Depletion of Tissue Angiotensin-Converting Enzyme Differentially Influences the Intrarenal and Urinary Expression of Angiotensin Peptides

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Abstract—The relative contribution of circulating versus tissue renin-angiotensin systems to the tissue expression of angiotensin peptides in the kidney remains unresolved. To address this issue, intrarenal and urinary levels of the peptide products of the renin-angiotensin system were assessed in a tissue angiotensin-converting enzyme knockout (tisACE−/−) mouse model. Systolic blood pressure was significantly lower (64.6±3.6 versus 81.4±4.5 mm Hg; P<0.02) and urinary volume was increased (7.25±0.86 versus 2.86±0.48 mL/d; P<0.001) in tisACE−/− mice compared with wild-type mice. Intrarenal angiotensin II was 80% lower in tisACE−/− mice compared with wild-type mice (5.17±0.60 versus 25.5±2.4 fmol/mg protein; P<0.001). Intrarenal angiotensin I levels also declined by a comparable extent (73%) in the tisACE−/− mice (P<0.01). Intrarenal angiotensin-(1–7) concentrations were similar between the strains, but the ratio of intrarenal angiotensin-(1–7) to angiotensin II and angiotensin I in tisACE−/− mice increased 470% and 355%, respectively, compared with wild-type mice. Urinary excretion of angiotensin II and angiotensin-(1–7) were not different, but the excretion of angiotensin I increased 270% in tisACE−/− mice (P<0.01). These studies suggest 2 potential mechanisms for the reduction of intrarenal angiotensin II in tisACE−/− mice: (1) an attenuated capacity to form angiotensin II by renal angiotensin-converting enzyme and (2) significant depletion of its direct precursor angiotensin I in renal tissue. Sustained intrarenal levels of angiotensin-(1–7) may contribute to chronic hypotension and polyuria in tisACE−/− mice, particularly in the context of depleted angiotensin II in the kidney. (Hypertension. 2004;43:849-853.)

Key Words: angiotensin II ■ angiotensin I ■ renin-angiotensin system ■ mice

The renin-angiotensin system (RAS) plays a central role in blood pressure (BP) regulation and electrolyte homeostasis. The RAS was initially described as a circulating endocrine system with angiotensin II (Ang II) as its only biologically active product. With cloning of the genes of the RAS, it became apparent that each of the components of the RAS is actively produced locally by tissue-specific RAS that may function in an autocrine–paracrine fashion within the target organs. It is presumed that the sum of systemic and local production of angiotensin II is a primary regulator of sodium reabsorption in the proximal tubule via its effect on activity of the sodium–hydrogen antiporter NHE-3. Intrarenal Ang II also increases vasomotor tone at the afferent and efferent arterioles, which may impact glomerular filtration rate. Angiotensin-(1–7) [Ang-(1–7)] is an alternative peptide product of the RAS that has vasodilatory effects in the kidney that may counterbalance the effects of Ang II on vasomotor tone. The relative concentration of the 2 angiotensin peptides determines vasomotor tone in the kidney and regulates filtration across the glomerulus.

The relative contribution of the circulating endocrine RAS versus local tissue-specific RAS to steady-state levels of angiotensin peptides in the kidney is unknown. To address this issue, the present study assessed the intrarenal and urinary concentrations of the peptide products of the RAS in a tissue angiotensin-converting enzyme (ACE) knockout (tisACE−/−) mouse model. Unlike traditional pharmacological blockade of the RAS with ACE inhibitors, which indiscriminately inhibits the endocrine and tissue RAS, the tisACE−/− mouse model affords the opportunity to selectively block tissue RAS. The tisACE−/− knockout mouse, previously termed ACE2, expresses a truncated form of ACE that lacks the carboxyl terminal domain required for insertion into cell membranes. The truncated ACE protein is catalytically...
active but entirely secreted from cells.\textsuperscript{11} Mice expressing the transgene have no tissue ACE and possess \textasciitilde 40\% of normal circulating levels of ACE.\textsuperscript{11,12} Unlike the profound phenotypic abnormalities observed in ACE null mice,\textsuperscript{13,14} \textit{tisACE}\textsuperscript{−/−} mice demonstrate only mild hypotension, urine-concentrating defects, and male infertility.\textsuperscript{11,12} As such, this knockout mouse model permitted a careful analysis of the relative contributions of local and endocrine RAS to steady-state levels of angiotensin peptides in the kidney.

Methods

An extended method section can be found in an online supplement available at http://www.hypertensionaha.org.

tisACE\textsuperscript{−/−} Knockout Mice

The \textit{tisACE}\textsuperscript{−/−} mice were generated as previously described.\textsuperscript{11} Wild-type (ACE\textsuperscript{+/+}) littermates were used as controls. Noninvasive BP was measured using a tail-cuff photosensor (IITC, Woodland Hills, Calif). The study was approved by the Institutional Animal Care and Use Committee.

Angiotensin Peptide Assays

Urine was collected in 1 N HCl to prevent ex vivo metabolism of angiotensin peptides\textsuperscript{11,12} and extracted on Sep-Pak C\textsubscript{18} cartridges (200 mg; Waters Corporation, Milford, Mass) as described previously. Blood was collected via cardiac puncture into chilled tubes containing peptide inhibitors (25 mmol/L EDTA; 0.44 mmol/L o-phenanthroline, 1 mmol/L 4-chloromercuribenzoic acid, 0.12 mmol/L pepstatin A, and 3 mmol/L acetyl-His-Pro-Phe-Val-Statine-Leu-Phe, a peptide renin inhibitor). Plasma was stored at \textasciitilde 80°C until radioimmunoassay (RIA).

Under general anesthesia, kidneys were removed rapidly, snap-frozen in liquid nitrogen, and stored at \textasciitilde 80°C until extraction as previously described. Immunoreactive Ang II and Ang-(1–7) in pooled tissue extracts of wild-type and \textit{tisACE}\textsuperscript{−/−} mice were evaluated by combined high-performance liquid chromatography (HPLC) and RIA analysis as described.\textsuperscript{16} Briefly, extracts were fractionated on a Waters C\textsubscript{18} narrow bore column (2.0×150 mm) using a linear gradient of 15% to 40% mobile phase B (0.1% HFBA/80% acetonitrile) for 20 minutes and isocratic (40% B) for 10 minutes at a flow rate of 0.3 mL/min. Fractions were collected at 1-minute intervals, evaporated, and assessed by the Ang-(1–7) (fractions 1 to 20) or Ang II (fractions 21 to 45) RIA.

RIAs for quantification of angiotensin peptides in urine, plasma, and renal tissue were performed as described previously.\textsuperscript{17} As described previously,\textsuperscript{17} Ang I was quantified using a modification of an RIA kit from Perkin Elmer (Boston, Mass). Ang II was quantified using a kit from ALPCO Diagnostics (Windham, NH), and Ang-(1–7) was measured as described previously.\textsuperscript{17}

Reverse-Transcriptase Polymerase Chain Reaction Assay

Total RNA was isolated from the renal cortex of each mouse with TRIZOL (BRL Products, Bethesda Md). Residual DNA was eliminated by treatment of total RNA with RQ1 DNase and then amplified with or without AMV reverse-transcriptase as described by Gallagher et al.\textsuperscript{18} Primer sequences were derived from the cloned mouse ACE gene, the ACE homolog that converts Ang II to Ang-(1–7) [forward primer 5′-TGTCAGCAAAAAGTGAAGTGG-3′, reverse primer 5′-ATCCGGGCTCACTGATGTGT-3′], mouse renin [5′-CGGACTTAGCATGCATCAGT-3′, reverse primer 5′-CCAGTATGCAGCATCAGTCG-3′], mouse angiotensinogen [forward primer 5′-CTTCCTGCTTCGAGCTTAC-3′, reverse primer 5′-CAGACTGTGGTCTCGG-3′], and the elongation factor 1α (EF1α) [forward primer 5′-GGAATGGTGAAGTGCAG-3′, reverse primer 5′-CTGGGGAAGAATTACACG-3′].\textsuperscript{14} Amplification conditions were denaturation for 60 seconds at 94°C, annealing for 60 seconds at 60°C and extension for 60 seconds at 72°C for 30 cycles, followed by a final extension at 72°C for 5 minutes. EF1α primers were added after a delay of 8 cycles for ACE2, 11 cycles for renin, or 12 cycles for angiotensinogen. Products were separated on a 6% polyacrylamide gel. Results were reported as the ratio of the RAS component to the control gene EF1α to account for variations in the reverse-transcriptase polymerase chain reaction assay. EF1α was used because Ang II is known to regulate other “control” genes such as GADPH.\textsuperscript{19}

Statistical Analysis

Continuous data were expressed as mean±SEM and compared between groups using an unpaired Student \textit{t} test; \textit{P}<0.05 defined statistical significance.

Results

Blood Pressure and Renal Function

To assess the physiological impact of the loss of tissue ACE expression, basal BP and renal function were assessed. Systolic BP was significantly lower in \textit{tisACE}\textsuperscript{−/−} mice than wild-type mice (Table). The urine-concentrating defect previously described in \textit{tisACE}\textsuperscript{−/−} mice was manifested by increased 24-hour urine volume in \textit{tisACE}\textsuperscript{−/−} mice (Table). Despite the urine-concentrating defect, calculated creatinine clearance and urinary excretion of sodium and creatinine were no different between \textit{tisACE}\textsuperscript{−/−} and wild-type mice (Table).

Intrarenal Angiotensin Peptides

To assess the contribution of local tissue-specific RAS to steady-state levels of angiotensin peptides in the kidney, angiotensin peptides were quantified in the kidneys of wild-type and \textit{tisACE}\textsuperscript{−/−} mice. Intrarenal Ang II levels were decreased 80\% in \textit{tisACE}−/− mice compared with wild-type mice (Figure 1A). This finding suggests that tissue RAS plays a pivotal role in the maintenance of intrarenal Ang II levels. The intrarenal levels of its precursor Ang I were also decreased by 80\% in \textit{tisACE}−/− mice (Figure 1A). To assess the levels of alternative peptide products of the RAS, intrarenal levels of the vasoactive peptide Ang-(1–7) were quantified. In contrast to Ang II or Ang I, the intrarenal Ang-(1–7) concentrations were similar between the strains (Figure 1A), but the ratio of intrarenal Ang-(1–7) to Ang II and Ang I in the kidneys of \textit{tisACE}−/− mice increased 470\% and 355\%, respectively, compared with wild-type mice (Figure 1B). As shown in Figure 2, the HPLC/RIA analysis of pooled tissue samples from the wild-type (Figure 2A) and \textit{tisACE}−/− (Figure 2B) mice confirmed the reduction in Ang II and the sustained levels of Ang-(1–7) in ACE-depleted kidneys.

Plasma Angiotensin Peptides

To determine if there is differential expression of angiotensin peptides in the plasma and kidneys of wild-type and
tisACE−/− mice, angiotensin peptides were also quantified in the plasma. As expected, plasma Ang II levels were decreased 50% in tisACE−/− mice (Figure 3). In contrast to intrarenal expression of Ang I (Figure 1A), plasma Ang I was increased 3-fold in the plasma of tisACE−/− mice (Figure 3). Plasma levels of Ang-(1–7) were also increased in tisACE−/− mice compared with wild-type mice (Figure 3). The ratios of angiotensin peptides in the plasma of tisACE−/− and wild-type mice are shown in online Figure I (http://www.hypertensionaha.org). These data suggest that there is differential regulation of angiotensin peptide expression in the plasma and kidneys of tisACE−/− mice.

**Urinary Angiotensin Peptides**

Urinary peptide levels were assessed to determine if urinary excretion of these peptides parallels intrarenal expression. The 24-hour urinary excretions of Ang II and Ang-(1–7) were not different between the strains (Figure 4). It is notable, though, that urinary excretion of Ang I increased 270% in the tisACE−/− mice (Figure 4). The concentration (pmol/mL urine) of urinary Ang I was unchanged in tisACE−/− mice, compared with wild-type mice (tisACE−/− = 1.27±0.035; ACE+/+ = 1.63±0.21; P=0.393), because of the increased urinary volume of the knockout mice. The ratios of angiotensin peptides in the urine of tisACE−/− and wild-type mice are shown in online Figure II (http://www.hypertensionaha.org).

**Renal Renin, Angiotensinogen, and ACE2 mRNA Levels**

The mRNA levels (in relative absorbance units) for renin in the kidney were markedly elevated (>600%) in the tisACE−/− mice (5.38±0.43 versus 0.65±0.09; online Figure III, http://www.hypertensionaha.org). In contrast, the mRNA levels for angiotensinogen in the kidney were not changed in tisACE−/− mice (0.70±0.07 versus 0.92±0.17; Figure III).

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** A, Intrarenal concentration of angiotensin peptides (fmol/mg protein) in wild-type (n=8) and tisACE−/− mice (n=8). *P<0.001; **P<0.01. B, Ratio of angiotensin peptides in the kidneys of wild-type and tisACE−/− mice.

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** A, High-performance liquid chromatography (HPLC)/radioimmune assay (RIA) analysis of pooled kidney samples from wild-type mice. B, HPLC/RIA analysis of pooled kidney samples from tisACE−/− mice.

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** Plasma levels of angiotensin peptides (fmol/mL) in wild-type (n=8) and tisACE−/− mice (n=8). *P=0.0001; **P<0.0001.
tends the possibility of a previously undefined positive feedback loop by which intrarenal Ang II augments angiotensinogen activity in the kidney. With decreased intrarenal Ang II, this feedback loop would be muted, resulting in decreased angiotensinogen activity and decreased Ang I production in the kidney. In an attempt to address this question, we found no difference in renal angiotensinogen mRNA levels between wild-type and tisACE−/− mice (Figure III). However, this finding does not preclude an affect on angiotensinogen protein because Campbell et al observed a marked reduction in angiotensinogen protein despite no change in angiotensinogen mRNA in the kidney after chronic ACE inhibitor treatment in normotensive rats.23

An alternative explanation for the reduction in intrarenal Ang I is enhanced luminal secretion of Ang I because urinary excretion of Ang I was significantly increased in tisACE−/− mice (Figure 4). Increased clearance of Ang I caused by the global concentrating defect in tisACE−/− mice seems unlikely because there was no change in the excretion of the remaining angiotensin peptides. It is also possible that enhanced excretion of Ang I noted in the present study could reflect an increase in the tubular release of angiotensinogen with subsequent urinary conversion to Ang I. Additional experiments are needed to address this possibility by measuring angiotensinogen in the urine of tisACE−/− mice. Finally, the present study cannot exclude the possibility that changes in Ang I and Ang II levels in the kidney are related to alterations in the half-life of these peptides in tisACE−/− mice. This possibility seems unlikely because neither of the major peptide products of Ang I metabolism [Ang II or Ang(1–7)] were increased in the kidney or urine of tisACE−/− mice, but further metabolism studies are necessary to exclude this possibility.

The tisACE−/− model also provided insight regarding alternative peptide products of the RAS, such as Ang(1–7). Recent studies demonstrated the importance of Ang(1–7) in promoting natriuresis and intrarenal vasodilatation in the kidney.10,24 Previous studies demonstrated that ACE inhibition increased plasma concentrations of Ang(1–7) severalfold, presumably by diverting Ang I through Ang(1–7)-forming pathways, as well as decreasing breakdown of the peptide by ACE.25,26 Based on these observations, we hypothesized that intrarenal Ang(1–7) levels in tisACE−/− mice would be increased. Moreover, our preliminary studies found that RAS blockade was associated with an increase in ACE2 expression, suggesting an enhanced capacity to convert Ang II to Ang(1–7).27 Although ACE2 mRNA levels and the absolute concentration of intrarenal Ang(1–7) were not different in tisACE−/− mice, the ratio of Ang(1–7) to Ang II was increased in the kidneys of these mice (Figure 1B). It is this relative balance of Ang(1–7) and Ang II in the kidney that likely determines whether sodium retention or natriuresis occurs and whether vasoconstriction or vasodilatation predominates.10 In fact, the imbalance of Ang(1–7) and Ang II in tisACE−/− mice may contribute to chronic hypertension and polyuria in tisACE−/− mice. Maintenance of intrarenal Ang(1–7) levels in tisACE−/− mice despite depletion of its precursors Ang I and Ang II indicates that the rate of

compared with wild-type mice. Similarly, the renal expression of ACE2, the ACE homolog that converts Ang II to Ang(1–7), was not altered by ACE depletion (1.35±0.20 versus 1.57±0.25; Figure III).

Discussion

Recognition of tissue-specific local RAS has prompted questions regarding the primacy of the intrarenal versus the systemic RAS in determining steady-state levels of Ang II in the kidney. The tisACE−/− model provided a unique opportunity to experimentally dissect the relative contributions of the local and systemic RAS to steady-state Ang II levels in the kidney by permitting complete blockade of ACE-dependent Ang II production by tissue RAS. Using this approach, the absence of tissue ACE activity in tisACE−/− mice induced a profound (≈80%) decrement in intrarenal Ang II levels. These data confirm the critical role of tissue RAS in the maintenance of steady-state Ang II levels in the kidney. The physiological relevance of this finding is underscored by the relative hypotension and polyuria in tisACE−/− mice that is presumed to be derived from decreased intrarenal Ang II in these mice.

These data further suggest that tissue ACE activity per se is critical for maintenance of steady-state Ang II levels in the kidney. This finding appears to contradict a previous study that used an alternate transgenic mouse model and noted that depletion of intrarenal ACE did not reduce intrarenal Ang II levels.20 This discrepancy may be explained by subtle differences in the transgenic mouse models used. Wei et al used a transgenic model in which intrarenal ACE was reduced to 20% of wild-type mice,20 while no tissue ACE activity is detectable in tisACE−/− mice.11 Taken together, these data indicate that even small amounts of residual tissue ACE activity are sufficient to maintain intrarenal Ang II levels. This observation corroborates previous studies that noted that incomplete ACE inhibition does not decrease intrarenal Ang II levels.21,22

An additional explanation for the marked reduction of intrarenal Ang II in tisACE−/− mice is the significant depletion of its immediate precursor Ang I in renal tissue (Figure 1). Depletion of intrarenal Ang I in ACE-depleted mice was unexpected because previous studies reported an increase in plasma Ang I with ACE inhibition.22 This observation por-
hydrolysis of Ang-(1–7) by ACE is an important determinant of steady-state levels of Ang-(1–7) in the kidney.

**Perspectives**

This study provided direct evidence of the relative contribution of the local RAS in the kidney in maintaining steady-state Ang II levels in the kidney, compared with the systemic endocrine RAS. Interruption of the tissue RAS in *tisACE*−/− mice induced a profound decrement in intrarenal Ang II levels, producing chronic hypotension and polyuria. In addition to augmenting our understanding of the RAS, this finding has direct clinical physiological and clinical relevance. These data explain the dissociation between plasma ACE activity during pharmacological ACE inhibition and intrarenal Ang II levels.21 These data suggest that future pharmacological strategies for modulation of the RAS should focus on impacting tissue RAS, recognizing that the circulating endocrine RAS represents only a small fraction of the functional RAS.

**Acknowledgments**

This work is supported in part by grants from the National Heart, Lung and Blood Institute (HL-51952, [P.E.G., K.B.B., M.C.C.], HL-56973 [M.C.C.]) and the American Heart Association (AHA-151521 [MCC]).

**References**

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Hypertension. 2004;43:849-853; originally published online February 23, 2004;
doi: 10.1161/01.HYP.0000121462.27393.f6
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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