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Role of the N-terminal Catalytic Domain of Angiotensin-converting Enzyme Investigated by Targeted Inactivation in Mice*

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Angiotensin-converting enzyme (ACE) produces the vasoconstrictor angiotensin II. The ACE protein is composed of two homologous domains, each binding zinc and each independently catalytic. To assess the physiologic significance of the two ACE catalytic domains, we used gene targeting in mice to introduce two point mutations (H395K and H399K) that selectively inactivated the ACE N-terminal catalytic site. This modification does not affect C-terminal enzymatic activity or ACE protein expression. In addition, the testis ACE isozyme is not affected by the mutations. Analysis of homozygous mutant mice (termed ACE 7/7) showed normal plasma levels of angiotensin II but an elevation of plasma and urine *N*-acetyl-Ser-Asp-Lys-Pro, a peptide suggested to inhibit bone marrow maturation. Despite this, ACE 7/7 mice had blood pressure, renal function, and hematocrit that were indistinguishable from wild-type mice. We also studied compound heterozygous mice in which one ACE allele was null (no ACE expression) and the second allele encoded the mutations selectively inactivating the N-terminal catalytic domain. These mice produced approximately half the normal levels of ACE, with the ACE protein lacking N-terminal catalytic activity. Despite this, the mice have a phenotype indistinguishable from wild-type animals. This study shows that, *in vivo*, the presence of the C-terminal ACE catalytic domain is sufficient to maintain a functional renin-angiotensin system. It also strongly suggests that the anemia present in ACE null mice is not due to the accumulation of the peptide *N*-acetyl-Ser-Asp-Lys-Pro.

The major role of the renin-angiotensin system (RAS)[1] is the regulation of electrolyte homeostasis and blood pressure in mammals. The major effector peptide of the RAS is the octapeptide angiotensin II, which raises blood pressure through a variety of mechanisms, including vasoconstriction and the control of aldosterone release. Angiotensin II is the product of two successive enzymatic cleavages of angiotensinogen. First, renin releases the intermediate decapeptide angiotensin I. Then the last two C terminus amino acids of angiotensin I are removed by the zinc-metallopeptidase, angiotensin-converting enzyme (ACE) (1). ACE inhibitors markedly reduce the formation of angiotensin II and are widely used in the treatment of hypertension, congestive heart failure, and diabetic nephropathy.

In mammals, two isozymes of ACE have been described (2). One isozyme, termed somatic ACE, is made by a variety of somatic tissues, including vascular endothelium, proximal tubular epithelium of the kidney, macrophages, and intestinal epithelium. The other isozyme is only produced by germ cells in the testis and is called testis ACE (3, 4). The study of ACE knock-out mice has provided convincing evidence that testis ACE is critical in male fertility. The two ACE isozymes are produced by the same gene from two different promoters (3).

In mouse, somatic ACE is a single polypeptide chain composed of 1,312 amino acids (5). Somatic ACE is an ectoenzyme where the majority of the protein is exported from cells by an N-terminal signal sequence. However, ACE remains bound to cell membranes by a C terminus hydrophobic domain and a short intracellular domain. Structural analysis of somatic ACE has shown that the extracellular region of the protein is composed of two homologous domains, each containing the HEXXH consensus active site motif typical of zinc-metallopeptidases. Each of these two domains, often termed the N- and C-terminal domains, is independently catalytic (6).

Although renin is an extremely specific enzyme that only cleaves angiotensinogen, ACE is active on a large number of natural substrates, including angiotensin I, bradykinin, Substance P, luteinizing hormone-releasing hormone, and the hemoregulatory peptide *N*-acetyl-Ser-Asp-Lys-Pro (AcSDKP). *In vitro* studies demonstrated that both the N- and C- domains can hydrolyze angiotensin I, bradykinin, and substance P (6–8). However, some significant biochemical differences exist between the two functional domains of ACE (9). First, while both the N- and C- domains are activated by chloride ions, the C-terminal site is highly dependent on their concentration and is inactive in their absence. In contrast, the N-terminal catalytic domain is maximally activated with lower concentrations of chloride ions and retains residual catalytic activity even in their absence. Second, the two catalytic sites show differences in binding and interaction with competitive inhibitors. For example, lisinopril is a more effective inhibitor of the C-terminal catalytic site, whereas captopril has a higher affinity for the N-terminal site. Third, and perhaps most important, the two catalytic sites differ in their affinity and effectiveness in cleav-

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[1] The abbreviations and trivial terms used are: RAS, renin-angiotensin system; ACE, angiotensin-converting enzyme; AcSDKP, *N*-acetyl-Ser-Asp-Lys-Pro; wt, wild-type; ES cells, embryonic stem cells.

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ing individual peptide substrates. For instance, whereas angiotensin I is cleaved with similar kinetics by the two catalytic domains, luteinizing hormone-releasing hormone is preferentially cleaved by the N terminus. AcSDKP, thought to be an inhibitor of bone marrow proliferation, is catalyzed almost exclusively by the N-terminal catalytic domain of ACE (10). Although the specificity of the two ACE catalytic domains has been studied extensively *in vitro*, virtually nothing is known about their relative roles *in vivo*. Thus, the physiologic relevance of each of the two catalytic domains is unknown.

Knock-out mice lacking all ACE activity present with a complex phenotype (11, 12). These animals have a reduction of systolic blood pressure greater than 35 mm Hg as compared with wild-type animals. ACE knock-out mice have reduced aldosterone production and elevated serum concentrations of potassium. The mice are unable to effectively concentrate urine and, even in the absence of water restriction, produce larger amounts of a more dilute urine than wild-type mice. These functional renal defects accompany structural kidney malformations typified by underdevelopment of the renal medulla and papilla. Finally, ACE knock-out mice have a significant and highly reproducible anemia. There are two controversial hypotheses about the mechanism of impaired hematopoiesis in ACE-deficient mice. The first mechanism involves the accumulation of the hemoregulator tetrapeptide AcSDKP in the plasma of these mice. AcSDKP has been described as an inhibitor of bone marrow proliferation, acting to prevent the recruitment of stem cells into S phase (13). The second hypothesis explains the anemia as only due to the lack of angiotensin II and is supported by the correction of the hematocrit in ACE knock-out mice treated with angiotensin II (14).

To assess the physiologic significance of each of the two ACE catalytic domains, we used gene targeting to create a mouse model where the ACE gene was mutated to specifically inactivate its N-terminal catalytic site. This modification does not affect the C-terminal site or the expression level of ACE protein. In addition, expression of testis ACE is normal in this model. Analysis of these mice showed that their blood pressure, renal function, and hematocrit were indistinguishable from wild-type mice. We also studied compound heterozygous mice in which one ACE allele was null (no ACE expression), and the second allele contained the mutations selectively inactivating the N-terminal catalytic domain. These animals produced roughly half the normal levels of the ACE protein and the ACE protein made lacks N-terminal catalytic activity. These mice also have a phenotype indistinguishable from wild-type mice. Thus, this study demonstrates that the presence of the C-terminal ACE catalytic domain is sufficient to maintain a functional RAS. It also strongly suggests that the anemia present in ACE null mice is not because of the accumulation of the peptide AcSDKP.

MATERIALS AND METHODS

Creation of Homozygous Mutant Mice—A 10.7-kb fragment of mouse genomic DNA was cloned from a mouse ES cell library derived from mouse strain 129. This contained 2.4 kb of the somatic ACE promoter and 8.3 kb encompassing somatic ACE exons 1–12. A 3.8-kb EcoR1 fragment containing exons 7–12 was subcloned, and site-directed mutagenesis was used to create the point mutations H395K and H399K in exon 8 of somatic ACE. These specific amino acid changes were previously reported to inactivate the enzymatic activity of the ACE N-terminal catalytic site (6). A second round of mutagenesis was then performed to create a BclI restriction site within intron 7 of somatic ACE. After reconstitution of the 10.7-kb segment of genomic DNA, a self-excising neomycin cassette (CAN-1, GenBank™/AF169416) (15) was cloned into the intron 7 BclI site. Finally, a thymidine kinase cassette was added at the 5′-end of the 10.7-kb segment. The targeting construct was linearized and electroporated into R1 ES cells (16). After positive and negative selection, individual ES cell clones were isolated and screened for targeted homologous recombination using a strategy of polymerase chain reaction (PCR) and genomic Southern blot analysis. Chimeric, heterozygote, and homozygous mutant mice were generated as previously described (12, 17). All studies were performed on F2 generation litters generated from the breeding of F1 heterozygous animals and on some animals obtained by breeding F2 homozygous ACE.7 mice with heterozygous ACE knock-out mice (ACE.1) (12). Age- and gender-matched littermate controls were used in all studies. Animal procedures were approved by the Institutional Animal Care and Use Committee and were supervised by the Emory University Division of Animal Research.

Genotyping—Mice were genotyped by PCR with primers 5′-GCCA-CATTAGCTCAGTATCCC-3′ and 5′-GCATTGCTTAATCCTGGAGTG-G-3′. The wild-type ACE allele amplified a DNA fragment of 241 bp, and the modified ACE.7 allele amplified a DNA fragment of 314 bp. A second set of primers (5′-GGTTGTTCAGACTACAATCTGACC-3′, 5′-A-GCTTCAGGGCTCTGCTTTCCTGC-3′, and 5′-CAGACGAGCTCGGG-ATCTGGGTTGTCTGGT-3′) was used to amplify a fragment of 287 bp in the presence of the null ACE.1 allele and a DNA fragment of 215 bp from the wild-type or the ACE.7-modified allele.

Characterization of the Mutant ACE Enzyme—Mice were exsanguinated by cardiac puncture, and blood was collected on ice in heparinized tubes. Plasma was immediately frozen and stored at −80 °C. ACE activity was then measured using different substrates to evaluate the separate activity of each ACE domain. Hip-His-Leu and *N*-acetyl-Ser-Asp-acetyl-Lys-Pro were used as C- and N- domain-specific substrates (6, 18). Angiotensin I is both a C and N domain substrate (17). Assays were performed at 37 °C with 5 or 10 μl of mouse plasma during 30 or 60 min. Substrate concentration was 2.5-fold K_m. The rate of hydrolysis of all substrates used was resolved and quantified by reverse HPLC (Waters Co, Milford, MA). Results were expressed in nmol/ml/min of generated hippuric acid, acetyl-Lys-Pro, and angiotensin II, respectively.

Tissue ACE Expression—Tissue ACE activity and Western blotting were performed as previously described (19). For the preparation of protein extracts, animals were euthanized under anesthesia and organs were weighted and then frozen in liquid nitrogen. Later, each organ was gently homogenized in ACE buffer (HEPES, pH 7.4, 50 mmol/liter; NaCl, 150 mmol/liter; $ZnCl_2$, 25 μmol/liter; and polyvinylidene difluoride, 1 mmol/liter). After centrifugation, the pellets were vigorously rehomogenized with ACE buffer containing 0.5% Triton X-100. After a second centrifugation, the supernatant was collected and protein concentration was measured with the BCA protein assay reagent kit (Pierce) following the manufacturer's instructions. ACE activity was measured using the ACE-REA kit from American Laboratory Products Company, Ltd. (ALPCO, Windham, NH). ACE activity was defined as that inhibitable by captopril.

For Western blotting, 20 μg of total proteins were separated by SDS-PAGE on a 10% gel and transferred to a nitrocellulose membrane. The membranes were probed with a 1:250 dilution of a rabbit polyclonal anti-mouse ACE antibody and exposed to X-ray film using enhanced chemiluminescence.

Blood Pressure—Systolic blood pressure was measured using a Visitech Systems BP2000 (Apex, NC) automated tail cuff system as previously described (12, 20). Briefly, mice were trained for 5 days prior to data collection. Systolic blood pressure was then measured in two sets of 10 measurements per day. The systolic blood pressure for an animal was the average blood pressure obtained over 4 days and consisted of 8 sets of 10 measurements.

To measure plasma levels of angiotensin I and II, mice were exsanguinated by cardiac puncture and blood was collected on ice in tubes containing potassium-EDTA (1.6 mg/ml), amastatin (100 μmol/liter), bestatin (100 μmol/liter), and lisinopril (4 μg/ml) (14). Plasma was immediately frozen and stored at −80 °C. Angiotensin I and II levels were measured by radioimmunoassay as previously described (21).

Blood Pressure after Infusion of Angiotensin I and II—Mice were anesthetized by an intraperitoneal injection of a ketamine and xylazine mixture (125 and 12.5 mg/kg, respectively). Animals were then placed on a 37 °C platform throughout the procedure. A polyethylene catheter (PE 10; Becton Dickinson) was inserted into the right carotid artery for direct blood pressure measurement using an MLT844 blood pressure transducer (ADInstruments, Colorado Springs, CO). A separate catheter was inserted into the left jugular vein for drug administration. After blood pressure stabilized, 1 μl/g body weight of heparinized saline (50 units/ml heparin in 0.9% saline) was injected as a control. 1 μl/g body weight of 1 μM Ang I or Ang II (Sigma) was then injected, and the increase of mean arterial blood pressure was recorded.

Plasma Renin Concentration—Plasma renin concentrations (PRC)

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were measured by enzymatic assay as previously described (22, 23). Briefly, PRC was determined by measuring the *in vitro* production of angiotensin I by radioimmunoassay after incubation with an excess of angiotensinogen from binephrectomized rat plasma.

Plasma and Urinary AcSDKP Measurement—For plasma and urinary AcSDKP determinations, 10^{-5} M lisinopril (Merck Sharp and Dohme) was added to heparinized tubes to prevent AcSDKP degradation by ACE. AcSDKP concentration was determined, directly in the urine or after methanol extraction from the plasma, by a competitive enzyme immunoassay (Spi Bio, Massy, France) as previously described (24). Urine creatinine was measured by automated enzymatic methods (Kodak Biolyzer; Eastman Kodak).

Anemia and Hematocrit—To induce anemia, phenylhydrazine (60 mg/kg) was administrated intraperiteoneally on two consecutive days (25). Hematocrit was measured 1, 3, 5, 8, and 13 days after the second injection. To measure hematocrit, mice were bled from the tail vein and blood collected in heparinized self-sealing capillary tubes (Safe-Tec; ClinicalProducts, Inc., Ivyland, PA). These were centrifuged for 4 min at 12,000 × *g* and read in a manual capillary reader.

Urine Osmolality—To determine urine concentrating ability, mice were water deprived for 24 h but given food *ad libitum*. Before and at the end of this 24-h period, a spot urine osmolality was determined using a Westcat 5500 vapor pressure osmometer (Wescor, Inc., Logan, UT). The mice were allowed to rehydrate for 3 weeks before any other experiments.

Statistical Analysis—All data were expressed as means ± S.E. The significance of the difference between two groups was evaluated by an unpaired Student's *t* test. The significance of the difference among multiple groups was obtained by analysis of variance analysis. A *p* value of <0.05 was considered statistically significant.

RESULTS

Generation of Genetically Modified Mice—Both the ACE N- and C-terminal domains contain the protein motif HEMGH that is necessary for zinc binding and catalytic activity (Fig. 1*A*). *In vitro*, the conversion of this motif to KEMGK eliminates zinc binding and all catalytic activity (6). To generate mice with a functional inactivation of the N-terminal domain, we used targeted homologous recombination to modify exon 8 as illustrated in Fig. 1*B*. A 10.8-kilobase portion of genomic DNA served as the template for the construct. *In vitro* mutagenesis was used to convert the zinc-binding histidines (amino acids 361 and 365) into codons encoding lysines. In addition, a self-excising neomycin cassette was positioned within intron 7. This cassette consists of a neomycin resistance gene driven by an RNA polymerase II promoter and Cre-recombinase driven by the testis ACE promoter (15). The cassette is flanked with Lox P sites. This neomycin cassette is stable in ES cells and in chimeric founder mice. However, it is excised by Cre-recombinase expression in the developing male germ cells of the chimeric animals and is therefore absent in F1 heterozygous mice. This strategy allowed generation of mice with an intact ACE gene except for the N-terminal catalytic site mutations and a 34-bp Lox P sequence in the seventh intron. Because these mice were the seventh strain of genetically mutated mice prepared in our laboratory, they are referred to as ACE.7; the genotype of homozygous mutated animals is termed ACE 7/7, whereas the heterozygous and wild-type mice have ACE genotypes termed 7/wt and wt/wt.

Targeted homologous recombination of mouse ES cells was carried out using standard techniques. Proper genomic targeting was verified by PCR, genomic Southern blot analysis using a DNA probe 3′ to the targeting construct, and selected sequence analysis of the ACE gene. To generate homozygous ACE 7/7 mice, we mated F1 heterozygous mice. Of 377 F2 pups, 53% were heterozygous (ACE 7/wt), 26% wild-type (ACE wt/wt), and 22% homozygous mutant (ACE 7/7). We observed no neonatal deaths between birth and weaning.

Compound Heterozygote Mice—Previously, our laboratory characterized ACE knock-out mice, called ACE.1, that were prepared using targeted homologous recombination. The ACE 1 allele is null, and homozygous mutant mice, termed ACE 1/1, lack all expression of somatic and testis ACE. The mating of ACE.1 heterozygous mice (genotype 1/wt) with ACE 7/7 mice generated compound heterozygous mice, termed ACE 1/7, with Mendelian fidelity. ACE 1/7 mice have one copy of the ACE null allele (the ACE 1 allele) and one copy of the ACE 7 allele (N-terminal catalytically inactive).

ACE Expression and Enzymology—To verify the inactivation of the N-terminal active site of ACE 7/7 mice, we measured the

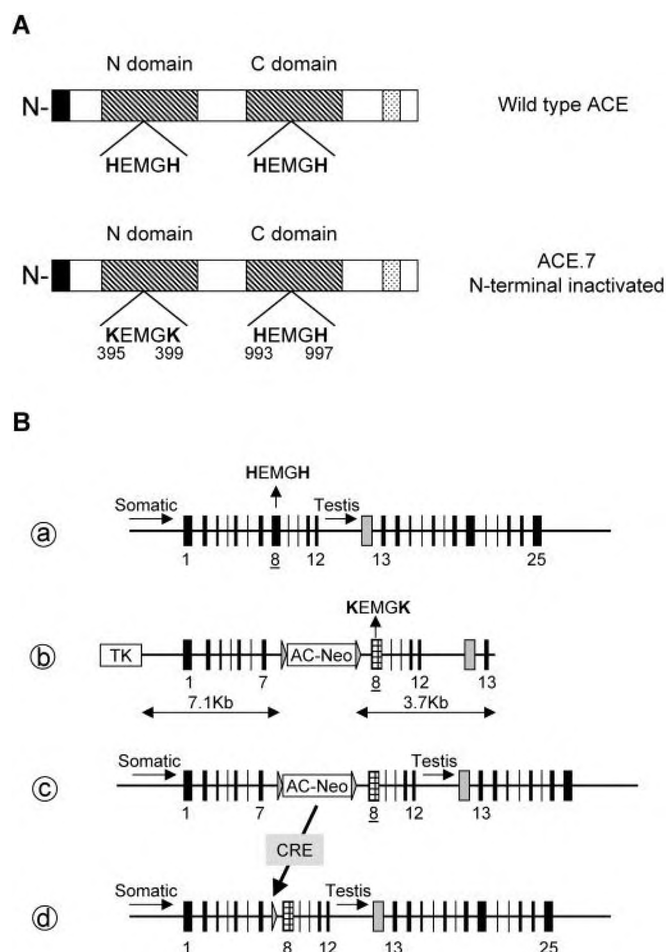

FIG. 1. **Creation of ACE.7 mice.** *A*, the wild-type ACE protein is an ectoenzyme with a signal sequence (*black bar*) at the N terminus of the protein. There are two homologous catalytic domains (*hatched boxes*) that each bear the amino acid sequence HEMGH. These domains are responsible for binding the two zinc molecules that catalyze enzymatic activity. Finally, at the C terminus of ACE, there is a hydrophobic domain (*dotted box*) that is responsible for the retention of the protein in cell membranes. *B*, the wild-type ACE allele is diagramed in the "*a*" portion of the figure. *Arrows* indicate both the somatic and testis ACE promoters. *Black boxes* represent exons 1–25 of somatic ACE. A testis-specific exon is indicated in the *gray box* located between exons 12 and 13. The N-terminal zinc-binding domain is found within exon 8 of somatic ACE. The targeting vector is diagramed in the "*b*" portion of the figure. The left and right arms of the targeting construct were 7.1- and 3.7-kb portions of ACE genomic DNA from mouse strain 129. Site-directed mutagenesis was used to convert the zinc-binding domain within the eighth intron from HEMGH to KEMGK (H395K and H399K). A self-excising neomycin cassette (*AC-Neo*) was inserted into the seventh intron of the ACE gene. A thymidine kinase cassette, used for negative selection, was inserted at the 5′-end of the construction. The "*c*" portion of the figure shows the ACE gene after successful gene targeting. When this modified allele was passed through a male animal, the testis ACE promoter, located within the self-excising neomycin cassette, induced Cre-recombinase expression and neomycin cassette excision. The final modified ACE allele is shown in "*d*." Point mutations are present in exon 8, and a residual 34-base pair loxP sequence (*gray triangle*) is present in the seventh intron.

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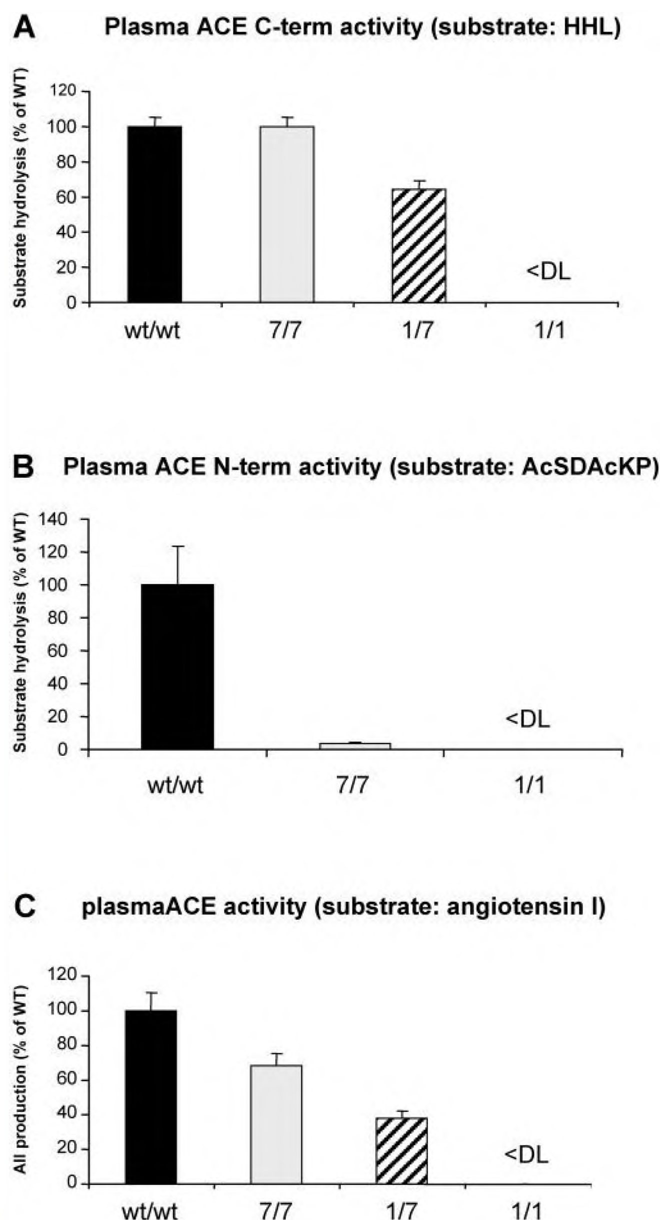

FIG. 2. **ACE substrate specificity.** ACE activity was measured in the plasma from wild-type (*wt/wt*), ACE 7/7, ACE 1/7, and ACE 1/1 mice using the C-terminal-specific ACE substrate HHL (*A*), the ACE N-terminal-specific substrate AcSDAcKP (*B*), and angiotensin I (*C*). Plasma from ACE 7/7 mice showed equivalent C-terminal activity to that in the plasma of wild-type mice. In contrast, plasma from ACE 7/7 mice had very little activity against the N-terminal-specific substrate. Plasma from ACE 1/7 mice showed 65 and 56% the activity of ACE 7/7 plasma against the C-terminal substrate HHL and angiotensin I, respectively. Plasma from 1/1 mice showed catalytic activity below the detection limit (*<DL*) against any ACE substrate. In each panel, $n = 11$ (wt/wt), 14 (7/7), 10 (1/7), and 5 (1/1).

catalytic activity of plasma using synthetic ACE substrates specific for either the C- or N-terminal catalytic domains (Fig. 2*A*). To measure C-terminal catalytic activity, the substrate HHL was used. As anticipated, there was no significant difference in enzymatic activity between plasma from wild-type (wt/wt) and ACE 7/7 mice with this substrate. In contrast, ACE 1/7 mice had a 35% reduction of catalytic activity, reflecting reduced ACE expression because of the ACE 1 allele. Plasma from ACE 1/1 mice showed no activity against HHL or any ACE substrate.

To investigate N-terminal ACE catalytic activity, hydrolysis of the N-terminal-specific peptide AcSDAcKP was measured (Fig. 2*B*). This peptide was efficiently cleaved by wild-type plasma, but very little enzymatic activity was exhibited by the plasma from ACE 7/7 mice (3.3 ± 1.1% of wild-type). We believe this residual activity is low-level cleavage of the substrate by the C-terminal domain and not nonspecific hydrolysis, because plasma from ACE 1/1 mice showed no such activity.

We also investigated the ability of plasma ACE to convert angiotensin I to angiotensin II (Fig. 2*C*). ACE activity in 7/7 plasma was 68% that of wild-type mice. A reduction of about this magnitude was expected, given the known K_{cat} values for angiotensin I hydrolysis by each of the two ACE catalytic domains (11 s^{-1} for the N terminus, 34 s^{-1} for the C terminus). Also as expected, the ACE 1/7 plasma showed activity that was 38 and 56% that of wild-type and ACE 7/7 plasma, respectively. Thus, the enzymatic characterization of ACE 7/7 and ACE 1/7 mice is consistent with our molecular genotype and establishes that these mice produce an ACE protein that lacks N-terminal catalytic activity. ACE 1/7 mice produce less of this enzyme because of the presence of the ACE 1 null allele.

Although ACE is found in plasma, the majority of the protein in an animal is bound to tissues such as the lung or kidney. To quantitate the tissue expression of ACE, we prepared detergent homogenates of lung, kidney, testis, brain, heart, and liver from wild-type, ACE 7/7, and ACE 1/7 mice (Fig. 3*A*). First, we measured ACE activity using [^{3}H]hippuryl-glycylglycine as a substrate. This showed a consistent pattern exemplified by lung ACE expression. Compared with wild-type, ACE 7/7 mice had a modest reduction of activity (20%). This is probably due to a lack of N-terminal activity against this substrate. An equivalent reduction was observed in all tissues examined. In contrast, lung ACE activity in ACE 1/7 mice was 46 and 58% of wild-type and ACE 7/7. As with plasma, these figures reflect reduced ACE protein.

To directly evaluate levels of protein expression, Western blot analysis was performed on the tissue homogenates using a polyclonal rabbit anti-mouse ACE antibody (Fig. 3*B*). Wild-type ACE is a protein of ~170 kDa, whereas testis ACE is 95 kDa. As with ACE activity, all organs showed a similar pattern in which there was no difference in ACE expression between wild-type and ACE 7/7 mice. In contrast, ACE 1/7 mice showed a reduction of somatic ACE that reflected the presence of the ACE.1 allele, which is null for ACE expression. All three groups of mice showed an equivalent amount of testis ACE. This is expected, because our strategy does not affect either the expression or activity of this isozyme.

Blood Pressure—To study the physiology of the mice, systolic blood pressure was measured in wild-type (wt/wt), heterozygous (7/wt), ACE 7/7, and ACE 1/7 mice (Fig. 4). Blood pressure was obtained in conscious, trained mice by tail cuff measurement. Fig. 4 shows the individual blood pressure determinations as well as the group mean for each genotype. Both ACE 7/7 and ACE 1/7 mice have systolic blood pressures indistinguishable from those of wild-type or heterozygous mice (7/wt). Specifically, whereas wild-type mice had an average blood pressure of 105.0 ± 2.2 mm Hg, ACE 7/7 and 1/7 mice had average systolic blood pressures of 110.1 ± 2.2 and 107.8 ± 1.9 mm Hg.

Angiotensin and AcSDKP Peptide Levels—To understand blood pressure control in ACE 7/7 and ACE 1/7 mice, we measured the plasma concentrations of angiotensin I and II. As seen in Fig. 5, these peptides are found in levels identical to those measured in wild-type mice. Thus, the inactivation of the N-terminal catalytic domain had no effect on the ability of ACE 7/7 mice to produce plasma angiotensin II. Indeed, the normal plasma levels of angiotensin I suggest that there is little compensation of the RAS necessary for maintaining normal blood pressure. We also measured plasma renin activity in wild-type,

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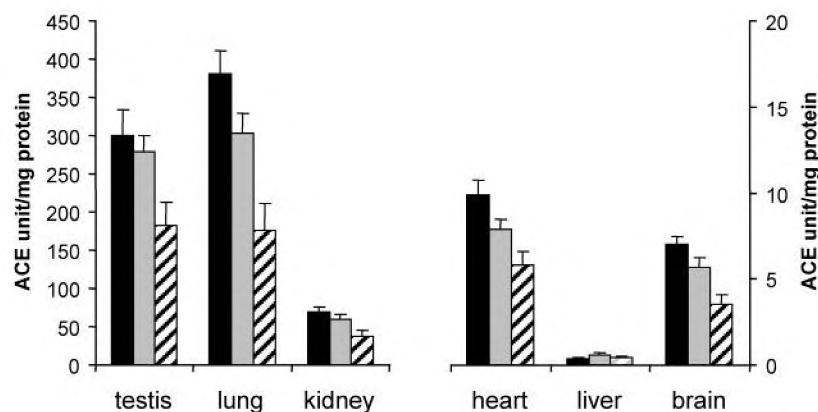

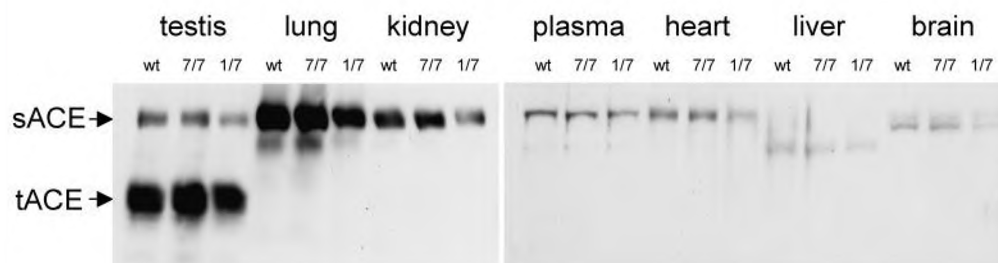

FIG. 3. **Tissue ACE expression.** *A*, ACE activity was measured in tissue homogenates using the substrate [^{3}H]hippuryl-glycyiglycine. In lung tissue, ACE 7/7 mice (*gray boxes*) had a modest reduction of ACE activity compared with wild-type animals (*black boxes*). A similar small reduction of ACE activity (compared with wild-type) was seen in all the organs from ACE 7/7 mice. ACE 1/7 mice (*hatched boxes*) showed a much more marked reduction of ACE activity. The number of mice measured in each group was 6. *B*, ACE protein was quantitated using Western blot analysis of tissue homogenates. ACE was detected using a polyclonal rabbit anti-ACE antibody. Somatic ACE (*sACE*) is a protein of ~170 kDa, whereas testis ACE (*tACE*) is 95 kDa. Tissue ACE levels in ACE 7/7 mice were no different from those of wild-type (*wt*) mice. In contrast, ACE 1/7 mice showed a significantly smaller amount of somatic ACE protein compared with either wild-type or ACE 7/7 mice. Testis ACE expression was not different between the three genotypes. The *left* and *right panels* were exposed to x-ray film for 5 s and 1 min, respectively.

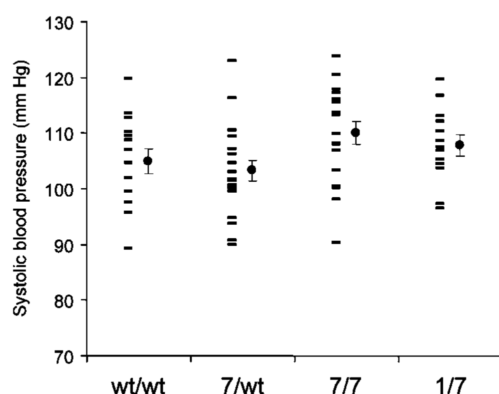

FIG. 4. **Blood pressure.** Systolic blood pressure was measured in conscious wild-type (*wt/wt*), heterozygous (*7/wt*), ACE 7/7, and ACE 1/7 mice. Blood pressure was measured using an automated tail cuff as previously described (12, 20). Individual blood pressure determinations are indicated (*dashes*) as well as group means (●) ± S.E. There was no significant difference in blood pressure between any of the four groups. The number of animals measured was greater than 13 for each genotype.

ACE 7/7, ACE 1/7, and ACE 1/1 mice. This confirms the above conclusion because the concentration of renin in the plasma of ACE 7/7 mice is not different from that of wild-type. Not surprising, the ACE 1/7 mice have an elevation of plasma renin in response to the reduced expression of ACE (2.3-fold wild-type, $p < 0.02$). However, this is far smaller in magnitude than the increase of renin levels observed in ACE 1/1 mice.

To evaluate ACE function in the ACE 7/7 and ACE 1/7 mice, we measured the change in blood pressure after the acute intravenous infusion of angiotensin I and compared this to the change in blood pressure after intravenous infusion of angiotensin II (Fig. 6). For this experiment, mice were anesthetized and prepared with an arterial catheter to continually monitor blood pressure. A venous catheter was used for peptide infusion. These studies showed no difference in response in the magnitude, time response, or shape of the pressure curves between wild-type and either ACE 7/7 or ACE 1/7 mice. As a control, we used a strain of ACE knock-out mice called ACE 4/4 (26). These mice are similar to ACE null mice (ACE 1/1) except that they retain normal expression of testis ACE. As expected for animals deficient in somatic ACE, their response to angiotensin I was markedly diminished. These experiments show that, functionally, ACE 7/7 and even ACE 1/7 mice are able to

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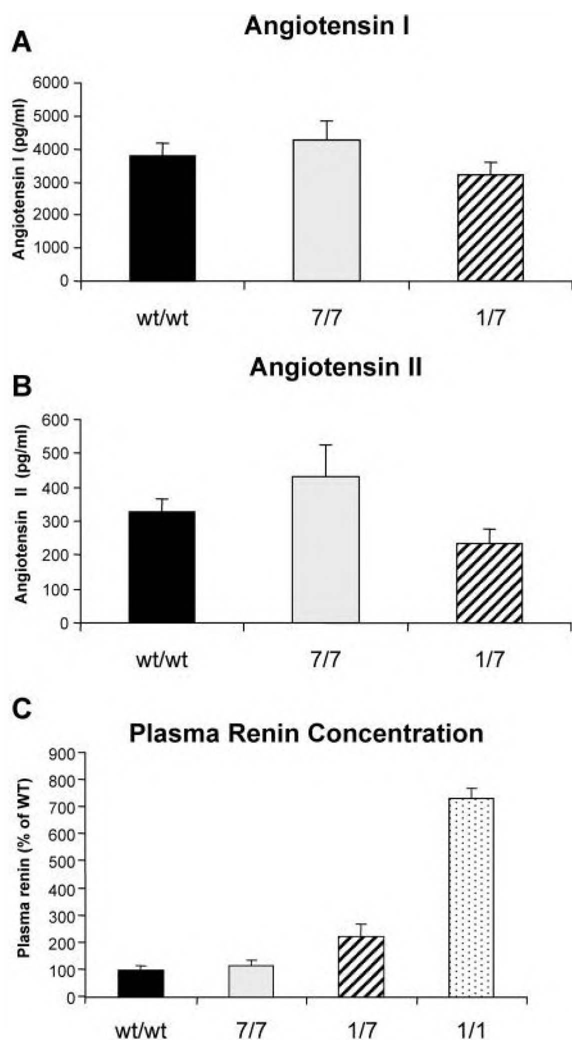

FIG. 5. **Plasma angiotensin and renin concentration.** *A* and *B*, plasma angiotensin I and II peptide levels were determined by radioimmunoassay for wild-type (*wt/wt*), ACE 7/7, and 1/7 mice. $n = 8$ (wt/wt), 12 (7/7), 11 (1/7). *C*, plasma renin concentration was measured in the presence of an excess of rodent angiotensinogen. Wild-type (*wt/wt*) and ACE 7/7 mice have the same plasma renin concentration. In contrast, plasma renin was elevated in ACE 1/7 mice (*hatched box*, $p < 0.02$) and markedly elevated in ACE knock-out mice (1/1). $n = 11$ (wt/wt), 14 (7/7), 10 (1/7), 4 (1/1).

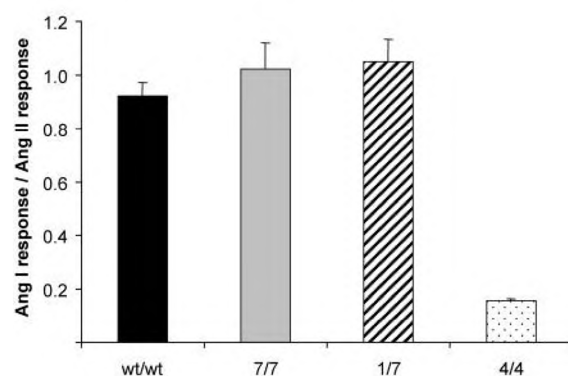

FIG. 6. **Infusion of angiotensin I and II.** Mice were anesthetized and prepared with both venous and arterial catheters. After stabilization of blood pressure, the increase in mean arterial blood pressure in response to angiotensin I and II infusions was measured in wild-type (*wt/wt*), ACE 7/7, ACE 1/7, and ACE knock-out (*4/4*) mice. The data are plotted as the pressure increase in response to angiotensin I divided by the increase in response to angiotensin II. There is no significant difference in the response of wild-type, ACE 7/7, and ACE 1/7 mice to the peptide infusion. In contrast, ACE 4/4 mice have a much reduced response to angiotensin I infusion. $n = 5$ (wt/wt), 6 (7/7), 4 (1/7), 3 (4/4).

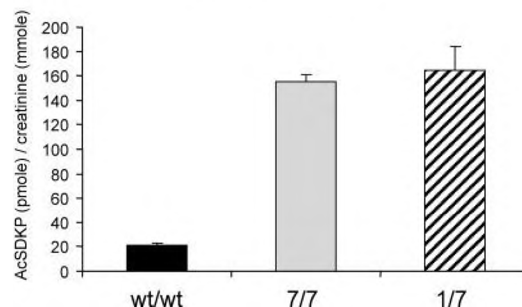

FIG. 7. **Urine concentration of AcSDKP.** The concentration of the tetrapeptide AcSDKP was determined by enzyme-linked immunosorbent assay in the urine of wild-type (*wt/wt*), ACE 7/7, and ACE 1/7 mice. AcSDKP concentrations were corrected for the creatinine concentrations. The concentrations of AcSDKP were increased in those mice lacking the N terminus catalytic site of ACE. $n = 8$ (wt/wt), 9 (7/7), 10 (1/7).

efficiently process angiotensin I. The lack of any difference between these mice and wild-type suggests that the ACE C-terminal catalytic site alone is sufficient for the processing of angiotensin I.

A specific substrate of the ACE N-terminal catalytic domain is the tetrapeptide AcSDKP (7). Clinical evaluation of patients treated with ACE inhibitors showed a marked elevation of plasma and urinary AcSDKP concentrations (27–29). Further, ACE null mice have elevated AcSDKP plasma concentrations, which raised the question as to whether the anemia in these animals may, in part, be due to this peptide. To study this question, we measured concentrations of AcSDKP in ACE.7 mice (Fig. 7). In plasma, the levels of this peptide were elevated in both ACE 7/7 and ACE 1/1 mice as compared with wild-type (data not shown). The AcSDKP levels in urine are also elevated in the groups lacking N-terminal ACE activity (ACE 7/7 and ACE 1/7). For example, whereas wild-type mice have urinary AcSDKP levels of about 21 pmol/mmol creatinine, ACE 7/7 mice have levels of 155 pmol/mmol creatinine. As discussed below, these differences in urinary AcSDKP are not because of differences in urinary concentrating ability. A possible explanation is that, normally, renal ACE may degrade urinary AcSDKP and, in the absence of ACE with a functional N-terminal catalytic activity, there is a marked elevation of peptide concentration.

AcSDKP has been suggested as an erythrocyte lineage bone marrow-suppressive peptide (30). Because levels of this peptide are elevated in ACE 7/7 and ACE 1/7 mice and reach a similar level to that noted in ACE 1/1 mice, we measured the hematocrit in these mice. As shown in Fig. 8*A*, there was no significant difference between the hematocrit of wild-type, heterozygous, ACE 7/7, and ACE 1/7 mice. We also studied the response to anemia. Red blood cell lysis was induced by injecting the mice with phenylhydrazine (Fig. 8*B*). In the recovery from the anemia, there was no significant difference between wild-type, ACE 7/7, and ACE 1/7 mice. These data suggest that, in mice, the elevations of AcSDKP seen in the absence of N-terminal ACE activity are not associated with major disturbances of erythropoiesis. Because AcSDKP is an N-terminal-specific substrate of ACE, the anemia observed in ACE null mice is probably due to factors other than AcSDKP.

ACE null mice show abnormal development of the kidney:

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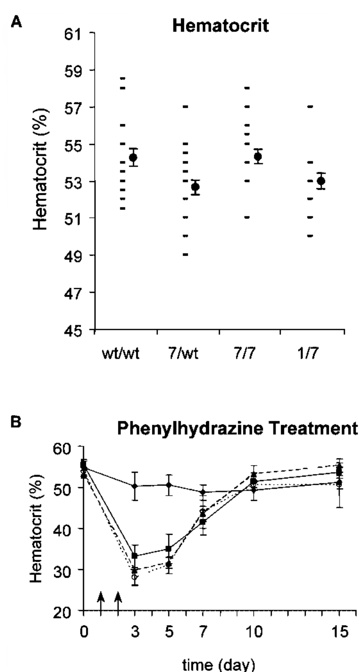

FIG. 8. **Hematocrit.** *A*, hematocrit was measured for wild-type (*wt/wt*), heterozygous (*7/wt*), ACE 7/7, and ACE 1/7 mice (n = 13–24 for each group). Individual measurements are indicated by a *dash*. The group mean is represented by a (●) ± S.E. No significant differences were noted. *B*, phenylhydrazine was administered intraperitoneally on days 1 and 2 (*arrows*) to wild-type (■), ACE 7/7 (▲), and ACE 1/7 (○) mice. Hematocrit was measured as indicated. Control, wild-type mice (◆), were injected with saline alone. No significant differences were observed for hematocrit recovery of wild-type, ACE 7/7, and 1/7 mice. n = 5 for wt/wt and ACE 7/7; n = 4 for ACE 1/7 and control mice.

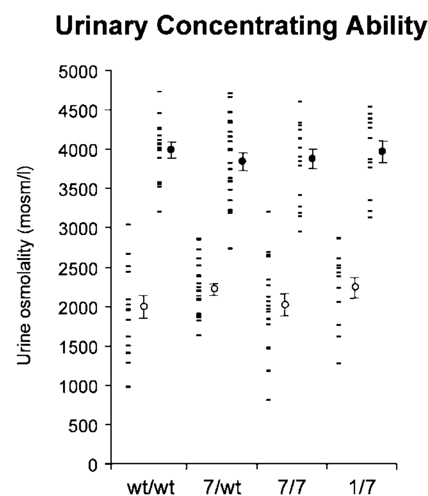

FIG. 9. **Renal concentrating ability.** Spot urine was collected and osmolality was measured for wild-type (*wt/wt*), heterozygous (*wt/7*), ACE 7/7, and ACE 1/7 mice under conditions of free water availability (○ ± S.E.) or after 24 h of water deprivation (● ± S.E.). Individual data values are shown by a *dash*. We found no difference in concentrating ability between the different groups of mice. n >13 for each group.

there is expansion of the renal calyx, underdevelopment of the medulla and papilla, thickening of renal arterioles, and the inability to concentrate urine (12). ACE 7/7 and ACE 1/7 mice were evaluated for renal concentrating ability by water restriction for 24 h (Fig. 9). These animals behaved identically to wild-type mice. With water present *ad libitum*, all groups of mice produced urine with an average osmolality of about 2000 mOSM/liter. In response to water deprivation, all groups elevated urinary osmolality to greater than 3,500 mOSM/liter. At sacrifice, careful evaluation showed normal gross renal development in the ACE 7/7 and ACE 1/7 mice. Histologic examination confirmed no significant differences between ACE 7/7, ACE 1/7, and wild-type mice.

DISCUSSION

For many years, ACE was thought to have a single catalytic domain (31), but the cloning of the mammalian gene showed a more complex structure (5, 32). It is now known that somatic ACE is composed of two homologous protein domains, each of which binds zinc and each of which is catalytic (8). Although the two domains of ACE are ~68% identical in amino acid sequence, higher conservation (89%) is present in the center of each domain where amino acids active in catalysis have been identified by comparison with the active sites of other metallopeptidases. ACE is encoded by a single genetic locus located on human chromosome 17 (mouse chromosome 11) where each of the two catalytic domains of the enzyme is encoded by a cluster of eight exons. In each domain, the corresponding exons are similar in size and in the codon phase at the exon/intron boundaries. The similarities of the exon/intron organization of the two clusters strongly suggest that the mammalian ACE gene is the result of an ancestral gene duplication. Interestingly, the ACE locus contains two ACE promoters that give rise to two distinct ACE isozymes: somatic and testis ACE. The testis ACE promoter is located in the twelfth intron of the somatic ACE gene and testis ACE corresponding to the C-terminal half of the somatic ACE protein.

The presence of testis ACE with a single catalytic domain and the identification of ACE homologs in *Drosophila*, also with a single catalytic domain (33), inevitably raises the question as to why mammalian somatic ACE has retained a gene duplication during evolution. Specifically, scientists have wondered whether the gene duplication resulted in specific properties of ACE that are critical for its diverse physiologic roles. Although the two domains of somatic ACE are highly homologous, their biochemical properties are slightly different in terms of chloride dependence, inhibitor affinity, and substrate kinetics (7, 9). For example, both angiotensin I and bradykinin are cleaved with near equal efficiency by the two ACE catalytic domains. In contrast, some substrates, such as AcSDKP, are much more efficiently hydrolyzed by one catalytic domain (in the case of AcSDKP, it is the N-terminal domain). To investigate the physiologic importance of the two ACE catalytic domains *in vivo*, we generated a new mouse model in which the N-terminal domain of ACE was inactivated without any alteration of protein expression. Because the ACE modification involved point mutations impairing the zinc-binding properties of the N-terminal catalytic site, the tissue pattern of expression and the level of protein expression are no different from wild-type mice. This approach is different from that used by Kessler *et al.* who created a transgenic mouse model in which rabbit testis ACE was expressed under the control of a tissue-specific promoter (Tie-1 or γ-glutamyltransferase) in a background mouse strain that was null for the endogenous ACE gene (an ACE knock-out mouse (40)). Evaluation of ACE 7/7 mice showed that ACE enzymatic properties were consistent with our genetic design. The conversion of angiotensin I to angiotensin II in plasma was 68% that of wild-type mice. In contrast, ACE 7/7 mice hydrolyzed AcSDAcKP with only 3% of the efficiency of control mice and had an elevation of both plasma and urine concentrations of AcSDKP. AcSDKP was initially purified from bone marrow and described as a regulator of hematopoiesis (30). It inhibits the proliferation of hematopoietic stem cells *in vitro* and when injected in mice (34–38). Because of this, it was hypothesized that the anemia observed in ACE knock-out mice might be the consequence of the accumulation of this peptide. ACE 7/7 mice have comparable AcSDKP levels with ACE knock-out mice but,

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surprisingly, have no reduction of hematocrit. Further, ACE 7/7 and ACE 1/7 mice reconstitute a normal hematocrit after chemically induced anemia with the same time course as wild-type mice. These data suggest that AcSDKP accumulation in ACE 7/7 mice has no significant effect on erythropoiesis. This conclusion is consistent with a study of ACE-deficient mice suggesting that the anemia in these animals may be because of reduced levels of angiotensin II (14). In fact, infusion of angiotensin II in these mice restored a normal hematocrit.

We also studied ACE 1/7 mice in which one of the two ACE alleles is null, resulting in only a single functional ACE allele that itself lacks N-terminal catalytic activity. In this model, tissues such as the lung have approximately half the ACE activity seen in ACE 7/7 mice and ~42% the activity of wild-type mice. Not surprising, plasma renin activity in ACE 1/7 mice was significantly greater than either wild-type or ACE 7/7 mice. However, comparison of the renin levels in ACE 1/7 mice *versus* those present in ACE 1/1 knock-out animals immediately demonstrated that the compensatory ability of the RAS was far from maximally engaged in the ACE 1/7 model. Perhaps because of this, the phenotype of ACE 1/7 mice was identical to wild-type in terms of blood pressure, renal structure, renal concentrating ability, and hematocrit.

In humans, the deletion/insertion (D/I) polymorphism of the ACE gene is associated with changes in plasma levels of ACE (39). Humans with the I/I phenotype have ~63% the plasma ACE of D/D individuals. Thus, for the conversion of angiotensin I to angiotensin II, the human I/I phenotype may be compared with the reduction of plasma ACE found in ACE 1/7 mice. Many human studies have examined the pathophysiologic effects of the ACE polymorphisms, and the literature is both complicated and contradictory. In our experience, we saw no increased mortality of ACE 1/7 mice as compared with wild-type. However, in these animals, we have not formally evaluated the disease models, such as heart disease and diabetes, that have been the subject of human studies.

In summary, we have created model strains of mice in which point mutations were introduced into the ACE N-terminal catalytic domain, rendering this domain catalytically inactive. In both the homozygous mutant mouse (ACE 7/7) and a compound heterozygous mouse (ACE 1/7), blood pressure control was no different from wild-type mice. Indeed, the normal phenotype of these mice suggests that the two ACE catalytic domains are functionally redundant. Although the N-terminal catalytic domain hydrolyzes unique substrates such as AcSDKP, we see no unique physiologic effects of this catalytic site under the present experimental conditions.

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