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Justin M. Cole, Nata Khokhlova, Roy L. Sutliff, Jonathan W. Adams, Kevin M. Disher, Hui Zhao, Mario R. Capecchi, Pierre Corvol, Kenneth E. Bernstein

Abstract—Recently, the concept of local renin-angiotensin systems (RAS) capable of generating angiotensin II apart from the circulation has received considerable attention. To investigate this, we generated ACE 1/3 mice in which one allele of ACE is null and the second allele was engineered to express ACE on the surface of hepatocytes. ACE 1/3 mice express no endothelial ACE and lack ACE within the lungs. Their kidneys contain <7.8% the enzyme levels present in control mice. Plasma conversion of angiotensin I to angiotensin II was 43.3% normal. The baseline blood pressure and renal function of the ACE 1/3 mice were normal, probably as a function of a marked increase of both plasma angiotensin I and angiotensin II. When exposed to 2 weeks of a salt-free diet (a stress diet stimulating the RAS), blood pressure in ACE 1/3 mice decreased to 92.3 ± 2.0 mm Hg, a level significantly lower than that of wild-type control mice. The ACE 1/3 mice demonstrate the plasticity of the RAS and show that significant compensation is required to maintain normal, basal blood pressure in a mouse with an impaired local vascular and renal RAS. (*Hypertension*. 2003;41:313-321.)

Key Words: mice, knockout ■ angiotensin-converting enzyme ■ angiotensin II ■ endothelium ■ blood pressure

The renin-angiotensin system (RAS) is a principle regulator of blood pressure and electrolyte homeostasis within the body. Inhibitors of this system have found broad use in the modern treatment of hypertension, congestive heart failure, and diabetic nephropathy. The RAS was originally characterized as a circulating, endocrine network, but in recent years the concept of a local RAS has gained considerable attention. In part, this is due to the finding that multiple organs express all of the components necessary to recapitulate a functional, local RAS in situ.¹⁻⁵ Although in theory, multiple tissues may regulate the production of angiotensin II independent from the circulation, the physiological and pathophysiological role of tissue RAS in organs such as the kidney and the vasculature is not known.

Recently, we engineered a mouse with an altered profile in its expression of ACE.⁶ This animal, termed ACE.3, does not produce vascular ACE but instead expresses the enzyme on the cell membrane of hepatocytes. This was accomplished by using targeted homologous recombination to substitute the control of ACE expression from its endogenous promoter to an albumin promoter.⁷ As such, the liver of the homologous recombinant mouse (termed ACE 3/3 to indicate two targeted alleles) produces 87-fold more ACE than that of a wild-type animal. The only extrahepatic source of tissue ACE in the

ACE 3/3 mouse is in the kidney, which has roughly 14% of wild-type levels. This expression is limited to the proximal tubule and is not observed within renal vascular endothelium or adventitia. This residual ACE expression is probably due to the specificity of the albumin promoter, since the original characterization of albumin promoter cassettes similar to that used in the ACE 3/3 mice showed low levels of reporter gene expression within the kidney. The systolic blood pressure of the ACE 3/3 is equivalent to wild-type at both baseline and after water deprivation, implying that endothelial ACE is not mandatory for the regulation of blood pressure, at least under these conditions.

The ACE.3 animal was created in a deliberate attempt to gain insight into the relative roles of the local versus endocrine RAS in mammalian physiology. We reasoned that an animal lacking vascular ACE but still capable of producing circulating angiotensin II would be a useful tool to address this issue. However, a limitation of the ACE.3 model is that it has plasma ACE activity that is 79% of wild-type, probably as a result of the release of hepatic ACE into the circulation. This level of plasma ACE in ACE 3/3 mice may provide substantial compensation for its lack of vascular ACE. To minimize this confounding variable and to minimize the renal tubular ACE found in the ACE.3 model, we have

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engineered a mouse that is a compound heterozygote at the ACE locus. This animal, termed ACE 1/3, contains one null allele for ACE (the ACE.1 allele) and one copy of the ACE.3 allele. Our analysis of this animal demonstrates that it has a systolic blood pressure equivalent to wild-type but that it has a substantial elevation in its circulating levels of both angiotensin I and angiotensin II. In this model, we believe the elevated plasma levels of angiotensin II compensate for the lack of local ACE production. The ACE 1/3 mice not only demonstrate the plasticity of the RAS but also show that a normal blood pressure can be associated with a marked elevation of plasma angiotensin II levels.

Methods

Creation and Genotyping of Compound Heterozygous Mice

To produce ACE 1/3 compound heterozygote animals, ACE.1 heterozygote mice (1/+) were mated with ACE.3 homozygous mutant mice (3/3). Both the ACE.1 and the ACE.3 line were created by using targeted homologous recombination in R1 embryonic stem cells (derived from a 129/SV \times 129/SvJ F₁ embryo) that were implanted in blastocysts from C57Bl/6 mice.⁸ The 1/+ animals used in matings were in the 7th generation of back-cross to C57Bl/6 and the 3/3 mice were of the F3 and F4 generation. Thus, the generated litters of ACE 1/3 and 3/+ mice were neither pure C57Bl/6 strain nor pure 129 strain.

Mice were genotyped through the use of two independent polymerase chain reactions (PCR). The first amplified a fragment of \approx 500 bp from the ACE.3 allele.⁶ As predicted, this reaction was positive for every animal examined. The second PCR reaction was designed to discriminate between the wild-type allele and the ACE.1 allele. Through the use of a 3-primer system, wild-type genomic DNA yielded a band of 215 bp and the ACE.1 mutant allele yielded a band of 287 bp.⁹

Western Blot and ACE Activity Assays

Samples for Western blot and tissue ACE activity were prepared in a manner designed to minimize plasma ACE contamination.⁶ ACE activity was measured in tissue homogenates and plasma with the ACE-REA kit from American Laboratory Products Company, Ltd. The measurement of plasma ACE conversion of angiotensin I to angiotensin II was performed as previously described.¹⁰

Blood Pressure Determination

Systolic blood pressure was determined with a Visitech automated tail-cuff system.¹¹ To measure the blood pressure response to a sodium-deficient diet, a baseline blood pressure was determined for each mouse. The mice were then placed on a zero-sodium rodent diet purchased from Dyets, Inc. Highly deionized water in clean plastic containers was available ad libitum. During the control period, the mice were fed an identical diet from Dyets, Inc that was supplemented with 0.4% NaCl. At the end of 2 weeks, the mice were reaccustomed to the blood pressure machine and their systolic blood pressure was evaluated.

Plasma Angiotensin I and II

Angiotensin I and II levels were measured by radioimmunoassay, as previously described.¹² The assay background was determined by measuring peptide levels in angiotensinogen knockout mice, animals genetically modified to lack angiotensin I and angiotensin II. Background values were subtracted from the measurements to obtain the final data.

Both plasma and urinary aldosterone were measured by using the Coat-a-Count radioimmunoassay from Diagnostic Products Corporation. Plasma renin concentration was determined as previously described.¹³ This assay measures renin concentration in the presence of an excess of angiotensinogen.

Plasma Volume

Plasma volume was measured as previously described, with minor changes.¹⁴ Mice were restrained and 0.1 mL of 1% Evans blue dye solution was infused into the tail vein. Blood samples were drawn retroorbitally 5 and 15 minutes after the dye was injected, and the OD₆₂₀ of a 1:100 dilution of plasma was measured. An initial OD value at the time of dye administration (time 0) was then extrapolated from the two time points. This value was compared with that of standard dye dilutions to determine the plasma volume. Plasma volume was not assessed on the same group of animals on the normal and zero-salt diets to eliminate error caused by residual dye in the circulation. Instead, a different cohort of mice was used for each diet.

Angiotensin Peptide Infusions

Angiotensin I and II infusions were performed as previously described, with slight modification.¹⁵ Mice were anesthetized with 125 mg/kg ketamine and 12.5 mg/kg xylazine, and the carotid artery and jugular vein were cannulated. Angiotensin I and II were infused separately, each at two concentrations (0.1 and 1.0 μ g/kg) in a volume equal to 0.1% of body weight and flushed in 50 μ L 0.9% saline/50 U/mL heparin. A saline control was also infused in an equivalent fashion. The mice were anesthetized during the course of the experiment. Arterial blood pressure was monitored with the Digi-Med BPA-400 (Micro-Med).

Hematocrit and Urine Collection

Hematocrit was measured manually in a microcapillary reader. Urine osmolality was determined with a Wescor 5500 Vapor Pressure Osmometer. For the measurement of urinary sodium, mice were housed without food and water in individual metabolic cages, and urine was collected for 24 hours. Urinary sodium concentration was determined with a Perkin Elmer 3110 atomic absorption spectrometer.

Statistical Analysis

ANOVA followed by the Tukey honest significant difference test and the Bonferroni *t* test were used for statistical comparisons between groups. A paired *t* test was used to analyze the changes in blood pressure within the same group. The probability value for significance was defined as $P < 0.05$.

Results

Creation of Compound Heterozygote Mice

We have previously described three strains of mice containing mutations of the ACE locus produced by using targeted homologous recombination in embryonic stem cells. The first strain (ACE.1) is a null in that homozygous animals (ACE 1/1) produce no ACE.⁹ The second strain (ACE.2) is a tissue ACE knockout; homozygous mice (ACE 2/2) have \approx 40% of normal plasma ACE, but tissues demonstrate no ACE protein or ACE activity.¹⁰ Both ACE.1 and ACE.2 homozygous mutant mice have a reduction in systolic blood pressure of \approx 35 mm Hg, are anemic, and cannot produce concentrated urine. In contrast, ACE.3 homozygous mice (ACE 3/3) have normal blood pressure and a phenotype apparently identical to wild-type mice.

To produce litters containing compound heterozygous animals, ACE.1 heterozygote mice (ACE 1/+) were mated with ACE.3 homozygous mutant mice. The resulting offspring were genotyped by using PCR reactions designed to detect the wild-type (+), ACE 1, and ACE 3 alleles. Of 110 pups born from these matings, 53 were ACE 3/+ in genotype and 57 were ACE 1/3, which did not differ from the expected mendelian ratio of 1:1. The physiological analyses described in this report were performed on the offspring from these

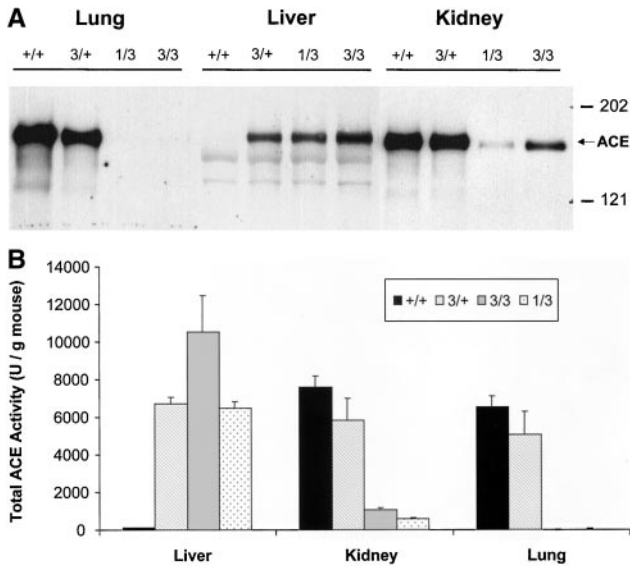


Figure 1. Tissue ACE. A, ACE.3 wild-type (+/+), heterozygote (3/+), knockout (3/3), and compound heterozygote (1/3) mice were killed and their lungs, liver, and kidneys homogenized. Ten micrograms of total protein homogenate was separated by SDS-PAGE and analyzed by Western blot, using a rabbit anti-mouse ACE antibody. Somatic ACE was detected at ≈ 170 kDa, as indicated by arrow. Wild-type mice have little ACE in the liver but abundant ACE in the lung and kidney. In contrast, ACE 3/3 and 1/3 mice express ACE in the liver but have no pulmonary ACE and a reduction in renal ACE. In particular, the 1/3 mouse expresses only a fraction of the renal ACE of the +/+ mouse. B, ACE.3 wild-type (+/+, black), heterozygote (3/+, striped), knockout (3/3, gray), and compound heterozygote (1/3, stippled) mice were killed and ACE activity measured in organ homogenates. Total organ ACE activity was then calculated for the liver, kidneys, and lungs of each mouse and normalized for the mass of the mouse. The livers of 3/+, 3/3, and 1/3 mice have abundant total ACE activity, partly as a result of the large size of this organ. ACE activity is not detected in the lung of the 3/3 and 1/3 mice and is significantly reduced in the kidney. Whereas ACE 3/3 mice have $\approx 14\%$ of the total renal ACE found in +/+ mice, ACE 1/3 mice average 7.8% of wild-type. The number of mice in each group was as follows: +/+ liver, 5; 3/+ liver, 14; 3/3 liver, 5; 1/3 liver, 9; +/+ kidney, 11; 3/+ kidney, 15; 3/3 kidney, 11; 1/3 kidney, 14; +/+ lung, 5; 3/+ lung, 14; 3/3 lung, 5; 1/3 lung, 9. All data presented are group mean \pm SEM.

litters (3/+ and 1/3) in conjunction with F2-F4 generation ACE.3 mice (+/+, 3/+, and 3/3) derived from matings of ACE.3 heterozygotes. Except as where specifically noted in the text, the two groups of ACE 3/+ mice were phenotypically equivalent.

ACE Expression

To verify the pattern of ACE expression in ACE 1/3 animals, organ homogenates were separated by SDS-PAGE and analyzed by Western blot, with a rabbit polyclonal antibody specific for murine ACE (Figure 1A). As expected, the lung of wild-type (+/+) animals contained a very large amount of ACE. The lung of ACE 3/+ animals was also rich in ACE expression because of the presence of a single wild-type allele in these mice. In contrast, ACE 3/3 and 1/3 animals did not express any pulmonary ACE. Likewise, the liver of wild-type animals did not express any ACE. However, ACE 3/+, 3/3, and 1/3 mice all contained hepatic ACE as the result

of the presence of the ACE.3 allele. In the kidney, wild-type and ACE 3/+ mice expressed abundant ACE protein, whereas 3/3 mice expressed substantially less. Kidney homogenates from ACE 1/3 animals had only a very faint band for ACE on Western analysis. Similar to what we have reported in the ACE 3/3 mouse, other tissues examined in the ACE 1/3 animal, including the aorta, intestine, heart, and testis, did not express any somatic ACE (data not shown).

Another method to examine the tissue distribution of ACE is to measure its enzymatic activity in various organs. A cohort of animals was killed and their organs were individually homogenized. The ACE activity was then determined in a known quantity of homogenized tissue. With the use of the weight of each organ, the total ACE activity in the entire organ was estimated for each animal. The average total organ ACE activity for each genotype, normalized for the weight of individual mice, is presented in Figure 1B. The level of ACE activity in the liver of wild-type (+/+) mice is essentially zero. The ACE 1/3 and 3/+ livers each contain an intermediate amount of ACE activity. There was a mild but statistically significant difference in hepatic ACE activity between the F2 generation ACE 3/+ mice produced from F1 3/+ \times 3/+ matings (5438 ± 613 U/g, $n=6$) and those produced from ACE 3/3 \times ACE 1/+ matings (7624 ± 389 U/g, $n=8$) ($P<0.05$). However, neither group individually (nor when combined) had hepatic ACE activity statistically different from that observed in ACE 1/3 mice. The highest level of hepatic ACE activity was found in 3/3 animals. In the kidney, both wild-type and ACE 3/+ mice had substantial ACE activity. As we have previously reported, the total renal ACE activity in 3/3 mice averaged 14% of wild-type. In ACE 1/3 mice, the renal ACE activity decreased to 7.8% of wild-type. When evaluated as ACE activity per microgram of solubilized protein, the ACE 1/3 mice averaged 5.3% of wild-type mice as opposed to 12.5% for ACE 3/3 mice.

In agreement with the data obtained from Western blots, the lungs of ACE 1/3 and 3/3 mice do not contain measurable levels of ACE activity, whereas the levels in ACE 3/+ and wild-type (+/+) mice are quite high. Therefore, the tissue distribution of ACE in the 1/3 animal closely matched our expectations, based on its genetic makeup.

An important factor in our decision to examine the ACE 1/3 model was the substantial level of plasma ACE in the 3/3 mouse. To measure circulating ACE levels, mice were bled from the tail vein and plasma was collected by centrifuging. ACE activity was then assayed by using the artificial substrate ^3H -hippuryl-glycyl-glycine (HGG) (Figure 2A). Under these conditions, ACE 1/3 mice had 47.3% the plasma ACE activity of wild-type mice.

The molecular structure of ACE is such that it consists of two highly homologous catalytic domains, each of which participates in the physiological conversion of angiotensin I to angiotensin II.¹⁶ The use of artificial substrates for ACE comes with caveats, one being that they are selectively cleaved by the carboxyl-terminal catalytic domain of ACE.¹⁰ The most physiologically appropriate method to measure ACE activity in the plasma of ACE 1/3 mice is to assess the rate of conversion of angiotensin I to angiotensin II (Figure 2B). In particular, this assay permits a comparison to be made

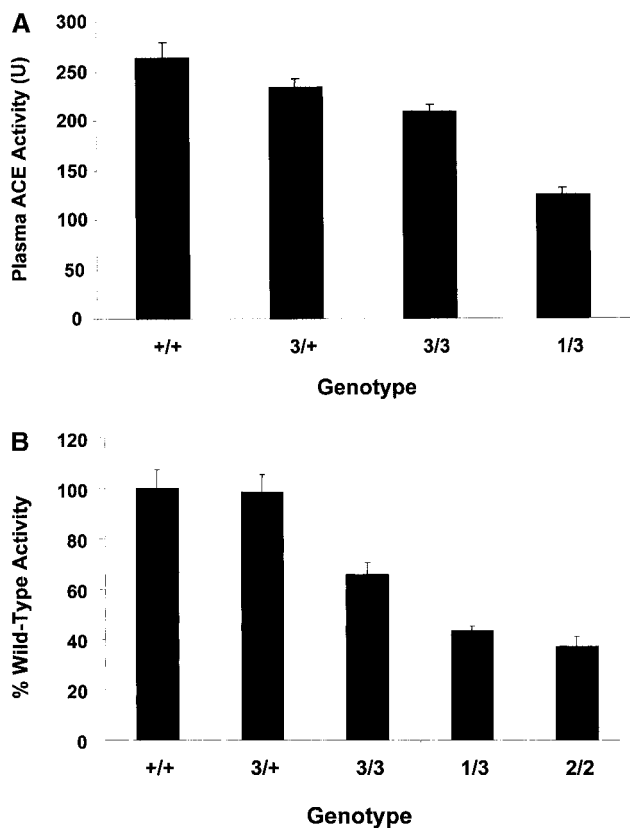


Figure 2. Plasma ACE. A, ACE.3 wild-type (+/+), heterozygote (3/+), knockout (3/3), and compound heterozygote (1/3) mice were bled from the tail vein, and plasma was isolated. Plasma ACE activity was assayed with the artificial substrate 3 HGG. Plasma ACE activity in 1/3 mice is \approx 47% of wild-type. Number of mice in each group was as follows: (+/+), 16; (3/+), 26; (3/3), 24; (1/3), 17. All data presented are the group mean \pm SEM. B, Plasma was collected from the groups shown in A along with ACE.2 knockout mice (2/2) and plasma ACE activity was determined by using the physiological substrate angiotensin I. Plasma of ACE 1/3 and 2/2 mice converts angiotensin I to angiotensin II at an approximately equal rate. Both groups have significantly less plasma ACE activity than +/+, 3/+, or 3/3 mice ($P < 0.01$ for all comparisons). The number of mice in each group was as follows: (+/+), 12; (3/+), 4; (3/3), 6; (1/3), 12; (2/2), 4. All data presented are group mean \pm SEM.

between the plasma ACE activity in the ACE 1/3 mouse and the ACE.2 knockout (2/2), an animal that secretes a truncated form of ACE containing only the amino-terminal domain. In the conversion of angiotensin I to angiotensin II, ACE 1/3 and 2/2 mice have 43.3% and 37.0% of wild-type plasma ACE activity, respectively. These values, although not statistically different from one another, are significantly decreased from the levels observed in ACE 3/3, 3/+, or +/+ mice ($P < 0.01$). This finding is relevant because the blood pressure and plasma angiotensin II levels of ACE 2/2 animals are quite low. Specifically, the blood pressure of ACE 2/2 mice averaged 75 mm Hg versus 110 mm Hg for wild-type control mice, and plasma angiotensin II levels averaged 21.8% of wild-type.^{10,17} Thus, the level of plasma ACE present in the ACE 2/2 animal is not sufficient for the normal regulation of blood pressure. This suggests that the residual plasma ACE activity in the ACE 1/3 mouse is also not sufficient to

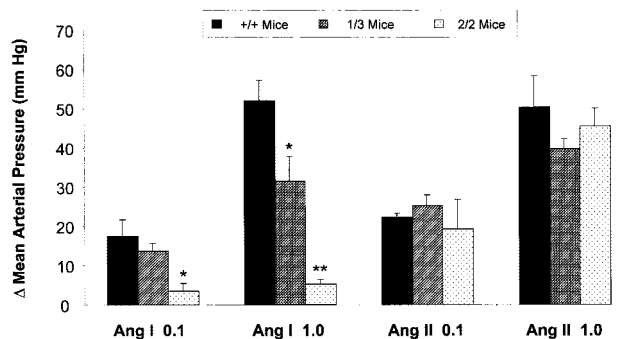


Figure 3. Response to angiotensin peptide infusion. The jugular vein of ACE +/+, ACE 1/3, and ACE 2/2 mice was cannulated ($n=4$ for each group) and angiotensin I and angiotensin II were infused separately, each at two concentrations (0.1 and 1.0 μ g/kg). The peak increase in mean arterial pressure after drug infusion was monitored with an arterial catheter. There was no difference in response among the three genotypes to either dose of angiotensin II. When angiotensin I was infused, the blood pressure increase in ACE 2/2 mice was significantly attenuated as compared with wild-type at both 0.1 μ g/kg ($*P < 0.05$) and 1.0 μ g/kg ($**P < 0.01$). Response of ACE 1/3 was also reduced compared with wild-type at 1.0 μ g/kg ($*P < 0.05$). Infusion of saline alone (vehicle control) led to an average pressure increase of 2.9 ± 0.7 mm Hg in the 12 mice studied. All data are presented as group mean \pm SEM.

generate angiotensin II in ample quantity for normal blood pressure homeostasis.

Physiological Analysis

As indicated, ACE 1/3 and ACE 2/2 mice have roughly equivalent plasma ACE activity. However, because of liver expression of ACE, the total enzyme available in an ACE 1/3 animal is greater than that found in the ACE 2/2 model. To quantify this, we measured the peak change in mean arterial pressure in wild-type, ACE 1/3, and ACE 2/2 mice after intravenous infusion of either angiotensin I or II (Figure 3). The effect of each peptide was assessed at two separate infusion dosages (0.1 and 1.0 μ g/kg). Although all three groups had a similar increase in blood pressure in response to angiotensin II, there was a large discrepancy in the response to angiotensin I. Wild-type mice showed a rise of blood pressure averaging 52.1 ± 5.2 mm Hg in response to 1.0 μ g/kg angiotensin I. In contrast, the ACE 2/2 animals showed a markedly reduced response, averaging just 5.2 ± 1.3 mm Hg in response to the same dosage. The response of the ACE 1/3 animal to infused angiotensin I was present but was more complicated. At the low dose of angiotensin I, the rise of blood pressure was similar to that of wild-type mice. However, at an infusion of 1.0 μ g/kg angiotensin I, the ACE 1/3 mice had an attenuated response compared with wild-type (31.5 ± 6.4 mm Hg, $P < 0.05$). Such a result suggests that the total body load of ACE present in the ACE 1/3 model is significantly more than in the ACE 2/2 animals but less than that present in a wild-type mouse.

In a wild-type animal, the vast majority of ACE activity is closely associated with the endothelium, permitting the production of angiotensin II in close proximity to underlying vascular smooth muscle. The ACE 1/3 animal completely lacks endothelial ACE and has a $>50\%$ reduction in circu-

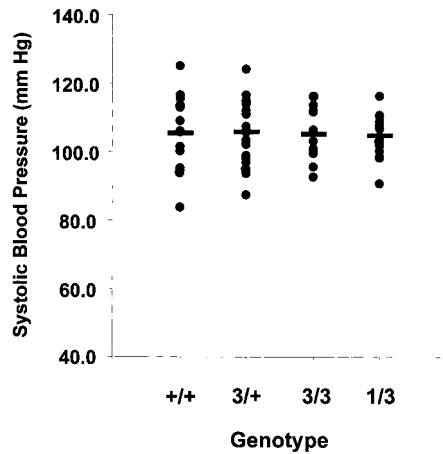


Figure 4. Systolic blood pressure. Baseline systolic blood pressure was determined for a cohort of ACE.3 wild-type (+/+), heterozygote (3/+), knockout (3/3), and compound heterozygote (1/3) mice over 4 consecutive days by using an automated tail-cuff manometer. Systolic blood pressure for each mouse (M) is the average of 80 individual measurements. Solid bar represents average for each group of mice. There is no significant difference in systolic blood pressure between the groups. The number of mice was as follows: (+/+), 16; (3/+), 25; (3/3), 16; (1/3), 17.

lating ACE. To assess the effect this has on blood pressure, the systolic blood pressure of a large group of mice was determined with an automated tail-cuff manometer (Figure 4). The blood pressure of each animal was recorded as the average of 80 individual data points taken over a 4-day period. The average blood pressure of 17 ACE 1/3 mice was 104.7 ± 1.4 mm Hg, as compared with 105.7 ± 1.7 mm Hg for 25 ACE 3/+ mice and 105.2 ± 2.6 mm Hg for 16 wild-type (+/+) mice. The blood pressure of individual +/+ mice ranged from 83.6 to 125.0 mm Hg and for ACE 1/3 mice the blood pressure ranged from 90.4 to 116.0 mm Hg. Thus, the blood pressure of 1/3 mice was not different from wild-type values either in average or in spread. These data indicate that the RAS is capable of compensating for the lack of local vascular production of angiotensin II in the control of blood pressure.

To gain further insight into the mechanism of blood pressure regulation in ACE 1/3 mice, we measured basal plasma angiotensin I and II levels (Figures 5A and 5B). The ACE 1/3 has significantly higher circulating angiotensin II peptide than wild-type (+/+), 3/+, or 3/3 mice (404 ± 82 pg/mL for 1/3 mice versus 137 ± 20 pg/mL for +/+, 184 ± 19 for 3/+, and 172 ± 18 for 3/3; $P < 0.01$ for all comparisons). Although the level of angiotensin II was the lowest in +/+ mice, this value did not significantly differ from ACE 3/+ or 3/3 mice. The ACE 1/3 animal also demonstrates a significant increase in plasma angiotensin I concentration as compared with the other genotypes (9046 ± 1202 pg/mL for ACE 1/3 mice versus 2911 ± 658 pg/mL for wild-type, 3004 ± 551 for ACE 3/+, and 4474 ± 986 for ACE 3/3; $P < 0.01$ for all comparisons). The trend toward an increase of angiotensin I in ACE 3/3 animals did not reach statistical significance.

Plasma renin concentration (PRC) was also measured in ACE +/+, 3/+, 3/3, and 1/3 mice while on a normal salt diet

(solid bars, Figure 5C). In agreement with the angiotensin peptide data, PRC was significantly elevated in the ACE 1/3 animal as compared with ACE +/+, ACE 3/+, and ACE 3/3 control mice ($P < 0.01$). The PRC was also increased in ACE 3/3 animals as compared with +/+ wild-type control mice ($P < 0.05$). These data provide evidence for the substantial plasticity of the RAS in the physiological regulation of blood pressure. The ACE 1/3 mouse not only has a shift in its distribution of ACE, but it also has a reduction in total body ACE activity. The combination of these stressors causes the RAS to greatly increase plasma renin and angiotensin I in order to generate sufficient angiotensin II for the maintenance of normal resting blood pressure.

As indicated above, we hypothesize that the increase of plasma angiotensin II present in the ACE 1/3 mice is a mechanism to maintain blood pressure in the face of an impaired local RAS. Alternatively, the elevation of angiotensin II could be in response to a reduction of plasma volume. To investigate this, we measured the plasma volume of ACE +/+, 3/+, 3/3, and 1/3 mice by the method of dye dilution (Figure 6). There was no difference in plasma volume between the four genotypes, leading to the conclusion that the elevation in circulating angiotensin II in the ACE 1/3 is not associated with volume-depletion.

ACE 1/3 mice have normal serum electrolytes including serum sodium, potassium, chloride, blood urea nitrogen, and creatinine (Table). The plasma aldosterone concentration and urinary aldosterone excretion of ACE 1/3 mice were also not significantly different from wild-type, ACE 3/+, or ACE 3/3 animals. Light microscopic examination of the kidneys of the ACE 1/3 mice showed a normal renal architecture, including a normal renal medulla and papilla. Normal rodents are able to produce urine that is concentrated in excess of 3000 mOsm/kg H₂O. In contrast, mice lacking all ACE (ACE 1/1) or all tissue ACE (ACE 2/2) cannot concentrate urine beyond ≈ 1000 mOsm/kg H₂O. Because ACE 1/3 compound heterozygotes are $>90\%$ deficient in renal ACE, we investigated whether they could generate a maximally concentrated urine. A spot of urine was collected from 17 ACE 1/3 and 6 3/+ mice both at baseline and after 24 hours of water deprivation. Under each condition, the osmolality of the ACE 1/3 urine was equivalent to that observed in ACE 3/+ mice (2036 ± 185 mOsm/kg H₂O at baseline and 3455 ± 27 mOsm/kg H₂O after water deprivation for 1/3 mice versus 2286 ± 143 mOsm/kg H₂O at baseline and 3481 ± 52 mOsm/kg H₂O for 3/+ mice). Thus, it is clear that despite a tremendous reduction in renal ACE ($>90\%$), the ACE 1/3 mice maintain a normal renal concentrating ability.

Another phenotype observed in ACE.1 and ACE.2 knockout mice was a normocytic, normochromic anemia.¹⁷ Although the cause underlying the anemia is not fully understood, it is a true anemia characterized by an $\approx 25\%$ reduction in circulating red cell mass. When we examined the hematocrit of the ACE 1/3 mouse, it averaged 0.548 ± 0.005 ($54.8 \pm 0.5\%$) ($n=17$) versus 0.552 ± 0.005 ($55.2 \pm 0.5\%$) ($n=9$) in littermate 3/+ mice ($P=NS$). Thus, the ACE 1/3 animal has sufficient residual ACE activity to prevent the development of anemia.

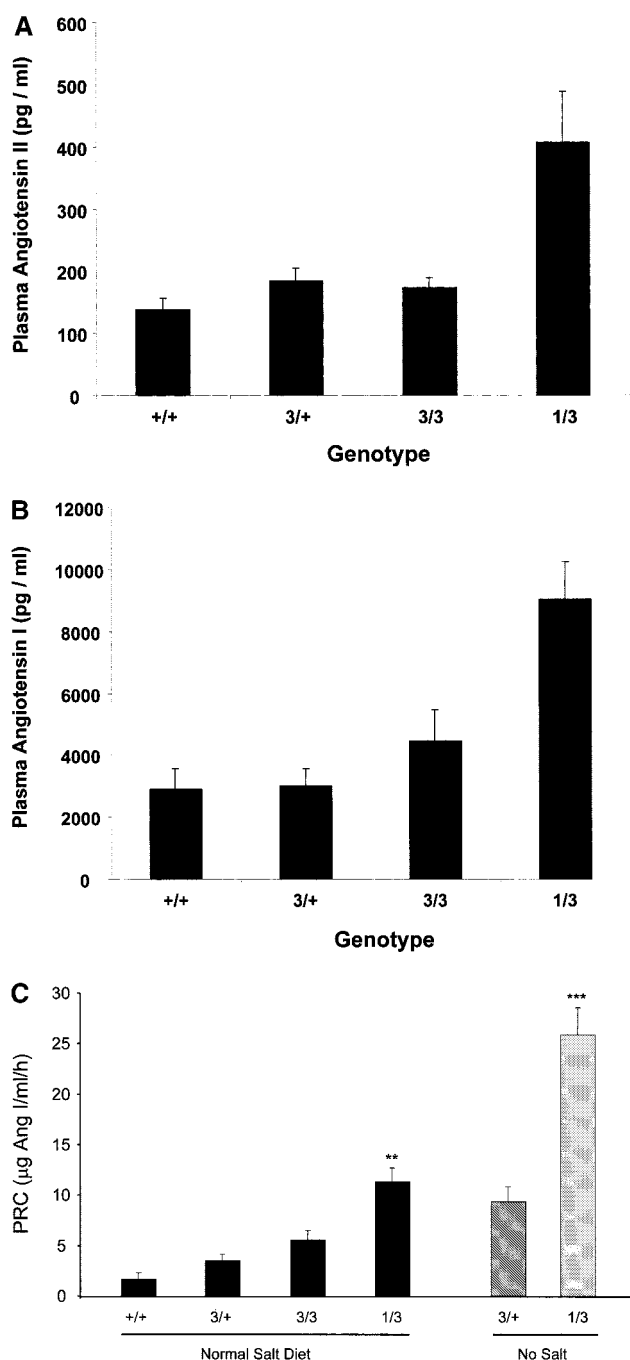


Figure 5. Plasma angiotensin peptide levels. Anesthetized mice were bled by cardiac puncture, and plasma was immediately frozen. Plasma angiotensin I and angiotensin II peptide levels were determined by radioimmunoassay. The number of mice in each group was as follows: (+/+), 14; (3/+), 27; (3/3), 13; (1/3), 13. A, ACE 1/3 mice have marked elevation of plasma angiotensin II as compared with +/+, 3/+, or 3/3 mice ($P<0.01$). B, Plasma angiotensin I levels are also significantly increased in ACE 1/3 mice as compared with the other groups ($P<0.01$). The data also show a trend toward higher plasma angiotensin I levels in ACE 3/3 mice as compared with +/+ and 3/+ mice, although this did not reach statistical significance. C, Mice on a standard (0.4% NaCl) diet were exsanguinated and plasma renin concentration (PRC) was measured (black bars). ACE 3/+ and 1/3 mice were also studied after >3 weeks on a zero sodium diet (striped bars). On the normal diet, the PRC of ACE 1/3 mice averaged $681\pm 83\%$, $323\pm 39\%$, and $204\pm 25\%$ that found in wild-type (+/+), ACE 3/+, or ACE 3/3 mice, respectively

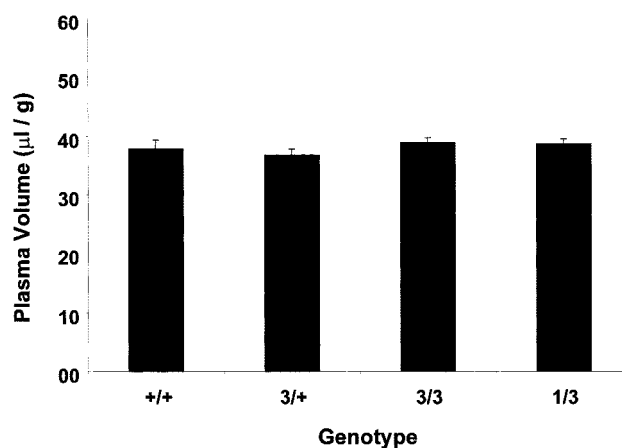


Figure 6. Plasma volume. The plasma volume of ACE +/+, 3/+, 3/3, and 1/3 mice was measured by the intravascular infusion of Evans blue dye. There is no difference in average plasma volume between the groups. The presence of a normal plasma volume and a normal hematocrit in the ACE 1/3 mouse indicates that the animal is not volume-depleted. All data are presented as group mean \pm SEM. The number of mice in each group was as follows: (+/+), 8; (3/+), 9; (3/3), 8; (1/3), 9.

Sodium Depletion

The ability of ACE 1/3 animals to maintain a wild-type phenotype indicates the tremendous flexibility of the RAS. However, to preserve this normal phenotype, the ACE 1/3 mouse must increase substantially both angiotensin I and angiotensin II. This suggests that the RAS may be approaching its maximum ability to compensate in this model and raises the question of how the ACE 1/3 animal would respond to physiological stress. To investigate this, we placed ACE +/+, 3/+, 3/3, and 1/3 animals on a diet lacking NaCl for 2 weeks, a condition that should maximally activate the RAS. The food intake and body weight were monitored for a subset of 1/3 and 3/+ mice ($n=6$ to 10 in each group). On both diets, the ACE 1/3 mice consumed a slightly larger percentage of their body weight daily than littermate 3/+ animals ($10.6\pm 2.0\%$ and $8.8\pm 1.7\%$ for ACE 1/3 mice on the normal-sodium and zero-sodium diets, respectively, versus $8.9\pm 1.2\%$ and $7.3\pm 2.3\%$ for 3/+ mice, $P<0.05$ for all comparisons). Neither group had a significant change in body weight during 2 weeks of sodium deprivation. The plasma volume was also assessed for a group of 7 3/+ and six 1/3 mice after >2 weeks of sodium restriction. The average plasma volume of 3/+ and 1/3 mice decreased by 12.5% and 9.1%, respectively, as compared with similar mice on the

(** $P<0.01$). Under these conditions, the PRC of ACE 3/3 mice was also significantly increased as compared with wild-type control mice ($P<0.05$). On the zero sodium diet, the PRC of ACE 1/3 mice was elevated >15-fold as compared with wild-type mice on a normal diet. This measurement was highly significant as compared with littermate ACE 3/+ mice on the zero sodium diet or ACE 1/3 mice at baseline (** $P<0.001$). The number of mice in each group was as follows: +/+ normal salt, 7; 3/+ normal salt, 8; 3/3 normal salt, 8; 1/3 normal salt, 8; 3/+ zero salt, 9; 1/3 zero salt, 6. The elevation of renin, angiotensin I, and angiotensin II indicates a physiological compensation by the RAS to sustain systolic blood pressure. All data presented are group mean \pm SEM.

Characteristics of ACE Mice

Parameter	+/+	3/+	3/3	1/3
Serum sodium, mEq/L	147.8±0.4 (8)	147.8±0.8 (8)	148.5±0.4 (6)	148.4±1.1 (7)
Serum potassium, mEq/L	4.41±0.12 (8)	4.29±0.16 (8)	4.33±0.23 (9)	4.20±0.21 (7)
Serum chloride, mEq/L	111.8±1.0 (8)	113.4±0.8 (8)	113.3±0.8 (6)	112.1±1.1 (7)
Serum BUN, mg/dL	25.3±1.3 (8)	26.9±4.9 (8)	24.0±2.6 (6)	23.1±1.3 (7)
Serum creatinine, mg/dL	0.24±0.02 (8)	0.24±0.02 (8)	0.24±0.02 (9)	0.27±0.02 (7)
Plasma Aldo (normal diet), pg/mL	913±97 (7)	699±60 (8)	779±129 (8)	565±31 (8)
Plasma Aldo (zero salt diet), pg/mL	ND	2041±520 (6)	ND	2088±767 (6)
Urinary Aldo (normal diet), pg/mL	1475±116 (6)	1583±120 (6)	1410±210 (6)	1715±152 (6)
Urinary Aldo (zero salt diet), pg/mL	4321±1047 (6)	4930±665 (6)	6994±1937 (6)	5945±2033 (6)

ACE.3 wild-type (+/+), heterozygote (3/+), knockout (3/3), and compound heterozygote (1/3) mice were studied for the parameters listed in the table. Number of animals in each group is given in parentheses and all data are presented as the mean±SEM. BUN, indicates blood urea nitrogen; Aldo, aldosterone; ND, no data.

normal sodium diet. Although this reduction in plasma volume was statistically significant as compared with mice on a normal salt diet ($P<0.01$), the magnitude of decrease was not significantly different between the 1/3 and 3/+ mice.

Twenty-four-hour urinary sodium excretion was determined for each mouse while on the normal salt diet and after 2 weeks of salt depletion (Figure 7A). On the baseline (normal sodium) diet, there was no significant difference in sodium excretion between the genotypes. All groups showed a marked reduction of sodium excretion on the salt-free diet. Under these conditions, the 1/3 mice appear to excrete the most salt, though the differences between all groups are small. In fact, the only statistical comparison attaining significance is between ACE 3/+ and ACE 1/3 mice ($P<0.05$). However, these trends in salt excretion parallel the changes in blood pressure described below.

Systolic blood pressure was determined over a 4-day period at baseline and after 2 weeks on the altered diet (Figure 7B). At baseline, there were no significant differences in average blood pressure between the 4 genotypes. After 2 weeks without NaCl, the average blood pressure of both 3/+ and 1/3 mice decreased significantly from baseline levels ($P<0.05$ and $P<0.01$ respectively). However, the drop in blood pressure was greater in ACE 1/3 mice than that observed in ACE 3/+ mice (9.7 ± 2.3 mm Hg for ACE 1/3 mice versus 5.0 ± 2.1 mm Hg for ACE 3/+ mice). Among +/+, 3/+, 3/3, and 1/3 animals, the ACE 1/3 mice also had the lowest average systolic blood pressure on the sodium-deficient diet, although the only comparison that attained statistical significance was between +/+ and 1/3 mice ($P<0.05$). After sodium restriction, the PRC of both ACE 1/3 and 3/+ mice was significantly elevated as compared with mice of the same genotype on a normal salt diet (Figure 5C, $P<0.001$). With a sodium-free diet, the PRC of the ACE 1/3 mice averaged $1552\pm 161\%$ that of wild-type mice on a normal salt diet and $277\pm 29\%$ that of littermate ACE 3/+ mice on the zero salt diet. Urinary aldosterone excretion showed a marked increase in all mice on the salt-free diet (Table). No statistical differences were noted between different genotypes. The salt depletion experiment demonstrated a subtle difference in the response of the ACE 1/3 mouse to physiological stress. However, it is noteworthy that even after

2 weeks of strict sodium depletion, ACE 1/3 mice sustain a systolic blood pressure of >90 mm Hg.

Discussion

The difference between a wild-type mouse and a knockout animal lacking all ACE is significant; the knockout has a striking reduction of blood pressure, serum electrolyte abnormalities, renal pathology, the inability to concentrate urine, anemia, and a marked reduction of male fertility.^{9,18} This complex phenotype indicates the many roles of the renin-angiotensin system. It also emphasizes that the animal null for all ACE expression is very different from a wild-type mouse. To study the function of the RAS in a more selective fashion, we used homologous recombination to place the structural portions of the ACE gene under the control of the albumin promoter. The ACE 3/3 mouse lacks endothelial ACE but produces large amounts of ACE in the liver. The idea was to investigate if the RAS could function if reconfigured with abundant ACE in an aberrant location. The answer is yes in that this animal model has a normal physiology, including a normal blood pressure. In analyzing the ACE 3/3 model, we were faced with two problems: the role of the residual renal ACE and the role of circulating plasma ACE. To address these issues, we created the ACE 1/3 mouse.

The ACE 1/3 animal is perhaps a more useful model to investigate the role of vascular and renal tubular ACE in blood pressure homeostasis. Like the ACE 3/3 mice, ACE 1/3 animals have no endothelial ACE but do express ACE on the surface of hepatocytes. Importantly, the ACE 1/3 model has much less circulating ACE than that present in ACE 3/3 animals. In fact, the plasma ACE levels are nearly equivalent to those in ACE 2/2 mice, animals lacking all tissue ACE. In the ACE 2/2 model, the circulating ACE is unable to elevate plasma angiotensin II levels much above the levels seen in mice totally deficient in ACE. Because of this, ACE 2/2 mice have a phenotype similar to ACE null mice, including a profound reduction of blood pressure. Thus, it seems very unlikely that the plasma ACE present in ACE 1/3 mice contributes much to the normal blood pressure in these animals.

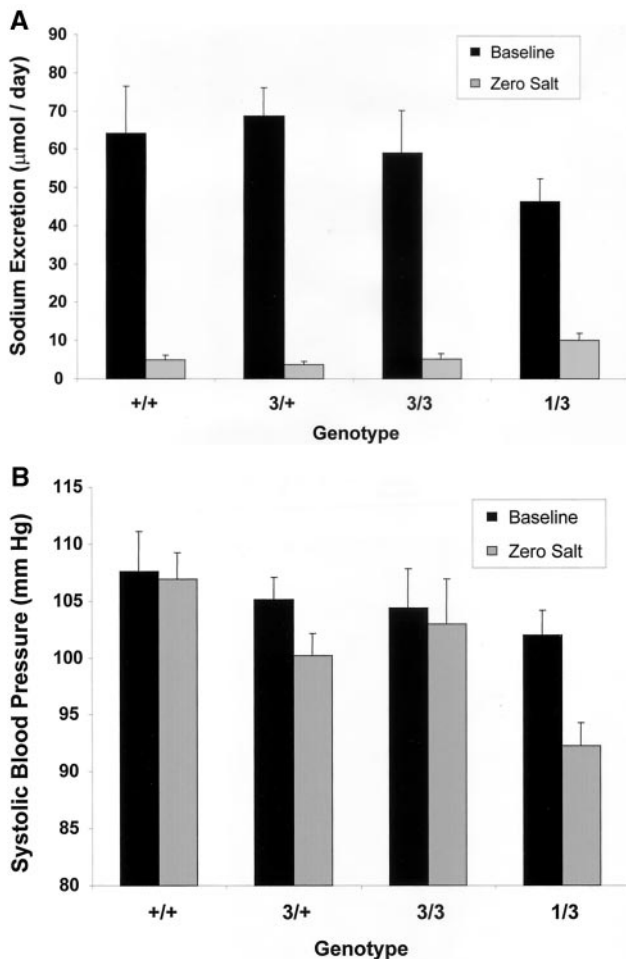


Figure 7. Response to sodium-deficient diet. Urinary sodium excretion and systolic blood pressure of ACE $+/+$, $3/+$, $3/3$, and $1/3$ mice were evaluated at baseline and after 2 weeks of a sodium-free diet. A, Urinary sodium excretion. On a normal sodium diet (black bars), there is no significant difference between the four genotypes. Daily sodium excretion is markedly decreased in all genotypes after 2 weeks of a salt-free diet (gray bars). In this condition, $1/3$ mice appear to excrete slightly more salt than $+/+$, $3/+$, or $3/3$ animals, although only the difference between $1/3$ and $3/+$ attained statistical significance ($P < 0.05$). The number of mice in each group was as follows: $+/+$ normal salt, 12, $+/+$ zero salt, 6; $3/+$ normal salt, 12, $3/+$ zero salt, 13; $3/3$ normal salt, 14; $3/3$ zero salt, 13; $1/3$ normal salt, 13; $1/3$ zero salt, 13. B, Systolic blood pressure. At baseline (black bars), the average blood pressure was not different between the groups. After 2 weeks on the altered diet (gray bars), the blood pressure of both ACE $3/+$ and $1/3$ mice significantly decreased from baseline levels ($P < 0.05$ and $P < 0.01$, respectively). In this state, the average blood pressure of the ACE $1/3$ mice was lower than the other three genotypes, although only the difference between $+/+$ and $1/3$ mice was statistically significant ($P < 0.05$). All data presented are the group mean \pm SEM. The number of mice in each group was as follows: ($+/+$), 9; ($3/+$), 14; ($3/3$), 11; ($1/3$), 8.

The kidney is another organ that expresses abundant ACE in endothelium, proximal tubular epithelium, and glomerular mesangial cells. All the components of the RAS are expressed in the kidney and it is logical to assume that a local, intrarenal RAS may participate in renal function and in the regulation of blood pressure. Mice that overexpress human angiotensinogen selectively in the proximal tubule (and human renin

systemically) have systolic blood pressure >20 mm Hg higher than either nontransgenic mice or those expressing either transgene alone,¹⁹ providing some evidence for the physiological significance of the local renal RAS in vivo. Angiotensin II is thought to promote renal concentrating ability both through direct effects on the nephron and through indirect effects, such as stimulating aldosterone release. Mice lacking angiotensinogen, ACE, the AT_{1A} receptor, or both AT_{1A} and AT_{1B} receptors cannot produce a concentrated urine, and in wild-type mice, the administration of the AT_1 antagonist losartan is sufficient to reduce urine osmolality (reviewed by Oliverio and Coffman²⁰). We have also recently reported that ACE $2/2$ mice have reductions in several medullary transporters, including UT-A1, CIC-K1, NKCC2/BSC1, and AQP1.²¹ However, the precise role of renal ACE and angiotensin II in the urine concentrating mechanism is not well understood. In the ACE $1/3$ mouse, the kidney contains roughly 5.3% to 7.8% the ACE level present in the kidney of a wild-type mouse. This level is significantly less than within the kidney of ACE $3/3$ mice. It is impossible to know if these very low renal ACE levels are of physiological consequence. However, the presence of apparently normal renal function in the ACE $1/3$ model suggests either no crucial role of a local RAS in the kidney or a system able to function with an extremely low threshold for renal ACE expression.

The most unusual feature of the ACE $1/3$ model is the presence of a marked elevation of plasma angiotensin I and angiotensin II peptides in animals with a normal blood pressure. This occurs in conjunction with a substantial elevation of plasma renin concentration, indicating a large increase in the activity of the circulating RAS. Although the precise signal that leads to this upregulation is not clear, it is possible that it is a compensatory response to a decrease in extracellular fluid volume. To examine this possibility, we measured the plasma volume of ACE $1/3$ mice and did not find it to be different from control. It seems logical that the increase in angiotensin I and angiotensin II peptides represent a response to the altered state of the RAS and emphasize the concept of ACE body load. In a wild-type mouse, ACE is abundant and is present both systemically and locally to produce appropriate levels of angiotensin II. In the ACE $3/3$ mouse, there is still abundant ACE with sufficient quantities in the liver and plasma to maintain ACE body load and produce appropriate angiotensin II. In contrast, both the ACE.1 heterozygote (ACE $1/+$) and the ACE.2 heterozygote (ACE $2/+$) have a reduction of ACE body load to $\approx 60\%$ normal. These mice respond with elevated angiotensin I, resulting in normal plasma angiotensin II levels and a normal blood pressure. A different situation is present in the ACE $1/3$ mice. Total body ACE levels are roughly equivalent to those in the ACE.2 heterozygotes. However, both the lack of endothelial ACE and the reduced ACE present in plasma preclude the local production of angiotensin II. The ACE $1/3$ mice respond by elevating both plasma angiotensin I and angiotensin II to levels sufficient to overcome any lack of local production and to establish a normal physiology under basal conditions. This includes not only a normal resting systolic blood pressure but

also normal basal aldosterone levels and renal concentrating ability.

The ACE 1/3 mice demonstrate the homeostatic capacity inherent in the RAS. However, any system will have a threshold beyond which it cannot compensate, and we postulated that ACE 1/3 mice might be vulnerable to conditions of stress associated with upregulation of the RAS. This was tested with a 2-week diet free of salt. Under these extreme conditions, we found a subtle difference in the phenotype of the ACE 1/3 mice from control mice. The ACE 1/3 mice appear to have a very slight reduction in the ability to maximally retain salt. After 2 weeks on a salt-free diet, this results in a slight reduction of blood pressure as compared with wild-type control mice.

That ACE 1/3 mice have an elevation of plasma angiotensin I can be predicted by the reduction in total ACE load in these animals. However, we did not predict that the reduction in ACE alone would induce elevated angiotensin II in the animals. As plasma angiotensin II levels may serve as a proxy for the activity of the endocrine RAS, it is probable that the increase in plasma angiotensin II in the ACE 1/3 mice is a compensation for the loss of local RAS. Thus, the ACE 1/3 animal indicates that the selective expression of hepatic ACE permits the normal regulation of systolic blood pressure and renal function in the mouse, but only after significant compensation by the RAS.

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