Identification of prolyl carboxypeptidase as an alternative enzyme for processing of renal angiotensin II using mass spectrometry

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The renin-angiotensin system (RAS) is a key regulator of renal and cardiovascular function. In diabetes and renal disease, angiotensin-converting enzyme (ACE) is activated and ANG II processing. Furthermore, the dual prolyl carboxypeptidase (PCP)-prolyl endopeptidase (PEP) inhibitor Z-prolyl-prolinal reduced ANG-(1–7) formation in ACE2 KO mice, while the ACE2 inhibitor MLN-4760 had no effect. Unlike the ACE2 KO mice, ANG-(1–7) formation from ANG II in PEP KO mice was not different from that in WT mice at any tested pH. However, at pH 5, this reaction was significantly reduced in kidneys and urine of PCP-depleted mice. In conclusion, results suggest that ACE2 metabolizes ANG II in the kidney at neutral and basic pH, while PCP catalyzes the same reaction at acidic pH. This is the first report demonstrating that renal ANG-(1–7) formation from ANG II is independent of ACE2. Elucidation of ACE2-independent ANG-(1–7) production pathways may have clinically important implications in patients with metabolic and renal disease.
if kidneys obtained from animals deficient in ACE2, PEP, or PCP generate ANG-(1–7) from ANG II.1

METHODS

Animals. Male mice were used at 16 wk of age. ACE2 KO breeding stocks were generously provided by Drs. T. Coffman and S. Gurley (Duke University, Durham, NC) (8, 57). PCPgt/gt animals were prepared by insertion of LacZ into intron 4 for gene interruption, as described previously (2). PEP KO mouse embryos were obtained from the European Mouse Mutant Archive with assistance from Martin D. Fray (Mammalian Genetics Unit, Medical Research Council, Harwell, UK). The targeted allele for Prep/H11022 tm1Dgen was obtained as follows: 10 μl of 6 N HCl were added to the urine collection tube before and 12 h after the urine collection was started. Urinary ANG peptide analysis. Twenty-four-hour urine samples were obtained as follows: 10 μl of 6 N HCl were added to the urine collection tube before and 12 h after the urine collection was started. Urinary ANG peptides were measured in the laboratory of Dr. K. Bridget Brossnihan (Wake Forest University Medical Center, Winston-Salem, NC). A cocktail of protease inhibitors, including 0.44 mM 1,20-p-phenanthroline monohydrate, 0.12 mM pepstatin, 1 μM rat renin inhibitor, and 1 mM Na p-hydroxymercuribenzoate, was added to the samples. Urine ANG was extracted using Sep-Pak columns and analyzed by radioimmunnoassay. ANG II was measured using an antibody obtained from Alpco Diagnostics (Windham, NH), and ANG-(1–7) was measured using an antibody described elsewhere (38). The minimum detectable levels of the assays were 0.8 and 2.8 fmol/ml for ANG II and ANG-(1–7), respectively. Intra- and interassay coefficients of variation were 12% and 22% for ANG II and 8% and 20% for ANG-(1–7).

Imaging MS assessment of in situ enzyme activity. Consecutive tissue sections were prepared from fresh frozen kidneys as previously described (16) and incubated with 0.01–1 mM ANG II at 37°C for 5–15 min. Inhibition of renal ANG-(1–7) formation was tested using reaction mixtures spiked with the ACE2 inhibitor MLN-4760 (80 μM; a gift from the former Millennium Pharmaceuticals, Cambridge, MA) or the PEP/PEP inhibitor Z-prolylprolinyl (ZPP, 0.4–40 μM; Enzo Life Sciences, Farmingdale, NY). The imaging technique is described in detail elsewhere (16). Briefly, a thin-layer chromatography nebulizer was used to spray-coat a matrix consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 60% HPLC-grade methanol, 10% HPLC-grade acetone, and 0.3% sequencing-grade trifluoroacetic acid onto kidney tissue sections. MS images were obtained using an AutoFlex III SmartBeam matrix-assisted desorption/ionization-time of flight (MALDI-TOF/TOF) instrument (Bruker Daltonics, Billerica, MA). Spectral analysis was performed with proprietary Bruker imaging software.

In vitro enzyme activity. ANG-(1–7) formation from ANG II was assessed as previously described with some modifications (12). Kidney homogenate from whole kidney (20 μg of protein) was incubated at 37°C in 50 mM buffer [sodium citrate buffer (pH 4–6), potassium phosphate buffer (pH 6–8), or glycine-NaOH buffer (pH 9–10)] containing 0.02–0.1 mM ANG II, 2 mM PMSF, and 20 μM bestatin. Inhibition of renal ANG-(1–7) formation was tested using reaction mixtures spiked with 80 μM MLN-4760, 0.4–40 μM ZPP, 100 μM captopril, or 100 μM thiorphan. For urinary ANG-(1–7) formation, 24-h urine samples were collected in the presence of 20 μl of inhibitor (Roche Diagnostics, Indianapolis, IN) containing 2.5 mM PMSF. Urine samples were collected every 12 h and kept at 4°C until the 24-h collection was completed. Urine (2 μl) was incubated for 1.5 h at 37°C in 50 mM sodium citrate buffer (pH 5) containing 0.5 μM ANG II, 2 mM PMSF, and 20 μM bestatin. The reaction was stopped by acidification with trifluoroacetic acid. Peptides were purified using micro C18 ZipTips (Millipore, Billerica, MA). Mass spectra were obtained using an Autoflex III SmartBeam MALDI-TOF/TOF instrument. Spectral analysis was performed with proprietary Bruker Flex Analysis software.

MALDI-TOF MS. The MS was operated with positive polarity in reflectron mode. A total of 2,000 laser shots were acquired randomly for each spot at a laser frequency of 100 Hz. Spectra were mass-calibrated by collection of 200 laser shots of spots containing Brüker peptide calibration standard II consisting of nine peptide standards covering a mass range of 700–3,200 Da.

Immunofluorescence. Paraffin-embedded kidney sections were deparaffinized in xylene, rehydrated in graded alcohols, and incubated overnight with primary antisera for ACE2, PCP, and PEP: goat anti-ACE2 (1:100 dilution; R & D Systems, Minneapolis, MN), goat anti-PCP [1:100 dilution, as described elsewhere (2)], or rabbit PEP (1:500 dilution; Abcam, Cambridge, MA) polyclonal antibody. Sections were then incubated with secondary antisera: donkey anti-goat labeled with Alexa Fluor 488 (1:100 dilution; Jackson ImmunoResearch, West Grove, PA) or donkey anti-rabbit labeled with Alexa Fluor 568 (1:200 dilution; Invitrogen, Grand Island, NY). Images were obtained using a confocal microscope (model FV300, Olympus, Center Valley, PA).

RESULTS

Urinary ANG peptides are undistinguishable in ACE2 WT and ACE2 KO mice. ANG II and ANG-(1–7) were measured in 24-h urine of ACE2 WT and ACE2 KO mice by radioimmunoassay. After normalization to urinary excretion per day, there was no significant difference in urinary ANG II levels in ACE2 KO compared with ACE2 WT mice (Fig. 1), nor was there a difference in urinary ANG-(1–7).

1This article is the topic of an Editorial Focus by Juan Carlos Q. Velez (43a).
In situ generation of renal ANG-(1–7) is dependent on ACE2 genotype, ANG II concentration, and incubation time. We previously used and optimized MS imaging to quantify enzymatic ANG II processing to ANG-(1–7) in situ (16). In the present study, ACE2 WT and ACE2 KO kidney sections (12 μm) were incubated at 37°C with ANG II at 0.01–1 mM per section for 5 or 15 min. As expected, ACE2 WT mice formed ANG-(1–7) in a dose- and time-dependent manner (Fig. 2). ACE2 KO mice were also capable of generating ANG-(1–7) from ANG II. At low concentrations of ANG II (≤0.1 mM) and 5 min of incubation, ANG-(1–7) formation was reduced in ACE2 KO mice (Fig. 2A). However, at >0.5 mM ANG II and 15 min of incubation, there was no significant difference in ANG-(1–7) levels between ACE2 WT and ACE2 KO mice (Fig. 2B). It is worth mentioning that these reaction conditions are optimal and the in situ reaction is quick, since at lower concentrations and longer incubation times ANG II and ANG-(1–7) were not detectable.

In vitro generation of renal ANG-(1–7) in ACE2 WT and ACE2 KO mice is dependent on pH. An in vitro MS approach was used to characterize the pH dependency of ANG-(1–7) formation in ACE2 WT and ACE2 KO mice. Kidney homogenates were incubated with ANG II in three different buffer systems over a pH range of 4–10. Generation of ANG-(1–7) in ACE2 WT mice was detected from pH 4 to pH 9 (Fig. 3A). At pH 4 and 5, there was no significant difference in ANG-(1–7) generation between ACE2 WT and ACE2 KO mice (Fig. 3A). However, at pH 6–9, ANG-(1–7) formation was significantly decreased in ACE2 KO mice (Fig. 3A). Results suggest that ACE2 is one of the predominant enzymes responsible for ANG-(1–7) formation in the kidney at pH 6–9. It is tempting to speculate that proteolytic enzyme(s) other than ACE2 catalyze(s) this reaction at pH 4–6. Since there is evidence that ANG-(1–7) can be further degraded by renal ACE and NEP (4), we tested the effect of the ACE inhibitor captopril and the NEP inhibitor thiorphan on in vitro ANG II processing in kidney homogenates obtained from ACE2 WT mice at pH 5 and 7. As illustrated in Fig. 3B, both inhibitors had no effect at pH 5 but showed significantly increased ANG-(1–7) formation at pH 7.

The dual PCP-PEP inhibitor ZPP reduces ANG-(1–7) formation in ACE2 KO mice, while the ACE inhibitor MLN-4760 has no effect. The effect of MLN-4760 on renal ANG-(1–7) formation in ACE2 KO mice under conditions of high ANG II concentrations (1 mM, in situ approach) or at pH 5 (in vitro approach) was examined. Under these conditions, incubation with MLN-4760 had no effect on in situ and in vitro renal ANG-(1–7) formation in ACE2 WT and ACE2 KO mice (Fig. 4). Since the ACE2 inhibitor did not block the detected ANG-(1–7)-forming enzyme activity at high substrate concentrations or at pH 5, potential contributions of two other peptidases capable of forming ANG-(1–7) from ANG II, PCP and PEP, were studied using the dual PCP-PEP inhibitor ZPP. At high ANG II concentrations or at pH 5, the in situ and in vitro MS-based assays showed that ZPP significantly inhibited ANG-(1–7) formation from ANG II in ACE2 WT and ACE2 KO mice (Fig. 5). These results suggest involvement of PCP-PEP in renal ANG-(1–7) formation. Comparison of both MS methods revealed a higher efficacy of the in situ approach to detect inhibition of ANG-(1–7) formation by ZPP.

Identification of PCP as an alternative renal ANG II-processing enzyme. To support the enzyme activity data, we used immunofluorescence staining and Western blotting to further analyze the presence and protein expression of renal PCP and PEP in ACE2 WT and ACE2 KO mice. PCP and PEP were localized to glomeruli and tubules in the renal cortex, as was ACE2 (Fig. 6, A–C). Immunofluorescence and Western blot analysis confirmed that ACE2 KO mice were deficient in ACE2 protein (Fig. 6, A and D). PCP and PEP were expressed in kidney cortex, and protein levels of PCP and PEP were unchanged in ACE2 KO mice compared with ACE2 WT mice (Fig. 6, E and F).

Although ACE2 was depleted (Fig. 7A), ACE2 KO mice were still capable of processing ANG II to ANG-(1–7) at pH 5 (Fig. 7B). To investigate whether renal PEP plays a role in

**A** 5 min incubation time  
ACE2 WT **ACE2 KO**  
Intensity ANG-(1–7)  
0.01 0.05 0.1 0.5 1  
***  
**  
•  
**B** 15 min incubation time  
ACE2 WT **ACE2 KO**  
Intensity ANG-(1–7)  
0.01 0.05 0.1 0.5 1  
**  
##  
•  

Fig. 2. Characterization of renal ANG-(1–7) formation in situ. Kidney sections from ACE2 WT and ACE2 KO mice were incubated with different concentrations of ANG II. Generated ANG-(1–7) was analyzed by in situ imaging mass spectrometry (MS). A: ANG-(1–7) formation at 5 min. Data were collected after 5-min incubation of tissue sections with ANG II. B: ANG-(1–7) formation at 15 min. Data were collected after 15-min incubation of tissue sections with ANG II. Note absence of bars for ANG-(1–7) formation in incubations containing 0.01, 0.05, and 0.1 mM ANG II. Values are means ± SE of 4 mice per group. Effect of genotype, ANG II concentration, and incubation time on ANG-(1–7) formation was analyzed by 2-way ANOVA. *P < 0.01, **P < 0.001, ANG-(1–7) formation in ACE2 WT vs. ACE2 KO mice or ANG-(1–7) formation at 0.5 and 1 mM ANG II vs. 0.01 mM ANG II. ##P < 0.05, ###P < 0.01, ANG-(1–7) formation at 5 min vs. 15 min.
ACE2-independent ANG-(1–7) formation, we examined ANG II processing in PEP WT and PEP KO mouse kidneys at pH 5 and 7. After confirmation by Western blotting that PEP KO mice completely lacked renal PEP protein (Fig. 7C), kidney homogenates were incubated with 0.05 mM ANG II at pH 5 and 7 for 30 min at 37°C and analyzed by MS. ANG-(1–7) formation in PEP WT and PEP KO mice was indistinguishable (Fig. 7D). Finally, PCP WT and PCPgt/gt mouse kidneys were examined for PCP protein expression and ANG II-processing activity at pH 5 and 7. PCPgt/gt animals showed reduced PCP protein levels (54.3 ± 14.3%) and enzyme activity (63.6 ± 7.3%) at pH 5, with no significant difference at pH 7 (Fig. 7, E and F). Addition of ZPP to the PCPgt/gt incubation mixtures produced a further decline in the enzyme activity at pH 5 (Fig. 7F). The in vitro MS assay detected urinary ANG-(1–7) formation at pH 5, which was significantly decreased in PCPgt/gt mice compared with PCP WT mice (Fig. 7G).

**DISCUSSION**

Our studies characterized renal ANG II processing to ANG-(1–7) by analyzing ANG peptide levels and ANG II metabolism in gene deletion models. First, urinary levels of ANG II and ANG-(1–7) were measured by radioimmunoas-

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**Fig. 3. Characterization of renal ANG-(1–7) formation in vitro.** A: kidney homogenates (20 μg of protein) from ACE2 WT and ACE2 KO mice were incubated with 0.1 mM ANG II for 30 min at 37°C in 50 mM buffer [sodium citrate buffer (pH 4–6), potassium phosphate buffer (pH 6–8), or glycine-NaOH buffer (pH 9–10)]. ANG peptides were purified and analyzed by MS. B: kidney homogenates from ACE2 WT mice (n = 4) were incubated with 0.1 mM ANG II for 20 min at 37°C in 50 mM citrate buffer (pH 5) or 50 mM phosphate buffer (pH 7) in the presence of 100 μM captopril (an ACE inhibitor) or 100 μM thiorphan (a neprilysin inhibitor). ANG-(1–7) formation at pH 5 and 7 without addition of inhibitors was set at 100% (i.e., control). Values are means ± SE of 3–4 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001, ANG-(1–7) formation at different pH in ACE2 WT vs. ACE2 KO mice (2-way ANOVA). #P < 0.001, ANG-(1–7) formation at pH 5 vs. pH 7 (2-way ANOVA).

**Fig. 4. Effect of the ACE2 inhibitor MLN-4760 on renal ANG-(1–7) formation in ACE2 WT and ACE2 KO mice at pH 5.** A: imaging MS of kidney sections incubated with 1 mM ANG II for 15 min without (control) or with 80 μM MLN-4760 (+MLN). Values are means ± SE of 4 mice per group. On 2-way ANOVA, there were no differences among the groups. B: kidney homogenate (50 μg) was incubated for 30 min in 50 mM citrate buffer (pH 5) containing 0.02 mM ANG II without (control) or with 80 μM MLN-4760 (+MLN), and generated ANG-(1–7) was evaluated using MS. m/z, Mass-to-charge ratio.
say, and no differences were noted between ACE2 KO and ACE2 WT mice. These findings agree with previous reports describing normal ANG II levels in plasma, heart, and kidney of ACE2 KO mice (18, 52, 57, 61). To elucidate whether ANG-(1–7) in ACE2 KO mice originates from ANG II by means of compensating mechanisms, we analyzed the ability of ACE2 KO mice to generate ANG-(1–7) from ANG II. Established MS-based protocols (12) allowed for the enzymatic characterization with respect to genotype, substrate concentration, incubation time, pH, and effect of inhibitors. This type of measurement is not possible with classical, commercially available systems that use nonnatural chromophore or fluorogenic substrates. For example, the substrates Mca-YVADAPK(Dnp) and Mca-APK(Dnp) are commonly used to measure ACE2 activity but are also used to measure activity of other peptidases such as caspase-1 (23, 46). The assay is based on cleavage of an amide bond between the fluorescent [methoxycoumarin (Mca)] and the quencher [dinitrophenyl (Dnp)] group, resulting in an increase in fluorescence. However, such a fluorescence signal could be produced by peptidases other than ACE2. Thus these artificial substrates may not be highly specific, may interact with other enzymes, and can generate conflicting results (16, 20, 46). In contrast, MS assays allow for the use of natural substrates and determination of alternate proteolytic cleavage sites (15). Possible multiple products can be easily detected and differentiated from one another. Therefore, the activity of specific enzymes can be precisely identified. Our laboratory first developed in vitro MS assays for ACE and ACE2 activities in tissue homogenates (11, 12). This was followed by an in situ tissue-imaging technique that allows for the anatomic localization of enzyme activity, showing ACE2 activity primarily in the outer renal cortex (16). The advantages of an MS-based enzyme assay over traditional techniques are their high specificity, sensitivity, and selectivity for the proteolytic products obtained with the endogenous peptide precursor (12, 16). In addition, MS imaging gives information on the spatial localization of enzyme activities within tissue sections (16). We utilized MS techniques to identify and characterize possible ACE2 compensatory pathways that are involved in renal ANG II processing. Kidney sections were analyzed by imaging MS. At >0.1 mM ANG II with 5 or 15 min of incubation, ANG-(1–7) was generated from ANG II to a similar extent in ACE2 KO and ACE2 WT mice. This is the first report to demonstrate renal ANG-(1–7) formation from ANG II that is independent of ACE2. Since the kidney plays a pivotal role in regulation of acid-base homeostasis and small changes in pH can severely affect enzyme activities, we examined the influence of pH on renal ANG-(1–7) formation in ACE2 WT and ACE2 KO mice. We used an in vitro MS approach, testing the processing of ANG II in renal homogenates. In ACE2 WT mice, ANG-(1–7) formation was found at a wide pH range (pH 4–9). In ACE2 KO mice, ANG-(1–7) formation was significantly reduced at pH ≥6 but was not significantly different from ACE2 WT mice at pH 4 and 5. Taken together, results suggest that ACE2 is the predominant enzyme responsible for ANG-(1–7) formation in the kidney under neutral and basic conditions (pH 6–9), while proteolytic enzymes other than ACE2 catalyze this reaction at acidic pH (pH 4–6). The flat pH response at pH 7–9 in ACE2 WT mice is most likely due to quick degradation of formed ANG-(1–7) by other renal peptidases active at neutral and basic conditions. Indeed, NEP and ACE are peptidases known to degrade ANG-(1–7) (4), and addition of NEP or ACE inhibitor to the assay caused a significant increase in ANG-(1–7) signal at pH 7 but had no effect at pH 5. Thiorphan also inhibits ACE at the concentrations used in this assay, which might explain why, at pH 7, both inhibitors increased the ratio of ANG-(1–7) to ANG II to a similar extent. Overall, these experiments indicate that NEP and ACE inhibitors are critical factors that allow for a quantitative comparison between ACE2 and ACE2-independent ANG-(1–7) formation.

The identity of the enzyme(s) catalyzing renal ANG-(1–7) formation in ACE2 KO mice was studied using peptidase inhibitors and genetic mouse models. As previously established for the in situ MS imaging approach (16), the specific ACE2 inhibitor MLN-4760 selectively inhibited ANG-(1–7) formation in ACE2 WT mice at low ANG II concentrations.
(≤0.1 mM). However, at higher ANG II concentrations, MLN-4760 had no effect on ANG-(1–7) formation in ACE2 WT mice. Here, we examined the effect of MLN-4760 on ANG-(1–7) formation in ACE2 KO mice at 1 mM ANG II or at pH 5. As expected, the specific ACE2 inhibitor MLN-4760 failed to reduce ANG-(1–7) formation in ACE2 KO mice in both experiments. In contrast, the dual PCP-PEP inhibitor ZPP, with a $K_i$ of 0.26 μM for PCP (40) and a $K_i$ of 0.35 nM for PEP (5), was effective. ANG-(1–7) formation was reduced to 4–14% by 40 μM ZPP in the in situ approach. When the in vitro method was used, 40 μM ZPP decreased ANG-(1–7) formation to 38–54%. Both experiments suggest a partial contribution of PCP or PEP to renal ANG-(1–7) formation. Western blotting confirmed that PCP and PEP were expressed in ACE2 WT and ACE2 KO mice. PEP has been proposed as a mediator in obesity and eating disorders (29, 49). PCP in the hypothalamus has been demonstrated to regulate α-MSH$_{1-13}$, and PCP$_{gt/gt}$ mice are lean (47). Unlike ACE2 KO mice, PCP$_{gt/gt}$ mice are constitutively hypertensive and prothrombotic, with vascular inflammation and increased reactive oxygen species (2, 37). Although plasma ANG peptide levels were unchanged (2, 37), renal ANG-(1–7) was increased threefold in PCP-deficient mice (37). This finding is in contrast to ANG-(1–7) levels in whole kidney extracts of ACE2 KO mice, which were not significantly different from ACE2 WT mice (61). It is worth noting that other studies reported significantly lower cortical ANG-(1–7) levels (42) and increased ANG II levels in kidney, heart, and plasma of ACE2 KO mice at baseline (8).

While PEP is most efficient at neutral/basic pH (48), acidic conditions at pH <6 are optimal for PCP (32). This enzymatic characteristic indicates that PCP is the predominant enzyme responsible for ANG II metabolism in ACE2 KO mice. To support this hypothesis, we conducted experiments in PEP KO and PCP$_{gt/gt}$ mice. ANG-(1–7) formation at pH <6 in PEP KO mice are lean (47). Unlike ACE2 KO mice, PCP$_{gt/gt}$ mice are constitutively hypertensive and prothrombotic, with vascular inflammation and increased reactive oxygen species (2, 37). Although plasma ANG peptide levels were unchanged (2, 37), renal ANG-(1–7) was increased threefold in PCP-deficient mice (37). This finding is in contrast to ANG-(1–7) levels in whole kidney extracts of ACE2 KO mice, which were not significantly different from ACE2 WT mice (61). It is worth noting that other studies reported significantly lower cortical ANG-(1–7) levels (42) and increased ANG II levels in kidney, heart, and plasma of ACE2 KO mice at baseline (8).

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mice was indistinguishable from that in PEP WT mice, while this reaction was reduced in PCPgt/gt animals. Moreover, addition of ZPP to the PCPgt/gt incubations resulted in a further decline of ANG-(1–7) formation at pH 5. These results suggest that PCP is an enzyme catalyzing renal ANG II conversion to ANG-(1–7) independent of ACE2. In fact, a possible involvement of PCP in renal ANG II processing has been identified recently (16) and confirmed by another independent study (44). A contribution of PCP to renal ANG II processing is likely, considering its localization to glomeruli and tubules in the renal cortex, which is similar to previous findings for ACE2 (2, 60). This also fits very well with the MS imaging results. Throughout the current study, MS imaging detected ANG-(1–7) formation exclusively in the renal cortex, regardless of genotype or treatment of samples, showing that the cortex is a key site of ANG-(1–7) formation in the kidney. This is consistent with our previous results (16). Moreover, the response of ZPP inhibition on ANG-(1–7) formation in ACE2 WT and ACE2 KO mice was more effectively revealed by MS imaging than by the in vitro method.

In addition to a reduced renal ANG-(1–7) formation, MS analysis revealed a decreased urinary ANG-(1–7) synthesis at pH 5 in PCPgt/gt mice, suggesting that enzymatically active PCP is excreted into the urine. We propose that intratubular ANG II is degraded to ANG-(1–7) by ACE2 and/or PCP, thereby preventing activation of cell signaling through luminal ANG II type 1 receptors. Recent clinical studies closely linked urinary acidity to diabetic patients and metabolic syndrome (1, 27, 28, 41), an association that has also been confirmed in a rat model of type 2 diabetes (7). Acidic urine can indicate changes in the biochemical composition and acidity of the intratubular micromilieu. These alterations, albeit small, can severely affect tubular enzyme activity and, as a result, establish PCP as one of the predominant, intratubular enzymes responsible for ANG II degradation. Therefore, ANG II processing by PCP may have clinical implications in patients with metabolic or renal pathologies.

In conclusion, this study shows that processing of ANG II to ANG-(1–7) is not unilaterally dependent on ACE2. Using novel MS enzyme tests in genetic mouse models, we identified and characterized a possible ACE2 compensatory pathway in kidney that is involved in pH-dependent ANG II processing. We suggest that this ACE2-independent ANG-(1–7)-forming enzyme activity escaped detection, possibly because different methodological approaches were used and the link between this enzyme reaction and its dependency on pH was not recognized. Since change in urinary pH is one of the underlying disturbances in renal and metabolic diseases, renal ANG II-degrading peptidases that prefer acidic conditions might play an important role in these pathologies.

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RENAL ANG II PROCESSING BY PROLYL CARBOXYPEPTIDASE


