

Angiotensin I Is Largely Converted to Angiotensin (1-7) and Angiotensin (2-10) by Isolated Rat Glomeruli

Juan Carlos Q. Velez, Kevin J. Ryan, Caroline E. Harbeson, Alison M. Bland, Milos N. Budisavljevic, John M. Arthur, Wayne R. Fitzgibbon, John R. Raymond, Michael G. Janech

Abstract—Intraglomerular renin-angiotensin system enzyme activities have been examined previously using glomerular lysates and immune-based assays. However, preparation of glomerular extracts compromises the integrity of their anatomic architecture. In addition, antibody-based assays focus on angiotensin (Ang) II detection, ignoring the generation of other Ang I-derived metabolites, some of which may cross-react with Ang II. Therefore, our aim was to examine the metabolism of Ang I in freshly isolated intact glomeruli using matrix-assisted laser desorption ionization time of flight mass spectrometry as an analytic method. Glomeruli from male Sprague-Dawley rats were isolated by sieving and incubated in Krebs buffer in the presence of 1 $\mu\text{mol/L}$ of Ang I for 15 to 90 minutes, with or without various peptidase inhibitors. Peptide sequences were confirmed by matrix-assisted laser desorption ionization time of flight tandem mass spectrometry or linear-trap-quadrupole mass spectrometry. Peaks were quantified using customized valine- ^{13}C - ^{15}N -labeled peptides as standards. The most prominent peaks resulting from Ang I cleavage were 899 and 1181 m/z , corresponding with Ang (1-7) and Ang (2-10), respectively. Smaller peaks for Ang II, Ang (1-9), and Ang (3-10) also were detected. The disappearance of Ang I was significantly reduced during inhibition of aminopeptidase A or neprilysin. In contrast, captopril did not alter Ang I degradation. Furthermore, during simultaneous inhibition of aminopeptidase A and neprilysin, the disappearance of Ang I was markedly attenuated compared with all of the other conditions. These results suggest that there is prominent intraglomerular conversion of Ang I to Ang (2-10) and Ang (1-7), mediated by aminopeptidase A and neprilysin, respectively. Formation of these alternative Ang peptides may be critical to counterbalance the local actions of Ang II. Enhancement of these enzymatic activities may constitute potential therapeutic targets for Ang II-mediated glomerular diseases. (*Hypertension*. 2009;53:790-797.)

Key Words: renin-angiotensin system ■ neprilysin ■ aminopeptidase A ■ angiotensin-converting enzyme ■ angiotensin II ■ podocytes ■ des-Asp (1)-angiotensin I

Overactivation of the renin-angiotensin system (RAS) exerts a pivotal role in the mechanisms of glomerular injury implicated in progressive kidney diseases. More importantly, an intrinsic RAS localized in the kidney seems to be critical for mediating those pathogenic processes. Research efforts focused on the intrarenal RAS have mainly addressed the local generation of angiotensin (Ang) II from its parent decapeptide Ang I,¹⁻³ because Ang II is the major vasoactive effector of the RAS. Evidence indicates that intrarenal Ang II formation is largely mediated by angiotensin-converting enzyme (ACE) under normal physiological conditions,⁴⁻⁶ although other non-ACE Ang II-forming enzymes have been recognized in certain types of renal disease.^{7,8} Most of the data that support this notion have been obtained using antibody-based methods to detect Ang II, such

as radioimmunoassays or ELISAs, which do not allow for a comprehensive and simultaneous examination of all of the metabolites that are generated from Ang I.

Indeed, the fate of Ang I depends on which truncating enzyme catalyzes its fragmentation (Figure 1). Recent studies performed in proximal tubular preparations using analytic methods, such as high-performance liquid chromatography and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (MS), have shown that Ang I is actively converted to peptides distinct from Ang II.^{9,10} Moreover, we showed recently that cultured mouse podocytes predominantly metabolize Ang I to Ang (1-7) and Ang (2-10) via conversion mediated by neprilysin (NEP) and aminopeptidase A (APA) respectively, with less detectable ACE-mediated Ang II formation.¹¹ Conversely, we observed

Received January 7, 2009; first decision February 8, 2009; revision accepted February 16, 2009.

From the Medical and Research Services (J.C.Q.V., M.N.B., J.M.A., J.R.R., M.G.J.), Ralph H. Johnson Veterans Affairs Medical Center; and the Division of Nephrology (J.C.Q.V., K.J.R., C.E.H., A.M.B., M.N.B., J.M.A., W.R.F., J.R.R., M.G.J.), Department of Medicine, Medical University of South Carolina, Charleston.

Portions of this article were presented in abstract form at the American Society of Nephrology Meeting, San Francisco, Calif, November 2007, and published as an abstract (*J Am Soc Nephrol*. 2007;18:A-F497), and at the American Heart Association High Blood Pressure Conference, Atlanta, Ga, September 2008, and published as an abstract (*Hypertension*. 2008;52:e92).

Correspondence to Juan Carlos Q. Velez, Division of Nephrology, 96 Jonathan Lucas St, CSB, Room 829, Medical University of South Carolina, Charleston, SC 29425. E-mail velezj@muscc.edu

© 2009 American Heart Association, Inc.

Hypertension is available at <http://hyper.ahajournals.org>

DOI: 10.1161/HYPERTENSIONAHA.109.128819

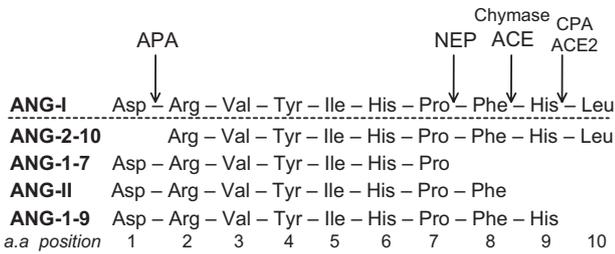


Figure 1. Schematic of intrarenal angiotensin-converting enzymes and the corresponding Ang metabolites.

that mesangial cells mainly convert Ang I to Ang II. These contrasting patterns of fragmentation are particularly interesting because of the alleged antagonistic actions of Ang (1-7) to those of Ang II.¹²⁻¹⁴

Thus, these observations led us to postulate that NEP and APA activities may play critical roles in the intraglomerular processing of Ang I. Therefore, the purpose of our study was to examine the relative contributions of the different RAS enzymes expressed in the glomerulus on the processing of Ang I in a preparation of freshly isolated glomeruli, using MALDI-TOF MS for peptide detection. We hypothesized that non-ACE pathways are largely responsible for the cleavage of Ang I in a glomerular unit as a whole.

Methods

Animals

Studies were performed on adult male Sprague-Dawley rats (160 to 240 g) purchased from Harlan Laboratories (Indianapolis, Ind). Animals were housed in the Medical University of South Carolina animal facility and fed using a standard chow, normal-sodium diet until sacrifice. Studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee.

Glomerular Isolation

Rats were anesthetized by an IP injection of pentobarbital (50 mg/kg), after which a midline abdominal incision and thoracotomy were performed. Then, the left cardiac ventricle was punctured with an 18-gauge, blunt-tipped needle for direct intracardiac injection of cold PBS solution to perfuse the kidneys at a rate of 400 μL/s. Blanching of both kidneys was visualized before organ resection.

Bilateral nephrectomies were performed quickly, and the harvested kidneys were placed onto a dissecting platform. Glomeruli were isolated by a standard mechanical sieving technique, as described previously.¹⁵ Briefly, kidneys were decapsulated, and sinus fat was excised. Isolated cortices were minced to a paste-like consistency with a surgical blade. The tissue paste then was forced through a series of mesh filters with 180, 100, 75, and 70 μm openings. Retained material on the last sieve was rinsed off and transferred to Krebs buffer at 37°C.

Verification of Sample Quality

Glomerular suspensions were centrifuged at 500g for 5 minutes. Pellets containing the glomeruli were washed and resuspended in fresh Krebs buffer for counting and verification of purity, according to the methods by Atiyeh et al,¹⁶ with minor modifications. Twenty-milliliter aliquots were placed on a hemacytometer for counting, as shown in Figure 2. Purity was determined by inspecting 20-μL aliquots of glomerular suspensions on glass slides at ×10 to ×40 magnification using a light microscope (American Optical Corporation). Purity was estimated by the number of glomeruli divided by the total number of particles counted, including tubular fragments, and averaged over 10 fields (Figure 2). Only preparations showing >95% purity and minimal tubular contamination were used for the experiments. Because some authors have reported that as many as 30% of glomeruli isolated by sieving retain their capsules,¹⁷ we confirmed the absence of Bowman’s capsule by scanning electron microscopy. We placed 25 μL of glomerular suspension on coated Thermanox coverslips (Electron Microscopy Sciences), incubated at 37°C for 2 hours and subsequently fixed with 2.5% glutaraldehyde for 1 hour. Preparations were treated with 2% osmium tetroxide, dehydrated with 50% to 100% ethanol, dried with hexamethyldisilazane, mounted on metal stubs, sputter coated with gold palladium, and then examined with a JEOL 5410 scanning electron microscope. None of the examined glomeruli had a visible capsule.

Experimental Conditions

To examine the processing of Ang substrates by isolated rat glomeruli, glomerular suspensions were aliquoted into 1.5-mL polypropylene tubes and incubated in the presence of 1 μmol/L of exogenous Ang I (Bachem Biochemicals) in Krebs buffer at 37°C in a rocking system for ≤90 minutes. The integrity of glomeruli after 90 minutes was corroborated by light microscopy. The optimal number of glomeruli needed per assay tube was determined based on the ability to detect metabolites with adequate resolution. Different numbers of glomeruli per assay tube were tested (Figure 3A). Two thousand glomeruli per microliter allowed detection of peaks of Ang metabolites with minimal noise, whereas 1000 glomeruli per microliter only allowed for minimal detection of metabolites. On the other hand, >4000 glomeruli per microliter produced a rapid disappearance of Ang I, not allowing for adequate examination of enzymatic

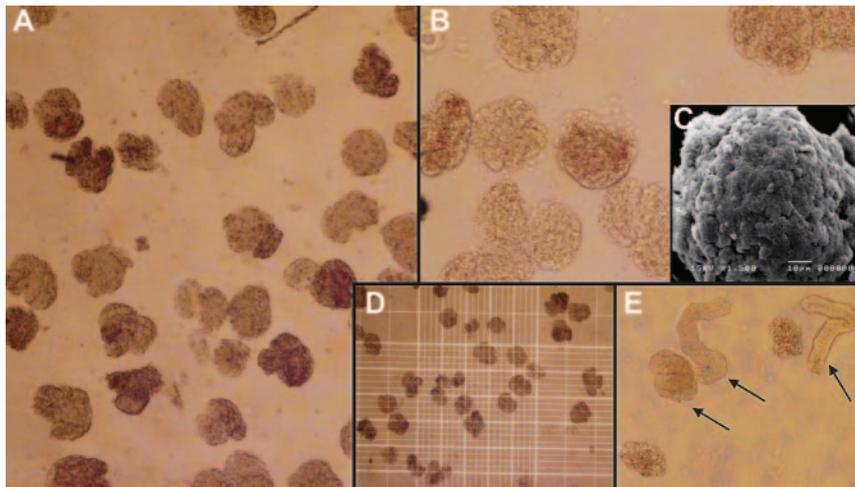


Figure 2. Rat glomeruli isolated by sieving visualized by light microscopy. A, Representative sample showing >95% purity (at ×10 magnification), therefore considered adequate. B, Additional example of an adequate sample (at ×20 magnification). C, Glomerulus examined by scanning electron microscopy showing lack of capsule. D, Glomeruli were counted using a hemocytometer. E, Inadequate sample contaminated with tubular fragments (arrows), which was discarded.

Downloaded from http://hyper.ahajournals.org/ by guest on January 23, 2018

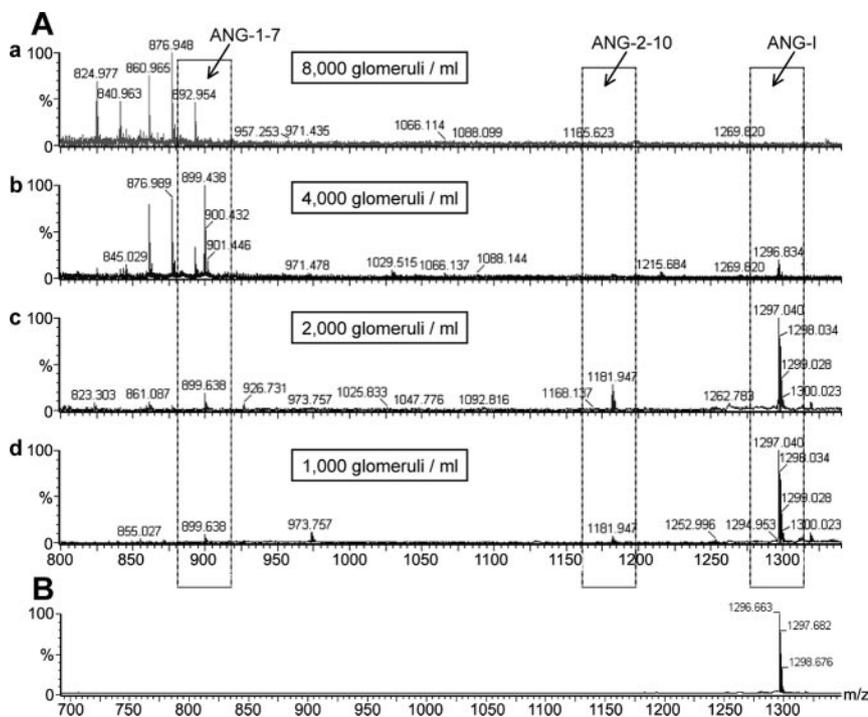


Figure 3. Top, A, Mass spectrum generated by suspensions containing different numbers of isolated rat glomeruli after incubation with 1 $\mu\text{mol/L}$ of Ang I for 60 minutes. a, 8,000 glomeruli per microliter; no Ang peak was identified. b, 4,000 glomeruli per microliter; rapid disappearance of Ang I and appearance of Ang (1-7) were observed. c, 2,000 glomeruli per microliter; Ang metabolites [Ang (2-10) and Ang (1-7)] were easily detected. d, 1,000 glomeruli per microliter; slower rate of Ang I disappearance was observed compared with suspensions with 2,000 glomeruli per microliter. Bottom, B, Mass spectrum from sample containing 1 $\mu\text{mol/L}$ of Ang I incubated in the absence of glomeruli for 60 minutes displaying no spontaneous cleavage.

conversions. Therefore, each experiment was performed with 2000 glomeruli per microliter of Krebs buffer. Aliquots of glomerular suspension (between 5 and 30 μL , depending on each glomerular count) were transferred into the assay tubes. To corroborate the specificity of our findings, we simultaneously examined the processing of Ang I by nonglomerular tissue. To that end, matching volumes of tubular fragments or rat plasma obtained from the same animal were aliquoted into separate tubes. In addition, incubation of Ang I in the absence of glomeruli or other tissue did not reveal artifacts because of postsource decay or evidence of spontaneous degradation of the peptide (Figure 3B). Samples (25 μL) were obtained from the surface of the assay tube at 15-minute intervals after gentle centrifugation of the suspensions at 1000g for 30 seconds and immediate resuspension for subsequent time points. Additional glomerular suspensions were preincubated in the presence of enzyme inhibitors or vehicle for 20 minutes before exposure to the Ang substrate. The following enzyme inhibitors were used: thiorphan 10 $\mu\text{mol/L}$ (NEP inhibitor), amastatin 100 $\mu\text{mol/L}$ (APA inhibitor), captopril 100 $\mu\text{mol/L}$ (ACE inhibitor), and benzylsuccinate 10 $\mu\text{mol/L}$ (carboxypeptidase-A [CPA] inhibitor), all from Sigma-Aldrich, as well as DX-600 10 $\mu\text{mol/L}$ (ACE-2 inhibitor) from Phoenix Pharmaceuticals. To examine the effect of Ang II type 1 receptor internalization, additional samples were preincubated with 10 $\mu\text{mol/L}$ of losartan. Samples collected were centrifuged and stored at -20°C for further analysis.

Ang Metabolite Analysis and Quantification

Samples obtained at various time points were assayed by MALDI-TOF MS, as described previously.¹¹ Briefly, peptides were purified by C18-Zip-Tip columns (Millipore). Columns were equilibrated with 100% acetonitrile, washed with 0.1% trifluoroacetic acid, loaded with 20 μL of sample, washed with 10 μL of 0.1% trifluoroacetic acid, and eluted with a low-pH MALDI matrix compound (10g/L α -cyano-4-hydroxycinnamic acid in a 1:1 mixture of 50% acetonitrile and 0.1% trifluoroacetic acid). The eluted matrix (1.5 μL) was applied to the surface of a target plate in triplicate. The plate was air dried before collecting spectra. Spectra were collected in reflectron mode using a M@LDI MALDI-TOF mass spectrometer (Waters Corp). Results were analyzed using MassLynx 2.0 software (Waters Corp). Peptide identities were confirmed by MS/MS de novo sequencing from an ion series generated by MALDI-TOF-TOF

(ABI 4700 Series) and linear trap quadrupole MS (Thermo Scientific). Quantifications of observed peaks were performed using customized absolute quantification (AQUA) peptides (Sigma-Aldrich) as internal standards, as described previously.¹¹ AQUA peptides are 6 Da larger than the native peptide as a result of [$^{13}\text{C}^{15}\text{N}$]-valine incorporation into the amino acid sequence. A solution of AQUA peptides was mixed with each sample of conditioned buffer before MALDI-TOF MS analysis. The final concentration of each AQUA peptide in the mix was set between 10 and 500 nmol/L, depending on the qualitative appearance of the peptide peaks on the mass spectra. For determination of the abundance of each native peptide, the intensity of the major monoisotopic peak was divided by the intensity of the major peak of the corresponding AQUA peptide. The ratio was multiplied by the known concentration of the AQUA peptide to estimate the native peptide concentration. This method has been validated previously by others.¹⁸ Peak abundances were normalized to the number of glomeruli rather than to total protein, because sample abundance was often below the level of detection by commercial protein assays. There was small variability in the glomerular diameter, confirmed by light microscopy and scanning electron microscopy (glomerular diameter in a representative sample: $80.9 \pm 3.0 \mu\text{mol/L}$ [mean \pm SD]). Therefore, we assumed that the glomerular size distribution was equal between animals. In addition, we compared the performance of MALDI-TOF MS with an antibody-based method for which samples were analyzed using a commercially available ELISA kit for Ang I (Peninsula Laboratories).

RAS Enzyme Expression

The presence of specific enzymes in the glomerular pellets was determined by immunoblotting by a method described previously.¹¹ The following primary antibodies were used: rabbit anti-NEP (dilution 1:500; Chemicon), goat anti-APA (anti-BP-1, dilution 1:500; Abcam), and goat anti-ACE (N-20, dilution 1:500; Santa Cruz Biotechnology).

Statistical Analyses

Data were expressed as means \pm SEs. Differences between pairs of means were analyzed by Student *t* test with the assumption of equal variance. Multiple group data were analyzed using ANOVA and then posthoc analysis of means by Student *t* test with Bonferroni's

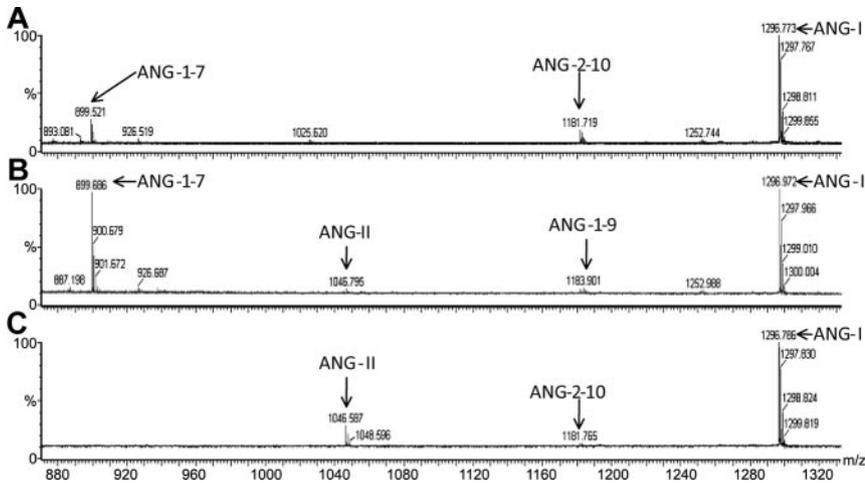


Figure 4. MALDI-TOF mass spectra generated by (A) glomerular suspensions, (B) volume-matched tubular fragments suspensions, or (C) volume-matched plasma aliquots, incubated in Krebs buffer in the presence of 1 $\mu\text{mol/L}$ of Ang I for 15 minutes.

correction. The effect on enzyme inhibition on Ang I disappearance was analyzed using 2-factor (inhibitor and time) ANOVA for repeated measures. Differences between pairs of enzyme inhibitors were tested using Scheffe test. $P < 0.05$ was considered significant.

Results

Conversion of Ang I to Ang (1-7) and Ang (2-10)

Incubation of glomerular suspensions in the presence of Ang I revealed evidence of rapid enzymatic processing of the decapeptide by isolated rat glomeruli. The most prominent peaks resulting from Ang I cleavage were detected at 899 and 1181 m/z , corresponding with Ang (1-7) and Ang (2-10), respectively (Figure 4A). Smaller peaks for Ang II, Ang (1-9), Ang (2-9), Ang (3-10), and Ang (4-10) were also detected. The observed spectra resembled those generated by cultured podocytes and differed from those observed in mesangial cells.¹¹ Preincubation with losartan did not enhance the detection of Ang II (data not shown), suggesting that internalization of Ang II did not substantially alter the spectra. Spectra generated by glomerular suspensions differed from those generated by either tubular fragments (Figure 4B) or plasma (Figure 4C).

Role of APA

Conversion of Ang I to Ang (2-10) was inhibited by an APA inhibitor. At 60 minutes, 100 $\mu\text{mol/L}$ of amastatin decreased the abundance of Ang (2-10) (27.61 ± 2.9 versus 11.57 ± 1.7 pg per glomeruli, for untreated and treated, respectively; $P < 0.01$) and favored the accumulation of Ang (1-7) (21.85 ± 1.8 versus 33.11 ± 2.9 pg per glomeruli, respectively; $P < 0.01$; Figure 5A). Furthermore, the decrease in the Ang (2-10) peak caused by amastatin was accompanied by the appearance of an Ang (1-9) peak, a nonsignificant increase in Ang II, and a relatively smaller peak height for Ang (3-10). Quantification of Ang (1-9) could not be performed because of overlap with Ang (2-10) monoisotopic peaks.

Role of NEP

Conversion of Ang I to Ang (1-7) was inhibited by a NEP inhibitor. At 60 minutes, 10 $\mu\text{mol/L}$ of thiorphan decreased the abundance of Ang (1-7) (21.85 ± 1.8 versus 13.41 ± 0.7 pg per glomeruli, for untreated and treated, respectively; $P < 0.05$) and increased the formation of Ang (2-10)

(27.61 ± 2.9 versus 46.62 ± 10.5 pg per glomeruli, respectively; $P < 0.01$; Figure 5B).

Effect of Dual Blockade of APA and NEP

Fragmentation of Ang I was largely inhibited by combining inhibitors for APA and NEP. After the initial 15 minutes of incubation, there was a 57.4% reduction in Ang I abundance in the untreated glomerular suspensions, which was significantly decreased to only 14.9% by combined APA and NEP inhibition ($P < 0.001$). In addition, dual blockade of APA and NEP significantly increased the abundance of Ang II (3.7 ± 2.9 versus 15.55 ± 3.9 pg per glomeruli, for untreated and treated, respectively; $P < 0.05$; Figure 5C and 6A). The appearance of Ang (1-9) and relative reduction in peak height for Ang (3-10) also were observed under these conditions (Figure 5C). Neither DX-600 (ACE-2 inhibitor) nor benzylsuccinate (CPA inhibitor) affected the qualitative appearance of Ang 1-9, suggesting that Ang I-to-Ang (1-9) conversion may not be mediated by ACE2 or CPA, as suggested by other authors,^{19,20} but by another unknown carboxypeptidase.

Ang I Disappearance From Glomerular Suspensions

The disappearance of Ang I was significantly reduced during inhibition of APA or NEP ($P < 0.05$ for each treatment versus untreated). In contrast, ACE inhibition by captopril did not inhibit Ang I degradation. Furthermore, simultaneous inhibition of APA and NEP significantly attenuated the disappearance of Ang I compared with all of the other conditions ($P < 0.0001$ for the combination of amastatin+thiorphan versus untreated or versus captopril-treated; $P < 0.005$ versus amastatin- or thiorphan-treated; Figure 6B). On the other hand, inhibition of ACE-2 or CPA failed to alter the degradation of Ang I. To compare the performance of our MALDI-TOF MS measurements with a standard antibody-based method, we also assayed samples from the untreated group by ELISA. Although similar curves of Ang I disappearance were obtained overall, there was a significant difference in the values obtained at 15 minutes ($P < 0.05$; Figure 7), possibly reflecting cross-reactivity of the ELISA antibody with other Ang peptides. The cross-reacting peptides are not present at time 0, because none have been created. It is likely that the

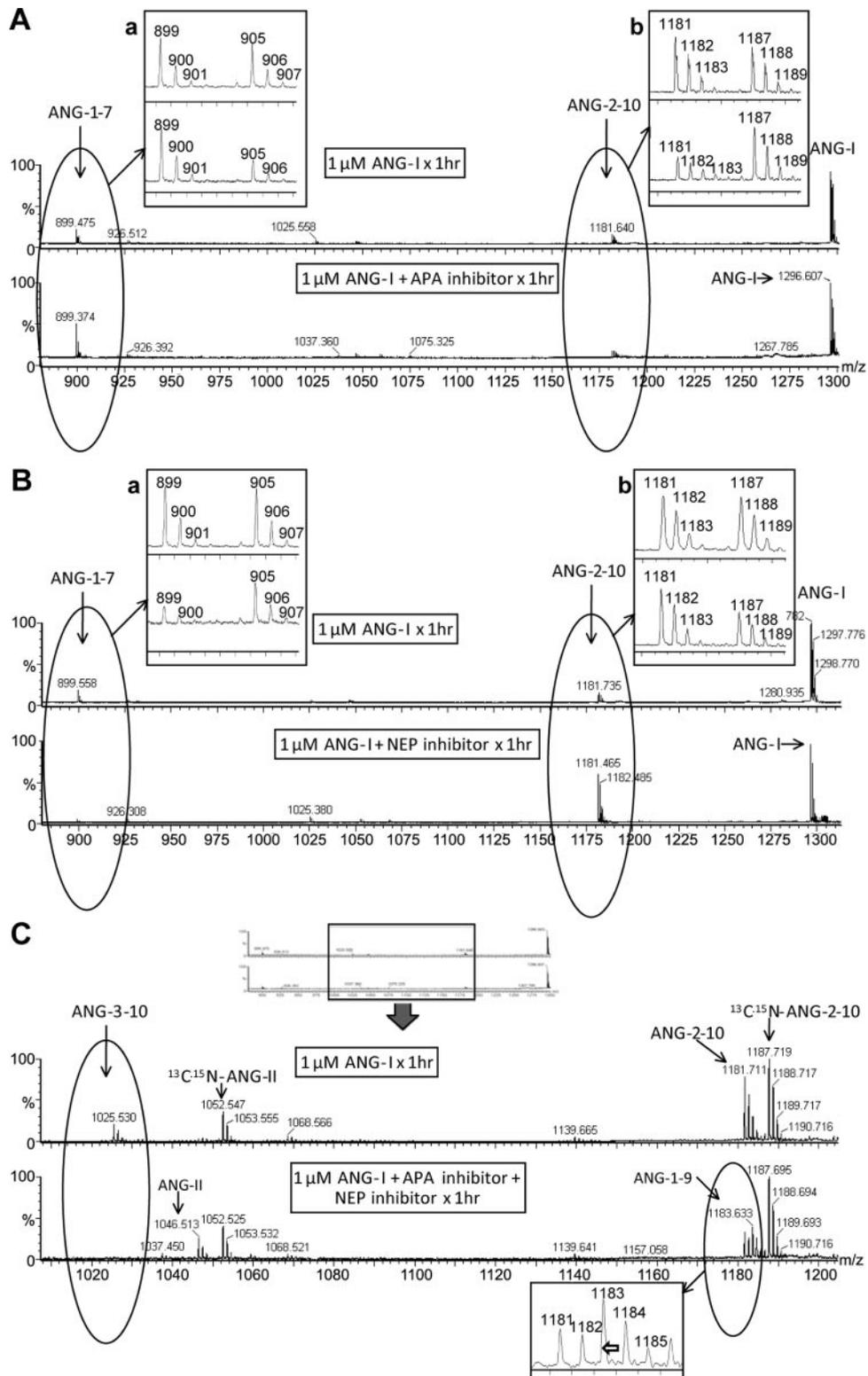


Figure 5. MALDI-TOF mass spectra generated from conditioned buffer samples collected from glomerular suspensions incubated with (A) 1 $\mu\text{mol/L}$ of Ang I (top) or 1 $\mu\text{mol/L}$ of Ang I+100 $\mu\text{mol/L}$ of amastatin (APA inhibitor, bottom) for 60 minutes. Inset a, Quantification of Ang (1-7) peaks using 50 nmol/L AQUA-Ang (1-7) as an internal standard. Inset b, Quantification of Ang (2-10) peaks using 50 nmol/L AQUA-Ang (2-10) as an internal standard. B, 1 $\mu\text{mol/L}$ of Ang I (top) or 1 $\mu\text{mol/L}$ of Ang I+10 $\mu\text{mol/L}$ of thiorphan (NEP inhibitor, bottom) for 60 minutes. Inset a, Quantification of Ang (1-7) peaks using 50 nmol/L AQUA-Ang (1-7) as an internal standard. Inset b, Quantification of Ang (2-10) peaks using 50 nmol/L AQUA-Ang (2-10) as an internal standard. C, 1 $\mu\text{mol/L}$ of Ang I (top) or 1 $\mu\text{mol/L}$ of Ang I+100 $\mu\text{mol/L}$ of amastatin (APA inhibitor)+10 $\mu\text{mol/L}$ of thiorphan (NEP inhibitor, bottom) for 60 minutes. AQUA-Ang II (10 nmol/L) and AQUA-Ang (2-10) (50 nmol/L) peptides are shown in the spectra (m/z 1052 and 1187, respectively). Dual APA+NEP inhibition qualitatively reduced the intensity of the Ang (2-10) and Ang (3-10) peaks and enhanced the detection of Ang II. Inset, APA inhibition unmasked a detectable Ang (1-9) peak (m/z 1183). Arrow indicates the predicted contribution of the 1181 monoisotopic peak to the total 1183 peak intensity.

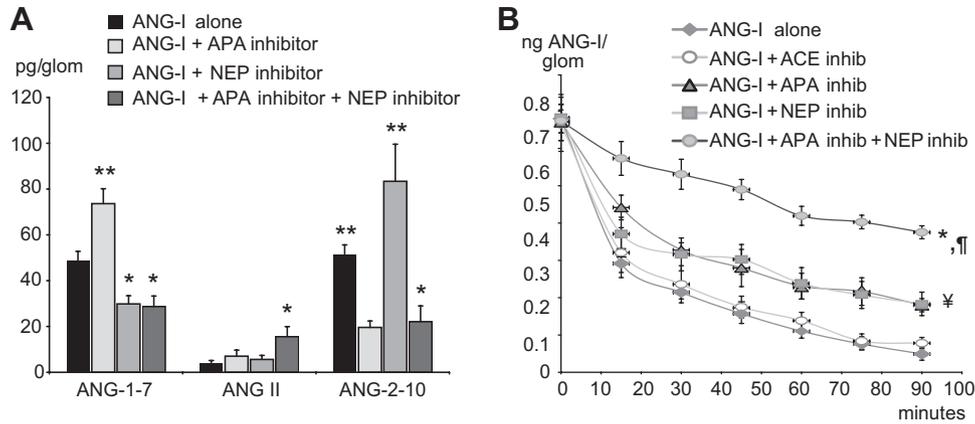


Figure 6. A, Quantification of Ang I metabolites after 60 minutes of incubation of glomerular suspensions with 1 μmol/L of Ang I, assayed by MALDI-TOF MS and calculated using AQUA peptides. **P*<0.05 vs untreated (Ang I alone), ***P*<0.01; n=7. B, Disappearance of Ang I determined for incubation of glomerular suspensions with 1 μmol/L of Ang I in the presence or absence of 100 μmol/L of amastatin (APA inhibitor), 10 μmol/L of thiorphan (NEP inhibitor), amastatin+thiorphan, or 100 μmol/L of captopril (ACE inhibitor). **P*<0.0001 vs Ang I alone or vs captopril-treated; †*P*<0.05 vs amastatin-treated or thiorphan-treated; ‡*P*<0.05 vs Ang I alone or captopril-treated; n=7.

peptides are themselves further metabolized at later time points, eliminating cross-reactivity. This ability to distinguish similar peptides specifically and simultaneously is a major advantage of MALDI-TOF MS analysis of Ang I metabolites.

ACE Expression

The presence of candidate RAS enzymes that were implicated by the MALDI-TOF MS assays was confirmed by Western blotting. APA, NEP, and ACE were detected in glomerular lysates (Figure 8).

Discussion

The fate of Ang I depends on catalytic reactions that convert it to smaller peptides. ACE is the most important renal carboxypeptidase that cleaves Ang I at its carboxy terminus to generate Ang II,^{4,5} although chymase also appears to play a role in Ang II formation in certain disease states.^{7,8,21} Endopeptidases, such as NEP or prolyl-endopeptidase, can also cleave Ang I to generate Ang (1-7), whereas conversion

of Ang I to Ang (1-9) is thought to occur via ACE2 or CPA. On the other hand, at the amino terminus domain, Ang I is a substrate for APA, which converts it to Ang (2-10), also known as Des-Asp¹-Ang I²² (Figure 1). To date, numerous studies have characterized the presence of an intrarenal RAS. However, few of those studies have attempted to describe a RAS localized in the glomeruli.^{16,23} Furthermore, most studies have solely focused on mechanisms of conversion of Ang I to Ang II,^{1,16,24} failing to explore the conversion of Ang I to other bioactive metabolites.

Herein, we examined the mechanisms of glomerular Ang I conversion. We used isolated rat glomerular suspensions to preserve the intrinsic architecture of the glomeruli and found that Ang I is rapidly metabolized to smaller fragments in this model. A similar model was used previously to examine endogenous formation of Ang II.¹⁶ However, the investigators did not explore the conversion of Ang I to other fragments. Our main finding is that the relative contribution of APA and NEP to the glomerular conversion of Ang I is significantly larger than that of ACE, resulting in prominent generation of Ang (1-7) and Ang (2-10). Individual inhibition of APA or NEP induced shunting of the enzymatic pathways for Ang I conversion, whereas combined inhibition of APA and NEP caused a robust decrease in the overall disappearance of Ang I from the glomerular suspensions. Combined APA and NEP inhibition did not abolish Ang I disappearance completely, possibly reflecting a shift toward Ang (1-9) and Ang II formation and some degree of nonspecific proteolytic activity. Furthermore, we observed that glomerular conversion of Ang I differs from that mediated by renal tubular

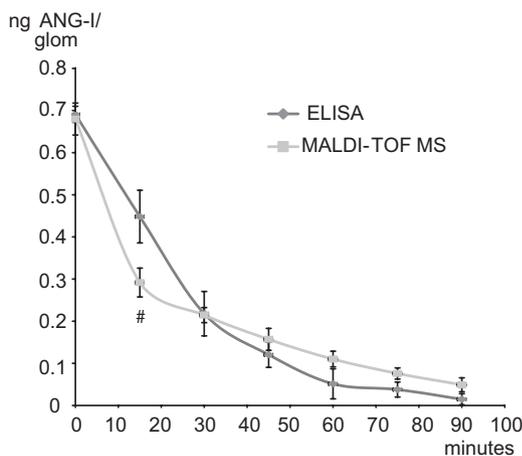


Figure 7. Comparison of the performance of MALDI-TOF MS and ELISA methods for measuring Ang I metabolites on incubation of glomerular suspensions with 1 μmol/L of Ang I. #*P*<0.05; n=3.

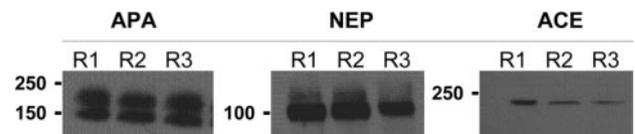


Figure 8. Expression of angiotensin-converting enzymes in rat glomeruli examined by Western blotting. Blots from 3 different animals (R1 through R3) are shown.

fragments in that Ang (1-7) was the only dominant fragment generated by the latter. In addition, Ang II was readily detectable in the tubular preparations. Similarly, incubation of Ang I with rat plasma led to prominent Ang II formation, most likely reflecting ACE activity. These findings emphasize the notion that the conversion of intraglomerular Ang I does not mimic the processing of the circulating peptide in plasma or in the tubular compartment.

Singh et al²³ also found significant glomerular Ang (1-7) formation by high-performance liquid chromatography, using lysed glomerular extracts. In their study, Ang I also was converted to Ang II and Ang (1-9), but they did not report Ang (2-10) formation. Nevertheless, our results are in agreement with previous studies that have demonstrated that there is substantial intrarenal conversion of Ang I that does not involve Ang II formation.^{5,6,25} Danser et al⁶ cannulated the renal vasculature of female pigs to measure the rate of degradation of Ang I within the renal circulation and found that >90% of Ang I was metabolized during ACE inhibition, suggesting rapid hydrolysis by other peptidases. Similarly, Rosivall et al⁵ studied mongrel dogs and found that <20% of Ang I was converted to Ang II during a single passage through the kidney. In contrast, they detected Ang (2-10) as a major metabolite in the renal venous effluent. Moreover, Bauer et al²⁶ also reported that Ang I is degraded by \approx 90% in the rat renal circulation and that 60% of that degradation was prevented by an APA inhibitor. However, none of those studies specifically examined Ang I metabolism within the glomerular compartment.

Our findings are novel in 2 regards. First, the magnitude of Ang I conversion to Ang (1-7) and Ang (2-10) by glomerular tissue has not been reported previously. These conversions are primarily mediated by NEP and APA, respectively. Both NEP and APA are expressed in podocytes,²⁷⁻²⁹ although others also have detected them in mesangial cells.³⁰ Secondly, our data unveil prominent Ang (2-10) formation by glomerular tissue. Few studies have attempted to examine the actions of Ang (2-10). Studying normotensive and hypertensive rats, Mustafa et al³¹ observed that femtomolar concentrations of Ang (2-10) attenuate the vasopressor responses induced by Ang III in mesenteric and renal vasculature. The same laboratory reported that Ang (2-10) binds to Ang II type 1 receptors in rabbit pulmonary arteries³² and opposes the proliferative actions of Ang II in rat aortic smooth muscle cells.³³ In contrast, 1 study found that Ang (2-10) has pressor and steroidogenic effects, mostly after being converted to Ang III.³⁴ Therefore, Ang (2-10) may be an active RAS peptide, not a mere waste metabolite. Recently, significant attention has been brought to the study of Ang (1-7) as a molecule that antagonizes the actions of Ang II, suggesting that Ang (1-7) may stimulate protective cardiovascular effects. Interestingly, measurements by high-performance liquid chromatography in rats revealed that plasma Ang (2-10) levels are comparable to those of Ang II and that they become elevated in the setting of ACE inhibition.³⁵ Furthermore, the same study also reported detection of Ang (2-10) in kidney tissue at a similar concentration to that of Ang (1-7). Thus, it seems logical to speculate that variations in the local profile and relative abundances of Ang I metabolites, ie, Ang II, Ang

(1-7), and Ang (2-10), ought to determine the net autocrine or paracrine effects of these Ang peptides, more so than individual peptide concentrations.

Our results must be interpreted in the context of the limitations of the study design. The assay was performed by incubating nonperfused, nonfiltering glomeruli. It is conceivable that renal hemodynamics or innervation may influence the activity of certain ectoenzymes, including ACE. Certain nonspecific peptidases could be released, shed, or activated during the isolation of glomeruli, thereby prompting peptide processing that does not entirely reflect in vivo glomerular enzymatic activity. In addition, because podocytes overlie the glomerular corpuscle and are situated at its periphery, they may be exposed more directly to the substrate-containing solution, thereby underestimating the activity of mesangial or endothelial cells that reside more centrally in the glomerular tuft. The similarity in Ang peptide profiles between isolated glomeruli and cultured podocytes supports this possibility,¹¹ although it is possible that podocytes play more prominent roles in glomerular Ang I metabolism than do mesangial cells. Nevertheless, modest effects of ACE inhibition on Ang I processing observed in in vivo models^{5,6,25} parallel our findings and support a critical role for APA and NEP in intraglomerular RAS homeostasis.

Perspectives

APA and NEP play significant roles in Ang I conversion within the glomerular compartment and may influence the homeostasis of the intraglomerular RAS and the net delivery of Ang peptides to the urinary space. Formation of Ang 2-10 is an overlooked component of the intrarenal RAS that may contribute to renal hemodynamics in normal or pathological states to counterbalance the actions of Ang II. Additional research is needed to discern the role of Ang 2-10 and the net effect of metabolites on glomerular disease.

Acknowledgments

We thank Dr Louis Luttrell for valuable discussion and Cory Woodworth for technical assistance.

Sources of Funding

This work was supported by a Research Enhancement Award Program grant to the Ralph H. Johnson Veterans Affairs Medical Center. J.C.Q.V. is supported by the Dialysis Clinics, Inc, Paul Teschan Research Fund, and National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases grant DK080944-01. M.G.J. is supported by a Veterans Affairs CDA-2 award and the Nephcure Foundation.

Disclosures

None.

References

1. Seikaly MG, Arant BS Jr, Seney FD Jr. Endogenous angiotensin concentrations in specific intrarenal fluid compartments of the rat. *J Clin Invest*. 1990;86:1352-1357.
2. Nishiyama A, Seth DM, Navar LG. Renal interstitial fluid concentrations of angiotensins I and II in anesthetized rats. *Hypertension*. 2002;39:129-134.
3. Navar LG, Lewis L, Hymel A, Braam B, Mitchell KD. Tubular fluid concentrations and kidney contents of angiotensins I and II in anesthetized rats. *J Am Soc Nephrol*. 1994;5:1153-1158.

4. Di Salvo J, Peterson A, Montefusco C, Menta M. Intrarenal conversion of angiotensin I to angiotensin II in the dog. *Circ Res.* 1971;29:398–406.
5. Rosivall L, Rinder DF, Champion J, Khosla MC, Navar LG, Oparil S. Intrarenal angiotensin I conversion at normal and reduced renal blood flow in the dog. *Am J Physiol.* 1983;245:F408–F415.
6. Danser AH, Koning MM, Admiraal PJ, Derckx FH, Verdouw PD, Schalekamp MA. Metabolism of angiotensin I by different tissues in the intact animal. *Am J Physiol.* 1992;263:H418–H428.
7. Huang XR, Chen WY, Truong LD, Lan HY. Chymase is upregulated in diabetic nephropathy: implications for an alternative pathway of angiotensin II-mediated diabetic renal and vascular disease. *J Am Soc Nephrol.* 2003;14:1738–1747.
8. Sadjadi J, Kramer GL, Yu CH, Burrell Welborn M III, Chappell MC, Gregory Modrall J. Angiotensin converting enzyme-independent angiotensin II production by chymase is up-regulated in the ischemic kidney in renovascular hypertension. *J Surg Res.* 2005;127:65–69.
9. Shaltout HA, Westwood BM, Averill DB, Ferrario CM, Figueroa JP, Diz DI, Rose JC, Chappell MC. Angiotensin metabolism in renal proximal tubules, urine, and serum of sheep: evidence for ACE2-dependent processing of angiotensin II. *Am J Physiol Renal Physiol.* 2007;292:F82–F91.
10. Li N, Zimpelmann J, Cheng K, Wilkins JA, Burns KD. The role of angiotensin converting enzyme 2 in the generation of angiotensin 1-7 by rat proximal tubules. *Am J Physiol Renal Physiol.* 2005;288:F353–F362.
11. Velez JC, Bland AM, Arthur JM, Raymond JR, Janech MG. Characterization of renin-angiotensin system enzyme activities in cultured mouse podocytes. *Am J Physiol Renal Physiol.* 2007;293:F398–F407.
12. Ren Y, Garvin JL, Carretero OA. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension.* 2002;39:799–802.
13. Chappell MC, Modrall JG, Diz DI, Ferrario CM. Novel aspects of the renal renin-angiotensin system: angiotensin-(1-7), ACE2 and blood pressure regulation. *Contrib Nephrol.* 2004;143:77–89.
14. Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A.* 2003;100:8258–8263.
15. Fong JS, Drummond KN. Method for preparation of glomeruli for metabolic studies. *J Lab Clin Med.* 1968;71:1034–1039.
16. Atiyeh BA, Arant BS Jr, Henrich WL, Seikaly MG. In vitro production of angiotensin II by isolated glomeruli. *Am J Physiol.* 1995;268:F266–F272.
17. Steinmetz OM, Panzer U, Fehr S, Meyer-Schwesinger C, Stahl RA, Wenzel UO. A pitfall of glomerular sieving: profibrotic and matrix proteins derive from the Bowman's capsule and not the glomerular tuft in rats with renovascular hypertension. *Nephrol Dial Transplant.* 2007;22:3055–3060.
18. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol.* 1999;17:994–999.
19. Igit R, Garber S, Sekosan M, Urbanska RA, Behnia R. Localization of carboxypeptidase A-like enzyme in rat kidney. *Peptides.* 2003;24:1237–1240.
20. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res.* 2000;87:E1–E9.
21. Cristovam PC, Arnoni CP, de Andrade MC, Casarini DE, Pereira LG, Schor N, Boim MA. ACE-dependent and chymase-dependent angiotensin II generation in normal and glucose-stimulated human mesangial cells. *Exp Biol Med (Maywood).* 2008;233:1035–1043.
22. Ardaillou R, Chansel D. Synthesis and effects of active fragments of angiotensin II. *Kidney Int.* 1997;52:1458–1468.
23. Singh R, Singh AK, Leehey DJ. A novel mechanism for angiotensin II formation in streptozotocin-diabetic rat glomeruli. *Am J Physiol Renal Physiol.* 2005;288:F1183–F1190.
24. Wysocki J, Ye M, Soler MJ, Gurley SB, Xiao HD, Bernstein KE, Coffman TM, Chen S, Battle D. ACE and ACE2 activity in diabetic mice. *Diabetes.* 2006;55:2132–2139.
25. Danser AH, Admiraal PJ, Derckx FH, Schalekamp MA. Angiotensin I-to-II conversion in the human renal vascular bed. *J Hypertens.* 1998;16:2051–2056.
26. Bauer J, Berthold H, Schaefer F, Ehmke H, Parekh N. Quantification of conversion and degradation of circulating angiotensin in rats. *Am J Physiol.* 1999;277:R412–R418.
27. Edwards RM, Pullen M, Nambi P. Distribution of neutral endopeptidase activity along the rat and rabbit nephron. *Pharmacology.* 1999;59:45–50.
28. Mentzel S, Van Son JP, De Jong AS, Dijkman HB, Koene RA, Wetzels JF, Assmann KJ. Mouse glomerular epithelial cells in culture with features of podocytes in vivo express aminopeptidase A and angiotensinogen but not other components of the renin-angiotensin system. *J Am Soc Nephrol.* 1997;8:706–719.
29. Song L, Ye M, Troyanovskaya M, Wilk E, Wilk S, Healy DP. Rat kidney glutamyl aminopeptidase (aminopeptidase A): molecular identity and cellular localization. *Am J Physiol.* 1994;267:F546–F557.
30. Ebihara F, Di Marco GS, Juliano MA, Casarini DE. Neutral endopeptidase expression in mesangial cells. *J Renin Angiotensin Aldosterone Syst.* 2003;4:228–233.
31. Mustafa MR, Dharmani M, Kunheen NK, Sim MK. Effects of des-aspartate-angiotensin I on the actions of angiotensin III in the renal and mesenteric vasculature of normo- and hypertensive rats. *Regul Pept.* 2004;120:15–22.
32. Chen WS, Sim MK, Go ML. Structure-activity and structure-binding studies of des-Asp(1)-angiotensin I analogues on the rabbit pulmonary artery. *Regul Pept.* 2002;106:39–46.
33. Min L, Sim MK, Xu XG. Effects of des-aspartate-angiotensin I on angiotensin II-induced incorporation of phenylalanine and thymidine in cultured rat cardiomyocytes and aortic smooth muscle cells. *Regul Pept.* 2000;95:93–97.
34. Campbell WB, Schmitz JM, Itskovitz HD. (Des-Asp1) angiotensin I: a study of its pressor and steroidogenic activities in conscious rats. *Endocrinology.* 1977;100:46–51.
35. Campbell DJ, Lawrence AC, Towrie A, Kladis A, Valentijn AJ. Differential regulation of angiotensin peptide levels in plasma and kidney of the rat. *Hypertension.* 1991;18:763–773.

Angiotensin I Is Largely Converted to Angiotensin (1-7) and Angiotensin (2-10) by Isolated Rat Glomeruli

Juan Carlos Q. Velez, Kevin J. Ryan, Caroline E. Harbeson, Alison M. Bland, Milos N. Budisavljevic, John M. Arthur, Wayne R. Fitzgibbon, John R. Raymond and Michael G. Janech

Hypertension. 2009;53:790-797; originally published online March 16, 2009;

doi: 10.1161/HYPERTENSIONAHA.109.128819

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

Copyright © 2009 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/53/5/790>

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/>