Intrarenal Angiotensin-Converting Enzyme Induces Hypertension in Response to Angiotensin I Infusion

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ABSTRACT
The contribution of the intrarenal renin-angiotensin system to the development of hypertension is incompletely understood. Here, we used targeted homologous recombination to generate mice that express angiotensin-converting enzyme (ACE) in the kidney tubules but not in other tissues. Mice homozygous for this genetic modification (ACE 9/9 mice) had low BP levels, impaired ability to concentrate urine, and variable medullary thinning. In accord with the ACE distribution, these mice also had reduced circulating angiotensin II and high plasma renin concentration but maintained normal kidney angiotensin II levels. In response to chronic angiotensin I infusions, ACE 9/9 mice displayed increased kidney angiotensin II, enhanced rate of urinary angiotensin II excretion, and development of hypertension. These findings suggest that intrarenal ACE-derived angiotensin II formation, even in the absence of systemic ACE, increases kidney angiotensin II levels and promotes the development of hypertension.


The renin-angiotensin system (RAS), and its main effector angiotensin II (Ang II), are key regulators of sodium and body fluid homeostasis and BP. Moreover, the presence of significant RAS alterations in the development of high BP is supported by the effectiveness of angiotensin-converting enzyme (ACE) inhibitors and AT1 receptor blockers in the treatment of hypertension and the prevention of organ damage.1–3 Because the vast majority of hypertensive patients lack consistent signs of systemic RAS activation,6 there is growing recognition that changes in tissue Ang II production in different organs may be of importance in the development and maintenance of hypertension.

Intrarenal Ang II generation might be of particular significance because of its critical role in regulating the kidneys’ handling of sodium balance, fluid homeostasis, and BP. Furthermore, high intrarenal Ang II levels are associated with profound changes in kidney function characterized by impairment of renal blood flow and GFR, reductions in sodium excretion, and suppression of the pressure-natriuresis relationship.7–11 As emphasized by Guyton,12 the presence of such changes has important consequences for long-term BP regulation because resetting of the pressure-natriuresis relationship and defective sodium handling by the kidneys leads to body fluid dysregulation and represents a final common pathway for maintenance of hypertension. Moreover, although the primary importance of the kidneys in hypertension does not negate the significance of various nonrenal mechanisms in the pathogenesis of this condition,13 a widely held premise is that hypertension cannot coexist in the presence of normal renal function.14

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ACE (EC3.4.15.1) is a zinc-containing dicarboxypeptidyl peptidase responsible for the cleavage of several substrates including Ang I to Ang II. The role of ACE as the main pathway for Ang II generation, in the systemic circulation and in the kidneys, has been substantiated by the presence of very low circulating and intrarenal Ang II levels in ACE knockout (KO) mice and wild-type (WT) mice after ACE inhibition.\textsuperscript{15} ACE KO mice also display very high levels of circulating Ang I and a reduced Ang II/Ang I ratio that supports the concept of impaired Ang II generation.\textsuperscript{15} Additionally, ACE KO mice fail to display BP increases in response to Ang I infusions.\textsuperscript{16}

We recently demonstrated that Ang II–infused mice treated with an ACE inhibitor (ACEi) had markedly attenuated increases in arterial pressure and lower intrarenal Ang II levels when compared with mice treated only with Ang II.\textsuperscript{17} Thus, endogenous ACE-derived Ang II formation contributes significantly to the augmentation of intrarenal Ang II and arterial pressure in Ang II–infused mice. Because ACEi reduces the activity of this enzyme throughout the body, it was not possible to separate the contribution of systemic ACE versus kidney ACE. Nevertheless, although systemic renin is markedly suppressed during chronic Ang II infusions, there is an augmented angiotensinogen expression as well as persistent renin and ACE activities in the kidneys.\textsuperscript{17,18} These findings suggest that kidney ACE-derived Ang II formation can be a major contributor to the generation of high local Ang II levels and hypertension. However, this issue needs to be properly addressed.

The objective of the present study was to determine the ability of intrarenal ACE to augment local Ang II content and BP levels while isolating its effects from those of systemic ACE. For this, targeted homologous recombination was used to generate mice with ACE expression restricted to the kidneys with simultaneous deletion from other tissues. Our findings indicate that chronic Ang I infusions augmented kidney-specific ACE-derived Ang II formation to an extent sufficient to increase intrarenal Ang II levels and lead to the progressive development of hypertension.

RESULTS

Creation and Characterization of Homozygous ACE 9/9 Mice

With use of the targeted homologous recombination approach depicted in Figure 1, the ACE gene was modified to place its expression under the control of a Ksp-cadherin/β-globin promoter. This strategy has shown to direct protein expression to the kidney tubules.\textsuperscript{19} Standard procedures were used to create mice homozygous for the mutant allele. Such mice were termed ACE 9/9 as this is the ninth ACE KO strain created by Bernstein and colleagues. All studies were performed in ACE 9/9 and WT littermates (F2 and F3 generation) obtained from the breeding of F1 heterozygous animals.

Tissue Distribution of ACE

As shown in Figure 2A, plasma ACE activity in ACE 9/9 mice was almost undetectable (0.02 ± 0.0 ACE unit per μl) and therefore significantly reduced when compared with that of WT mice (4.86 ± 1.7 ACE unit per μl). ACE activity in the lungs of ACE 9/9 mice was also reduced by 99% (6.13 ± 0.2 ACE unit per μg) when compared with WT mice (436.04 ± 32.1 ACE unit per μg). In turn, ACE activity in the kidneys of ACE 9/9 mice (157.07 ± 13.1 ACE unit per μg) was equivalent to that of WT mice (149.95 ± 21.2 ACE unit per μg). These results were confirmed by Western blot analysis, demonstrating that total ACE protein expression was similar in the kidneys of ACE 9/9 and WT, while absent in other tissues like the brain.

Figure 1. Generation of ACE 9/9 mice. (A) Structure of the ACE wild-type allele. Arrows indicate somatic and testis promoters. Boxes indicate exon 1 through exon 25 of somatic ACE. (B) Homologous recombination construct. With use of a BssHII restriction site on the ACE gene, a neomycin resistance (Neo\textsuperscript{R}) cassette, and kidney-specific (Ksp) cadherin promoter upstream of a human β-globin minimal promoter (Ksp cadherin/Bg) were inserted sequentially. (C) ACE 9 modified allele. As a result of homologous recombination, the structural portion of the ACE gene is now under the control of the Ksp cadherin/Bg promoter. The effects of the somatic ACE promoter are minimized because of the presence of the Neo\textsuperscript{R} cassette as any transcripts originated by this promoter would terminate with the neomycin construct.
and barely detectable in lungs of ACE 9/9 mice (Figure 2B). Thus, ACE protein and activity in ACE 9/9 mice is preserved in the kidneys while simultaneously deleted from other tissues. Within the kidneys of this model, ACE expression as determined by immunohistochemistry (Figure 2C, right) was observed throughout the nephron including proximal tubules, distal tubules, collecting ducts, and glomeruli. In the latter, ACE expression was restricted to cells in the parietal epithelium of the Bowman’s capsule. In contrast, ACE expression was not present in the vascular endothelium, mesangial cells, or podocytes. This pattern was different from that of WT mice (Figure 2C, left) in which ACE was present mainly in proximal tubules, and in the endothelium of glomeruli and arterioles. Also, lower levels of ACE expression were observed in some interstitial cells and segments of the distal nephron.

Physiologic Studies
Systolic BP, as determined by tail-cuff plethysmography, averaged 20 mmHg lower in ACE 9/9 mice (99 ± 3.0 mmHg) when compared with WT mice (119 ± 2.8, P < 0.05) (Figure 4A). ACE 9/9 mice have a higher urine output (4.5 ± 0.4 versus 1.1 ± 0.2 ml/24 h in WT) and water intake (8.54 versus 3.8 ± 0.8 ml/24 h in WT) as well as lower urine osmolality (830 ± 81.1 versus 1950 ± 127.4 mOsm/kg in WT) (Figure 3A). Furthermore, when subjected to water deprivation for 24 hours (Figure 3B), ACE 9/9 failed to increase urine osmolality (1497 ± 146 mOsm/kg) to an extent similar to WT mice (3665 ± 397 mOsm/kg). In contrast, food intake was similar in ACE 9/9 mice (2.83 ± 0.42 g/24 h) and WT mice (3.19 ± 0.22 g/24 h). The higher urine output and water intake, the lower osmolality, and the failure to increase urine osmolality in the presence of water deprivation indicate an impaired concentrating ability by the kidneys of ACE 9/9 mice.

Renal Morphology
The alterations in renal function are also associated with structural renal abnormalities. Kidneys from ACE 9/9 display variable degrees of pelvic expansion with thinned medullas as well as thickening of intrarenal arteries and perivascular infiltrates. Examination of other organs did not reveal other abnormalities.

Plasma Angiotensin Peptides
Blood was collected from the inferior vena cava of anesthetized mice to assess plasma angiotensins and renin concentration (PRC) in control conditions and to allow comparison with previous ACE KO models. Plasma Ang II was lower in ACE 9/9 mice (30.94 ± 4.10 fmol/L) than in WT mice (96.83 ± 13.10 fmol/L, P < 0.05). In turn, PRC was higher in ACE 9/9 mice.
(13.20 ± 1.32 μg of Ang I per ml/h) than in WT mice (3.49 ± 0.73 μg of Ang I per ml/h, P < 0.05). Thus, ACE 9/9 mice have an impaired capacity to generate normal circulating Ang II levels because of the absence of systemic ACE.

In summary, ACE 9/9 display low BP and plasma Ang II levels as well as a combination of structural and functional renal defects. This phenotype is similar to that of systemic ACE KO mice,20,21 indicating the importance of systemic ACE or the ACE present in other structures of the kidney for normal renal development and BP control.

Responses to Chronic Ang I Infusions
ACE 9/9 mice and WT littermates were subjected to sham-operation or minipump implantation to infuse Ang I subcutaneously (1000 ng per kg/min) for 3 weeks. Additionally, mice were housed individually in metabolic cages for 72-hour periods before and two times during the experiment. After 21 days, mice were subjected to conscious decapitation and trunk blood and tissue samples were collected.

Systolic Blood Pressure and Body Weight
As observed in Figure 4A, Ang I–infused WT mice had a rapid increase in BP that was significant after 1 week of infusion and sustained during the rest of the experiment. The BP increased from 119 ± 2.8 to 155 ± 7.8 mmHg after 21 days of infusion. Ang I–infused ACE 9/9 mice also exhibited a significant increase in BP, from 99 ± 3.0 to 140 ± 6.6 mmHg after 21 days of infusion. However, a significant difference was observed after 2 weeks of infusions, and thus, the hypertensinogenic response developed at a slower rate when compared with the response of WT mice.

WT and ACE 9/9 control mice did not show significant differences in body weight when compared in each group before and after the experiment (Figure 4B, gains of 1.40 ± 0.39 and 1.34 ± 0.678 g, respectively, NS). On the other hand, WT and ACE 9/9 Ang I–infused mice showed significant increases (gains of 2.34 ± 0.68 and 2.88 ± 0.69 g, P < 0.05 each).

Plasma Angiotensin Peptides
When compared with plasma Ang I concentrations in WT mice (9 ± 1.8 fmol/ml), ACE 9/9 mice values were much higher (126 ± 31 fmol/ml, P < 0.05). Moreover, the plasma Ang II/Ang I ratio was significantly reduced in ACE 9/9 (0.98 ± 0.2 mol/mol) when compared with that of WT mice (17 ± 6.4 mol/mol, P < 0.05), thus demonstrating an impaired capacity for Ang I conversion to Ang II because of the absence of systemic ACE. Chronic Ang I infusions increased plasma values of Ang I in WT mice (32 ± 7.1 fmol/ml, P < 0.05 versus control WT) but not in ACE 9/9 mice (82 ± 38.1, NS versus control ACE 9/9). In turn, plasma Ang II levels were increased by chronic Ang I infusions in WT mice to 196 ± 48 fmol/ml and ACE 9/9 mice to 189 ± 44.5 fmol/ml (P < 0.05 versus respective controls). Chronic Ang I infusions also decreased the Ang II/Ang I ratio in WT mice (6 ± 1.8 mol/mol, P < 0.05 versus respective controls) and increased the Ang II/Ang I ratio in ACE 9/9 mice (5 ± 1.6 mol/mol, P < 0.05 versus respective controls).

Kidney and Urinary Angiotensin Peptides
Kidney Ang I levels (Figure 5, left) were similar in WT and ACE 9/9 control mice (177 ± 20.5 and 176 ± 21.1 fmol/g, respectively). Kidney Ang II levels (Figure 5, middle) were also comparable in WT and ACE 9/9 control mice (842 ± 101.4 and 832 ± 124.3 fmol/g, respectively). As a result, the Ang II/Ang I ratios were also similar in WT and ACE 9/9 mice (4.86 ± 0.95.
versus 4.15 ± 0.56 mol/mol, respectively). Thus, in contrast to what was observed in plasma, the presence of ACE in the tubules is sufficient to maintain normal intrarenal Ang peptide levels. Chronic Ang I infusions caused significant increases in intrarenal Ang II content in WT mice (225 ± 18.9 fmol/g, a 26% increase) and in ACE 9/9 mice (249 ± 17.2 fmol/g, a 41% increase). Importantly, Ang I infusions caused pronounced increases in intrarenal Ang II content in WT mice (1405 ± 202.3 fmol/g, a 75% increase) and ACE 9/9 mice (1420 ± 147.2 fmol/g, a 75% increase). Finally, chronic Ang I infusions significantly increased the Ang II/Ang I ratio in WT mice (8.07 ± 1.62 mol/mol, 66% increase) and ACE 9/9 mice (7.18 ± 0.90 mol/mol, 73% increase). The higher intrarenal Ang II levels and Ang II/Ang I ratios suggest an enhanced conversion of Ang I to Ang II in WT and ACE 9/9 mice. Because ACE expression in the kidneys of ACE 9/9 mice is localized mostly along the nephrons, we hypothesized that Ang II generation in the tubular lumen would be a good indicator of differences between ACE 9/9 and WT mice. Moreover, Ang II spillover and accumulation in the urine might be enhanced in Ang I–infused ACE 9/9 mice and perhaps also in WT. As depicted in Figure 6A, basal levels of urinary Ang II excretion were higher in ACE 9/9 mice (8249 ± 596.4 fmol/24 h) than in WT mice (4482 ± 216.4 fmol/24 h, P < 0.05). Nevertheless, in response to Ang I infusions, both WT and ACE 9/9 mice had significant increases in urinary Ang II excretion (10975 ± 1295 and 14443 ± 2868 fmol/24 h, respectively). Because the progressive increase in Ang II excretion resembled the BP response in ACE 9/9, a correlation between urinary Ang II excretion and SBP was explored (Figure 6B). Such relationship was not detected in WT mice. In contrast, a significant correlation was observed between urinary Ang II excretion and SBP in Ang I–infused ACE 9/9 mice.

ACE and AT1R Expression in the Kidneys
Ang II–dependent hypertension changes ACE and AT1R expression in the mouse kidney.22 Therefore, experiments were conducted to analyze the expression of these two genes (Figure 7). When compared with ACE mRNA in WT controls (ACE/β-actin mRNA expression normalized as 1.00 ± 0.05), renal ACE mRNA expression in ACE 9/9 controls was lower (0.59 ± 0.05); chronic Ang I infusions reduced ACE mRNA expression in WT mice (0.72 ± 0.08) but not in ACE 9/9 mice (0.57 ± 0.06). In contrast, ACE protein expression was similar in WT controls (ACE/β-actin protein expression normalized as 1.00 ± 0.06) and ACE 9/9 controls (1.00 ± 0.06). Chronic Ang I infusions reduced ACE protein expression in WT (0.81 ± 0.06) but not in ACE 9/9 (1.04 ± 0.09) mice. AT1aR mRNA expression was similar in WT controls (1.00 ± 0.06) and ACE 9/9 control (1.00 ± 0.09) mice. Chronic Ang I infusions caused a small but significant reduction in AT1aR expression in WT mice (0.81 ± 0.04) and ACE 9/9 mice (0.89 ± 0.07). However, AT1aR protein expression was similar in WT control mice (1.02 ± 0.03) and ACE 9/9 control mice (1.09 ± 0.05), and was not altered by chronic Ang I infusions in WT mice (1.02 ± 0.05) or ACE 9/9 mice (1.12 ± 0.04).

In brief, the observed responses to chronic Ang I infusions in ACE 9/9 mice indicate that tubular ACE possess the capacity to increase local Ang II, perhaps circulating Ang II, and to enhance urinary Ang II excretion and that such increments are associated with the development of hypertension.

DISCUSSION
The presence of ACE in the kidneys is critical for normal development and function of this organ as well as for maintaining local Ang II levels.15,20 However, it is less clear whether kidney ACE can serve a unique role in the development of hypertension. In support of this possibility, this study demonstrates that an enhanced intrarenal ACE-dependent Ang II formation is sufficient to increase kidney and urinary Ang II levels and to

![Figure 4](https://journals.c梅l/journals/images/basick/article-figures/45324.png)
**Figure 5.** Kidney Ang I and Ang II increase during chronic Ang I infusions in ACE 9/9 mice. Ang I and Ang II content were measured by radioimmunoanalysis in total kidney homogenates obtained from ACE 9/9 or WT mice after 3 weeks of infusions. The results are expressed as per gram of kidney weight (KW). *P < 0.05 versus respective controls by unpaired t test analysis, n = 5 to 8 for Ang I analysis and 11 to 15 for Ang II analysis.

**Figure 6.** Urinary Ang II excretion increases during chronic Ang I infusions in ACE 9/9 mice. Urine samples were collected by housing the mice individually in metabolic cages for 72 hours, during which the mice had free access to food and water (results are expressed as daily average). Ang II concentration was measured by radioimmunoanalysis. Systolic BP was determined by tail-cuff plethysmography. *P < 0.05 versus respective genotype and time-matched controls by two-way ANOVA, n = 7 to 13. The Pearson’s test was used to determine a correlation in (B).
lead to the development of hypertension even in the absence of systemic ACE.

The expression of a complete RAS along the nephron has been recognized for many years. However, the role of this system in the development and maintenance of hypertension has remained uncertain. The present results indicate that activation of the intrarenal RAS alone is sufficient to increase BP. These findings are in agreement with the high intrarenal Ang II levels, hypertension, and kidney damage observed in transgenic mice overexpressing human angiotensinogen in proximal tubules of the kidney. Our observations are also consistent with those reported by Crowley et al., who used a cross-transplantation model to demonstrate that the AT1R in the kidneys are essential for Ang II actions to cause hypertension. If the intrarenal RAS were to be responsible for the development of high BP levels, then the expression of its components should be increased, or at least maintained, during hypertension. This has been reported previously in several experimental models. In agreement with this, we observed that chronic Ang I infusions reduced only partially ACE expression (in WT mice) and did not change AT1R expression. Importantly, it is likely that an overactive intrarenal RAS can cause human hypertension as urinary angiotensinogen excretion was recently shown to be increased in hypertensive subjects.

Intrarenal Ang II stimulates sodium and water reabsorption in proximal and distal segments of the nephron directly or through stimulation of aldosterone, reduces renal plasma flow and GFR, and increases the sensitivity of the tubuloglomerular feedback mechanism. Hence, it follows that augmenting Ang II synthesis within the kidneys will have significant long-term consequences favoring sodium and water retention, an increase in total extracellular fluid volume, and higher BP levels. In accord with this, an increased local Ang II generation in the kidneys of ACE 9/9 mice was associated with the development of hypertension. Furthermore, a significant correlation between urinary Ang II excretion and systolic BP was found in ACE 9/9 mice, suggesting a causal relationship between these parameters. Such a correlation was not detected in WT mice, but this is probably because of substantial Ang II generation occurring in the systemic circulation with consequent clearance in the urine by the kidneys. In view of the presence of renin and ACE in distal nephron segments, as well as the spillover of AGT from proximal tubules, the ability to generate Ang II in these segments might be of importance. Indeed, Ang II can directly stimulate sodium reabsorption in collecting duct cells. Furthermore, Zhao et al. demonstrated that, in Ang II–infused mice, there is an enhanced distal sodium reabsorption associated with increased urinary Ang II concentrations that was not prevented by aldosterone blockade. The slowly progressive hypertensive response observed in ACE 9/9 mice, while providing additional support to a renal mechanism, can also be explained by the lack of early gener-

Figure 7. Changes in kidney ACE (left) and AT1R (right) mRNA and protein expression in response to chronic angiotensin I infusions. ACE and AT1aR mRNA expression were determined by quantitative real time-PCR. Protein expression was determined by Western blot. In both cases, whole kidney homogenates were used and the results normalized against β-actin expression. *P < 0.05 versus respective controls by unpaired t test analysis, n = 11 to 15.
alized vasoconstrictor or other extra renal effects because of the absence of systemic Ang II generation. With regard to the changes in renal function and body fluids, we observed increases in body weight in WT and ACE 9/9 Ang I–infused mice that are probably caused by fluid retention. We intend to explore renal function in more detail with future clearance studies in Ang I–infused ACE 9/9 mice, as well as metabolic studies performed early during chronic Ang I infusions.

In the absence of ACE or during its inhibition, the ACE substrate bradykinin accumulates. Bradykinin induces vaso-dilatation and natriuresis, and it is present in high concentrations in ACE KO mice. The role of bradykinin in the phenotype of these mice has been assessed by the generation of double KO mice lacking ACE and the bradykinin B2 receptor. The B2 receptor is the main pathway for bradykinin activation of eNOS. The observed phenotype in the double KO mouse was indistinguishable from mice lacking only ACE. Hence, it is likely that the phenotype of ACE KO mice is primarily due to the lack of Ang II generation and not to bradykinin accumulation. However, it has also been reported that bradykinin has a buffering effect during Ang II–dependent hypertension as B2 receptor KO mice subjected to chronic Ang II infusions display an exacerbated hypertensive response. Because we did not measure bradykinin levels in the ACE 9/9 mice, we cannot disregard the contribution of bradykinin to some of the differences observed between ACE 9/9 and WT mice.

As mentioned in the introduction, utilizing ACE 9/9 mice offered the major advantage over pharmacologic approaches of isolating the contributions of kidney ACE from those of systemic ACE. However, targeting ACE expression exclusively to the kidney tubules has important consequences that confound our analysis. Most notably, ACE 9/9 display low BP, urine concentrating impairment, and kidney abnormalities. These alterations, although slightly less severe, are consistent with those observed in mice with targeted disruption of RAS components, including total ACE KO mice, indicating that Ang II generated by systemic ACE or locally by structures other than the tubules is required for normal kidney development and function. Thus, the presence of ACE in the tubules is not sufficient to completely rescue the phenotype induced by the total absence of this enzyme. The low BP, as well as the low Ang II and high PRC and Ang I concentrations, provides evidence that the presence of ACE solely in the kidney tubules is not sufficient to maintain normal plasma Ang II and BP levels. Thus, it is likely that, under basal conditions, Ang II generated in the tubular lumen does not sufficiently enter into the systemic circulation. They also offer additional evidence supporting the nonredundant contributions of the systemic RAS and the kidney RAS to the control of body fluids and BP. However, the increases in plasma Ang II observed in Ang I–infused ACE 9/9 mice indicate that, in conditions where there is an overactive intrarenal RAS, some of the Ang II generated by the kidneys is released into the general circulation, contributing to direct vasoconstrictor effects and the increased BP. This interpretation is supported by reports demonstrating that endogenous Ang II generation contributes to the rise of plasma Ang II during Ang II–dependent hypertension. A different possibility is that, in the presence of excess substrate, Ang II might be generated by ACE-independent pathways such as chymase.

In summary, our data demonstrate that an enhanced intrarenal ACE-derived Ang II formation, in the absence of systemic ACE, increases kidney, and urinary Ang II levels and induces the progressive development of hypertension. The findings presented here suggest that ACE inhibitors and other RAS blockers with enhanced kidney tissue distribution or specificity may provide a better approach to treat patients with certain forms of hypertension or kidney disease with increased intrarenal RAS activity.

**CONCISE METHODS**

**Creation of Homozygous Mutant Mice**

A 10.7-kb fragment of mouse genomic DNA was cloned from a mouse C12.1 embryonic stem cell library derived from mouse strain 129 DNA. This contained 2.4 kb of the somatic ACE promoter, the somatic ACE transcription start site, and 8.3 kb of genomic sequence encompassing somatic ACE exon 1 through exon 12. With use of a unique BssHII restriction site, a neomycin cassette was inserted 3′ to the somatic ACE transcriptional start site but 5′ to somatic ACE exon 1. Next, 1341 bp of the Mus musculus Ksp-cadherin 5′ flanking region (nucleotides 2430 through 3770 of GenBank accession number AF118228) upstream of a human β-globin minimal promoter (−31 to +12 bp) was placed immediately 3′ to the neomycin cassette. This Ksp/Bg promoter was a gift from Dr. Peter Igarashi (Southwestern Medical School, Dallas, Texas).

The targeting construct was linearized and electroporated into R1 ES cells. These cells were derived from a 129/SV129/SwJ F1 embryo. After positive and negative selection, individual ES cell clones were isolated and screened for targeted homologous recombination using a strategy of PCR and genomic Southern blot analysis. The generation of chimeric, heterozygote, and homozygous mutant mice was performed as described previously. Blastocysts were obtained from C57BL/6 mice. Chimeric mice were mated to C57BL/6 mice.

Although the genetic background of ACE 9/9 mice is mixed between 129J and C57BL/6, the mice have been selected to harbor only one renin gene. This has been done by breeding and selecting the renin locus. 129 mice harbor two renin genes (Ren-1d and Ren-2); whereas C57BL/6 have only one gene (Ren-1d); several reports indicate major phenotypic differences associated with this variation. Therefore, we selected our experimental mice to harbor only one renin gene (Ren-1d), a model more similar to humans.

**Genotyping**

The genotyping of genomic DNA was based on a single PCR reaction. The presence of the WT ACE allele was revealed by the amplification of a 793-bp fragment using the primers WT sense (5′-CAAGGGT-GAACTGGTGGTGCCTGA-3′) and antisense (5′-TTCTCTCCGTO-GATGTGGT-3′). The mutant allele was amplified with the primers
mice were subjected to sham operation (ACE 9/9 controls, J Am Soc Nephrol 22: 849–859, 2011 Kidney-Specific ACE and Hypertension) and were homogenized in methanol to be processed and analyzed by RIA as described previously.

For Southern blot analysis, DNA was digested to completion with the restriction enzyme Thl11I and was probed with a 1.1-kb genomic construct. This probe hybridizes to the ACE gene but not to the targeting construct. This method of euthanasia was selected because it avoids the confounding effects of anesthesia to elevate renin secretion. Such an increase in renin can potentially mask changes in Ang I and Ang II induced by chronic Ang I infusions. Mice were also housed individually for 72 hours in metabolic cages (Hatteras Instruments, Cary, NC) before minipump implantation (basal measurement, days −3 to −1) and on days 8 to 10 and 18 to 20. Water and food ingestion and urine output were monitored and calculated as daily averages. Urine samples were collected in tubes prefilled with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and spun down, and the supernatant was collected and frozen at −80°C until further use.

Determinations of Angiotensin Peptides
For the initial characterization, blood from anesthetized mice (ketamine 125 mg/kg, xylazine 12.5 mg/kg) was collected from the inferior vena cava in syringes containing 4 M guanidine thiocyanate and immediately frozen at −80°C. Angiotensins were measured by HPLC-based RIA. After the experimental protocol, plasma Ang II from trunk blood collected in chilled tubes containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and spun down, and the supernatant was collected and frozen at −80°C until further use.

ACE Activity and Plasma Renin Concentration
For measurements of ACE activity, plasma samples were collected in heparin-containing tubes. Kidneys and other tissues were collected in ACE assay homogenization buffer. Plasma and tissue ACE activities were determined using a ACE REA kit by ALPCO (Salem, NH) following manufacturer’s recommendations and protocols described by others. In this protocol 1 unit of ACE activity is defined as the amount of enzyme required to release 1 μmol of hippuric acid per min/L of sample at 37°C. Plasma renin concentration was measured by enzymatic assay, as described previously.

Urinary Osmolality
Urinary osmolality was determined using a vapor pressure osmometer by Wescor (Model 5500; Wescor Inc., Logan, UT).

Kidney ACE and AT1R Expression
Whole kidney extracts were used to isolate total RNA and protein. ACE and AT1R mRNA expression were determined by quantitative real-time PCR using β-actin as normalizer as described before. ACE and AT1R protein expression were determined by Western blotting with previously established protocols except that an anti-ACE antibody was used at 1:1000 concentration with overnight incubation (SC-12187; Santa Cruz Biotechnology, Santa Cruz, CA).

Kidney Histology
Paraffin-embedded kidney sections (3 μM) representative from ACE 9/9 mice and WT littermates were immunostained by the immunoperoxidase technique by sequential incubation with normal blocking rabbit serum, an anti-ACE primary antibody (SC-12187; Santa Cruz Biotechnology) at 1:1000 concentration for 90 minutes, a biotin-conjugated rabbit anti-mouse secondary antibody for 30 minutes, and a rabbit HRP-Polymer kit (Biocare Medical, LLC), followed by 0.1% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis) to visualize peroxidase activity. Additionally, samples were also counterstained with hematoxilin before analysis.

Statistical Analysis
All data are presented as means ± SD. Two-way ANOVA with Bonferroni post test was used when analyzing changes of data collected over time. Unpaired t test was used to analyze differences between controls and Ang I–infused mice within the same genotype or to assess the differences between WT and ACE 9/9 mice when appropriate. The Pearson test was used to determine a correlation between systolic BP and urinary Ang II excretion. All statistical tests were calculated using GraphPad Prism 5.00 (GraphPad Software, San Diego). A value of P < 0.05 was regarded as significant.

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DISCLOSURES
None.
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