Characterization of renin-angiotensin system enzyme activities in cultured mouse podocytes

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Characterization of renin-angiotensin system enzyme activities in cultured mouse podocytes. Am J Physiol Renal Physiol 293: F398–F407, 2007. First published April 7, 2007; doi:10.1152/ajprenal.00050.2007.—Intraglomerular ANG II has been linked to glomerular injury. However, little is known about the contribution of podocytes (POD) to intraglomerular ANG II homeostasis. The aim of the present study was to examine the processing of angiotensin substrates by cultured POD. Our approach was to use matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry for peptide determination from conditioned cell media and customized AQUA peptides for quantification. Immortalized mouse POD were incubated with 1-2 μM ANG I, ANG II, or the renin substrate ANG-(1-14) for different time intervals and coincubated in parallel with various inhibitors. Human mesangial cells (MES) were used as controls. POD incubated with 1 μM ANG I primarily formed ANG-(1-9) and ANG-(1-7). In contrast, MES incubated with ANG I primarily generated ANG II. In POD, ANG-(1-7) was the predominant product, and its formation was inhibited by a nephrilysin inhibitor. Modest angiotensin-converting enzyme (ACE) activity was also detected in POD, although only after cells were incubated with 2 μM ANG I. In addition, we observed that POD degraded ANG II into ANG III and ANG-(1-7). An aminopeptidase A inhibitor inhibited ANG III formation, and an ACE2 inhibitor led to ANG II accumulation. Furthermore, we found that POD converted ANG-(1-14) to ANG I and ANG-(1-7). This conversion was inhibited by a renin inhibitor. These findings demonstrate that POD express a functional intrinsic renin-angiotensin system characterized by nephrilysin, aminopeptidase A, ACE2, and renin activities, which predominantly lead to ANG-(1-7) and ANG-(1-9) formation, as well as ANG II degradation. These findings may reflect a specific role of POD in maintenance of intraglomerular renin-angiotensin system balance.

angiotensin II; angiotensin-(1-7); nephrilysin; ACE2; aminopeptidase A

The renin-angiotensin-system (RAS) plays an indisputable role in the pathogenesis of kidney disease. A substantial amount of evidence demonstrates that blockade of the RAS provides renoprotection (22, 28, 31). In the classical view of the RAS, ANG II is considered the target for blockade. This molecule is an octapeptide that results from cleavage of ANG I by angiotensin-converting enzyme (ACE) (and by other non-ACE peptidases, e.g., chymase and cathepsins). However, recent studies have revealed that other active metabolites, such as ANG-(1-9) and ANG-(1-7), may result from the metabolism of ANG I through the action of ACE2 and nephrilysin (also called neutral endopeptidase) (7, 42). Interestingly, these alternative peptides appear to have effects that oppose those of ANG II (21). For instance, ANG-(1-7) has been recognized as a vasodilator and an anti-inflammatory (18, 39, 43). In terms of tissue specificity, the majority of the studies focused on the generation of these alternative peptides have been performed in vascular smooth muscle cells or cardiac tissue (5, 55), but tissue specificity has also been described in renal cells. In particular, formation of ANG-(1-7) has been detected in rat and sheep proximal tubular extracts (29, 45), whereas ANG-(1-9) formation has been found in rat mesangial cells (48).

More recently, there has been growing interest in the mechanistic role of podocytes in glomerular injury. Numerous studies have attempted to unravel the importance of podocytes in progressive kidney diseases, including models of diabetic nephropathy and focal segmental glomerulosclerosis (2, 46). Nevertheless, the relationship between the RAS and podocyte biology remains largely understudied. Recent reports suggest that podocytes may contain an intrinsic RAS and endogenously form ANG II without the addition of exogenous substrate (15, 30). However, other groups have challenged those findings by demonstrating that podocytes express ACE2, but not ACE, suggesting that pathways opposing ANG II synthesis may be more active in these cells (54). A clear understanding of which RAS enzymatic pathways are functionally present in podocytes is lacking.

The purpose of this investigation was to characterize the enzymatic processes involved in a local RAS in podocytes. Our hypothesis is that podocytes express functional RAS enzymes that constitute a distinct system within the glomerulus. Alternative metabolites such as ANG-(1-7) and ANG-(1-9) can be formed and may predominate over ANG II. This unique handling of angiotensin substrates by podocytes may represent a specific function to maintain intraglomerular RAS balance.

METHODS

Cell culture. An immortalized mouse podocyte cell line was generously provided by Dr. Peter Mandel (Albert Einstein College of Medicine, Bronx, NY). Cells were harvested as described previously (35). Briefly, cells were cultured with interferon-γ-enriched medium at 33°C for two to four passages and subsequently transferred to 37°C for 14 days to allow differentiation. Cells were grown on RPMI 1640 with 10% FBS and 1% penicillin-streptomycin. Phenotype was verified by immunostaining for synaptopodin and WT-1. Undifferentiated and differentiated podocytes showed 95–100% staining for WT-1. Undifferentiated podocytes did not show staining for synaptopodin. Differentiated podocytes showed 70–80% staining for synaptopodin.
Passages 8–14 were used for the experiments. For comparison of podocytes with other resident glomerular cells, human mesangial cells (generously provided by Dr. Hannah Abboud, University of Texas, Houston, TX) were harvested and cultured as described previously (32) and grown in low-glucose DMEFM with 17% FBS, 3.36 g/l HEPES, 13 mg/l insulin, and 1% penicillin-streptomycin. Phenotype was verified by a spindle-shaped appearance and immunostaining for α-smooth muscle actin. Passages 8–24 were used for our experiments.

Experimental conditions. Mouse podocytes were grown in 12- or 24-well plates to subconfluence. Cells were then incubated in 0.5% FBS culture medium for 24 h before incubation in serum-free medium with 1 μM ANG I, ANG II, or the renin substrate ANG-(1-14) (Sigma-Aldrich, St. Louis, MO) for 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, and 8 h. Simultaneously, selected wells were coincubated with specific RAS enzyme inhibitors: 100 μM captopril (ACE inhibitor), 100 μM chymostatin (chymase inhibitor), 1 μM aprotinin (kallikrein inhibitor), 1 μM thiophan (neprilysin inhibitor), 100 μM amastatin (aminopeptidase A inhibitor), and 10 μM pepstatin A (acid protease inhibitor; Phoenix Pharmaceuticals, Burlingame, CA), 100 μM CMK-008 (cathepsin G inhibitor; MP Biomedicals, Solon, OH), or 1 μM CaaH3H2H3O2 (renin inhibitor; Bachem, Torrance, CA). Additional cells were treated with the angiotensin type 1 (AT1) receptor antagonist losartan (gift from Merck). Cells were preincubated with each enzyme inhibitor or receptor antagonist for 20 min before addition of the substrate. Conditioned media were collected at specified times and stored at −20°C until processed. Human mesangial cells were treated under similar conditions in selected experiments for comparison. To control for spontaneous peptide degradation, angiotensin substrates were incubated for the same time intervals in cell-free wells. No spontaneous degradation was observed for up to 24 h. Enzymes of interest are membrane-bound peptidases; therefore, cell media samples were considered adequate for examination of conversions at the cell surface.

Angiotensin peptide determination. Aliquots of conditioned cell culture medium were obtained from each well for analysis. Angiotensin peptide abundance was determined using matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS). Peptides were purified by C18 Zip-Tip columns (Millipore, Billerica, MA), which were equilibrated with 100% acetonitrile, washed with 0.1% trifluoroacetic acid, and loaded with 30 μl of cell medium. Columns were washed with 10 μl of 0.1% trifluoroacetic acid and eluted with a low-pH MALDI matrix compound (10 g/l α-cyano-4-hydroxycinnamic acid in 1:1 50% acetonitrile-0.1% trifluoroacetic acid). Eluted matrix (1.5 μl) was applied to the surface of a MALDI plate in triplicate. After the plate was air-dried, spectra were collected in reflectron mode using a M6/LDI MALDI-TOF MS (Waters, Milford, MA), and 20 spectra were combined for analysis. Results were analyzed using MassLynx 2.0 software (Waters). Angiotensin-derived peptides were analyzed on an ABI 4700 MALDI-TOF-TOF MS at the Nevada Proteomics Center (University of Nevada, Reno, NV) to confirm the identity of the peptides by de novo sequencing.

For quantification of peptide abundance, customized isotopically labeled AQUA peptides were purchased from Sigma-Genosys (St. Louis, MO). This method has been previously validated for quantification of peptide abundance (20). AQUA peptides are 6 Da larger than the native peptide as a result of [13C]valine incorporation into the amino acid sequence. AQUA-ANG I has a molecular weight (MW) of 1,302, AQUA-ANG-(1-9) an MW of 1,189, AQUA-ANG II an MW of 1,052, AQUA-ANG-(1-7) an MW of 905, and AQUA-ANG III an MW of 937. For standardization purposes, various concentrations of each AQUA peptide were mixed in separate tubes, with their matching native peptide also distributed in consecutive tubes at escalating concentrations. For determination of the abundance of the native peptide, the sum of the intensities of the three major peaks [monoiso-
of ANG II to its receptor and subsequent internalization as a potential cause of underestimation of abundance. The AT\textsubscript{1} receptor antagonist had no effect on the composition of the mass spectra (data not shown), suggesting that receptor binding does not explain the low abundance of ANG II in cell media.

**Evidence of ANG II degradation.** We hypothesized that the limited detection of ANG II may be due to rapid degradation of the peptide. To explore the ability of podocytes to degrade ANG II, cells were incubated with 1 μM ANG II in the presence or absence of amastatin. The ANG II metabolite ANG

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**Fig. 1.** Quantification of angiotensin peptide abundance in conditioned cell culture medium with use of AQUA peptides. A: mass spectrum generated by cultured mouse podocytes incubated with 1 μM ANG II. Samples were mixed with the corresponding cocktail of AQUA peptides (AQUA-ANG II and AQUA-ANG III) before they were applied to the matrix-assisted laser desorption/ionization (MALDI) plate. B: magnification of 3 isotopic ANG-(1-7) peaks detected from a different sample spotted with 200 nM AQUA-ANG-(1-7). Calculated concentration of ANG-(1-7) was 80 nM.

**Fig. 2.** Mass spectrum generated by cultured mouse podocytes (POD) or human mesangial cells (MES) incubated with 1 μM ANG I for 4 h. Conditioned cell medium was collected and subjected to MALDI time-of-flight mass spectroscopy (MALDI-TOF MS). Top: formation of ANG-(1-9) and ANG-(1-7) in mass spectrum generated by POD. Bottom: ANG II as the predominant peak in mass spectrum generated by MES. Spectra represent results from 3 separate batches of cells for each cell type.
III was detected by 15 min, suggesting rapid degradation of ANG II. Amastatin significantly decreased the formation of ANG III (169.9 ± 35 and 35.0 ± 22 nM without and with amastatin, respectively, \( P < 0.001 \); Fig. 5), suggesting that ANG II degradation in podocytes is mediated by aminopeptidase A. ANG II was nearly undetectable after 8 h (Fig. 5C).

Fig. 3. A: mass spectrum generated by cultured mouse podocytes incubated with 1 \( \mu \text{M} \) ANG I (top) or 1 \( \mu \text{M} \) ANG I + 1 \( \mu \text{M} \) thiorphan [a neprilysin inhibitor (NEPi); bottom] for 2 h. Conditioned cell medium was collected and subjected to MALDI-TOF MS. Inset: quantification of difference in ANG-(1-7) peaks, with 300 nM AQUA-ANG (1-7) used as internal standard. B: quantification of effects of thiorphan and DX-600 [an angiotensin-converting enzyme isoform 2 (ACE2) inhibitor (ACE2i)] with use of AQUA peptides. *\( P < 0.0001 \) vs. control. Similar data were obtained from 3 separate batches of cells.

Fig. 4. A: mass spectrum generated by cultured mouse podocytes incubated with 2 \( \mu \text{M} \) ANG I or 2 \( \mu \text{M} \) ANG I + 100 \( \mu \text{M} \) captopril (an ACE inhibitor) for 2 h. Conditioned cell medium was collected and subjected to MALDI-TOF MS. Inset: quantification of difference in ANG II peaks, with 100 nM AQUA-ANG II used as internal standard. B: quantification of differences in ANG II and ANG-(1-7) peaks with use of AQUA peptides. *\( P < 0.001 \) vs. control. **\( P < 0.004 \) vs. control. Similar data were obtained from 3 separate batches of cells.
Incubation of podocytes with ANG II resulted in the generation of ANG III and ANG-(1-7) after 4 h (Fig. 5). Coincubation of ANG II in the presence of DX-600 significantly decreased the degradation of ANG II (378.6 ± 42.4 and 744.7 ± 107.4 nM without and with DX-600, respectively, P < 0.01; Fig. 6). ANG-(1-7) levels were close to the lower limit of detection; therefore, we were not able to reliably quantify the differences in ANG-(1-7) concentration between samples with and without DX-600, although ANG-(1-7) generation appeared to be qualitatively lower. In addition, treatment with DX-600 led to the accumulation of ANG III, perhaps as a result of the increase in the amount of substrate (ANG II) for aminopeptidase A. Furthermore, to examine the role of ACE in the degradation of ANG-(1-7), cells were coincubated with ANG II in the presence of captopril. Although there was a trend for accumulation of ANG-(1-7), it did not reach statistical significance (34.4 ± 6.6 and 48.1 ± 2.8 nM without and with captopril, respectively, P < 0.08).

Evidence of renin activity. Podocytes incubated with the renin substrate ANG-(1-14) (1 μM) generated ANG I, ANG-(1-9), ANG II, and ANG-(1-7) (Fig. 7A). The predominant product generated was ANG-(1-7), which was inhibited by a renin inhibitor (C₆₀H₅₉H₁₅O₁₂) and pepstatin A (Fig. 7B). Formation of ANG I was decreased by the renin inhibitor from 28.2 ± 2.0 to 20.6 ± 4.3 nM (P < 0.05) and by pepstatin A to 16.8 ± 4.7 nM (P < 0.01), whereas ANG-(1-7) formation was decreased by the renin inhibitor from 62.8 ± 6.2 to 20.9 ± 5.6 nM (P < 0.005) and by pepstatin A to 28.7 ± 5.9 nM (P < 0.01).

Protein expression of RAS enzymes. To confirm the presence of the RAS enzymes implicated by the MALDI-TOF MS assays, protein abundance was examined by Western blotting. Renin, cathepsin D, ACE, ACE2, neprilysin, and aminopeptidase A were detected in mouse podocytes (Fig. 8). Angiotensinogen expression was not detected, even after prolonged (up to 24 h) film exposure.

DISCUSSION

Evidence has accumulated to support the concept that the kidney is a major reservoir for a complete and independent RAS. Pioneering work in this area was done by Navar et al. (36), who demonstrated the intrarenal formation of ANG II. Subsequently, Anderson et al. (1) characterized the expression of angiotensinogen and ACE in diabetic rat kidneys. Furthermore, membrane-bound ACE has been found to reside in proximal tubular cells (26, 33, 51). In fact, the luminal concentration of ANG II at the level of the proximal tubule is ~1,000-fold higher than the plasma concentration (25) by virtue of local ACE activity. Although evidence for an intratubular RAS appears solid, functional evidence for an intraglomerular RAS is somewhat less definitive. In a model of streptozotocin-induced diabetic rats, Singh et al. (49) demonstrated increased ANG II and renin content from glomerular extracts. Other studies have attempted to implicate specific cell
types within the glomerulus and found that mesangial cells are capable of endogenously synthesizing ANG II (23, 27, 48, 50); however, evidence for a functional RAS in podocytes is limited. The importance of an intraglomerular RAS has been highlighted by numerous publications demonstrating the up-regulation of the RAS in kidneys with diabetic nephropathy, focal segmental glomerulosclerosis, and IgA nephropathy (4, 9, 47, 52).

Interest in the study of podocyte biology has exploded over the last few years. The development of an immortalized podocyte cell line by Mundel et al. (35) provided a significant step toward a better understanding of the mechanisms mediating glomerular permeability and injury (14). Few reports have examined the presence of a RAS in podocytes. Durvasula et al. (15) reported that mechanical strain induces de novo ANG II production, as demonstrated by increased signaling through the AT1 receptor. Liebau et al. (30) reported that human podocytes contain mRNA for angiotensinogen, renin, and ACE, although the detection of these proteins was not reported. The same study presented immunoreactive evidence for the endogenous formation of ANG I and ANG II by human podocytes, which suggested the existence of an unidentified ANG II-forming pathway. A thorough characterization of the components of an intrinsic RAS in podocytes is still lacking. Therefore, we decided to examine the ability of cultured podocytes to metabolize RAS substrates.

Our approach utilized MALDI-TOF MS, instead of immunoreactive methods, to maximize the specificity of our findings. A similar method has been successfully used to qualitatively analyze RAS enzyme activity (16); however, our method of quantification with AQUA peptides has not been previously used in this area. The concentrations of substrates chosen for our experiments reflect those reported for ANG II in the renal interstitium and proximal tubular lumen (37) but were also selected so that metabolites of ANG I could be detected by MALDI-TOF. The selected concentrations are not unprecedented, since similar amounts of ANG I have been used in previous studies of intact cells or tissue preparations (29, 48). We observed that podocytes primarily synthesize ANG-(1-9) and ANG-(1-7), the latter being the most abundant ANG metabolite. The pattern of peptide generation observed in podocytes is striking in contrast to the pattern observed in human mesangial cells, which preferentially synthesize ANG II. Formation of ANG-(1-7) has been described to occur mainly through two catalytic reactions: 1) direct cleavage of ANG I by neprilysin or 2) cleavage of ANG II by ACE2 (6, 14, 42). Therefore, we explored the contribution of ACE2 and neprilysin to ANG-(1-7) formation. We observed that the formation of ANG-(1-7) was significantly blocked by a neprilysin inhibitor when cells were incubated with ANG I. This finding suggests that neprilysin plays an important role in ANG-(1-7) generation in podocytes. Expression of neprilysin in podocytes has not been previously described. Neprilysin is an 87-kDa transmembrane endopeptidase widely expressed in tissues and is also responsible for the degradation of natriuretic peptides, bradykinin, and enkephalins (19). To explore the effect of ACE2 on ANG-(1-7) formation, podocytes were preincubated with the ACE2 inhibitor DX-600. We found that ANG-(1-7) formation was not affected by ACE2 inhibition. In contrast, Li et al. (29) reported a decrease in ANG-(1-7)
formation by ACE2 inhibition in proximal tubular extracts. This difference could represent a true difference in the enzymatic machinery between cell types, but we cannot overlook the fact that this divergence could correspond to differences in experimental conditions.

Our data also suggest that ANG-(1-9) is formed by podocytes; however, we were not able to identify the responsible peptidase. Donoghue et al. (13) reported that ACE2 can convert ANG I to ANG-(1-9). In our experiments, the ACE2 inhibitor DX-600 did not block this reaction, suggesting that an unknown carboxypeptidase mediates the conversion of ANG I to ANG-(1-9) in podocytes. The formation of ANG-(1-9) was previously reported in mesangial cells (48) and isolated proximal tubule (29), but the existence of ANG-(1-9) in cultured podocytes is a new finding.

We demonstrated for the first time direct functional evidence of ACE activity in mouse podocytes. It is noteworthy that ANG II formation was detectable only when podocytes were provided with a higher concentration of substrate and that ANG-(1-7) and ANG-(1-9) continued to be the predominant ANG peptide formed by mouse podocytes. Nevertheless, our findings suggest that ANG II is predominantly formed by ACE, as evidenced by the remarkable inhibitory effect observed with captopril, in contrast to a previous publication that reported endogenous ANG II formation that was not inhibited by captopril (30). Furthermore, we did not find evidence for expression of other non-ACE, ANG II-forming enzyme candidates, such as chymase or cathepsin G. Degradation of ANG-(1-7) to ANG-(1-5) is also mediated by ACE; however, we observed only a small increase in ANG-(1-7) when cells were spiked with ANG I and incubated with captopril. Lack of a robust ACE-mediated degradation of ANG-(1-7) may underlie the observed accumulation of ANG-(1-7) in podocytes.

Avid degradation of ANG II has been previously described to occur in the kidney, where 90% of filtered ANG II is degraded (3, 40, 53). However, the kidney cell types that are involved in ANG II degradation are not well established. Our data revealed that cultured mouse podocytes are able to degrade ANG II. We observed ~50% degradation of exogenous ANG II after 120 min of incubation. The conversion of ANG II to ANG III was inhibited by amastatin, an aminopeptidase A inhibitor. Aminopeptidase A is a 250-kDa transmembrane protein that cleaves ANG II to ANG III (40), and ANG III is subsequently converted to ANG IV by aminopeptidase N. The expression of aminopeptidase A in podocytes has been previously reported in developing kidneys (11). Highlighting the

Fig. 7. A: mass spectrum generated by cultured mouse podocytes incubated with 1 μM ANG-(1-14) (a renin substrate; top) or 1 μM ANG-(1-14) + 10 μM C6H12O6H3 (a renin inhibitor; bottom) for 4 h. Conditioned cell medium was collected and subjected to MALDI-TOF MS. Note formation of ANG I, ANG-(1-9), ANG II, and ANG-(1-7) with ANG-(1-14). ANG I and ANG-(1-7) were the predominant peaks. Inset: quantification of difference in ANG I peaks, with 100 nM AQUA-ANG I used as internal standard.

B: quantification of observed differences with use of AQUA peptides. *P < 0.05 vs. control. **P < 0.01 vs. control. ***P < 0.005 vs. control. Similar data were obtained from 3 separate batches of cells.

Fig. 8. Gene expression of renin-angiotensin system (RAS) enzymes in mouse podocytes examined by Western blotting from cell lysates. NEP, neprilysin; APA, aminopeptidase A; Cath D, cathepsin D. Blots are representative of those obtained from 3 separate cell batches.
role of aminopeptidase A in the metabolism of ANG II and glomerular injury, Dijkman et al. (12) found that mice injected with antibodies against aminopeptidase A developed proteinuria. Therefore, aminopeptidase A activity in podocytes may be critically important in regulating intrarenal ANG II concentration.

Incubation of podocytes with ANG II also resulted in generation of ANG-(1-7). Coincubation of podocytes with an ACE2 inhibitor led to the accumulation of ANG II, reflecting the inhibition of ANG II degradation. However, we failed to document a consistent difference in ANG-(1-7) levels upon ACE2 inhibition. The levels of ANG-(1-7) were close to the lower limit of detection and, thus, limited our ability to perform a reliable quantification. When they utilized an MS-based assay, other authors experienced similar limitations in their ability to detect ANG-(1-7) converted from ANG II (29).

The presence of glomerular renin activity has been proposed by some authors (49, 50). No study has directly explored renin activity in podocytes. Our results suggest the existence of renin activity in podocytes, as evidenced by the formation of ANG I, ANG-(1-9), ANG II, and ANG-(1-7) after cells were incubated with ANG-(1-14). This conversion was inhibited by a renin inhibitor, demonstrating that the formation of angiotensin peptides from ANG-(1-14) is, in part, attributable to renin. Once more, ANG-(1-7) was the predominant product. Previous studies using immunohistochemistry have consistently failed to detect renin inside the glomerulus (8, 10, 17), and the idea that podocytes express renin may be difficult to reconcile on the basis of the previous reports. However, an elegant study by Sequeira-Lopez et al. (44) has proposed that renin-expressing cells are progenitors of multiple cell types that can revert to the renin-expressing phenotype under certain insults or stimuli. It remains possible that cultured mouse podocytes can revert to the renin-expressing phenotype by virtue of their immortalized status, but it is interesting to speculate that diseases resulting in podocyte injury may lead to phenotype changes that elicit the expression of renin and the alteration of RAS homeostasis. Conversely, a non-renin pathway for ANG I formation may be present in podocytes, as evidenced by the expression of cathepsin D. Cathepsin D is known to cleave angiotensinogen (34); however, the inhibitory effect of pepstatin A (a nonspecific acid protease inhibitor) was not different from that of the renin inhibitor and, therefore, did not help elucidate this possibility. A cathepsin D-specific inhibitor was not available to further address this question.

In summary, to our knowledge, we have demonstrated for the first time that ANG-(1-7) is the predominant product of cleavage of ANG I in mouse podocytes and that nephrilysin is the enzyme responsible for the direct conversion of ANG II to ANG-(1-7). ACE2 also appears to generate ANG-(1-7) on cleavage of ANG II. In addition, ACE-mediated ANG II formation does not result in the accumulation of ANG II to a high concentration in podocyte culture, possibly because of ANG II degradation by aminopeptidase A. Furthermore, evidence for podocyte renin activity was also found. A comprehensive diagram of the podocyte RAS that incorporates these findings is depicted in Fig. 9. Overall, the significance of our observations is supported by recent publications demonstrating that ANG-(1-7) may represent an ANG II-counteracting peptide. In blood vessels, ANG-(1-7) acts as a vasodilator (43). It has been shown to produce relaxation in various vascular beds, including the renal circulation (41). Moreover, a recent report showed that ANG-(1-7) reduces migratory responses of macrophages (18). Inhibition of ACE2 caused proteinuria in db/db diabetic mice in one study (54), and ACE2 knockout mice developed glomerulosclerosis in another report (38). These findings underscore the importance of ANG-(1-7) in renal physiology and pathophysiology.

Our data reveal that podocytes primarily degrade ANG II and synthesize peptides that are known to counterbalance the actions of ANG II. These novel observations could implicate specific glomerular diseases with podocyte injury, when such intraglomerular regulatory functions might be lost. We speculate that podocytes may have a previously unrecognized function as an "ANG II gateway" that separates systemic ANG II from the intrarenal RAS. The anatomic localization of podocytes at the glomerular filtration barrier is optimally situated to execute this role. In this case, podocytes would regulate the entrance of systemic ANG II by degrading filtered ANG II or by promoting the conversion of filtered ANG I or angiotensinogen to ANG-(1-7). As well, the opposing actions of ANG-(1-7) might have an additional effect: they might "buffer" the actions of intraglomerular ANG II formed by mesangial cells and, perhaps, play a role in normal physiology, such as regulation of glomerular filtration rate. These hypotheses deserve further investigation.

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