Overexpression of Renin in the Collecting Duct Causes Elevated Blood Pressure

Nirupama Ramkumar,* 1 Jian Ying,* 2 Deborah Stuart,1 and Donald E. Kohan1

BACKGROUND
Renin is synthesized in the collecting duct and is regulated differently than renin in the juxtaglomerular apparatus. However, the physiological relevance of collecting duct renin remains unknown, particularly with regard to its ability to regulate blood pressure.

METHODS
We used gene targeting to generate mice with overexpression of renin in the collecting duct. A conditional mutant mouse line was created with the mouse renin transcript distal to a “transcriptional stop sequence” such that gene expression only occurred when the stop sequence was excised. These mice were bred with mice transgenic for the aquaporin-2 promoter driving Cre recombinase in order to achieve collecting duct–specific overexpression of renin.

RESULTS
RNA analysis confirmed kidney-specific recombination, and medullary renin mRNA levels were increased 5-fold in collecting duct renin mice. Blood pressure was recorded by telemetry and plasma and urine was collected in 24-hour metabolic cages on normal, high-, and low-Na+ diets. Although no significant differences in 24-hour urinary Na+ excretion between targeted and control mice were detected, renin overexpresser mice had elevated blood pressure compared with controls on a high-Na+ diet. Urinary renin excretion was 2-fold higher in targeted mice as compared with controls on normal and low-Na+ diets. Plasma renin concentration was significantly suppressed in targeted mice as compared with controls on normal and high-Na+ diets.

CONCLUSION
Taken together, these results suggest that collecting duct–derived renin has the potential to modulate blood pressure independent of the systemic renin-angiotensin system.

Keywords: blood pressure; collecting duct; gene targeting; hypertension; kidney; renin.

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The intrarenal renin-angiotensin system (RAS) has been implicated in control of blood pressure (BP) under physiological conditions and in the setting of hypertension. One component of this system, collecting duct (CD)–derived renin, has been the subject of numerous studies in recent years. Renin production by the CD was definitively identified in 1999 and was hypothesized to hydrolyze tubule fluid angiotensinogen (AGT), ultimately leading to production of intraluminal angiotensin II (AngII). Since luminal AngII can stimulate CD Na+ transport,3,4 the idea developed that CD-derived renin played a role in controlling Na+ reabsorption and BP. Ensuing studies determined that CD-derived renin production was regulated differently than juxtaglomerular apparatus (JGA)–derived renin; in particular, AngII increased renin release by CD cells, while inhibiting renin secretion by the JGA.4 This suggested that CD renin might contribute to AngII-induced hypertension. In 2009, CD intercalated cells were described to express luminal prorenin receptors (PRR).5 Since binding of renin or prorenin to PRR can increase AGT catalysis and activate intracellular signaling pathways, further support was lent to the notion that a system existed wherein CD-derived renin modulated CD function to ultimately affect BP. Over the past decade, a number of studies have investigated changes in CD and urinary renin, prorenin, and/or PRR expression in response to varying Na+ diets,1,6 AngII infusion,7,8 knockout of natriuretic peptide receptors9 or kallikrein,10 diabetes,11,12 and various models of hypertension.13,14 The essence of these studies is that expression of the CD renin system is modifiable and hence changes in activity of this system might lead to altered renal function. However, despite the substantial effort invested in understanding the biological role of CD renin, no studies to date have actually determined if CD-derived renin can and does control Na+ reabsorption and BP. To address this fundamentally important question, we developed a mouse model that overexpresses renin selectively within the CD. Herein, we

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report that overexpression of renin in the CD causes elevated BP, lending support to the notion that CD-derived renin has the potential to regulate BP.

**METHODS**

**Animal care**

All animal studies were conducted with the approval of the University of Utah Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Generation of targeting vector**

The cloning steps are shown in *Supplementary Figure 1*. A polylinker containing PacI and Ascl restriction sites was inserted into the Rosa26/1 vector at the Xbal restriction site. Renin cDNA was cloned into the pBigT vector between Sall and NotI restriction sites. The resulting construct, Renin/pBigT, was then inserted into the modified Rosa26-1 vector (Rosa-polylinker) using restriction sites PacI and Ascl to generate the targeting vector Renin/pBigT/Rosa26. This vector contained, from 5′ to 3′: 1.2 kb of the Rosa26 gene, a splice acceptor site, loxP, phosphoglycerate kinase 1 promoter, neomycin resistance gene, transcriptional stop sequence, loxP, Ren1 cDNA, bovine growth hormone polyA, 4.8 kb of the Rosa26 gene, and the diphertheria toxin gene (the latter as a negative selection marker). Following validation of the vector by sequencing, linearized DNA was provided to the University of Utah core transgenic facility for electroporation into 129x1/SVJ embryonic stem (ES) cells.

**ES cell screening**

ES cell clones were screened by Southern blotting. The genomic DNA was digested with EcoRV restriction endonuclease before blotting. A fragment located 5′ upstream of the 5′ homolog arm was amplified by polymerase chain reaction (PCR) and labeled with P32 to serve as a Southern blotting probe. The targeted allele was characterized by a 3.8 kb band located 5′ upstream of the Rosa26 gene, a splice acceptor site, loxP, phosphoglycerate kinase 1 promoter, neomycin resistance gene, transcriptional stop sequence, loxP, Ren1 cDNA, bovine growth hormone polyA, 4.8 kb of the Rosa26 gene, and the diphertheria toxin gene (the latter as a negative selection marker). Following validation of the vector by sequencing, linearized DNA was provided to the University of Utah core transgenic facility for electroporation into 129x1/SVJ embryonic stem (ES) cells.

**Blastocyst injection**

The targeted ES cells identified above were injected into C57BL/6j blastocysts. Chimeras with approximately 75% or more agouti coat color were backcrossed to C57BL/6j mates. The F1 progeny from these crosses were genotyped by Southern blotting and PCR to determine whether germ line transmission of the transgene had occurred. Mice containing transgene were bred for at least 10 generations with C57BL/6j mice.

**Generation of CD-specific renin-overexpressing mice**

Mice heterozygous for the Rosa26-renin allele were bred with mice transgenic for the aquaporin-2 promoter driving expression of Cre recombinase (AQP2-Cre).

**Genotyping**

For genotyping the Rosa26-renin allele, three primers were used for PCR of tail DNA: primer 1: CCCCCAGATGACTCC TATCCTC; primer 2: GCTGAGCCAGCTCCATC; and primer 3: TCACTAGGAAACCCCTGGAC. Primer 1 and primer 2 amplify the wild-type allele (product size of 487 bp), while primer 2 and primer 3 amplify the targeted allele (product size is 350 bp). The presence of the AQP2 transgene was determined using primers mAQP2 F 5′-GAGACGTCAATCCCTATCTGAGAG-3′ and creTag R 5′-GCGAACATCTTTAGGTTCTGGCG-3′. This amplifies the junction between the mouse AQP2 promoter and the Cre gene.

**Verification of organ-specific expression**

Total RNA was isolated from a variety of organs and reverse-transcription (RT)-PCR was performed to examine the organ-specific expression of targeted renin gene. The primers used to amplify the Rosa26-renin mRNA were GTTGGACGCGTCTGGTAGAC (5′ primer in the Rosa26 gene) and CTTTTGATATAAGCAGCCCACT (3′ primer in Ren1 exon 2); these yielded a 396 bp product. The primers for GAPDH were TGGGAACTCTGTACAAAG and ATGCAGGATGATGTCTGG. The two pairs of primers were designed to span at least 1 intron to avoid amplification of genomic DNA. PCR were performed for 40 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s.

**Quantitation of renin mRNA**

Renal cortex and medulla were dissected from CD renin and control mice for RNA isolation. Reverse transcription was performed on 2 μg of total RNA with oligo(dt) and Superscript III reverse transcriptase according to the manufacturer’s protocol (Invitrogen). The resulting cDNA was then assayed for relative expression of renin mRNA in CD renin and control mice using the Taqman Gene Expression Assay (renin probe: cat# Mm02342889_g1, GAPDH probe: cat# Mm03302249_g1, Applied Biosystems, Carlsbad, CA).

**BP monitoring**

BP was recorded via telemetry (TA11-PAC10, Data Sciences International, St. Paul, MN). Targeted and control (wild-type) mice aged 3 months were anesthetized with 2% isoflurane, implanted with a radio transmitter, and allowed to recover in individual cages for 5 days. Automated BP and heart rate were recorded continuously for 3 weeks with measurements taken every 10 minutes. Animals were maintained on a normal Na+ diet (0.15% Na+) for 5 days, high-Na+ diet (3.2% Na+) for 6 days, and low-Na+ diet (0.01% Na+) for 3 days. Animals were not handled during the BP recording period as even small stimuli may markedly affect BP in mice. Mean arterial pressure was calculated as (1/3 × pulse pressure) + diastolic pressure.
Plasma and urine assays

At the conclusion of BP studies, targeted and control mice were placed in metabolic cages for measurement of weight, food, and water intake and 24-hour urine collection. A small amount of blood (35 μl) was collected from the dorsal pedal vein in chilled polypropylene tubes containing heparin lithium. Plasma was separated and chilled at −80 °C until assay. Urine samples were centrifuged at 15,000 rpm for 15 minutes and supernatant was frozen in aliquots at −80 °C until assay. In a separate experiment, acute excretion of urinary Na+ and K+ was evaluated by intraperitoneal injection of isotonic saline (1 ml). Urine was collected hourly for 6 hours from the time of injection by placing the mice in metabolic cages. Plasma renin concentration (PRC) and urine renin activity was measured as the amount of angiotensin I (Ang-I) generated after incubation with excess porcine AGT using the Ang-I EIA Kit (Bachem, San Carlos, CA). PRC and urine renin activity were expressed as the amount of Ang-I generated per hour per microliter of plasma or urine. Urinary Na+ and K+ were determined using the EasyVet Analyzer (Medica, Bedford, MA). Urinary renin excretion was obtained by multiplying 24-hour urine volume times concentration and expressed as ng/day. Urinary creatinine was measured by colorimetry (non-Jaffe method).

Statistical analysis

All results are expressed as mean ± standard error of the mean. The paired t test was used to compare differences between targeted and control animals within a given diet. We initially analyzed BP as daytime and nighttime separately to reduce the effects caused by the heterogeneity in activity during the two periods. Since daytime and nighttime results parallel the BP trends from 24-hour readings, we present all BP values as 24-hour readings. One-way analysis of variance was used to compare differences in BP between targeted and control animals within each diet.

RESULTS

Generation of animals

The targeting vector for the Rosa26-renin allele was cloned as shown in Supplementary Figure 1. After amplification in host bacteria, the plasmid was linearized and trimmed. Plasmid DNA was purified and transferred to a core gene targeting facility to generate ES clones enriched in targeted events by positive−negative selection. The ES clones were screened by Southern blotting (Supplementary Figure 2) and 3 clones among about 500 clones were identified as targeted. One of the targeted clones with normal chromosome count was selected to generate chimeras, following standard protocols. As a result, 12 male chimeras with approximately 75% or more agouti coat color were generated by the core facility. These male chimeras were crossed to B6 female mice to generate progeny. Transmission of the targeting gene to the progeny was tested by Southern blotting and/or PCR genotyping. The line with the highest ratio of transmission was used for further studies.

To achieve overexpression of renin in connecting tubule (CNT) and CD, the following strategy was used: (1) the conditionally expressed gene, a promoterless renin minigene, was placed downstream from a strong transcriptional stop in the ubiquitously expressed Rosa26 gene using gene targeting (Figure 1). This conditional mutant remains silent until excision of the transcriptional stop occurs; (2) the transgene of the effector animal expresses Cre recombinase under the control of the AQP2 promoter. Upon crossing and Cre-mediated excision, targeted renin overexpression occurs only in cells with AQP2, i.e., principal cells of CD (Figure 1). This is in accordance with numerous previous studies demonstrating principal cell-specific expression of Cre recombinase by the AQP2-Cre transgene.15–19 Male and female mice of C57BL/6J genetic background were backgrounded in all experiments.

Verification of kidney-specific expression of renin

A multitissue RNA panel was prepared from targeted animals and screened by RT-PCR using primers that only amplify the transgene with NeoR cassette removed. The targeted gene was detected only in the kidney (Figure 1). Within the kidney, renin mRNA levels by RT-PCR were similar in the cortex of CD renin mice and control mice (wild-type mice) (Figure 1). In contrast, renin mRNA levels in the medulla were 5-fold higher in CD renin mice compared with control mice, confirming targeted overexpression of renin in the distal nephron.

Assessment of BP and urinary water and Na excretion

Mean 24-hour BP in CD renin mice was 4–5 mm Hg higher on a normal Na+ diet (not statistically different) and 8–10 mm Hg higher on a high-Na+ diet (P < 0.05) as compared with control mice (Figure 2a). Mean BP was not different between CD renin and control mice on a low Na diet. Systolic and diastolic BP were higher in CD renin compared with control mice with high Na intake (P < 0.05; Figure 2b,c). There were no differences in body weight, urine volume, or urinary Na+, K+, or creatinine excretion between CD renin and control mice on day 2 after switching to a new diet (Table 1), nor was there any change in these parameters on days 3 and 4 after switching to a new diet (data not shown). Similarly, we did not see any differences in urinary volume or Na+ or K+ excretion either hourly or in the total excretion between CD renin mice and control mice after intraperitoneal injection of isotonic saline up to 6 hours following injection (Supplementary Figure 3).

Plasma and urine measurements

PRC was significantly reduced in CD renin (29.3 ± 5.8 ng Ang-I/μl/hr) compared with control mice (61.9 ± 5.8 ng Ang-I/μl/hr) on a normal Na diet (Figure 3a). High Na+ intake decreased PRC in both groups, with significantly lower levels in CD renin mice (7.7 ± 2.0 ng Ang-I/μl/hr) than control mice (37.1 ± 8.3 ng Ang-I/μl/hr). PRC was not
Ramkumar et al.

Different between CD renin and control mice fed a low-Na+ diet (CD renin: 80.6 ± 15.8 ng Ang-I/µl/hr; control: 81.9 ± 16.8 ng Ang-I/µl/hr).

Urinary renin excretion was higher in CD renin mice on normal (1218.9 ± 199.3 ng/day) and low-Na+ diets (403.5 ± 34.5 ng/day) compared with control mice (normal Na+: 595.8 ± 114.3 ng/day; low Na+: 199.2 ± 38.3 ng/day; Figure 3b). There was no significant difference in urinary renin excretion between CD renin and control mice fed a high-Na+ diet, although renin excretion increased in both groups with high Na+ intake as compared to a low Na+ diet (CD renin mice 1,553.9 ± 173.3 ng/day; control mice 1,647.1 ± 384.1 ng/day).

**DISCUSSION**

The current study examined, for the first time, the ability of renin in the CD to modulate physiological parameters. The finding that overexpression of renin in the CD increases BP indicates that CD-derived renin has the potential to modulate BP. These studies were not designed to determine whether or not normal levels of endogenous CD renin do, in fact, regulate BP; such an analysis would most likely require CD-specific knockout of renin. Nonetheless, the observation that CD renin overexpression elevates BP indicates that, in the setting of increased CD renin, all the necessary components exist to have such increased distal nephron renin activity lead to increased BP.

The magnitude of BP increase was modest in CD renin mice. We did not have the ability to control absolute levels of CD renin overexpression, hence the meaning of the degree of BP elevation is uncertain. It may be that CD renin expression under physiological and, in particular, pathophysiological conditions, such as diabetes, could be substantially greater than that in the current study."11,12 Furthermore, we do not know the degree to which normal CD renin production (in control animals) contributes to BP; it may be that further increments in CD renin cannot alter BP to any great extent. Again, using mice with CD-specific knockout of renin may shed light on this issue.

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*Figure 1.* Construct and verification of renin expression in collecting duct (CD) renin mice. Top panel shows AQP2-Cre–mediated recombination of ROSA26-renin allele. The PGK-neo-pA and STOP sequence are excised, permitting renin transcription driven by the Rosa26 promoter. Abbreviations: bGH, bovine growth hormone polyA; PGK-neo-pA, phosphoglycerate kinase 1 promoter driving the neomycin resistance gene; Renin, Ren1 cDNA; SA, splice acceptor site; STOP, transcriptional stop sequence. Bottom left shows relative renin mRNA expression in kidney cortex and medulla as assessed by reverse-transcription-polymerase chain reaction (RT-PCR) in control and renin overexpresser (CD renin) mice. N = 9 both genotypes *P < 0.05 compared with control mice. Bottom right shows verification of kidney-specific expression of the targeted renin mRNA by RT-PCR. Representative blots from four animals are shown.
No effect of CD renin overexpression was observed in 24-hour urinary Na+ excretion or in the acute natriuretic response to intraperitoneal saline injection. This result is not surprising in that the BP elevation was modest, hence only a relatively small degree of Na+ and fluid retention would be required to increase BP to the degree observed. Further, urinary Na+ and water excretion in mice are intrinsically highly variable, making detection of small changes in these parameters problematic. However, despite the inability to detect Na+ and fluid retention in CD renin mice, they showed evidence consistent with fluid retention. In addition to the BP elevation, CD renin mice had a substantial reduction in plasma renin activity (PRA) when fed a normal or high-Na+ diet, conditions under which the mice either tended to be (normal salt intake) or were significantly (high salt intake) hypertensive. This latter finding is of additional interest in that it indicates that CD renin overexpression does not lead to increased PRA, i.e., CD renin appears to remain confined...
Table 1. Plasma and urine measurements in wild type (control) and renin overexpressor mice (collecting duct renin) mice on normal (0.15%), high- (3.2%), and low- (0.01%) Na diets

<table>
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<th>CD renin</th>
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<tr>
<td><strong>Normal Na+ diet</strong></td>
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<tr>
<td>Weight, g</td>
<td>25.1 ± 0.8</td>
<td>26.6 ± 0.7</td>
<td>25.6 ± 0.7</td>
<td>25.9 ± 0.7</td>
<td>25.8 ± 0.7</td>
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<tr>
<td>Urine volume, ml/day</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>4.8 ± 0.7</td>
<td>4.3 ± 1.1</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
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<tr>
<td>Urinary Na+ excretion, µEq/day</td>
<td>113 ± 12</td>
<td>95 ± 14</td>
<td>1627 ± 268</td>
<td>2277 ± 854</td>
<td>23 ± 4</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>Urinary potassium excretion, µEq/day</td>
<td>198 ± 24</td>
<td>177 ± 35</td>
<td>417 ± 63</td>
<td>303 ± 54</td>
<td>175 ± 21</td>
<td>191 ± 24</td>
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<tr>
<td>Urinary creatinine, mg/day</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
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Data are shown on the third day of transitioning to each diet. N = 12 for CD renin group and N = 8 for control mice.

to the kidney. While not specifically investigated, these findings raise the possibility the CD renin is primarily secreted into the tubule lumen wherein it exerts biologic effects. This is in accordance with previous findings showing that renin immunostaining localizes to the apical side of the CD lumen.1

The mechanism(s) by which CD renin modulates BP are speculative, but at least two major possibilities exist. First, CD renin might exert effects, at least in part, via binding to PRR located on intercalated cells.2,20 Binding of renin to PRR induces intracellular signaling, including activation of ERK1/2 and upregulation of cyclooxygenase-2 and other inflammatory mediators.20 An interesting possibility is that CD renin, via PRR binding on intercalated cells, might modulate chloride transport by pendrin, thereby potentially influencing BP. In addition to cell signaling, binding of CD-derived prorenin and/or renin to the PRR could increase the catalytic efficiency of the conversion of AGT to angiotensin I. The proximal tubule synthesizes AGT, which in turn enters the tubule lumen and is excreted in the urine,21,22 hence it is highly likely that CD renin has the potential to increase luminal AGT hydrolysis. Since the distal nephron contains abundant angiotensin-converting enzyme,23,24 it is reasonable to hypothesize that CD renin could augment luminal AngII content. Studies have indicated that luminal AngII potently stimulates CD Na+ transport (more so than basolateral AngII), suggesting that CD renin might lead to enhanced CD epithelial Na+ channel activity.2 The current study was not intended to address the mechanisms by which CD renin influences BP; however, ultimately studies using AngII receptor blockers in CD renin mice would be informative. Specific PRR antagonists do not exist, while PRR knockout is lethal to cells due to PRR serving a dual role as a renin receptor and a modulatory protein necessary for vacuolar ATPase acidification of endolysosomal vesicles,25,26 hence, studying the role of PRR in the effects of CD renin will be problematic.

The CD renin mouse manifested clear evidence for overexpression of renin in the CD since medullary renin mRNA
levels were much higher in the targeted mice compared to control animals. Cortical renin mRNA levels were not different between targeted and control mice, most likely due to the present of renin in the juxtaglomerular apparatus.

The CD renin mice demonstrated a significant increase in urinary renin excretion as compared to control animals on either a normal or low-Na+ diet. This suggests that urinary renin derives, at least in part, from the CD. Such a finding is in agreement with previous studies wherein AngII infusion increased urinary renin, while decreasing systemic renin. Curiously, urinary renin was not different between targeted and control mice fed a high-Na+ diet. In addition, CD renin mice were most apparently hypertensive on a high-Na+ diet when differences in urinary renin between groups were not detected. The meaning of urinary renin is therefore uncertain; possibly CD-derived renin primarily acts locally and its production is not greatly reflected by changes in urinary renin. In this regard, the sources of urinary renin are uncertain. Urinary renin excretion was significantly increased by a high-Na+ diet as compared with a low-Na+ diet in both groups of mice. This latter finding is in accordance with previous studies. However, CD renin has been reported to increase during a low-Na+ diet or amiloride treatment, but not by high Na+ intake. Thus, it may be

Figure 3. Renin activity in control and targeted renin overexpresser (collecting duct [CD] renin) mice. Panel A shows plasma renin concentration and panel B shows urine renin excretion on normal (0.15%), high- (3.2%), and low- (0.01%) Na+ diets. N = 12 (CD renin mice) and N = 8 (control mice). *P < 0.05 vs. control on same Na+ diet.
that during high Na+ intake, there is reduced reabsorption of filtered renin, resulting in increased contribution to urinary renin from the circulation. A high-Na+ diet suppresses PRA, so one might imagine that even if filtered renin entered the urine, it might not be in substantial amounts. This issue is clearly unresolved, clarification might be obtained by using mice with CD renin knock-out (KO) and assessing urinary renin excretion under varying salt intakes.

In conclusion, the results from this study indicate that CD renin has the potential to modulate BP independently of systemic renin. Further studies are needed to examine the role of endogenous renin, the mechanism by which CD renin modulates BP, and the factors responsible for urinary renin excretion.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at American Journal of Hypertension (http://ajh.oxfordjournals.org).

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DISCLOSURE

The authors declared no conflict of interest.

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972 American Journal of Hypertension 26(8) August 2013