Genetic Variation in Angiotensin-Converting Enzyme Does Not Prevent Development of Cardiac Hypertrophy or Upregulation of Angiotensin II in Response to Aortocaval Fistula

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**Background**—Experimental and clinical evidence suggests that angiotensin II may be an important mediator of cardiac hypertrophy in response to hemodynamic stress. We investigated the effect of genetic variation in angiotensin-converting enzyme (ACE) on the development of cardiac hypertrophy and left ventricular (LV) dysfunction in response to volume overload.

**Methods and Results**—Male heterozygous ACE knockout (1/0) and wild-type (1/1) mice were studied 4 weeks after the creation of an aortocaval fistula (ACF). The LV weight/body weight ratio increased 74% in ACF versus sham-operated control mice but did not differ between genotypes. Echocardiographic circumferential stress versus rate-corrected velocity of circumferential shortening curves demonstrated depressed LV function in ACF versus sham-operated mice but no difference between genotypes. LV ACE activity was higher in 1/1 versus 1/0 mice and in ACF versus sham-operated mice, and it increased significantly more in the 1/1 versus the 1/0 mice after ACF (P<0.001 for effect of genotype, ACF/sham operation, and interaction term). LV angiotensin II was higher in ACF versus sham-operated mice but did not differ between genotypes, despite 3-fold higher LV ACE activity in ACF 1/1 versus ACF 1/0 mice.

**Conclusions**—ACE underexpression does not prevent cardiac hypertrophy or LV dysfunction in response to volume overload. LV angiotensin II is unaffected by ACE genotype, both at baseline and after volume overload, indicating that the heart can maintain angiotensin II levels across a broad range of genetic ACE variation under both physiological and pathophysiological conditions. *(Circulation. 2001;103:1012-1016.)*

**Key Words:** hypertrophy ■ angiotensin ■ fistula ■ genes

Recent studies suggest that locally produced angiotensin-converting enzyme (ACE), which catalyzes the conversion of angiotensin I (Ang I) to angiotensin II (Ang II) as well as the degradation of bradykinin and other biologically active peptides,¹ may be a critical component in the signal transduction linking hemodynamic stress and cardiac remodeling. Cardiac ACE activity is upregulated and correlates with the degree of hypertrophy in volume- and pressure-overload models of congestive heart failure (CHF) in the rat²–⁴ and with diastolic wall stress in mitral regurgitation in the dog.⁵ Both clinical and experimental studies demonstrate a favorable impact of ACE inhibitor therapy on the progressive remodeling and ventricular dysfunction of CHF.⁶–⁸ Additionally, genotypes linked to high ACE activity have been associated with adverse outcomes in dilated cardiomyopathy⁹ and post–myocardial infarction remodeling.¹⁰ Thus, either pharmacological or genetic downregulation of ACE slows the development and progression of CHF of diverse causes. The mechanism whereby genetic variation in ACE confers clinical benefit remains unknown, inasmuch as blood pressure is unaffected, and it is uncertain whether the range of ACE variation observed is sufficient to alter tissue Ang II. Transgenic mice demonstrating variation of ACE activity have recently been developed, providing a unique opportunity to study the effect of genetic variation of ACE activity on cardiac hypertrophy and remodeling in response to experimental CHF.¹¹ Heterozygous ACE knockout mice (1/0) demonstrate 50% of normal plasma and tissue ACE activity but show no difference in blood pressure compared with 1/1 wild-type mice.¹²,¹³ We hypothesized that 1/0 mice would develop less cardiac hypertrophy and left ventricular (LV) dysfunction in response to an aortocaval fistula (ACF).
Methods

Generation of Transgenic Mice
F2 transgenic mice (strain 129×C57BL/6J) heterozygous for an insertional disruption of exon 14 of the ACE gene were provided by Dr Oliver Smithies and Dr John Krege11 (Department of Pathology, University of North Carolina, Chapel Hill). Male 1/1 and 1/0 offspring bred at the University of Alabama at Birmingham animal research center were used for the present investigation. Mice were provided chow (Teklad LM-485 sterilizable mouse diet) and water ad libitum and were maintained on a 12-hour light/dark cycle. Experiments were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee and were consistent with the National Research Council Guide for the Care and Use of Laboratory Animals (National Academic Press, revised 1996). From mice aged 6 to 8 weeks, a tail specimen was obtained for determination of ACE genotype by Southern restriction analysis.11 The DNA fragment containing the mutant (nonfunctional) ACE gene fragment contains an insertion of a 1.8-kb Neo gene and thus migrates to 8.5 rather than 6.7 kb. Genomic DNA was digested with the restriction enzyme BamHI, separated by electrophoresis in 0.8% agarose gels, and blotted onto Nytran plus membranes (0.45 μm, Schleicher & Schuell). [α-32P]dCTP ACE 12-T probes were used to detect the normal and mutated ACE gene fragments.

Creation of ACF
Mice underwent sham or ACF surgery at 10 to 14 weeks of age. With mice under sodium pentobarbital (30 mg/kg IP) anesthesia, the abdominal cavity was opened via a midline incision. The inferior vena cava and aorta were exposed, gently separated, and clamped distal to the renal vasculature. A 2-mm side-to-side anastomosis between the inferior vena cava and aorta was made with a 30-gauge disposable needle and scissors, and 10-0 Dermalon suture (American Cyanamid Co). Shunt patency was verified visually by swelling of the inferior vena cava and by mixing of arterial and venous blood. The peritoneal cavity was closed with 5-0 Chromic gut stitches (Ethicon, Inc), and the skin was closed with metallic clips.

Echocardiographic Assessment of LV Size and Function
Echocardiography with simultaneous monitoring of arterial pressure was performed 4 weeks after surgery. Under trichloroethanol anesthesia (375 mg/kg IP), the left carotid artery was exposed and cannulated with a polyethylene cannula (PE-10 fused to PE-50 tubing, Becton Dickinson) containing 10 U/mL heparinized saline. The catheter was exteriorized to the posterior neck and fixed with dental acrylic (Dental Manufacturing Co). Arterial pressure was recorded on a Biopak MP100 (Biopac Systems, Inc) with output to a PC sampling at 100 Hz, with use of AcqKnowledge software. LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), and septal and posterior wall diastolic thickness (PW) were measured by 2D guided M-mode echocardiography from the parasternal long-axis view by using a 10- to 12.5-MHz vascular probe (ATL 5000 HDI, ATL). The echocardiographer was blinded to genotype and the presence or absence of a fistula. The high-definition zoom feature was used to maximize the area of interest, allowing online measurement of LV dimensions. Ejection time (EjT) and RR interval were measured from pulsed Doppler of LV outflow at the aortic valve level. Echocardiographic LV mass was calculated as 2×[PW(LVEDD)2−LVESD2] (LVESD/2+PW/2)3/2 (first 10 mice) or 2×[PW(LVEDD)2−LVESD2] (LVESD/2+PW/2)3/2 (last 30 mice) in normal saline containing 0.01% Tween 80. The digest was then vortexed and vacuum-filtered. The filter was transferred to a microcentrifuge tube, and 100 μm dimethylformamide was added to break the microspheres. The solution was vortexed for 30 seconds, fol-
lowed by centrifugation at 2000g for 3 minutes. The absorption of the solution at 672 nm was determined by spectrophotometry (Beckman DU 650) and compared with standard curves to determine the number of microspheres. Lung uptake of microspheres was normalized to the total number of microspheres injected. Lung microsphere recovery was 0.8±0.08% (range 0.3% to 1.0%) in sham-operated control mice (n = 12), reflecting bronchial circulation, versus 29±4% (range 1% to 74%) in ACF mice (n = 28), validating the concept of using microspheres to confirm shunt patency. Three of the above ACF mice had <3% microsphere recovery in the lungs. These mice had no cardiac hypertrophy, were presumed to have no significant ACE, and were excluded from further analysis.

Cardiac ACE and Chymase Activity

At the completion of the echocardiographic study and microsphere injection, mice were euthanized by cervical dislocation. The heart was immediately excised, blotted dry, and placed on an iced Petri dish. The ventricles were separated and weighed, and the LV was immediately placed on dry ice. ACE tissue activity was measured by using the artificial substrate hippuryl-His-Leu as previously described.19 Chymase activity was assayed by using a modification of the procedure of Urata et al.20 as previously reported by our laboratory.21

Cardiac Angiotensin Peptide Levels

To avoid anesthesia, which might acutely affect LV Ang II levels, cardiac Ang II was measured in a separate group of mice that did not undergo echocardiographic or shunt quantification before euthanasia. The heart was rapidly excised, and the LV was immediately separated, weighed, and placed on dry ice. Cardiac angiotensin peptide concentrations were measured by high-performance liquid chromatography and radioimmunoassay, as previously described.2 Two hundred milligrams of tissue was necessary to ensure sufficient tissue for LV angiotensin peptide analysis, necessitating the pooling of 1 or 2 ACF or 3 or 4 sham-operated LVs for each measurement.

Statistical Analysis

Results are expressed as mean±SEM. Differences between ACE and sham-operated groups for 1/1 and 1/0 genotypes were evaluated by 2-way ANOVA. Post hoc comparisons were performed by the Student-Newman-Keuls test where appropriate. Correlations between continuous parameters of interest were determined from linear regression. Power estimates for the 2-way ANOVA interaction term to detect a 20% difference (ACF 1/1 versus ACF 1/0) in the LV weight/body weight (BW) ratio, VCFr, and the difference between observed and predicted VCF (VCFdif) and a 25% difference in Ang II were calculated retrospectively on the basis of sample size and observed standard deviation. All statistics were calculated by using SigmaStat statistical software (Jandel Scientific). Differences were considered statistically significant at P<0.05.

Results

Effect of ACE Genotype on Development of Cardiac Hypertrophy and LV Dysfunction

Shunt patency at the time of hemodynamic study and euthanasia was confirmed in all mice either by microspheres (n=25) or by significant cardiac hypertrophy (n=5). There was no significant difference in lung microsphere recovery between the 1/0 and 1/1 genotypes, indicating similar degrees of shunting in each group (3.2% versus 32.6%, P=NS). Lung microsphere recovery was correlated with the LV weight/BW ratio determined at autopsy, providing evidence that it reflects the degree of aortocaval shunting (r=0.58, P=0.002).

There was significant cardiac hypertrophy and an increase in the lung weight/BW ratio after ACF (Table). Cardiac hypertrophy and the lung weight/BW ratio increased to a similar extent in the 1/1 and 1/0 genotypes, indicating that the 1/0 ACE genotype does not prevent hypertrophy or the development of pulmonary congestion due to volume overloading (power 0.81 to detect a 20% difference in LV weight/BW between ACF 1/0 and ACF 1/1).

The echocardiographic LV mass correlated well with the LV mass at euthanasia (y=1.04x−14.2; r=0.89, P<0.0001). LVEDD, LVESD, and PW all increased in response to ACE. The LV wall thickness/diameter ratio was lower in the 1/0 genotype versus the 1/1 genotype in both the sham and ACF groups, suggesting that the ACE genotype may have an effect on LV structure. However, there was no significant effect of ACE genotype on the response of any of the echocardiographic parameters of LV structure to ACE (interaction terms, Table.)

Heart rate and MAP were close to the expected awake values and did not differ between groups. LV systolic function was moderately depressed after ACF, as evidenced by a decreased echocardiographic VCFr, in ACF versus sham-operated animals. VCFdif, which was calculated to correct for differences in wall stress between groups, was lower in ACF than in sham-operated mice, indicating that the depressed LV chamber function was not a result of altered loading conditions. There was no significant effect of ACE genotype on VCFr or VCFdif. Thus, LV function was depressed after 4 weeks of ACF, as evidenced by decreased VCFr and VCFdif and an increased lung weight/BW ratio. The 1/0 ACE genotype did not prevent the development of LV dysfunction (power 0.82 for VCFr and 0.99 for VCFdif to detect a 20% difference between ACF 1/0 and ACF 1/1).

LV ACE, Chymase, and Angiotensins

There was dramatic upregulation of LV ACE activity in response to ACF in the 1/1 mice, whereas there was very little increase in ACE activity in response to ACF in the 1/0 mice. LV chymase activity and Ang II peptide were significantly increased in ACF versus sham-operated animals but did not differ between genotypes. Importantly, LV Ang II was similarly elevated in the 1/0 and 1/1 ACF groups, despite 3-fold higher LV ACE activity in the latter (power 0.72 to detect a 25% difference in Ang II between ACF 1/0 and ACF 1/1). LV Ang I peptide did not differ between groups.

Discussion

The effect of genetic variation in ACE on the response to hemodynamic stress has not previously been studied in an experimental animal preparation. On the basis of the detrimental effects of genotypes associated with high ACE activity in several human disease states and the protective effects of ACE inhibitors in multiple experimental trials, we hypothesized that genetically lower ACE activity would protect against the development of cardiac hypertrophy and LV dysfunction. To our surprise, we found that mice with only 1 functioning ACE gene developed a similar amount of cardiac hypertrophy and dysfunction in response to an ACF compared with mice with 2 functioning ACE genes, despite significantly lower cardiac ACE levels in the former. ACE genotype did not affect Ang II peptide levels either at baseline or in response to volume overload, indicating that the heart is capable of maintaining Ang II levels across a wide range of genetic ACE variation under both physiological and pathophysiological conditions.
Cardiac Ang II is upregulated in both the canine mitral regurgitation and the rat ACF models of volume-overload CHF.\textsuperscript{21} The mechanisms regulating cardiac Ang II concentration remain uncertain. Plasma renin activity and cardiac renin, ACE, and angiotensin type 1 mRNA are elevated after ACF in the rat,\textsuperscript{3} whereas cardiac angiotensinogen mRNA is very low and does not increase significantly after ACF.\textsuperscript{2} Cardiac ACE activity is upregulated and correlates with regional diastolic wall stress in a canine model of mitral regurgitation and with the LV weight/BW ratio in the rat after ACF.\textsuperscript{2,5} It has not been possible from these prior studies to determine whether ACE upregulation is an integral and necessary feature regulating cardiac hypertrophy and Ang II or simply a marker of hemodynamic stress. We found a similar amount of cardiac hypertrophy, depressed myocardial function, and increased lung weight in I/0 and I/1 ACE genotypes after ACF, despite a 3-fold difference in LV ACE activity between these groups. Thus, although local ACE is upregulated in response to volume overload, our results indicate that this is not a prerequisite for the development of LV hypertrophy, early LV dysfunction, or elevation of LV Ang II. The insensitivity of LV Ang I and II levels to variation in LV ACE indicate that ACE is not rate limiting for conversion of Ang I to Ang II in the range studied. This could be explained either by a constitutive excess of ACE or by alternate pathways for conversion of Ang I to Ang II, such as chymase.

Several points need to be considered in reconciling our findings with the multiple epidemiological and experimental trials demonstrating a beneficial effect of genetic or pharmacological ACE downregulation on outcomes in CHF.\textsuperscript{2–4,7–10} The mechanism by which ACE inhibitors are efficacious is uncertain and may vary depending on the underlying pathological physiology. Quinapril, an ACE inhibitor with high affinity for ACE indicate that ACE is not rate limiting for conversion of Ang I to Ang II in the range studied. This could be explained either by a constitutive excess of ACE or by alternate pathways for conversion of Ang I to Ang II, such as chymase.
Ang II of the 2 agents.5,23 These findings suggest that attenuation of volume-overload hypertrophy by ACE inhibitors requires high-level suppression of tissue ACE, and they are consistent with our findings that incomplete cardiac ACE suppression does not prevent hypertrophy or suppress cardiac Ang II upregulation due to ACF. However, the results stand in contrast to experimental animal models demonstrating the efficacy of low-dose ACE inhibitor in reversing hypertrophy from aortic banding.24 Clinical trials demonstrating the efficacy of nonselective ACE inhibitors, and epidemiological trials demonstrating the benefit of modest genetic variation in ACE, possibly reflecting important pathophysiological differences between experimental volume overload and the clinical states in the above trials. Patients in the major ACE inhibitor CHF trials and in the ACE genotype epidemiological studies have predominantly had coronary artery disease, hypertension, or idiopathic dilated cardiomyopathy as the underlying cause of CHF.9,10 Conditions characterized by collagen deposition and fibrosis in the extracellular matrix of the myocardium.25 In contrast, in short-term ACF, there is no fibrosis and either no change in collagen or decreased collagen levels.26,27 Thus, the lack of a beneficial effect of low ACE genotype in the present model, as opposed to its beneficial effect in a variety of pathophysiological states in humans, may in part reflect the absence of fibrosis as an important pathophysiological feature in early ACF. Although caution must be used in extrapolating results from mice to humans because of the multiple inherent differences between the 2 species, the present study may provide useful insights into the mechanisms underlying the beneficial effect of genetic ACE variation in humans. Our results indicate that incomplete suppression of tissue ACE is ineffective in preventing hypertension and LV dysfunction due to pure volume overload. Additionally, our findings of maintained LV Ang II in the heterozygous ACE knockout mouse indicates that ACE is not rate limiting for Ang II production within the range studied and suggests that a non–Ang II mechanism may mediate the beneficial effect of low ACE genotype observed in humans.

In conclusion, mice with 1 and 2 ACE genes develop a similar amount of cardiac hypertrophy and dysfunction and similar elevation of LV Ang II and chymase after ACF, despite 3-fold higher LV ACE levels in the latter. Variation in LV ACE activity within the range studied is not an important regulator of steady-state LV Ang II concentration.

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