Angiotensin-Converting Enzyme C-Terminal Catalytic Domain Is the Main Site of Angiotensin I Cleavage In Vivo

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Abstract—Angiotensin-converting enzyme (ACE) plays a central role in the production of the vasoconstrictor angiotensin II. ACE is a single polypeptide, but it contains 2 homologous and independent catalytic domains, each of which binds zinc. To understand the in vivo role of these 2 domains, we used gene targeting to create mice with point mutations in the ACE C-domain zinc-binding motif. Such mice, termed ACE13/13, produce a full-length ACE protein with tissue expression identical to wild-type mice. Analysis of ACE13/13 mice showed that they produce ACE having only N-domain catalytic activity, as determined by the hydrolysis of domain specific substrates and by chloride sensitivity. ACE13/13 mice have blood pressure and blood angiotensin II levels similar to wild-type mice. However, plasma renin concentration is increased 2.6-fold and blood angiotensin I levels are increased 7.5-fold. Bradykinin peptide levels are not different from wild-type levels. ACE13/13 mice have a reduced increase of blood pressure after intravenous infusion of angiotensin I. ACE13/13 mice have a normal renal structure, but they are not able to concentrate urine after dehydration as effectively as wild-type mice. This study shows that the C-domain of ACE is the predominant site of angiotensin I cleavage in vivo. Although mice lacking C-domain activity have normal physiology under laboratory conditions, they respond less well to the stress of dehydration. (Hypertension. 2008;51:267-274.)

Key Words: angiotensin-converting enzyme ■ angiotensin ■ blood pressure ■ male infertility ■ catalytic sites ■ bradykinin ■ genetic mice model

Angiotensin-converting enzyme (ACE; EC3.4.15.1) is a di-carboxypeptidyl peptidase that is an integral part of the renin-angiotensin system.1 ACE converts Ang I to the vasoconstrictor peptide Ang II. It also cleaves bradykinin and other peptides, including substance P, gonadotropin-releasing hormone, the tetrapeptide N-acetyl-Ser-Asp-Lys-Pro, and angiotensin 1–7. The localization of ACE on endothelial cell membranes generates Ang II in close proximity to the vascular smooth muscle, a critical target organ. This and several other physiological mechanisms are part of the critical role of the renin-angiotensin system in the regulation of blood pressure and electrolyte balance. In addition to cardiovascular effects, ACE influences several other physiological systems, including reproduction, hematopoiesis, and renal development. Although the exact biochemistry of these additional actions is not completely understood, the physiological consequences of ACE action are observed in the complex phenotype of mice lacking all ACE expression.2,3

The ACE protein is a single polypeptide chain, composed of 2 separate and independent catalytic domains. Each domain contains the zinc-binding site HEMGH; these domains, called N- and C- domains, have a high conservation of sequence and exon structure, arguing that they originated from a gene duplication event during the course of evolution.4 Further complicating the story, there are 2 isozymes of ACE called somatic and testis ACE.5 The larger somatic isoform (sACE) is the enzyme expressed in somatic tissues, and it is this form that is composed of 2 catalytic domains. The testis isoform (tACE) is expressed from an alternate promoter found within the 12th intron of the ACE gene. Because of this, tACE is composed of only 1 catalytic domain, identical to that of the C-domain of sACE. Although the N- and C-terminal domains of sACE are similar in amino acid sequence, several significant biochemical differences have been described in vitro. First, although both domains are sensitive to chloride, the C-domain requires a much higher chloride concentration for optimal activity than the N-domain.6 Second, the 2 catalytic domains exhibit different sensitivities to individual ACE inhibitors.7 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, the 2 catalytic sites differ in their in vitro ability to process peptides. Gonadotropin-releasing hormone is preferentially cleaved by...
the N-terminal catalytic domain, and N-acetyl-Ser-Asp-Lys-Pro is almost exclusively cleaved by this domain. In contrast, in vitro, angiotensin I (Ang I) is better cleaved by the C-terminal site than the N-terminal domain. The complexity of this system is demonstrated by the heptapeptide angiotensin 1–7, an important regulator of cardiovascular function, which is cleaved by the ACE N-domain but inhibits the C-domain. Finally, as noted above, the tACE isozyme is only composed of the C-terminal domain of sACE. We now know that it is the dipeptidyl peptidase function of tACE that is essential for normal male fertility. However, the precise peptide substrates important in this process are, at present, not known.

To understand the detailed biochemistry and physiological function of each of the 2 catalytic domains of sACE, our group has created mouse models with point mutations that inactivate one or the other of the sACE catalytic domains. We postulated that this would be advantageous in that point mutations would not be expected to interfere with the expression of the ACE protein but would alter enzyme cleavage of N- or C-specific peptide substrates. Previously, we published the phenotype of mice lacking N-terminal catalytic activity. These mice were essentially normal in their cardiovascular function. Here, we present the analysis of mice with point mutations inactivating the C-domain. Although such mice have normal blood pressure, this is only achieved through the activation of renin and the enhanced production of Ang I. This in vivo study establishes that it is the C-domain of ACE that plays the major role in the conversion of Ang I to angiotensin II (Ang II). We also found that, under conditions of dehydration, mice lacking C-domain activity cannot concentrate urine as effectively as wild-type mice, despite normal kidney structure. This may provide an explanation for why sACE bearing 2 catalytic domains was established in birds and mammals.

Methods

This information can be found in the online data supplement, available at http://hyper.ahajournals.org.

Results

Generation of C-Terminal Domain–Inactivated Mice

To inactivate the C-terminal catalytic site of ACE, we mutated the zinc-binding motif of this domain to KEMGK by targeted homologous recombination in embryonic stem cells (Figure 1A and 1B). The mutated ACE locus contains the mutated exon 20 and a neomycin cassette flanked by lox P sites within intron 19 (Figure 1C). Mice homozygous for this genetic modification (termed “ACE13/13 mice”) expressed virtually no ACE protein, as assessed by Western blot analysis (data not shown). Also, the phenotype of these mice was similar to that of ACE-null mice (data not shown). The neomycin cassette was excised by breeding with transgenic mice expressing CRE recombinase ubiquitously. In double transgenic embryos (CRE transgene and ACE mutated locus), CRE recombinase triggered the elimination of the neomycin cassette in all of the cells, including the germ cells, with only a single lox P site left within intron 19 (Figure 1D). This was verified by PCR and Southern blot analysis (data not shown). Homozygous mutated animals are named ACE13/13. ACE13/13 mice produce somatic ACE with a wild-type N-terminal catalytic domain but a mutated C-terminal domain. In these mice, tACE is catalytically inactive (Figure 1E).

After the recombination of the ACE locus, the CRE transgene was dispensable. Therefore, we selectively bred ACE13/13 mice without this transgene, eliminating any bias because of
continued CRE expression. Also, we paid particular attention to the mouse renin locus polymorphism. C57BL/6 mice, and most of the other mammals, have only 1 renin gene in their genome. In contrast, the source of the embryonic stem cells, strain 129, has 2 functional renin genes. Because several reports have found that this renin polymorphism has a significant influence on the cardiovascular and renal phenotype, we bred and studied mice with only the 1 renin gene.14 Because of the sterility of the homozygote males, we generated ACE13/13 mice by breeding heterozygote parents.10 This procedure also allows us to generate homozygote mutant ACE13/13 mice with wild-type control mice in the same litter.

ACE Enzymology and Expression

A previous report showed that, in vitro, the mutation of the C-terminal ACE zinc-binding motif to KEMGK resulted in the complete enzymatic inactivation.15 To verify that ACE13/13 mice contain a catalytically inactive C-terminal domain, we used high-performance liquid chromatography to study ACE activity in plasma using domain-specific substrates (Figure 2). The C-terminal substrate Hip-His-Leu (HHL) was efficiently cleaved by wild-type plasma but relatively poorly cleaved by ACE13/13 plasma (26.1 ± 15.9 nmol of hippuric acid from HHL and 45.9 ± 1.1 nmol of AcKP from AcSDAcKP per minute. Data are mean ± SEM. n = 8 in each group; **P < 0.001.

Figure 2. Plasma ACE substrate specificity. ACE activity was measured in the plasma of wild-type (WT) or ACE13/13 mice using the C-terminal–specific ACE substrate HHL and the ACE N-terminal–specific substrate AcSDAcKP. Plasma from ACE13/13 mice showed an equivalent N-terminal activity to that found in wild-type mice. In contrast, plasma from ACE13/13 mice had very little activity against the C-terminal–specific substrate. One milliliter of wild-type mice ACE plasma releases 261.4 ± 15.9 nmol of hippuric acid from HHL and 45.9 ± 1.1 nmol of AcKP from AcSDAcKP per minute. Data are mean ± SEM. n = 8 in each group; **P < 0.001.

wild-type plasma was measured, there was a clear dependence on chloride concentration, with plateau levels achieved at 300 mmol/L. Plasma from ACE13/13 mice showed ACE activity that was essentially independent of the chloride concentration, plateauing at ~20 mmol/L. This behavior is characteristic of the ACE N-terminal domain.

To determine the expression level and tissue distribution of ACE in the ACE13/13 mice, homogenates from a variety of tissues were analyzed by Western blot using a rabbit polyclonal anti-ACE antibody. Similar levels of ACE immunoreactivity were evident in wild-type and ACE13/13 mice for both the somatic (170-kDa) and the testis (95-kDa) isoforms (Figure 4). As shown in Figure 3, when cleavage of HHL by ACE enzyme was assayed, there was a clear dependence on chloride concentration, with plateau levels achieved at 300 mmol/L. Plasma from ACE13/13 mice showed ACE activity that was essentially independent of the chloride concentration, plateauing at ~20 mmol/L. This behavior is characteristic of the ACE N-terminal domain.

Figure 3. Chloride sensitivity. The effect of chloride concentration was measured on the hydrolysis of HHL by ACE from wild-type (WT) or ACE13/13 mice. The insert panel shows the enzymatic activity at various NaCl concentrations expressed as a percentage of its maximum activity. ACE activity in wild-type mice was broadly sensitive to chloride concentration. In contrast, ACE13/13 mice plasma showed little chloride sensitivity, reaching maximum activity at ~20 mmol/L. This behavior is characteristic of the ACE N-terminal domain.
ACE is unchanged, supporting the idea that the introduction of point mutations inactivating the C-terminal domain does not affect the size, level, or localization of ACE protein expression.

Angiotensin and Bradykinin Peptide Concentrations in the Blood

To assess ACE efficiency to process natural substrates in vivo, we measured the concentration of angiotensin and bradykinin peptides in the blood by high-performance liquid chromatography–based radioimmunoassay (Figure 5A). We found a small increase of Ang II concentration in the blood of ACE13/13 mice, as compared with blood from wild-type mice (6.19 ± 0.81 and 3.91 ± 0.87 fmol/mL, respectively). In contrast, the concentration of Ang I was 7-fold higher in the ACE13/13 blood as compared with wild-type blood (72.3 ± 8.8 versus 9.6 ± 1.5 fmol/mL). Therefore, the inactivation of the C-terminal domain of ACE limits the in vivo ability of the enzyme to convert Ang I to Ang II, as confirmed by Ang II/Ang I ratios of 0.11 ± 0.03 in ACE13/13 mice compared with 0.47 ± 0.09 in wild-type mice (P < 0.005). In comparison, we also measured a group of mice with a genetic inactivation of the N-terminal ACE catalytic site. Such mice are termed “ACE7/7.” Under identical conditions as described for the ACE13/13 mice, the ACE7/7 mice averaged Ang II blood concentrations of 4.29 ± 0.65 fmol/mL and Ang I concentrations of 10.5 ± 2.6 fmol/mL. Here, the ratio of Ang II/Ang I was 0.53 ± 0.08 mol/mol, which was not significantly different from the wild-type control mice. In summary, the inactivation of the ACE C-terminus reduces the in vivo efficiency of sACE to convert Ang I to Ang II. The animals respond by elevating Ang I concentrations to maintain homeostasis of Ang II. In contrast, the inactivation of the ACE N-terminus in ACE7/7 mice seems to have no major effects on the in vivo cleavage of Ang I.

We also measured the concentration of bradykinin peptides in ACE13/13 mice. As indicated in Figure 5A, there was no significant difference in the concentration of either bradykinin 1–7 or bradykinin 1–9. Also, the ratio of these peptides was no different from that found in wild-type mice.

Plasma Renin Concentration

In maintaining homeostasis, the most important compensatory mechanism of the renin-angiotensin system is the regulation of renin release by the juxtaglomerular apparatus. To assess such compensation in ACE13/13 mice, we measured plasma renin concentrations (Figure 5B). In these mice, the renin activity was increased 2.6-fold as compared with wild-type animals. Not surprisingly, this elevation of renin was not nearly as high as that observed in ACE-null mice (8.6-fold). Previous analysis has examined this question in ACE7/7 mice (N-terminus knockout). Such animals have...
renin levels that are not significantly different from wild-type.11

Systolic Blood Pressure
To evaluate the physiological impact of ACE C-terminal inactivation, we measured the systolic blood pressure of ACE13/13 and wild-type mice (Figure 6). The blood pressure was determined in conscious mice by tail-cuff measurement. This study showed no significant difference between the systolic pressure of ACE13/13 or wild-type mice (106 ± 2.9 and 104 ± 2.6 mm Hg, respectively). The automated tail-cuff apparatus also allowed us to measure the heart rate of the mice. Again, there was no significant difference between ACE13/13 mice (60.8 ± 21.5 pulse per second) and wild-type animals (60.4 ± 25.8 pulse per second). These data indicate that ACE13/13 mice compensate for the loss of the C-terminal catalytic activity of ACE. Normal blood pressure in ACE13/13 mice is achieved through the elevation of renin and enhanced Ang I concentrations sufficient to maintain Ang II levels and, ultimately, normal cardiovascular physiology.

Peptide Infusion
To evaluate the in vivo function of ACE in wild-type and ACE13/13 mice, we measured the change in blood pressure elicited by an acute intravenous infusion of Ang I. To standardize the results and minimize the variation from mouse to mouse, the blood pressure elevation after Ang I injection was reported as the percentage increase of the blood pressure obtained by the injection of the same amount of Ang II (Figure 7). For these experiments, the mice were anesthetized and prepared with an arterial catheter to continually monitor blood pressure. A different venous catheter was used for peptide infusions. These studies showed that the response of ACE13/13 mice to Ang I infusion was reduced by 48.3%, as compared with wild-type mice (P < 0.001). As a control, we used mice that are null for all somatic ACE expression. As expected, these mice showed little blood pressure elevation after Ang I infusion (16% the response of wild-type animals). Similarly, we evaluated in vivo bradykinin catabolism by measuring the reduction of blood pressure generated by bradykinin (1–9) venous infusion (Figure 7B). In ACE13/13 mice, the bradykinin-elicited blood pressure reduction was not different from that observed in the wild-type animal: 36.7 ± 6.7 mm Hg versus 44.0 ± 6.0 mm Hg, respectively.

Figure 6. Blood pressure. Systolic blood pressure was measured in conscious wild-type and ACE13/13 mice using an automated tail-cuff system. Individual mice blood pressures are indicated (dashes), as well as group means (filled circle) ± SEM. There was no significant difference in blood pressure between the 2 groups of mice. n = 8 in each group.

Figure 7. Blood pressure response to peptide infusion. A, 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 Ang I and Ang II infusion was measured in wild-type, ACE13/13, and ACE null mice. The data are plotted as the percentages of the blood pressure increase in response to Ang I as compared with the increase of blood pressure in response to Ang II infusion. In ACE13/13 mice the response to Ang II infusion is reduced by 50%, as compared with wild-type mice. In ACE null mice, the increase of blood pressure elicited by Ang II was only 18% of that observed in wild-type mice. B, The decrease of blood pressure induced by bradykinin infusion was measured in a separate group of mice. There was no difference in the blood pressure response to bradykinin infusion between wild-type and ACE13/13 mice. n = 7 in each group; *** P < 0.001.

Hematocrit
We reported that mice lacking ACE have a low hematocrit, probably because of the lack of Ang II generation.17 To evaluate whether the inactivation of the C-terminal catalytic domain of ACE would influence erythropoiesis, we measured the hematocrits of ACE13/13 and wild-type mice. These values were 54.6 ± 1.0% in ACE13/13 mice and 54.3 ± 1.3% in wild-type mice, which were not significantly different.

Kidney Function
ACE-null mice are unable to effectively concentrate urine, and this is associated with vascular thickening, as well as a thinned renal medulla, papillary atresia, and dilated renal calyces.2,3 To study renal development and function in ACE13/13 mice, their kidneys were first examined by light microscopy. This showed no difference from wild-type mice in overall morphology. In particular, blood vessels were normal in thickness, and there was normal development of the
medulla. We carefully assessed the length and width of the renal papilla; no differences were found between ACE13/13 and wild-type mice. Immunostaining of the kidneys with a rabbit anti-ACE polyclonal antibody showed a normal pattern of ACE protein expression (data not shown).

To evaluate concentrating ability in ACE13/13 mice, we measured urine osmolality with water available ad libitum or after 24 hours of water deprivation (Figure 8). With normal access to water, the urine osmolality of ACE13/13 and wild-type mice was similar: 1519.8±117.3 mosmol/L for the ACE13/13 mice and 1504.1±142.7 mosmol/L for wild-type mice. When mice were water deprived for 24 hours, urine osmolality significantly increased. However, water deprivation revealed a small but significant difference between the 2 groups. ACE13/13 mice averaged a urinary concentration of 2979.2±146.9 mosmol/L, less than that of wild-type mice, which averaged 3721.8±138.3 mosmol/L (P<0.001). Stated differently, water deprivation resulted in an average increase of urine osmolality of 2217.7±159.6 mosmol/L in wild-type mice but only 1459.5±146.2 mosmol/L in ACE13/13 mice (P<0.001).

Discussion

It was the cloning of ACE that first showed that this protein is composed of 2 homologous catalytic domains.4,18 Both catalytic domains are centered around a zinc-binding site, and it is in these regions that amino acid sequence homology is highest.19 Each domain is catalytic.6 The ACE gene is present throughout phylogeny.20–25 In insects, which lack a cardiovascular system, ACE homologues have only 1 catalytic domain and control fertility and sperm development. The testis ACE isoform in mammals is similar in function in that the lack of tACE or the inactivation of its catalytic activity results in male sterility.10

In contrast to male germ cells, somatic tissues produce ACE with 2 catalytic domains. However, even today, there is little insight into the specific advantage that this conferred in vertebrate evolution. To investigate the physiological importance of the 2 sACE catalytic domains in vivo, we generated 2 new mouse models with either the N- or the C-terminal domain of ACE specifically inactivated.11 In each case, the inactivation was achieved by introducing point mutations into the catalytic site that eliminated zinc-binding activity. As expected, these mice make a full-length ACE protein with levels of protein expression no different from wild-type mice.

In ACE7/7 mice, the inactivation of the N-domain of ACE did not modify the blood pressure, renal function, or the hematocrit, suggesting that, in vivo, the C-terminal domain alone is physiologically sufficient. This idea was reinforced by the measurement of plasma renin concentration, which was not significantly different in ACE7/7 mice as compared with wild-type (5.90±0.88 μg of Ang II/mL per hour versus 4.34±0.73 μg of Ang II/mL per hour).14 This implies that the C-terminal site of ACE alone is able to generate Ang II in vivo as efficiently (or nearly so) as the wild-type enzyme. These data contrast to the situation found in the C-domain–inactivated mice. Although ACE13/13 mice have normal blood pressure at steady state, this is achieved only with an upregulation of renin and the resulting 7-fold increase in the plasma concentration of Ang I. This high concentration of ACE substrate drives Ang II formation by the less efficient N-domain, resulting in homeostatic reestablishment of a normal blood pressure.

In fact, our in vivo data are consistent with the in vitro analysis of sACE. Such studies found that the 2 catalytic sites of sACE have the same affinity for Ang I but different catalytic constants. The catalytic constant of the C-domain was ~75% of the value of the wild-type enzyme (measured at pH 7.5 and 50 mmol/L of NaCl), whereas the catalytic constant of the N-domain was ~25% that of the wild-type enzyme.6 In vivo, the diminished ability of ACE13/13 mice to process Ang I was confirmed by the blood pressure response to the acute injection of this peptide. ACE13/13 mice have a limited response to Ang I injection, reduced by roughly half as compared with the response observed in wild-type animals. This is very different from the N-terminal–inactivated mice, ACE7/7, which had a response to Ang I infusion equivalent to wild-type mice.11 This observation is also in accordance with a previous publication from van Esch et al.26 They also showed a predominant role of the C-terminus in the in vivo conversion of Ang I to Ang II.

In contrast to Ang I, the metabolism of bradykinin was unchanged by C-terminal inactivation of ACE. In ACE13/13 mice, the blood concentrations of bradykinin 1–9 and bradykinin 1–7, as well as their ratio, were identical to those found in wild-type mice. The same situation was true in the N-terminal–inactivated ACE7/7 mice (unpublished data). To study whether this reflected a steady-state situation or a more dynamic phenomenon, we acutely injected bradykinin 1–9 into ACE13/13 and wild-type mice. This study showed no significant differences between wild-type and ACE13/13 animals in either the amplitude or the duration of the elicited blood pressure reduction. One explanation for these findings

![Figure 8. Renal concentrating ability. Spot urine was collected from wild-type (WT) or ACE13/13 mice under condition of free water availability or after 24 hours of water deprivation and osmolality was measured. Data for individual mice are shown, as well as the group mean±SEM for osmolality with free water availability (open circles) and after water deprivation (filled circles). With free water availability, there is no difference between wild-type or ACE13/13 mice. However, a significant reduction (P<0.001) in the ability to concentrate after 24-hour water deprivation was observed in the ACE13/13 mice as compared with the wild-type mice. n=19 for wild-type mice and n=20 for ACE13/13 mice.](http://hyper.ahajournals.org/DownloadedFrom/77x572to269x726)
may be that sACE is only 1 of the many peptidases that metabolize bradykinin. These included neutral endopeptidase, which also cleaves bradykinin 1–9 to bradykinin 1–7.27 There are also aminopeptidases, such as aminopeptidase P, which would not alter the bradykinin 1–7:bradykinin 1–9 ratio.28 This is in contrast to angiotensins where sACE is the dominant pathway of the conversion of Ang I to Ang II in mice.29

The structure of the kidney was normal in ACE13/13 mice. In particular, no abnormality was found in the structure of the cortex, medulla, or papilla. To further analyze renal function, the mice were deprived of water for 24 hours. In this situation, both wild-type and ACE7/7 mice concentrate urine (0530136N). D.J.C. is recipient of a Senior Research Fellowship Development Grant from the American Heart Association (K99-HL088000). H.D.X. is supported by a Scientist Pathway to Independence Award from the Health R01 DK039777 and R01 DK051445. S.F. is supported by a Scientist of dehydration.

Perspectives

Our studies of ACE7/7 and ACE13/13 mice show that only 1 catalytic site of somatic ACE is sufficient to maintain a normal blood pressure. This is because of the plasticity of the renin-angiotensin system and the regulation of renin production, which is elevated in ACE13/13 mice. In contrast, this system remains normal in ACE7/7 mice, indicating the predominant role of the C-terminal domain in the production of Ang II. The increase of renin in ACE13/13 mice allows an increase of Ang I concentration to compensate for the relative inability of the N-terminal ACE catalytic site alone to process this peptide. In turn, this maintains a normal Ang II concentration and a normal blood pressure. If the regulation of the renin-angiotensin system was similar in hypertensive patients, we would speculate that sustained pharmacological inactivation of one or the other catalytic site of ACE (similar to the genetic alteration induced in ACE7/7 or ACE13/13 mice) would result in compensatory changes of plasma renin concentration and ultimately only minor effects on blood pressure. Others have expressed contrary views.30 Finally, tests ACE is composed of only the C-terminal domain of ACE. An ACE C-terminal site-specific inhibitor, able to cross the blood/testis barrier, could in theory inhibit sperm function. Thus, C-terminal ACE inhibition might be a possible nonhormonal approach to male contraception.

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Disclosures

None.

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