An angiotensin-(1–7) peptidase in the kidney cortex, proximal tubules, and human HK-2 epithelial cells that is distinct from insulin-degrading enzyme

Bryan A. Wilson, Nildris Cruz-Diaz, Allyson C. Marshall, Nancy T. Pirro, Yixin Su, TanYa M. Gwathmey, James C. Rose, and Mark C. Chappell

1Hypertension and Vascular Research Center, Wake Forest University School of Medicine, Winston-Salem, North Carolina; and 2Department of Obstetrics and Gynecology, Wake Forest University School of Medicine, Winston-Salem, North Carolina

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Wilson BA, Cruz-Diaz N, Marshall AC, Pirro NT, Su Y, Gwathmey TM, Rose JC, Chappell MC. An angiotensin-(1–7) peptidase in the kidney cortex, proximal tubules, and human HK-2 epithelial cells that is distinct from insulin-degrading enzyme. Am J Physiol Renal Physiol 308: F594–F601, 2015. First published January 7, 2015; doi:10.1152/ajprenal.00609.2014.—Angiotensin 1–7 [ANG-(1–7)] is expressed within the kidney and exhibits renoprotective actions that antagonize the inflammatory, fibrotic, and pro-oxidant effects of ANG II. We previously identified an peptidase that preferentially metabolized ANG-(1–7) to ANG-(1–4) in the brain medulla and cerebrospinal fluid (CSF) of sheep (Marshall AC, Pirro NT, Rose JC, Diz DI, Chappell MC, J Neurochem 130: 313–323, 2014); thus the present study established the expression of the peptidase in the kidney. Utilizing a sensitive HPLC-based approach, we demonstrate a peptidase activity that hydrolyzed ANG-(1–7) to ANG-(1–4) in the sheep cortex, isolated tubules, and human HK-2 renal epithelial cells. The peptidase was markedly sensitive to the metalloendopeptidase inhibitor JMV-390; human HK-2 cells expressed subnanomolar sensitivity (IC50 = 0.5 nM) and the highest specific activity (123 ± 5 fmol-min⁻¹·mg⁻¹) compared with the tubules (96 ± 12 fmol-min⁻¹·mg⁻¹) and cortex (107 ± 9 fmol-min⁻¹·mg⁻¹). The peptidase was purified 41-fold from HK-2 cells; the activity was sensitive to JMV-390, the chelator o-phenanthroline, and the mercury-containing compound p-chloromercuribenzoic acid (PCMB), but not to selective inhibitors against neprilysin, neurolysin and thimet oligopeptidase. Both ANG-(1–7) and its endogenous analog [Ala1]-ANG-(1–7) (alamandine) were preferentially hydrolyzed by the peptidase compared with ANG II, [Asp1]-ANG II, ANG I, and ANG-(1–12). Although the ANG-(1–7) peptidase and insulin-degrading enzyme (IDE) share similar inhibitor characteristics of a metallothiolendopeptidase, we demonstrate marked differences in substrate specificity, which suggest these peptidases are distinct. We conclude that an ANG-(1–7) peptidase is expressed within the renal proximal tubule and may play a potential role in the renal renin-angiotensin system to regulate ANG-(1–7) tone.

ANG-(1–7); endopeptidase; HK-2 epithelial cells; proximal tubules

We recently identified a peptidase that metabolized angiotensin-(1–7) [ANG-(1–7)] to ANG-(1–4) in the brain medulla and cerebrospinal fluid (CSF) of sheep (20–22). The purified peptidase exhibited a very high affinity (IC50 <1 nM) to the metalloendopeptidase agent JMV-390 but was insensitive to other metalloendopeptidase inhibitors including neprilysin (NEP; EC. 3.4.24.11), neurolysin (EC 3.4.24.16), and thimet oligopeptidase (TOP; EC 3.4.24.15) (20). The peptidase hydrolyzed ANG-(1–7) at a 12-fold higher rate than ANG II, but failed to metabolize other biologically active peptides apelin, bradykinin, and neurotensin (20). Interestingly, ANG-(1–7) peptidase activity was threefold higher in the CSF of glucocorticoid-exposed sheep, an experimental model of in utero fetal programming that expresses higher blood pressure with associated baroreceptor dysfunction (21). Moreover, peptidase activity significantly correlated with the elevated blood pressure in glucocorticoid-exposed sheep but was negatively associated with endogenous levels of ANG-(1–7) in the CSF (9). The central expression of an ANG-(1–7) peptidase may regulate ANG-(1–7) tone within key cardiovascular centers of the brain to influence blood pressure and baroreflex function (9).

Although ANG-(1–7) was originally identified as an endogenous component of the brain renin-angiotensin system (RAS) over 25 years ago (8), the peptide and its receptor Mas were subsequently identified in various cell types of the kidney, as well as other peripheral tissues (3, 14, 28). In contrast to the ANG II-AT1 receptor pathway within the kidney, ANG-(1–7) increases renal blood flow and acts directly as a natriuretic to enhance sodium excretion likely through the regulation of nitric oxide (7). ANG-(1–7) also exhibits renoprotective actions and antagonizes the inflammatory, fibrotic, and pro-oxidant actions of ANG II (2, 3, 9, 24, 28, 34). Since the proximal tubule is considered a key site for an intrarenal RAS, the present study sought to establish the ANG-(1–7) peptidase activity in the renal cortex and isolated proximal tubules from adult sheep, as well as in the HK-2 human epithelial cell line, a well-characterized model of the human proximal tubule that expresses a complete RAS (1, 29, 32). Using a sensitive HPLC-based assay, we report that the sheep cortex, isolated tubules, and human HK-2 cells express an ANG-(1–7) peptidase activity that metabolizes the peptide to ANG-(1–4). Similar to the brain peptidase, the HK-2 ANG-(1–7) peptidase was potently blocked by the inhibitor N-[3-[hydroxyamino]carbonyl]-1-oxo-2(R)-benzylpropyl]-L-leucine (JMV-390; IC50 of 0.5 nM), as well as by mercury-containing agents and metallochelators that are characteristic of the M16A family of metallothioleptidases and include insulin-degrading enzyme (IDE; 3.4.24.56). The peptidase activity was subsequently enriched ~40-fold from the human HK-2 cells; the purified preparation hydrolyzed ANG-(1–7) and [Ala1]-ANG-(1–7) at a 9- to 10-fold greater rate than ANG II, [Ala1]-ANG II, or ANG-(1–9), but did not metabolize ANG I or ANG-(1–12). A comparative analysis between human IDE and the HK-2 ANG-(1–7) peptidase revealed marked differences to hydrolyze 125I-ANG-(1–7) and the fluorescently quenched peptide Abz-ANG-(1–7)-[Tyr(1)NO2], suggesting the peptidases are distinct. From the current results, we conclude that an ANG-(1–7) peptidase is expressed within the...
proximal tubules of the kidney and may play a potential role in the renal RAS to regulate ANG-(1–7) tone.

METHODS

Animals

Mixed breed sheep (obtained from a private local vendor) were delivered at term, farm-raised, and weaned at 3 mo of age. Sheep (10–12 mo of age) were anesthetized with ketamine and isoflurane and euthanized by exsanguination. The kidneys were removed immediately, and the renal cortex was dissected out on ice for immediate isolation of proximal tubules as previously described (30). Cortical tissue or isolated tubules were stored at −80°C. All procedures were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee.

HK-2 Cells

HK-2 cells derived from human proximal tubular cells were obtained from American Type Culture Collection (Manassas, VA). The cells were incubated at 37°C under 5% CO₂, humidified atmosphere and were routinely maintained in DMEM/F12 supplemented with 10% FBS, insulin-transferrin-selenium-cortisol, 100 μg/ml streptomycin. HK-2 cells were placed in serum-free DMEM/F12 media for 24 h before the metabolism and purification studies.

ANG-(1–7) peptidase activity was purified from HK-2 cells using a similar approach to that for the brain peptidase (20). Sensitivity of the purified HK-2 peptidase was assessed to various inhibitors including para-chloromercuribenzoic acid (PCMB; 10 μM), E-64 (10 μM), ortho-phenanthroline (10 μM), N-[N-[1-(S-carboxyl-3-phenylpropyl)-(S)-phenyl-alanyl]-]-(S)-isoserine (SCH; 10 μM), N-[N-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-p-aminobenzoate (CPP; 10 μM), the dipeptide proline-isoleucine (Pro-Ile; 1 mM), and the metallopeptidase inhibitor JMV-390 (5 and 50 nM) as described for the brain and CSF peptidase (20). All inhibitors were obtained from Sigma (St. Louis, MO) except SCH (gift from Schering Plow), CPP (Bachem, King of Prussia, PA), and JMV-390 (Tocris Bioscience, Bristol, UK).

Tissue and Cell Preparation

Kidney cortex (1 g) was homogenized in HEPES buffer (25 mM Na⁺-free HEPES, 10 μM ZnCl₂, 125 mM NaCl, 0.01% Triton X-100, pH 7.4) using a Power Gen 1000 tissue grinder (Fisher Scientific, Pittsburgh, PA) on setting 5 for 60 s and centrifuged at 100,000 × g for 30 min at 4°C. Frozen proximal tubules and HK-2 cell pellets were resuspended in the HEPES buffer and sonicated briefly utilizing an ultrasonic processor (model W-380 Heat Systems-Ultrasonics) and centrifuged at 100,000 × g for 30 min at 4°C. Supernatants were stored on ice and utilized for peptide metabolism experiments or enzyme purification (HK-2 supernatant).

Peptidase Assays

Metabolism reactions were conducted at 37°C in the HEPES assay buffer with the HK-2 purified peptidase (0.3 μg) and 100 μM of unlabeled ANG I, ANG-(1–12), ANG-(1–9), ANG II, [Ala¹]-ANG II, ANG-(1–7), [Ala¹]-ANG-(1–7), and Abz-ANG-(1–7)-[Tyr⁵(NO₂)]₃. The higher peptide concentration was necessary to detect the ANG-(1–4) product by UV analysis as opposed to the 125I-labeled product (20). Reactions were stopped after 20 h at 37°C with 1.0% phosphoric acid and separated on a Shimadzu Prominence HPLC using a NovaPak C₁₈ column (2.1 × 150 mm, Waters, Milford, MA) under gradient conditions (20). Peptides were monitored at 220 nm and the products identified by the retention time of standard peptides.

125I-ANG I metabolism was performed at 37°C in the HEPES reaction buffer using the concentrated HK-2 supernatant (10 μg) in a final volume of 250 μl. Each reaction contained a final concentration of 0.5 nM 125I-ANG I and 100 nM ANG I with or without the inhibitors JMV-390 (1 nM) and JMV-390 (1 nM)/CPP (10 μM) and PCMB (10 μM). The reactions were stopped after 60 min by addition of ice-cold 1.0% phosphoric acid and centrifuged at 16,000 g. The supernatants were immediately filtered, and the products were detected by HPLC-γ detection.

Statistics

Data are expressed as means ± SE. One-way repeated-measures ANOVA with Bonferroni posttests were used for the statistical analysis of data (GraphPad Prism 5, San Diego, CA). The inhibitory constants (IC₅₀) for the peptidase activity were determined by non-linear regression one-site competition with no constraints (GraphPad Prism 5 statistical program). The criterion for statistical significance was set at P < 0.05.

RESULTS

To determine whether ANG-(1–7) peptidase activity in the sheep CSF and brain medulla was evident in peripheral tissues, peptidase activity was assessed in the 100,000-g cytosolic fraction of the sheep cortex by HPLC-based detection of 125I-ANG-(1–4) (19). As shown in the chromatograph in Fig. 1A, the cortical activity hydrolyzed 125I-ANG-(1–7) to 125I-ANG-(1–4) that was reduced by the inhibitor JMV-390 (Fig. 1B). The cytosolic fraction from the sheep isolated proximal tubules also hydrolyzed 125I-ANG-(1–7) to 125I-ANG-(1–4) and was sensitive to the inhibitor JMV-390 (Fig. 1, C and D, respectively). We next assessed the activity in the human HK-2 proximal tubule cell line, a well-characterized tubule model that contains a complete RAS. As shown in Fig. 2, the ANG-(1–7) peptidase activity was expressed in the cell cytosol (Fig. 2A) and the collected cell media (Fig. 2C) of the HK-2 cells. The addition of JMV-390 essentially abolished the peptidase activity in both fractions (Fig. 2, B and D, respectively). Competition studies in the HK-2 cytosol revealed IC₅₀ values of 5 and 2 μM for unlabeled ANG-(1–7) and [Ala¹]-ANG-(1–7) (Fig. 3, A and B, respectively) to block the hydrolysis of 125I-ANG-(1–7) to 125I-ANG-(1–4). [Ala¹]-ANG-(1–7) is an endogenous analog of ANG-(1–7) that may arise from the decarboxylation of aspartic acid to alanine or conversion from [Ala¹]-ANG II by ACE2; however, the metabolism pathways for the peptide are not currently known (17). In comparison, the inhibitor JMV-390 exhibited IC₅₀ values of 0.5 and 0.2 nM for 125I-ANG-(1–7) hydrolysis in the HK-2 cytosol and the cell media (Fig. 3, C and D, respectively).

Comparison of the specific activity of the three preparations revealed that the HK-2 cells expressed slightly higher activity than the cortical tissue or isolated tubules (Table 1). The
majority of activity in all three preparations was sensitive to the inhibitor JMV-390 (Table 1). Moreover, we did not detect activity in the membrane fraction of the sheep tissue or HK-2 cells (data not shown). We enriched the peptidase activity from the HK-2 cell cytosol using dye absorption (Cibacron Blue 3AG) and ion exchange chromatography (DEAE and Sepharose Q), an approach previously utilized to purify the brain peptidase (20). HK-2 peptidase activity was purified ~40-fold with an overall yield of 29% (Table 2). The purified activity that eluted from the Sepharose Q column in 250 mM NaCl ("Q fraction") was subsequently used to characterize the enzyme regarding the hydrolysis of unlabeled angiotensins known to be expressed endogenously, as well as the sensitivity to various inhibitors. As shown in Fig. 4A, incubation of 100 μM unlabeled ANG-(1–7) with the Q fraction yielded a single peak that was reduced by JMV-390 (Fig. 4B). [Ala1]-ANG-(1–7) was hydrolyzed to a similar extent as ANG-(1–7) (28 vs. 30 nmol·min⁻¹·mg⁻¹); Figs. 4, A and C, and 5); however, the C terminally extended peptides ANG II, ANG I, and ANG-(1–12) were hydrolyzed to a lesser extent or not at all by the HK-2 peptidase (Fig. 4, D–F). These and other angiotensin metabolism data are summarized in Fig. 5; the sequence for the peptides that share the Tyr4-Ile5 bond is shown in Table 3. ANG-(1–7) was hydrolyzed at approximately a 10-fold higher rate than ANG-(1–9) (3·min⁻¹·mg⁻¹) or ANG II (2·min⁻¹·mg⁻¹). In regard to the sensitivity toward various enzyme inhibitors, the activity of the enriched Q fraction was markedly inhibited by 5 nM JMV-390 (>90%) and abolished with a 10-fold higher concentration (Fig. 6A). The thiol inhibitor PCMB and the metal chelator o-phenanthroline inhibited 92 and 94% of the peptidase activity, respectively (Fig. 6A). Other selective inhibitors against TOP (CPP, 10 μM), neuropeptide (Pre-Ile, 1 mM), and NEP (SCH, 10 μM), as well as the thiol epoxide agent E64 (10 μM) did not significantly attenuate activity (Fig. 6B).

Based on the sensitivity of the ANG-(1–7) peptidase to the metallochelator o-phenanthroline and the mercury-based inhibitor PCMB, as well as the lack of inhibition by the thiol agent E64 and other peptidase inhibitors, we noted that IDE shared similar inhibitor characteristics (4). We obtained recombinant human IDE and compared its activity to the ANG-(1–7) peptidase. As shown in Fig. 7A, the purified HK-2 Q fraction (0.3 μg) hydrolyzed 125I-ANG-(1–7) to 125I-ANG-(1–4); however, human IDE (2 μg) failed to metabolize 125I-ANG-(1–7) (Fig. 7C). In contrast, the ANG-(1–7) peptidase did not metabolize the fluorescent substrate [Abz]-ANG-(1–7)-[Tyr7-(NO2)] while human IDE hydrolyzed the fluorescent peptide (Fig. 7, B and D). The large void peak in the UV chromatographs (Fig. 7, B and D) is the DMSO solvent for the fluorescent peptide.

Finally, we examined the processing of 125I-ANG I in the 100,000-g cytosol fraction of the human HK-2 cells. As shown in the chromatographs for Fig. 8, 125I-ANG I was metabolized primarily to 125I-ANG-(1–7) and 125I-ANG-(1–4). Addition of JMV-390 reduced ANG-(1–4) and enhanced the 125I-ANG-(1–7) peak (Figs. 8B and 9). The coaddition of JMV-390 and the TOP inhibitor CPP reduced the 125I-ANG-(1–7) peak and preserved the 125I-ANG I peak (Figs. 8C and 9), while the thiol inhibitor PCMB abolished metabolism of 125I-ANG I (Figs. 8D and 9). In Fig. 9, the 125I-ANG I metabolism studies were quantified as the extent of 125I-ANG-(1–7) or 125I-ANG-(1–4) formation in the absence or presence of JMV-390, JMV-390/CPP, and PCMB (Fig. 9, A and B, respectively). We did not attempt higher concentrations of JMV-390 to completely block 125I-ANG-(1–4) as this inhibitor may attenuate TOP activity (IC₅₀ = 30 nM) and subsequently reduce the conversion of 125I-ANG I to 125I-ANG-(1–7) (15).
Abundant evidence strongly supports a local or tissue-based RAS in the kidney that contributes to renal function, as well as the regulation of blood pressure and water and sodium balance (24, 25). The proximal tubule is one site within the kidney that contains many, if not all, elements of a complete RAS capable of generating and responding to the bioactive peptide ANG II (24, 33). The present study provides evidence for an enzymatic pathway that specifically metabolizes the Tyr4-Ile5 bond of ANG-(1–7) to form ANG-(1–4) within the proximal tubules of the sheep kidney and human HK-2 tubule cells. The isolation and characterization of the peptidase from the HK-2 tubule cells revealed essentially identical characteristics to the ANG-(1–7) peptidase isolated from the sheep brain and CSF (20). Similar to the brain peptidase, the HK-2 cellular activity and that isolated from the cell media exhibited a very low IC50 (0.2–0.5 nM) for the metallopeptidase inhibitor JMV-390. The peptidase was purified from the HK-2 cells using a similar chromatographic approach to that of the brain peptidase; we achieved a 40-fold increase in specific activity that likely reflects the lower initial protein content of the cells compared with the brain tissue (20). Nevertheless, the purified peptidase revealed essentially an identical specificity as the brain peptidase regarding the hydrolysis of angiotensins. ANG-(1–7) was hydrolyzed at a greater rate than ANG II and ANG-(1–9), while the hydrolysis of ANG I or ANG-(1–12) was not detectable under the present conditions. Although the endogenous ANG-(1–7) analog [Ala1]-ANG-(1–7) exhibited a lower IC50 than ANG-(1–7) for hydrolysis of 125I-ANG-(1–7) (2 vs. 5 μM), we noted little difference in the metabolism of these two peptides by the purified HK-2 peptidase. The peptidase also hydrolyzed ANG II and [Ala1]-ANG II in a similar manner, albeit at a 10-fold lower rate than that of ANG-(1–7) or [Ala1]-ANG-(1–7). Importantly, these data suggest that substitution at the N terminus of the peptide (alanine to aspartic acid) does not have a major influence on the hydrolysis of the peptide; however, additions to the C-terminal end appear to markedly reduce metabolism. The quenched fluorescent ANG-(1–7) peptide with a substituted tyrosine (NO2) group was not hydrolyzed, suggesting that the C-terminal proline may be a critical residue in the recognition and catalysis of ANG-(1–7) by the peptidase.
The purified HK-2 peptidase activity was sensitive to both the metallochelator \(\beta\)-phenanthroline and the thiol inhibitor PCMB, but not the thiol epoxide inhibitor E-64. The brain peptidase was also quite sensitive to \(\beta\)-phenanthroline, PCMB, and another mercurial agent, APMA, but not E-64 or leupeptin (20). Moreover, the chelating agent EDTA was significantly less potent than \(\beta\)-phenanthroline to inhibit the brain peptidase (20). Interestingly, the metallothiolendopeptidase IDE exhibited essentially an identical pattern of sensitivity to this group of inhibitors (4, 31). Originally identified as the primary peptidase that metabolizes insulin, IDE contributes to the in vivo metabolism of amylin and glucagon (18). IDE also hydrolyzes a number of other peptides in vitro including ANP, \(\gamma\)-endorphin, bradykinin, and relaxin (5, 19, 23, 27); however, human IDE failed to metabolize radiolabeled ANG-(1–7). IDE hydrolyzed the quenched Abz-ANG-(1–7) fluorescent peptide to Abz-ANG-(1–4), but this substrate was not metabolized by the HK-2 peptidase. Replacement of tyrosine (NO\(_2\)) for proline in the seventh position of the fluorescent peptide may obviate recognition of this substrate by the HK-2 peptidase, while the tyrosine (NO\(_2\)) substitution does not appear critical for IDE hydrolysis. IDE belongs to the M16 family of soluble metallothiolpeptidases or inverzincins; other members include the bacterial enzyme pitrilysin (EC.3.4.24.55), nardilysin (EC 3.4.24.61), and mammalian metallopeptidase I that is predominantly localized to the mitochondria (10). We have not obtained sequence data on the enriched ANG-(1–7) peptidase from brain or the HK-2 cells to unequivocally identify the protein. The ANG-(1–7) peptidase may belong to the M16 family of soluble metallothiolpeptidases or inverzincins.

**Table 3. Angiotensin peptide sequences**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>ANG-(1–7)</td>
<td>DRVYHP</td>
</tr>
<tr>
<td>[Ala(^{1})]-ANG-(1–7)</td>
<td>ARVYHP</td>
</tr>
<tr>
<td>Abz-ANG-(1–7)[Tyr(^{7})(NO(_2))]</td>
<td>Abz-DRVYHY-NO(_2)</td>
</tr>
<tr>
<td>ANG II</td>
<td>DRVYHF</td>
</tr>
<tr>
<td>[Ala(^{1})]-ANG II</td>
<td>ARVYHF</td>
</tr>
<tr>
<td>ANG-(1–9)</td>
<td>DRVYHFFL</td>
</tr>
<tr>
<td>ANG-(1–12)</td>
<td>DRVYHFFLLY</td>
</tr>
</tbody>
</table>

Fig. 4. Purified HK-2 peptidase preferentially hydrolyzes ANG-(1–7) to ANG-(1–4). Purified peptidase (0.3 \(\mu\)g) from the HK-2 100,000-\(\times\) g cytosol fraction was incubated with 100 \(\mu\)M of ANG-(1–7) (A7; A); A7+1 \(\mu\)M JMV-390 (D); [Ala\(^{1}\)]-ANG-(1–7) (AAl\(^{1}\)-A7; C); ANG II (AII; D); ANG I (AI; E); and ANG-(1–12) (A12; F). Reactions occurred for 20 h at 37°C, and the products were detected by HPLC-UV (220 nM) under identical gradient conditions.

Fig. 5. Comparison of the peptidase velocities for the hydrolysis of angiotensin peptides by purified HK-2 peptidase. Purified peptidase (0.3 \(\mu\)g) was incubated with angiotensin peptides (100 \(\mu\)M), and metabolism was detected by HPLC-UV (220 nM) under gradient conditions. Data are from a single experiment.
class of metallothiolendopeptidase based on the inhibitor profile; however, complete purification and identification of the peptidase are necessary to address this issue. In addition, it remains to be determined whether the ANG-(1–7) peptidase is expressed in the kidney in other species such as the rat or mouse.

The ANG-(1–7) peptidase was prominent in the 100,000-g cytosolic fraction of the sheep cortex, proximal tubules, and HK-2 cells. The kidney exhibits a high content of ANG-(1–7) comparable to ANG II (26), and immunocytochemical studies localized ANG-(1–7) predominantly to the proximal tubules (13). In the NRK52-E rat epithelial cell line, the advanced

Fig. 6. Inhibitor profile of purified HK-2 peptidase. Peptidase activity was blocked by JMV-390 (JMV, 5 and 50 nM) and the thiol inhibitor para-chloromercuribenzoic acetate (PCMB; 10 μM), and metallochelator ortho-phenanthroline (O-Phen; 10 μM; A). Other inhibitors including CPP (10 μM), Pro-Ile (10 μM), SCH (10 μM), and E-64 (10 μM) did not significantly inhibit activity (B). Peptidase reactions were performed with 125I-ANG-(1–7) for 60 min at 37°C. Values are means ± SE; n = 3. *P < 0.05 vs. control conditions.

Fig. 7. ANG-(1–7) peptidase from HK-2 cells is distinct from insulin-degrading enzyme (IDE). Purified HK-2 peptidase (0.3 μg) hydrolyzed 125I-A7 to 125I-A4 (A) but did not hydrolyze Abz-ANG-(1–7)[Tyr7(NO2)] (Abz-A7; B). Human recombinant IDE (2 μg) did not hydrolyze 125I-A7 (C) but hydrolyzed Abz-A7 to Abz-ANG-(1–4) (Abz-A4; D). Products were separated by HPLC under isocratic (A and C) or gradient (B and D) conditions.

Fig. 8. HK-2 processing of 125I-ANG I. Chromatograph reveals 125I-ANG I (125I-AI) is metabolized to 125I-A7 and 125I-A4 in the supernatant from HK-2 100,000-g cytosol fraction (A). 125I-A7 metabolism to 125I-A4 is reduced by 1 nM JMV-390 (B), and 125I-A7 metabolism to 125I-A7 is reduced by 10 μM CPP in combination with 1 nM JMV-390 (C). AI metabolism is abolished by 10 μM PCMB (D). 125I-labeled products were separated by HPLC under gradient conditions.

Fig. 9. 125I-ANG I metabolism in HK-2 cells. JMV-390 (JMV, 1 nM) increased 125I-A7, while CPP (10 μM) and JMV reduced 125I-A7 (A). JMV reduced 125I-A4 levels while addition of JMV and CPP did not further influence 125I-A4 (B). The mercury-containing inhibitor PCMB (10 μM) abolished the metabolism of 125I-ANG I to 125I-A7 and 125I-A4. Values are means ± SE; n = 3. *P < 0.05 vs. control (CON). #P < 0.05 vs. JMV. αP < 0.05 vs. JMV/CPP.
glycation end product (AGE) methylglyoxal-albumin reduced intracellular levels of ANG-(1–7) which were associated with an enhanced metabolism of the peptide to ANG-(1–4), myofibroblast transition, and chronic MAPK activation (2). Moreover, the intracellular expression of functional ANG-(1–7) and ANG II receptors is evident within the kidney, proximal tubules, and other tissues, and the peptidase may be a novel component of an intracellular RAS to regulate the cellular levels of ANG-(1–7) (6, 11, 12, 14, 16, 32).

**Perspectives and Significance**

ANG-(1–7) constitutes a key bioactive component of the nonclassic or alternative RAS in the kidney, circulation, and other tissues (7). In general, the ACE2/NEP/TOP-ANG-(1–7)-AT7/MasR axis opposes or functionally antagonizes a stimulated ACE-ANG II-AT1R pathway. The renal actions of ANG-(1–7) include natriuresis, diuresis, and reduced vascular resistance, as well as anti-inflammatory and antifibrotic effects that may encompass the release of nitric oxide, activation of cellular phosphatases, and the reduction of oxidative stress (9, 28).

The presence of an ANG-(1–7) peptidase within the proximal tubule of the kidney may constitute a novel therapeutic target to maintain the intracellular levels of the peptide and potentially provide renoprotective effects.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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