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Soluble ACE2 is Filtered into the Urine

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Jonathan Nelson, David Ortiz-Melo, Natalie Mattocks, Jacqueline Emathinger, Jessica Prescott, Katherine Xu, Robert Griffiths, Rumie Wakasaki, Paul Pichowski, Michael Hutchens, Thomas Coffman, and Susan Gurley

Key Points:
*Combining unique genetic and surgical models, we demonstrate that both renal and systemic sources contribute to ACE2 detected in the urine.

*Micropuncture-coupled with nano-proteomics confirm detection of ACE2 in early glomerular filtrate obtained from Bowman’s capsule in mice.

*Kidney-derived ACE2 and soluble ACE2 may be useful clinical targets in kidney disease.

Abstract:
Background: ACE2 is a key enzyme in the renin-angiotensin system (RAS) capable of balancing the RAS by metabolizing angiotensin II (AngII). First described in cardiac tissue, ACE2 has highest levels of abundance in the kidney with expression in a number of extra-renal tissues as well. Previously, we reported an association between enhanced susceptibility to hypertension and elevated renal AngII levels in global ACE2 knockout mice. Methods: To examine the impact of ACE2 expressed in the kidney, relative to extra-renal expression, on the development of hypertension, we utilized a kidney cross-transplantation strategy with ACE2 KO and WT mice. In this model, both native kidneys are removed and renal function is provided entirely by the transplanted kidney such that 4 experimental groups with restricted ACE2 expression are generated: WT→WT (WT), KO→WT (KidneyKO), WT→KO (SystemicKO), and KO→KO (TotalKO). Additionally, we utilized nano-scale mass spectrometry-based proteomics to identify ACE2 fragments in early glomerular filtrate of mice. Result: Although significant differences in BP were not detected, a major finding of our study is that shed or soluble ACE2 (sACE2) was present in urine of KidneyKO mice which lack renal ACE2 expression. Detection of sACE2 in the urine of KidneyKO mice during AngII hypertension suggests that sACE2 originating from extra-renal tissues is able to reach the kidney and can be excreted in urine. To confirm glomerular filtration of ACE2, we used micropuncture and nano-scale proteomics to detect peptides derived from ACE2 in Bowman's space. Conclusions: Our findings suggest that both systemic and renal tissues may contribute to sACE2 in urine, identifying the kidney as a major site for ACE2 actions. Moreover, filtration of sACE2 into the lumen of the nephron may contribute to the pathophysiology of kidney diseases characterized by disruption of the glomerular filtration barrier.

Disclosures: R. Wakasaki reports the following: Ownership Interest: FUJI FILM Holdings, LION, TOYOTA, KDDI. P. Pichowski reports the following: Employer: Pacific Northwest National Laboratory; Research Funding: Pfizer worldwide research PI: Jacobs; and Patents or Royalties: Pacific Northwest National Laboratory. M. Hutchens reports the following: Ownership Interest: Exxon, ATT, Verizon, Frontier; and Patents or Royalties: ProjectLite, LLC. T. Coffman reports the following: Advisory or Leadership Role: Editorial Boards: Cell Metabolism, JCI; Singapore Health Services, Board of Directors; Singapore Eye Research Institute; Kidney Research Institute University of Washington. S. Gurley reports the following: Employer: United Therapeutics; and Ownership Interest: United Therapeutics. The remaining authors have nothing to disclose.

Author Contributions: Jonathan Nelson: Data curation; Formal analysis; Visualization; Writing - original draft; Writing - review and editing David Ortiz-Melo: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing - original draft; Writing - review and editing Natalie Mattocks: Investigation Jacqueline Emathinger: Investigation; Visualization; Writing - review and editing Jessica Prescott: Investigation Katherine Xu: Investigation Robert Griffiths: Investigation Rumie Wakasaki: Investigation Paul Piehowski: Formal analysis; Investigation; Methodology Michael Hutchens: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing - original draft; Writing - review and editing Thomas Coffman: Conceptualization; Supervision; Writing - review and editing Susan Gurley: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing - original draft; Writing - review and editing

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Soluble ACE2 is Filtered into the Urine

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Key Points

• Combining unique genetic and surgical models, we demonstrate that both renal and systemic sources contribute to ACE2 detected in the urine in AngII hypertension.

• Micropuncture-coupled with nano-proteomics confirm detection of ACE2 in early glomerular filtrate obtained from Bowman’s capsule in mice.

• Kidney-derived ACE2 and soluble ACE2 may be useful clinical targets in kidney disease.
Abstract

Background: ACE2 is a key enzyme in the renin-angiotensin system (RAS) capable of balancing the RAS by metabolizing angiotensin II (AngII). First described in cardiac tissue, ACE2 has highest levels of abundance in the kidney with expression in a number of extra-renal tissues as well. Previously, we reported an association between enhanced susceptibility to hypertension and elevated renal AngII levels in global ACE2 knockout mice.

Methods: To examine the impact of ACE2 expressed in the kidney, relative to extra-renal expression, on the development of hypertension, we utilized a kidney cross-transplantation strategy with ACE2 KO and WT mice. In this model, both native kidneys are removed and renal function is provided entirely by the transplanted kidney such that 4 experimental groups with restricted ACE2 expression are generated: WT→WT (WT), KO→WT (KidneyKO), WT→KO (SystemicKO), and KO→KO (TotalKO). Additionally, we utilized nano-scale mass spectrometry-based proteomics to identify ACE2 fragments in early glomerular filtrate of mice.

Result: Although significant differences in BP were not detected, a major finding of our study is that shed or soluble ACE2 (sACE2) was present in urine of KidneyKO mice which lack renal ACE2 expression. Detection of sACE2 in the urine of KidneyKO mice during AngII hypertension suggests that sACE2 originating from extra-renal tissues is able to reach the kidney and can be excreted in urine. To confirm glomerular filtration of ACE2, we used micropuncture and nano-scale proteomics to detect peptides derived from ACE2 in Bowman’s space.

Conclusions: Our findings suggest that both systemic and renal tissues may contribute to sACE2 in urine, identifying the kidney as a major site for ACE2 actions. Moreover, filtration of sACE2 into the lumen of the nephron may contribute to the pathophysiology of kidney diseases characterized by disruption of the glomerular filtration barrier.
**Introduction**

First reported just twenty years ago, angiotensin converting enzyme 2 or ACE2 is a monocarboxypeptidase that is distinct from its homolog, the dicarboxypeptidase ACE.\(^1\) ACE2 hydrolyzes several angiotensin peptides but has highest affinity for AngII.\(^1\) By removing a single amino acid from AngII, ACE2 generates Ang(1-7), which signals via the Mas receptor\(^2\), and thereby plays an integral role in AngII metabolism and balancing overall tone of the renin-angiotensin system (RAS).\(^3\) ACE2 has been implicated in a number of clinical cardiovascular and renal pathologies, including heart failure where it was first described\(^1\) along with hypertension and chronic kidney disease.\(^4\)-\(^7\) Because of its pivotal position in the RAS, ACE2 remains the focus of studies aimed at understanding the regulation of cardiovascular and renal function. Furthermore, although not directly examined in these studies, ACE2 also serves as the receptor for SARS-CoV2 and as such plays a significant role in COVID-19 infectious complications which include a high incidence of kidney dysfunction.\(^8\)-\(^11\)

Previously our group leveraged the use of gene-targeting in mice to study cardiovascular and renal functions of ACE2.\(^12\) We generated global ACE2 knockout mice (ACE2 KO) and observed that lack of ACE2 was associated with an exaggerated blood pressure (BP) response and elevated AngII peptide levels in the kidney during AngII hypertension.\(^12\) We also showed that under baseline conditions, reduced ability to degrade AngII was associated with increased oxidative stress in kidney tissue from ACE2 KO mice.\(^13\) Furthermore, research by others using ACE2 KO mouse models has revealed that in addition to modulating BP,\(^14\)-\(^16\) ACE2 also protects against cardiac hypertrophy\(^17\) and plays a role in diabetic renal injury.\(^4\)

ACE2 exists as a membrane-bound protein but can be cleaved by ADAM17 (a disintegrin and metalloproteinase domain-containing protein) to produce a smaller, shed or soluble form (sACE2) which maintains catalytic activity.\(^18\) Shedding can functionally relocate ACE2 activity moving ACE2 from cells where it was originally expressed to allow ACE2 to gain access to new sites where it can regulate AngII
levels.\textsuperscript{19, 20} As an example, cleavage of ACE2 from cells in the CNS into the cerebrospinal fluid exacerbates hypertension due to loss of local brain ACE2 activity.\textsuperscript{20} While these studies by Xia et al indentified regulatory mechanisms for the local brain RAS, it remains unknown how shedding of ACE2 functions in the kidney.\textsuperscript{21} Here we examine the relative contributions of renal versus systemic ACE2 by genetically restricting expression to renal or systemic tissues. Our kidney cross-transplant studies combined with micropuncture-guided nanoproteomics demonstrate that extra-renal sources of sACE2 are able to reach the kidney where it may play a role to modulate the renal RAS during angII hypertension.
Methods

Experimental Animals. The generation of ACE2 knockout mice (ACE2 KO) has been previously described. Kidney cross-transplant experiments were performed in male (129 x C57BL/6)F₁ ACE2 KO mice 2-4 months of age, along with their age-matched, wild-type (WT) littermates (Figure 1A). Aspiration of glomerular filtrate was performed on 8-12 week old, male C57BL/6 mice purchased from Jackson Laboratories.

All animals were bred, housed, or maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility at the Durham Veterans Affairs Health Care Center, Oregon Health & Science University, or the Portland Veterans Affairs Medical Center according to NIH Guidelines for Care and Use of Laboratory Animals. Approval for animal care and experiments was granted by Institutional Animal Care and Use Committees.

Renal Cross-transplantation: Mouse renal transplantation surgeries were performed as previously described. Briefly, the donor kidney, ureter, and bladder were harvested en bloc, including the renal artery and vein with a small aortic and vena cava cuff, respectively. End-to-side anastomoses were created, below the level of the native renal vessels. In the same way, the recipient vena cava was anastomosed to the donor vena cava cuff. Total ischemic time averaged 20 minutes. Donor and recipient bladders were anastomosed dome-to-dome. The left native kidney was removed at the time of transplant surgery, and the right native kidney was removed through a flank incision 1–3 days later. The adrenal glands and adrenal blood supply were preserved.

Measurement of Blood Pressures. BPs were measured in conscious, unrestrained mice using a radiotelemetry system as described previously. Briefly, mice were anesthetized with inhaled 1.5% isoflurane and a non-thrombogenic pressure-sensing catheter connected to a small radiotelemetry device (DSI PAC-10) was inserted into the carotid artery of the mouse and advanced into the aortic arch. The transducer unit was then inserted into a subcutaneous pouch along the right flank. Before
measurements were recorded, mice were given 1 week to recover from surgery and regain normal circadian rhythm. During the measurement period, mice were housed unrestrained in individual cages, in a quiet monitoring room. BPs were measured continuously over a 10-second interval every 5 minutes and data were collected, stored, and analyzed using Dataquest ART software (version 4.1; Transoma Medical). Telemetry values were averaged before or after AngII osmotic pump implantation to determine baseline and AngII HTN values, respectively, for mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR).

**Experimental Protocol.** Baseline BPs were measured continuously for two weeks while the animals ingested a normal chow diet (LabDiet 5001) and had free access to water. After baseline recordings, AngII peptide (Sigma-Aldrich A9525) was infused at a dose of 1000ng/kg/min for two weeks using subcutaneous osmotic minipumps (Alzet 1004). During the infusion period, BPs were monitored by radiotelemetry as described above. Urine was collected as 24-hour samples in individual metabolic cages (Hatteras Instruments, Cary, NC). At the end of the experiment, serum was collected, and the kidney was harvested. Animals were euthanized under 4.5% isoflurane by exsanguination.

**Enzymatic ACE2 Activity.** ACE2 activity was determined following incubation with the intramolecularly quenched synthetic ACE2-specific substrate Mca-APK-Dnp (Anaspec) as previously described.

**Analysis of ACE2 Protein in Kidney and Urine by Immunoblot.** Approximately 2-3 mg of kidney cortex were homogenized in a buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.3 mol/L sucrose, 1.0% NP-40, 0.1% sodium dodecyl sulfate and 1:100 dilution of a protease inhibitor cocktail (Sigma, P8340). Kidney homogenates were centrifuged for 30 minutes at 1000xg, and supernatants were stored at -80°C. Protein concentration was determined by Bradford assay (Bio-Rad Protein Assay). For urine samples, equal volumes of undiluted urine were loaded into a 4-12% polyacrylamide gel. Detection of ACE2 protein by Immunoblot was carried out as previously described.
**RNA isolation and analysis.** Relative levels of mRNA for Ace2 were measured in kidney cortices from each group. RNA was isolated and reverse transcription performed using qScript cDNA Supermix (Quanta Biosciences). Quantitative PCR was carried out using the SYBR Green PCR Master Mix (Applied Biosystems). The amount of target gene relative to endogenous control was determined by the ΔΔCT method. The following primer sequences were used:

ACE2 Forward-5’-ACTCACAGCAACCTCCAAG-3’
ACE2 Reverse-5’-ATCACCACCAAGCTGTTTCC-3’ (236BP)
GAPDH Forward-5’-TCACCACCATGGGAGGC-3’
GAPDH Reverse-5’-GCTAAGCAGTTGGTGCTGGA-3’ (168BP)
18S Forward-5’-TCACCACCATGGGAGGC-3’
18S Reverse-5’-GCTAAGCAGTTGGTGCTGGA-3’ (168BP)

**Measurement of ACE2 in Early Glomerular Filtrate.** Samples (n=4) were obtained via micropuncture from Bowman’s space and analyzed as described. 22

**Statistical Analysis.** Statistical analysis was performed using Graphpad Prism. Values for each parameter are expressed as the mean±standard error of the mean (SEM) with individual data points plotted when possible. The statistical analysis used for each comparison is described within each figure legend. Statistical significance was defined as a p-value less than 0.05. Peptide identifications from mass spectrometry were filtered against a reversed decoy database at false discovery rate=0.01. Peptide BLAST alignments were filtered for statistical significance at evalue<0.05. Binary comparisons of peptide origin (sequences corresponding to endo- or ectodomain) in plasma and glomerular filtrate samples was carried out by chi square analysis.
Results

Effect of renal ACE2 on BP regulation, renal and cardiac injury.

To examine the relative contribution of renal ACE2 to the pathogenesis of AngII-mediated hypertension, we utilized a kidney cross-transplantation approach. Specifically, we transplanted kidneys from genetically-matched (129 x C57BL/6)F1 wild-type (WT) and ACE2 KO mice into (129 x C57BL/6)F1 WT and ACE2 KO recipients, such that 4 kidney cross-transplant groups were generated: WT, KidneyKO, SystemicKO, and TotalKO (Figure 1A). Since all mice underwent bilateral native nephrectomy, expression of ACE2 was determined experimentally by genotype of transplanted kidney and recipient. As controls, a group of WT recipients were transplanted with kidneys from WT donors (WT). In KidneyKO mice, ACE2 expression is maintained in systemic tissues but absent from the kidney; conversely, in SystemicKO mice, ACE2 expression is maintained within the single transplanted kidney, but is absent from all other systemic tissues. Lastly, ACE2 KO mice were transplanted with ACE2 KO kidneys to generate TotalKO mice, which lacked ACE2 expression in all tissues. These models were validated by measuring renal ACE2 mRNA and protein expression and renal cortical ACE2 activity which followed genotype of recipient and donor as expected (Figure 1B, C, D).

At baseline, there were no differences in BPs among the four groups, consistent with our previous findings in ACE2 KO mice (Figure 2A, Table 1). During infusion of AngII, BPs increased significantly from baseline in all four groups and mean MAPs during AngII infusion for WT (132±7 mmHg), KidneyKO (140±4 mmHg) and SystemicKOs (141±13 mmHg) were nearly identical (p=.83) (Figure 2A, Table 1). The mean MAP response in the TotalKO group was higher than that of the other groups (148±5 mmHg) throughout the infusion period, but this did not reach statistical significance. Additionally, there were no differences in systolic blood pressures (SBP), diastolic blood pressures (DBP), or heart rates (HR) (Table 1). Nonetheless, compared to the WT group, TotalKO mice developed significantly more pronounced cardiac hypertrophy (6.3±.2mg/g and 7.2±.4mg/g, p<0.05 vs WT; Heart Weight/Body Weight; Figure 2B),
a surrogate marker for chronic BP elevation. Furthermore, urinary albumin excretion was significantly increased in TotalKO mice (3224±663µg and 1519±457µg, p<0.05 vs WT; Figure 2C). Thus, expression of ACE2 in either the kidney or extra-renal compartment appears similarly capable of modulating BP responses and attenuating hypertensive complications during chronic AngII infusion.

**Extra-renal source of soluble ACE2 in serum and urine**

Based on our previous studies using conventional ACE2 knockout mice, we had hypothesized that ACE2 actions within the kidney to metabolize AngII were critical to abrogate hypertension. To examine potential mechanisms explaining lack of impact of ACE2-deficiency in the kidney alone on AngII-dependent hypertension, we examined circulating and urinary levels of ACE2 in the transplanted groups. While our primary goal was to confirm that ACE2 expression and activity followed the expected patterns based on genotype, we considered the possibility that ACE2 arising from extra-renal tissues might enter the kidney circulation and/or urine to provide a compensatory source of ACE2 contributing to the attenuated BP response observed in the KidneyKOs during AngII hypertension. As shown in Figure 3A, plasma levels of ACE2 activity were significantly reduced in SystemicKOs and TotalKOs, indicating a major contribution of extra-renal tissues to circulating ACE2 levels as predicted by genotype. Yet, reduced plasma ACE2 activity alone in SystemicKOs did not impact baseline blood pressure or increase hypertensive responses to AngII infusion, indicating an ability of ACE2 in kidney to compensate for its absence in systemic tissues.

We next compared ACE2 activity in the urine among the four groups. As expected, ACE2 activity in urine was absent in TotalKO mice (Figure 3B). Urinary ACE2 activity was present in the SystemicKO group (127.62±65.91RFU/µl/hr) but tended to be reduced compared to WT (289.49±72.37RFU/µl/hr, p=0.49) indicating that the kidney is a major source of ACE2 in urine. Unexpectedly, ACE2 activity was also easily detected in urine from KidneyKO mice at levels not different from controls (306.95±179.52RFU/µL/hr,
p=0.99) despite genetic elimination of ACE2 from the transplanted kidney. Thus, in KidneyKO mice, enzymatically active ACE2 derived from systemic (non-renal) sources reaches the kidney and is excreted in the urine during AngII hypertension.

To further characterize the nature of ACE2 in urine during AngII hypertension, we measured ACE2 protein by immunoblot with an antibody generated against the extracellular N-terminus domain, expected to be present in both full-length- and soluble-ACE2 (Figure 3C). In the WT group, we detected two species of immunoreactive ACE2 at 98 and 62 kDa, consistent with full-length and sACE2 moieties, respectively.30, 31 Similarly, both forms of ACE2 were detected in urine of SystemicKOs, although the relative amounts of the smaller ACE2 fragment appeared to be reduced, in parallel with activity levels (Figure 3C). While neither full-length nor sACE2 was detected in urine from TotalKO mice as expected, prominent immunoreactive bands representing sACE2 were detected in urine from KidneyKO mice (Figure 3C). We did not detect any full-length ACE2 in the urine from KidneyKO mice suggesting that in the absence of endogenous kidney ACE2, only smaller sACE2 is able to reach the lumen of the nephron. Furthermore, given that the transplanted kidney in KidneyKO mice lack all ACE2 expression, the sACE2 detected in the urine must have originated outside the kidney.

Detection of ACE2 Fragments in Early Glomerular Filtrate

The finding of sACE2 in urine of KidneyKOs suggests that sACE2 is being filtered at the glomerulus into the nephron and is excreted in the urine of mice. To examine this possibility, we directly sampled early glomerular filtrate from Bowman’s space using multiphoton micropuncture followed by nanoproteomics. In samples obtained with this technique, systemic peptides are typically over-represented relative to the very low levels of kidney-specific peptides.22 In addition, this methodology allows the use of mass spectrometry in fluid specifically aspirated from the urinary space in a single nephron. Following tryptic digest and mass spectrometry of the aspirated early glomerular fluid,
sequence analysis demonstrated multiple, highly-specific peptides of ACE2 in the filtrate. Comparison of peptides recovered from plasma and recombinant ACE2 demonstrates identical peptides in plasma and glomerular filtrate, and similar patterns of coverage, particularly that centered around the most highly significant ACE2 identification peptide (R.QLQALQQSGSSALSADKNK.Q), with sequence start at ACE2 amino acid position 95 (aa95). Together these data strongly support the presence of sACE2 in the glomerular filtrate. To assess the relative contribution of the soluble fraction of ACE2 within samples, we compared recovered peptides from the soluble ectodomain (those with sequence endings at position < aa709) with those from the endodomain (sequence ending >= aa 709). As expected, no peptides were identified in the recombinant ACE2 sample after the ADAM17 cleavage site since this preparation is truncated at aa740. The relative presence of endodomain- vs. ectodomain-derived peptides was similar between plasma and glomerular filtrate samples (p=0.3), suggesting that plasma can serve as the source of ACE2 in glomerular filtrate. **Figure 4** summarizes the sequence alignments of peptides recovered from the three types of samples. Our direct sampling of ACE2 peptides from the glomerular filtrate strongly supports the filtration of systemically-derived sACE2.
Discussion

As an integrated network of signaling cascades with broad expression throughout the body, the renin-angiotensin system (RAS) coordinates key renal and cardiovascular functions. Here, we have uncovered an additional and novel layer of RAS regulation involving ACE2. This study builds on our prior work which identified ACE2 as a modulator of intrarenal RAS and hypertension. Here, we show that in response to AngII hypertension, sACE2 is shed from cells outside of the kidney, enters the circulation, and crosses the glomerular filtration barrier, ultimately being excreted in the urine.

Following our earlier findings of enhanced hypertension and elevated renal AngII levels in mice completely deficient in ACE2, we hypothesized that ACE2 within the kidney serves as a critical regulator of BP through its actions to metabolize AngII and diminish its effects to promote renal sodium reabsorption. We reasoned that mice lacking ACE2 in the kidney (KidneyKO and TotalKO) would have an exaggerated BP response to AngII infusion, compared to SystemicKO and WT groups which retained normal expression of ACE2 in kidney. While mean BPs in the TotalKO group were numerically higher than the other 3 groups throughout the AngII infusion period (Figure 2A, Table 1), these differences did not reach statistical significance. This may have been due to unusual variability in BP values and high mortality during this complex study requiring multiple surgeries. Nonetheless, cardiac hypertrophy was significantly exaggerated in TotalKOs compared to WT, which was also seen in global ACE2 KOs and typically correlates with increased pressure load in experimental models of hypertension. Another surrogate marker suggesting higher sustained blood pressures in TotalKO mice was the significantly elevated levels of albuminuria, also likely indicative of elevated blood pressures and consequent renal injury. While we do not have other markers of damage to the filtration barrier such as nephrin levels, the markedly elevated levels of albuminuria indicate significant damage as we have previously reported that nephrinuria correlates with both albuminuria and podocyte dropout.
A truncated, circulating form of ACE2 (sACE2) can be produced by cleavage of the extracellular domain of ACE2 by metalloproteinases such as ADAM17, also known as TACE. Studies by Xiao et al demonstrated that ACE2 exhibits constitutive shedding of enzymatically active fragments, which can be stimulated by AngII or glucose. We reasoned that the somewhat unexpected finding of ACE2 enzymatic activity in the urine of KidneyKO mice might be due to the presence of a cleaved, soluble ACE2 (sACE2) originating outside of the kidney, since renal expression of ACE2 is absent in KidneyKOs.

Although it has been identified as a potential biomarker, the biological and clinical significance of ACE2 ectodomain shedding is yet to be fully characterized. We propose that ACE2 shed from extra-renal sources, as in KidneyKO mice, can be filtered to reach the luminal surface of the nephron to impact disease processes dependent on upregulation of the intrarenal RAS. Furthermore, modulation of shedding by enhancement or inhibition of enzymes such as ADAM17 could have therapeutic potential in hypertension and other kidney diseases. While Lazartigues demonstrated that shedding of ACE2 into the CSF effectively removes it from exerting actions in the RAS centers of the CNS, our studies suggest that shedding of ACE2 in the systemic circulation actually allows it to gain access to the kidney where it can impact actions of AngII and the intra-renal RAS. Thus, ACE2 shedding appears to influence physiological functions in a system-dependent manner.

Based on our findings, we suggest that enzymatically active fragments of ACE2 in urine may play a key role in BP control, such as defending against acute increases in BP induced by AngII. ACE2 shed into the lumen of the nephron would be optimally positioned to metabolize AngII along the length of the nephron, extinguishing its physiological actions, while also generating Ang1-7, which may have independent effects to ameliorate hypertension. Limited samples prevented us from assessing full peptide profiles in these transplanted animals, but our prior report of strong correlation between renal angiotensin II levels and hypertension, suggest that renal angiotensin peptides play a key role in regulating blood pressure and the intra-renal RAS. The presence of full-length ACE2 (in addition to
soluble ACE2) in the urine of WT and SystemicKO mice may represent another mechanism by which ACE2 can be released from kidney cells such as the proximal tubule, through cell shedding, to extend its activity along the nephron to regulate the RAS. The presence of urinary sACE2 in the KidneyKOs, derived from systemic sources, may represent a means of extra-renal compensation against sustained, exaggerated hypertension. Our proteomics analysis confirm that ACE2 peptides are present in early glomerular filtrate, likely originating from plasma which demonstrated a similar profile of sACE2 fragments. The direct micropuncture approach circumvents potential confounding influence of ACE2 peptides originating from renal epithelial cells or more distal urologic sources, including the bladder. Our assays of the early filtrate provide a clear explanation for our findings in the cross-transplantation studies, establishing a pathway by which sACE2 reaches the lumen of kidney tubules and is excreted in urine.

In the future, it will be important to identify the specific cellular origins of extra-renal circulating sACE2 as well as the signaling pathways controlling its shedding. ACE2 was originally identified from the cardiac ventricular tissue and is highly expressed in kidney, indicating robust expression in cardiovascular tissues which may serve as important sources of sACE2. Future studies could address the mechanism(s) of shedding and release of ACE2 from the cell surface, likely mediated by enzymes such as ADAM17 as in other tissues. Based on our findings, we anticipate that the modulators of this regulatory process might be promising therapeutic targets in hypertension and other kidney diseases.
Disclosures

R. Wakasaki reports the following: Ownership Interest: FUJI FILM Holdings, LION, TOYOTA, KDDI. P. Piehowski reports the following: Employer: Pacific Northwest National Laboratory; Research Funding: Pfizer worldwide research PI: Jacobs; and Patents or Royalties: Pacific Northwest National Laboratory. M. Hutchens reports the following: Ownership Interest: Exxon, ATT, Verizon, Frontier; and Patents or Royalties: ProjectLite, LLC. T. Coffman reports the following: Advisory or Leadership Role: Editorial Boards: Cell Metabolism, JCI; Singapore Health Services, Board of Directors; Singapore Eye Research Institute; Kidney Research Institute University of Washington. S. Gurley reports the following: Employer: United Therapeutics; and Ownership Interest: United Therapeutics. The remaining authors have nothing to disclose.

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Author Contributions

Jonathan Nelson: Data curation; Formal analysis; Visualization; Writing - original draft; Writing - review and editing. David Ortiz-Melo: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing - original draft; Writing - review and editing. Natalie Mattocks: Investigation. Jacqueline Emathinger: Investigation; Visualization; Writing - review and editing. Jessica Prescott: Investigation. Katherine Xu: Investigation. Robert Griffiths: Investigation. Rumie Wakasaki: Investigation. Paul Piehowski: Formal analysis; Investigation; Methodology. Michael Hutchens: Data
Data Sharing Statement

All data is included in the manuscript and/or supporting information.
References


pressure overload-induced cardiac dysfunction by increasing local angiotensin ii. *Hypertension.*

2006;47:718-726


2011;50:5182-5194


(type 1 angiotensin) receptors in cardiomyocytes do not contribute to cardiac hypertrophy.

_Hypertension._ 2021;77:393-404


38. Santos RA. Angiotensin-(1-7). _Hypertension._ 2014;63:1138-1147
**Table 1. Summary of Telemetry Measurements.** Mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) measurements at baseline and during AngII-mediated hypertension (AngII HTN).

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>KidneyKO</th>
<th>SystemicKO</th>
<th>TotalKO</th>
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<tr>
<td><strong>Baseline</strong></td>
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<tr>
<td>MAP (mmHg)</td>
<td>112±2</td>
<td>113±2</td>
<td>107±5</td>
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<td><strong>AngII HTN</strong></td>
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<tr>
<td>MAP (mmHg)</td>
<td>132±7</td>
<td>140±4</td>
<td>141±13</td>
<td>148±5</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>150±7</td>
<td>159±5</td>
<td>155±13</td>
<td>161±5</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>120±6</td>
<td>122±4</td>
<td>127±14</td>
<td>136±6</td>
</tr>
<tr>
<td>HR (BPM)</td>
<td>519±22</td>
<td>535±26</td>
<td>553±20</td>
<td>549±11</td>
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</tbody>
</table>
Figure Legends

Figure 1. Kidney cross-transplantation of ACE2. A. Schematic depicting experimental strategy. Wildtype (+/y) or ACE2 KO mice (-/y) were transplanted with either a wildtype or ACE2 KO kidney. Wild Type (WT) cohort had a full complement of ACE2, the KidneyKO group expressed ACE2 systemically, with no expression of ACE2 in the transplanted kidney. Mice in the SystemicKO group expressed ACE2 in the kidney, but not systemically, and the TotalKO group lacked ACE2 expression globally. B. Renal Ace2 mRNA expression confirms expression of ACE2 within the cross-transplanted groups according to genotype. C. Immunoblot demonstrating expression of full length ACE2 in the kidneys of WT and SystemicKO mice, but not in KidneyKO or TotalKO animals. D. Cortical ACE2 activity assay demonstrates a lack of ACE2 within the kidneys of the KidneyKO and TotalKO cohorts. Data expressed as mean±SEM, analyzed as one-way ANOVA with a Dunnett’s multiple comparison post hoc analysis. N= 3-7 animals per group. *p<0.05, **p<0.01.

Figure 2. Renal ACE2 and hypertension. A. Mean arterial pressures at baseline and with continuous AngII infusion for 15 days. B. Heart weight/body weight (HW/BW) ratios of cross-transplanted mice at the end of the experiment. C. 24h Albumin Excretion during AngII infusion. Data expressed as mean ±SEM, HW/BW and Albuminuria were analyzed as student’s t-test between Wild Type and TotalKO groups. N= 6-11 animals per group. *p<0.05.

Figure 3. ACE2 in plasma and urine during AngII infusion. A. Plasma ACE2 activity. B. ACE2 activity in the urine (24 hour urine collection). C. Immunoblot demonstrated full length ACE2 in the urine of Wild Type and SystemicKO mice, but not TotalKO or KidneyKO animals. In contrast, soluble, N-terminal extracellular catalytic domain ACE2 is detectable in the urine of Wild Type, KidneyKO and SystemicKO mice. Data expressed as mean ± SEM. Plasma and Urine ACE2 Activity data analyzed as one-way ANOVA with Dunn’s multiple comparison test. N= 5-7 animals per group. *p<0.05, vs. Wild Type.
Figure 4. Detection of ACE2 in glomerular filtrate. Highly significant (e-value <0.05) ACE2-aligned peptides are similar in glomerular filtrate, plasma, and recombinant ACE2-derived samples. The x-axis is the amino acid position within the sequence of ACE2_MOUSE. Each significantly aligned peptide is displayed as a bar with length equal to the peptide sequence length and starting position along the x axis equal to the ACE2_MOUSE alignment starting position. The y-axis displays the ranked frequency of each identified peptide, with more frequently-identified peptides appearing at higher rank. A vertical line at position 709 represents the ADAM17 cleavage site between endo- and ecto-domain. sACE2 – soluble ace2; FL-ACE2 – full length ACE2.
Figure 1

A. WT vs ACE2 KO

B. Ace2 mRNA Expression

C. ACE2 Expression

D. Cortical ACE2 Activity
Figure 2
Figure 4

Peptide alignments to ACE2_MOUSE sequence

Normalized Frequency Rank

Sequence start

Adam17 Cleavage Site

sACE2

FL-ACE2