptly added to a solution of 1.2 mM Na₂EDTA and 1.2 mM phenanthroline. All star vessel plasma samples from each rat were pooled to ensure that sufficient peptide was present for detection by RIA. Samples of carotid plasma were processed as described earlier.

Experiment 3

Specific measurement of angiotensin II and angiotensin III concentrations in glomerular filtrate and late proximal tubule fluid. As indicated below, the RIA used in these studies had the capacity to detect angiotensin II, angiotensin III, and two smaller angiotensin fragments. To specifically measure concentrations of angiotensins II and III, micropuncture samples obtained from seven additional rats were purified further by HPLC before radioimmunoassay. Samples were obtained from Bowman's space and late proximal tubule as before; samples from all rats were pooled according to the site of collection (to provide sufficient peptide for detection after HPLC separation), added to 1.2 mM Na₂EDTA/phenanthroline, and stored at -20°C.

Extraction of samples

Experiments 1-3. Angiotensin peptides in samples from all three experiments were partially purified by reversible adsorption and extraction from C₁₈ Sep-Pak cartridges (Waters Associates, Millipore Corp., Milford, MA). Cartridges were purged with 20 ml distilled water, 5 ml methanol, and another 10 ml of water. ¹²⁵I-angiotensin II (New England Nuclear, Boston, MA) was added to all samples for determination of recovery. Then each sample was loaded onto a cartridge, rinsed with 10 ml of distilled water followed by 10 ml of 4% glacial acetic acid in water, and eluted in 6 ml of 4% glacial acetic acid in redistilled ethanol. Eluates were dried at 45°C under a stream of nitrogen. In experiments 1 and 2, the RIA for angiotensin was performed on extracts reconstituted in tromethamine acetate buffer. In experiment 3, extracts were further purified by HPLC before RIA.

HPLC separation of samples

Experiment 3. Sep-Pak extracts of glomerular filtrate and late proximal tubule fluid were processed by isocratic reverse phase HPLC. First they were reconstituted in 300 μ l of mobile phase (a 1:3 mixture of acetonitrile and 34.5 mM NaH₂PO₄ buffer, pH 6.0), vortexed for 2 min, and centrifuged (2,000 g) at 4°C for 10 min. Then supernatants were passed through 0.2- μ m nylon filters before HPLC injection. The HPLC system contained a gradient liquid chromatograph (model 334; Beckman Instruments, Inc., Palo Alto, CA), a 250- μ l injection loop, a

C₁₈ Microsorb column (5- μ m particles, 4.6 \times 250 mm; Rainin Dynamax, Woburn, MA), and mobile phase pumped at 1 ml/min. Finally, the RIA was performed on HPLC eluates obtained continuously every 8.6 s. Retention times of standards (Peninsula Laboratories, Inc., Belmont, CA) were established by ultraviolet spectrophotometry (spectrophotometer model 100-40; Hitachi Ltd., Tokyo, Japan). These standards and their retention times are shown in Table I.

Radioimmunoassay for angiotensin. In experiments 1 and 2, dried Sep-Pak extracts and nonradioactive angiotensin II standards were reconstituted in 1.0 ml of 0.1 M tromethamine acetate buffer (pH 7.4) containing 0.2% BSA. Aliquots (0.2 ml) were removed for determination of internal recovery. Other 0.2-ml aliquots were removed for RIA, combined with 0.2 ml of ¹²⁵I-angiotensin II (14,000 cpm/0.2 ml) and 0.1 ml of angiotensin II antiserum (1:5,000 initial dilution), and allowed to incubate at 4°C for 72 h. Incubated material was then mixed with 0.5 ml of BSA-free buffer containing 6 g activated charcoal and 0.75 g dextran (molecular weight 70,000)/liter. The resulting mixtures were centrifuged for 15 min at 2,000 g and radioactivity in the supernatant counted (Compugamma 1282; LKB-Wallac, Turku, Finland). In experiment 3, the same assay was performed directly on HPLC eluates.

The angiotensin II antiserum was prepared in the manner previously described (18). As shown in Table I, it cross-reacted with angiotensin III and the angiotensin fragments comprising amino acids 3-8 and 4-8. However, it was possible to separate angiotensin II from cross-reacting species with the use of HPLC combined with frequent collection of eluates. The antiserum did not cross-react with angiotensin I, nor did it react with the two tetrapeptide angiotensin fragments. Interassay variability was 9%; intraassay variability was 7%. The detection limit of the assay was < 1 pg.

Amounts of immunoreactive angiotensin were calculated by interpolation from standard curves, which yielded linear results between 2.5 and 1,000 pg of angiotensin II. Immunoreactive angiotensin concentrations in micropuncture samples were calculated as the ratio of total amount of angiotensin per sample and total sample volume. Total angiotensin averaged 5-10 pg per individual sample (experiment 1) and 100-200 pg per pooled sample (experiments 2 and 3).

Validation of collection technique and assay methodology. Recovery of angiotensin II in standard solutions was determined four ways. First, to determine peptide recovery after the initial phase of sample processing, 10⁻⁹ and 10⁻⁷ M solutions of ³H-angiotensin II (New England Nuclear) were aspirated into siliconized collection pipettes, transferred to the constant bore capillary, and added to scintillation vials: recovery of the isotope (scintillation counter LS3801; Beckman Instruments, Inc., Fullerton, CA) averaged 99%. Second, the effect of Sep-Pak extraction and subsequent RIA on recovery of nonradioactive 10⁻⁹, 10⁻⁸, and 10⁻⁷ M angiotensin II (Peninsula Labs) was assessed: recovery averaged 97%. Third, the effect of HPLC on recovery of 10⁻⁹ M ¹²⁵I-angiotensin II was assessed: recovery averaged 87%. And finally, to determine overall recovery, unlabeled 10⁻⁸ M angiotensin II was

Table I. Evaluation of Angiotensin Standards by HPLC and RIA

Peptide	HPLC retention time min	% Detected by RIA
Angiotensin (ANG) II	4.8	97
ANG III	13.2	79
ANG II ₍₃₋₈₎	6.2	66
ANG II ₍₄₋₈₎	5.5	66
ANG I	NT	0
ANG II ₍₁₋₄₎	4.3	0
ANG II ₍₅₋₈₎	4.8	0

NT, not tested.

aspirated into siliconized collection pipettes, transferred to the constant bore capillary, extracted from Sep-Pak cartridges, and analyzed by RIA. Overall recovery averaged $82.1 \pm 2.6\%$, thus leading to slight underestimation of the true angiotensin concentration in the original solution. Angiotensin concentrations are reported without correction.

To test whether angiotensin II could have been generated from angiotensin I *in vitro*, siliconized pipettes containing known volumes (168 ± 27 nl) of 2 nM ^{125}I -angiotensin I (New England Nuclear) were used to collect samples from Bowman's space in four additional animals. These pipettes did not contain EDTA or phenanthroline. The total volume of the resultant samples, reflecting the combined volumes of collected and pre-aspirated fluid, averaged 471 ± 131 nl. Samples were then added to 1.2 M EDTA and 1.2 M phenanthroline, pooled, stored at -20°C , and processed by Sep-Pak extraction as before. Next, samples were processed by HPLC, which was modified to effect clear separation of ^{125}I -angiotensin I from ^{125}I -angiotensin II. The mobile phase for this separation consisted of acetonitrile (0–40% gradient) and 50 mM NaH_2PO_4 buffer (pH 6.0) pumped at 1 ml/min; the stationary phase was as previously described. Measurement of radioactivity in eluates collected over 30-s intervals demonstrated that no angiotensin I was converted to angiotensin II *in vitro*. All of the recovered isotope (recovery averaged 85%) comigrated with ^{125}I -angiotensin I standards. Similar results were obtained when pipettes were rinsed with 0.125 mM EDTA and phenanthroline before collection.

Reagents were supplied by Sigma unless otherwise specified. Results are expressed as mean \pm 1 SEM.

Results

Experiment 1. Concentrations of total immunoreactive angiotensin in systemic arterial plasma, glomerular filtrate, and tubule fluid are shown in Table II. Plasma concentrations in animals prepared for micropuncture and in their corresponding decapitated controls were not significantly different ($P > 0.5$, unpaired *t* test). Thus, anesthesia and surgery had no major effect on plasma concentrations. Angiotensin concentrations in glomerular filtrate and in fluid from the proximal tubule were approximately 1,000-fold higher than in arterial plasma and did not decline detectably from the beginning to the end of the proximal tubule.

Experiment 2. Concentrations of total immunoreactive angiotensin were measured in systemic arterial and star vessel plasma in a separate group of animals (Table II). These animals had slightly higher plasma angiotensin concentrations than the experiment 1 animals, which were studied several months earlier under identical conditions. As in experiment 1, however, intrarenal angiotensin concentrations were much higher than systemic levels: star vessel concentrations exceeded systemic concentrations by 1,000-fold, a factor identi-

cal to that observed in glomerular filtrate and proximal tubule fluid.

Experiment 3. To characterize the peptides detected in glomerular filtrate and in late proximal tubule fluid, samples partially purified by Sep-Pak extraction were further purified by HPLC in a manner that separated angiotensin II from all known cross-reacting species. RIA of glomerular filtrate revealed two immunoreactive fractions, which were identified by their retention times as angiotensin II (23% of total immunoreactive material, 8 nM) and angiotensin III (77%, 25 nM). Assay of late proximal tubule fluid revealed three substances. Two were identified as angiotensins II (15% of total immunoreactive material, 6 nM) and III (36%, 14 nM). The third peptide, a likely breakdown product of angiotensin II accounting for 49% of immunoreactive material, is unidentified at this time.

Discussion

The effects of angiotensin II on the kidney are critically concentration dependent. This is particularly evident in the proximal tubule, where application of exogenous angiotensin II can stimulate or inhibit the fluxes of water, salt, and hydrogen/bicarbonate ions, depending on whether picomolar or micromolar concentrations are applied (3, 5, 6, 9, 10). It is clear that angiotensin II enters the kidney in arterial plasma and leaves it in lymph and venous plasma (19–21). It is also clear that non-vascular compartments of the kidney contain angiotensin II, which is present in renal homogenates and certain types of renal cells including the granular cell of the juxtaglomerular apparatus (22–28). Despite this information, however, the precise concentrations of angiotensin II and related peptides in fluid compartments near receptors on the luminal and basolateral sides of proximal tubule cells, glomeruli, and vasa recta are not known. Neither is it a straightforward exercise to predict local renal angiotensin concentrations owing to the compartmental complexity of the kidney and interactions between local angiotensin production, degradation, sequestration, and clearance. The present studies show (a) that angiotensins II and III are normally present in glomerular filtrate, proximal tubule fluid, and star vessel plasma, and (b) that the angiotensin II concentrations in these compartments are significantly higher than in the systemic circulation, thereby directly supporting previous suggestions that a "local" angiotensin system operates in the kidney.

In contrast to the high concentrations in glomerular filtrate, proximal tubule fluid, and star vessel plasma, it is not-

Table II. Total Immunoreactive Angiotensin Concentrations (nM)

	Systemic plasma	Glomerular filtrate	Proximal tubule			Star vessel plasma
			Early	Mid	Late	
Experiment 1						
17 rats	0.032 ± 0.005	32.8 ± 3.8 (40)	33.9 ± 9.1 (14)	29.0 ± 6.1 (13)	40.5 ± 7.6 (18)	
5 unanesthetized rats	0.029 ± 0.005					
Experiment 2						
6 rats	0.089 ± 0.026					95.4 ± 34.5 (19)*

Values expressed as mean \pm 1 SEM. Values in parentheses indicate number of collections. *Samples from each rat combined: n = 6.

able that angiotensin II concentrations in renal venous blood are often similar to those in the systemic circulation (19, 21). Given that there are high concentrations of angiotensin II in star vessel plasma, it follows that most of the angiotensin II present in star vessel plasma is removed from the circulation at one or more sites along the extensive vascular system interposed between star vessels and the renal vein. Whether this process solely involves local extraction and degradation in postglomerular renal blood vessels or requires additional means of disposal in the interstitium, renal lymph, renal epithelia, or tubule fluid is not determined by the present studies. That it certainly can occur, however, is supported by the following lines of reasoning. First, the kidney extracts nearly all intravascular angiotensin II from the blood flowing through it in a single passage (21, 29–32). Destruction of filtered angiotensin in the proximal tubule undoubtedly contributes to this process (33). However, because glomerular filtration fractions generally range between 20 and 30%, it is clear that the majority of intravascular angiotensin II in the kidney must be removed from plasma elsewhere in the renal circulation, which is most extensive at sites downstream to the star vessel. Second, it has been shown that certain nonrenal organs (liver, heart, hindlimb) remove angiotensin II from the circulation very rapidly (29–31). We believe that similar mechanisms allow the kidney to maintain a large gradient in angiotensin concentrations between star vessels and the renal vein, thereby serving to minimize the systemic effects of intrarenally released peptide.

The information in Table III shows that there are several potential sources for the angiotensin in glomerular filtrate, proximal tubule fluid, and star vessel plasma. The present studies do not distinguish between these sources, nor do they exclude augmentation from possible sources in the interstitium. However, the very high angiotensin concentrations in star vessel plasma suggest that angiotensin II can be released directly into or formed within the lumina of glomerular arterioles (and perhaps glomerular capillaries). Granular cells in the afferent arteriole contain abundant angiotensin II (24, 28). Thus, if direct release of angiotensin into plasma does occur, it is especially likely that it occurs in the afferent arteriole. It must also be kept in mind that endothelial cells in afferent arterioles, efferent arterioles, and, to a lesser extent, glomerular endothelial cells contain angiotensin-converting enzyme, thereby indicating that intravascular formation might occur at these sites.

The present studies also strongly suggest that angiotensin II is added to proximal tubule fluid. Because exogenously provided angiotensin II is rapidly degraded by the proximal tubule (33), it is likely that filtered endogenous angiotensin II is handled similarly, a conclusion supported by the presence of an unidentified peptide and probable degradation product of angiotensin II in proximal tubule fluid but not glomerular filtrate. The present studies show, however, that endogenous angiotensin concentrations do not fall rapidly in the lumen of the proximal tubule. Therefore, we believe that angiotensin II concentrations in proximal tubule fluid are kept high either by secretion of preformed peptide from proximal tubule cells, by intraluminal generation from precursors, or by influx from the interstitium. Proximal tubule cells contain angiotensinogen and the mRNA necessary for its production (37, 40); they contain renin in at least some situations, apparently due to endocytosis of renin produced elsewhere (24, 25); and they contain angiotensin-converting enzyme activity near their luminal surfaces (38, 39). Thus, the possibility of local angiotensin II production in the proximal tubule is especially appealing.

In considering the significance of nanomolar concentrations of angiotensin II in the intrarenal fluid compartments examined in this study, we note that angiotensin II receptors in proximal tubule preparations have binding constants of ~ 2–10 nM (12, 14, 16). These values closely match our measurements of 6–8 nM angiotensin II in glomerular filtrate and late proximal tubule fluid. We believe, therefore, that small variations in luminal angiotensin concentrations are likely to play an important role in the modulation of proximal tubule functions independently of the very low levels of angiotensin in the systemic circulation. Since most evidence indicates that glomerular angiotensin II binding constants are also in the low nanomolar range (13, 14, 16), we believe that a similar conclusion can be extended to the regulation of glomerular function. This is not to say that systemically applied angiotensin II does not affect renal function. It has been well established, for example, that systemic infusion of a small amount (20 ng/min) of Asn¹, Val⁵-angiotensin II can stimulate bicarbonate reabsorption in the early proximal tubule even though systemic concentrations do not appear to rise higher than 10⁻¹¹ M (9). However, the latter finding may not be directly applicable to the present results for several reasons. First, the present studies measured concentrations of Asp¹, Ile⁵-angiotensin II, which is the native form of angiotensin II in the rat (41).

Table III. Angiotensin II in Glomerular Filtrate, Proximal Tubule Fluid, and Star Vessel Plasma: Possible Sites of Production and/or Release

	Angiotensinogen		Renin		ACE	Angiotensin II	Ref.
	Peptide	mRNA	Peptide	mRNA			
Afferent arterioles			+	+	+(E)	+	24, 25, 28, 34–36
Glomerulus		±	±	+	±(E)	+	24, 27, 28, 34, 36, 37
Efferent arterioles			±		+(E)		24, 25, 28, 34
Peritubular capillary					±(E)		34
Proximal tubule	+	+	+		+(BB)		24, 25, 37–40
Distal nephron segments		±	+				24, 37

ACE, angiotensin converting enzyme; E, endothelium; BB, brush border.

Second, the results of the present study show that systemic angiotensin concentrations cannot be used to predict angiotensin concentrations near intrarenal angiotensin receptors. It is quite possible that any direct effect of systemically administered angiotensin II is attributable to transient augmentation of ambient intrarenal angiotensin concentrations. Third, even small doses of systemically applied Asn¹, Val⁵-angiotensin II exert potentially important systemic effects, as evidenced by 9–11 mm Hg increments in systemic blood pressure (2, 9). Such systemic effects make it difficult to conclude that the effects of systemically administered angiotensin II on the kidney must be solely the result of direct renal actions. We therefore believe that the direct effects of angiotensin II on the kidney are more likely to correlate with local rather than systemic concentrations of this peptide.

We conclude that nanomolar concentrations of angiotensins II and III are normally present in glomerular filtrate, proximal tubule fluid, and star vessel plasma. These concentrations approximate known angiotensin receptor affinities. However, they are substantially higher than angiotensin II concentrations in the systemic circulation, thus directly supporting the notion that local angiotensin II effects regulation of renal function independently of systemic angiotensin II. We further conclude that intrarenal angiotensin II concentrations are substantially higher than previously reported angiotensin concentrations in the renal vein. We interpret this to mean that angiotensin II is cleared from the circulation at one or more sites between star vessels and the renal vein, thereby serving to minimize the systemic effects of intrarenally released angiotensin II.

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