Point Mutation in the Stalk of Angiotensin-Converting Enzyme Causes a Dramatic Increase in Serum Angiotensin-Converting Enzyme But No Cardiovascular Disease

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Background—Angiotensin-converting enzyme (ACE) metabolizes many small peptides and plays a key role in blood pressure regulation. Elevated serum ACE is claimed to be associated with an increased risk for cardiovascular disease. Previously, two families with dramatically increased serum ACE were described, but no systematic survey of affected individuals was performed, and the molecular background of this trait is unknown.

Methods and Results—Eight families were identified with autosomal dominant inheritance of a dramatic (5-fold) increase of serum ACE activity. Strikingly, no clinical abnormalities were apparent in the affected subjects. Isolated blood cells were used for genetic and biochemical analysis. The level of ACE expression on the blood leukocytes and dendritic cells and total cell-associated ACE of the affected individuals was similar to that in nonaffected relatives; however membrane-bound mutant ACE was much more efficiently clipped from the cell surface compared with its wild-type counterpart. A point mutation causing Pro1199Leu in the stalk region of the ACE molecule cosegregates with the increase in serum ACE (LOD score, 6.63).

Conclusions—A point mutation in the stalk region of the ACE protein causes increased shedding, leading to increased serum ACE, whereas cell-bound ACE is unaltered, and affected individuals exhibit no clinical abnormalities. These findings qualify the importance of serum ACE and establish a new determinant of ACE solubilization. (Circulation. 2001;104:1236-1240.)

Key Words: genetics ■ angiotensin ■ proteins ■ blood pressure

Angiotensin-converting enzyme (ACE) (kininase II, EC 3.4.15.1, CD 143) is a membrane-bound Zn+-metalloendopeptidase that is involved in the metabolism of many small peptides, such as the conversion of angiotensin I to angiotensin II or hydrolysis of bradykinin. It is expressed on the cell surface and plays a key role in blood pressure regulation and vascular remodeling. Its importance is best illustrated by the impact that ACE inhibitors have had on the treatment of hypertension and heart failure. Somatic ACE has two homologous domains at its N-terminus, each with an active center with distinctive catalytic properties. Somatic ACE also exists in a catalytically active soluble form, derived from endothelial cells by proteolytic cleavage at the juxtamembrane stalk region. This cleavage/secretion process is catalyzed by an unidentified membrane-bound secretase. The activity of this protease, and thus ACE release, is stimulated by various agonists such as phorbolesters, calcium ionophores, and unidentified serum factors. Therefore, proteolytic cleavage of ACE appears to be a highly regulated process, suggesting that membrane-bound and soluble ACE may have different functions.

The concentration of soluble serum ACE appears to be genetically determined. A 287-bp Alu-repeat sequence insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene has been proposed to be associated with a modest elevation in serum ACE activity and with cardiovascular disease. This increase in serum ACE levels does not exceed the upper limit of normal, however, and the association with cardiovascular disease has not been found in other studies.
More pronounced elevation of serum ACE activity is found in granulomatous disorders such as sarcoidosis and in some other diseases. This increase is rarely >3 times the upper limit of normal. Extreme elevation of serum ACE activity (>4 times the upper limit of normal) has been described in two families. In both families, the distribution of high serum ACE levels (hyper-ACE) among the family members suggested autosomal dominant inheritance. No known disease could explain or could be ascribed to the increased ACE level. Until now, a systematic study into the genetic and biochemical basis of this familial hyper-ACE phenotype has not been performed.

In this study, we describe 8 new hyper-ACE families and demonstrate that the molecular basis of the hyper-ACE phenotype is a point mutation located in the stalk region of the ACE molecule.

Methods

Clinical Evaluation

The study was approved by the hospital ethics committee. The procedures followed were in accordance with institutional guidelines. All plasma ACE activities determined in the last 5 years in the UMC Nijmegen (n=2900) were reviewed, and 4 unrelated patients were identified with serum ACE activities exceeding 4 times the upper limit of normal. In all of them, other unrelated patients were identified elsewhere in the Netherlands. First-degree (and in some instances second-degree) relatives of these patients were asked to participate in the clinical evaluation.

After giving written informed consent, these family members (not the probands) were given an interview and physical examination. An ECG was recorded, and blood pressure was recorded automatically every 3 minutes during 20 minutes (Dinapmap, Critikon Inc). Serum samples were taken for determination of ACE activity, erythrocyte sedimentation rate, hematology and blood chemistry (including blood minerals, kidney function, liver enzymes, albumin, and glucose), and thyroid-stimulating hormone. Blood samples for renin, angiotensinogen, aldosterone, and angiotensin I and II determination were taken and processed as described. Aldosterone was measured in plasma by a radioimmunoassay kit (DPC). In one family (No. 6, Figure 1) an echocardiography (Diasonics Vingmed Ultra, Sound, System V) was performed in all 12 family members (8 with elevated ACE). In the left parasternal short- and long-axis views, the cardiac dimensions were assessed; in the apical 2-, 4-, and 5-chamber views, the wall motions and valvular function were assessed. In 7 selected individuals 300 mL of blood was drawn in tubes containing heparin for additional in vitro analysis of isolated blood cells.

Serum ACE and ACE genotype were determined in 166 randomly selected, unrelated individuals from an epidemiological survey in Rotterdam.

Blood Cell Isolation Cultivation and Fluorescently Activated Cell Sorter Analysis

Activated peripheral blood lymphocytes (PBL) and immature dendritic cells (DC) were generated from 4 individuals with elevated ACE and 3 nonaffected family members (subjects indicated in Figure 1) as described. Cells were cultured for 6 days, with a complete change of medium at day 4. Nonadherent PBL were activated for 3 days with PHA (1 μg/mL) and IL-2 (20 U/mL) in the same culture medium. Flow cytometric analysis of the leukocyte populations was performed with the primary antibody (10 μg/mL) and FITC-conjugated GAM IgG (μ)4 (Zymed) as the secondary antibody directly on a FACScan flow cytometer (Becton and Dickinson & Co). An isotype-matched mAb was used as a control.

ACE Activity Measurements

Colorimetric determination of ACE activity in the plasma was performed with a kit by Fujirebio Inc, which uses a p-hydroxy-Hip-His-Leu substrate. The reference range in our laboratory is 8.3 to 31.4 U/L (n=215). Fluorometric assay of ACE activity in serum, plasma, culture fluids, or lysate of cultured cells was performed by measuring the release of His-Leu from the substrates Hip-His-Leu and Z-Phe-His-Leu. In isolated cells, ACE levels were corrected for the amount of cells and volume of culture medium.

ACE Plate Precipitation Assay and ACE ELISA

These assays were performed as described in References 24 and 25, respectively.

DNA and RNA Characterization

The ACE gene was sequenced in 3 unrelated individuals by means of an automatic sequencer (ABI). After the discovery of the mutation, genotyping of other individuals was performed by polymerase chain reaction (PCR)-based restriction fragment length polymorphism assessment. Genomic DNA was amplified with Goldstar Taq (Eurorgenet), with the use of a forward primer ATGTTGAGCTACTCTCAAGGGG and a reverse primer GCTAGGGCCTGCGGTGTT, resulting in a 141-bp fragment. This fragment was digested with AciI (Boehringer Mannheim). The C-to-T mutation at position 3705 eliminates this AciI restriction site. The same primers were used for reverse transcription-PCR to confirm the mutation at mRNA level and to estimate the amount of ACE mRNA isolated from the cultured cells described above. Haplotyping of mutation-containing alleles of the ACE gene was performed by typing of two single nucleotide polymorphisms, 577 bp upstream (C/T, frequencies 0.54/0.46) and 154 bp downstream (A/G, frequencies 0.50/0.50) of the mutation, respectively. A PCR (forward primer CAGCCTTGAATGGAGATTT, reverse CAGGTTCTCCATCCAGCTTG) spanning 1508 bp around the mutation and the two flanking polymorphisms was performed. The wild-type amplifier was digested by AciI, and the resulting fragments were removed by agarose electrophoresis. The 5’ polymorphic marker on the undigested fragment was typed by single base extension with a commercial kit (Snipshot, Applied Biosystems). The 3’ marker was characterized by digestion with BpiI (New England Biolabs).

Statistical Evaluation

Linkage

We calculated 2-point LOD scores by using the subroutine MLINK of the LINKAGE program (version 5.1). A mutated ACE allele frequency of 0.01 (a robust estimate, based on the fact that in 166 randomly selected, unrelated individuals, this mutation was not found) and a penetrance of 95% were assumed.

The data collected in the clinical study were analyzed by the use of a Mann-Whitney U test and data of the in vitro analysis by a Student’s t test.

Results

Clinical Data

Eight Dutch individuals were identified with ACE values exceeding 4 times the upper limit of normal. In all of them,
ACE activity was measured because of a suspicion of sarcoidosis. The patients had a variety of complaints, including fatigue, arthralgia, nephrolithiasis and hypercalciuria, restrictive pulmonary disease, and premature stroke. Despite an extensive workup, this diagnosis could be made in none. Subsequent analysis revealed additional individuals with an extensive workup, this diagnosis could be made in none. The hyper-ACE segregation pattern was compatible with autosomal dominant inheritance (Figure 1).

Elevated ACE was not accompanied by any apparent clinical abnormality. In each group, 2 individuals had hypertension (diastolic blood pressure $>90$ mm Hg), 1 individual (female, 68 years of age, with normal ACE level) had heart failure and angina pectoris, and 1 individual (female, 72 years of age, with elevated ACE) had angina pectoris and proven coronary artery disease. Renin, aldosterone (Table), and angiotensinogen (not shown) levels did not differ significantly. Angiotensin II levels were slightly increased in subjects with high serum ACE (Table). The results of all other blood analyses performed were without abnormalities (not shown). ECG recordings were normal, except for the 2 patients with known heart disease. There were no signs of left ventricular hypertrophy on ECG (Table). In one family, echocardiography of all 12 members (8 with elevated ACE) was without abnormalities.

**Defect in Hyper-ACE Patients Occurs at the Level of ACE Secretion**

We determined the activity, amount, and possible structural abnormalities of serum ACE in affected and nonaffected family members. The increase of ACE activity (4- to 6-fold) was similar with application of different substrates (Table comparing 20 affected with 21 nonaffected individuals, $P<0.0001$; Figure 2A comparing 4 affected with 3 nonaffected individuals, $P<0.01$) and correlated with the increase found in the ACE ELISA (not shown). Moreover, the ratio between the hydrolysis of the substrates Hip-His-Leu and Z-Phe-His-Leu in affected subjects was normal (not shown), suggesting that neither one of two catalytic domains of ACE was altered.25,28 The binding characteristics of a panel of anti-ACE mAb (9B9) were obtained with other ACE-specific mAbs (not shown). Cell-surface ACE was expressed with anti-ACE mAb (9B9). Similar results were obtained with other ACE-specific mAbs (not shown). Cell-surface ACE expression was indicated as mean fluorescence relative to isotype control. D, Rate of ACE secretion. ACE activity23 in culture medium of immature DC and PHA/IL-2 activated PBL, from which cell pellets were used in part B. Rate of ACE cleavage was determined as ratio between ACE in culture medium and total amount of cell-associated ACE. Data are expressed as percentage from mean of normal individuals. Determination of ACE amount27 gave similar results.

### Table: Comparison of Subjects With Elevated and Normal ACE Levels

<table>
<thead>
<tr>
<th></th>
<th>Elevated ACE</th>
<th>Normal ACE</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>20</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>ACE, U/L†</td>
<td>105 (23)</td>
<td>16 (6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age, y</td>
<td>37 (17)</td>
<td>45 (17)</td>
<td>NS</td>
</tr>
<tr>
<td>Male/female</td>
<td>8/12</td>
<td>12/9</td>
<td>NS</td>
</tr>
<tr>
<td>SBP autom, mm Hg</td>
<td>130 (14)</td>
<td>129 (14)</td>
<td>NS</td>
</tr>
<tr>
<td>DBP autom, mm Hg</td>
<td>78 (11)</td>
<td>74 (11)</td>
<td>NS</td>
</tr>
<tr>
<td>S V&lt;sub&gt;1&lt;/sub&gt;+R V&lt;sub&gt;5&lt;/sub&gt;, mm</td>
<td>27 (8)</td>
<td>23 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>Renin, mU/L</td>
<td>17.8 (18.8)</td>
<td>9.6 (5.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Angiotensin I, pmol/L</td>
<td>7.3 (5.8)</td>
<td>9.3 (5.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Angiotensin II, pmol/L</td>
<td>5.0 (5.0)</td>
<td>2.2 (1.4)</td>
<td>0.034</td>
</tr>
<tr>
<td>Aldosterone, ng/L</td>
<td>67 (45)</td>
<td>60 (44)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Data are given as mean (SD). Proband data are not included. SBP autom indicates systolic blood pressure and DBP autom, diastolic blood pressure, mean of 3 automatic recordings; S V<sub>1</sub>+R V<sub>5</sub>, R wave in V<sub>1</sub> lead + S wave in V<sub>5</sub> lead of the ECG. Obtained by Mann-Whitney U test; $P<0.05$ was considered not significant. ACE activity was measured colorimetrically, with p-hydroxy-Hip-His-Leu as substrate.23

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**Figure 2.** Biochemical and immunologic characterization of ACE in hyper-ACE individuals ($n=4$) and nonaffected family members ($n=3$) from 2 independent families (see also Figure 1). Data are represented as mean±SEM. *$P<0.05$, **$P<0.01$ (Student’s t test). A, Serum ACE activity. ACE activity was measured fluorimetrically with Hip-His-Leu as substrate. Data are expressed as percentage from normal individuals (28 mU/mL). B, Cell-associated ACE activity. Lysates from thoroughly washed cell pellets from peripheral blood mononuclear cells (PBMC), activated PBL, and immature DC were prepared with 3-[3-cholamidopropyl]dimethyl-ammonio]-1-propane sulfonate (CHAPs) as detergent. Cell-bound ACE activity was determined with Hip-His-Leu. C, Cell-surface ACE expression: FACS analysis of immature DC and freshly isolated peripheral blood mononuclear cells (gated monocytes and lymphocytes are shown separately) with anti-ACE mAb (9B9). Similar results were obtained with other ACE-specific mAbs (not shown). Cell-surface ACE expression is indicated as mean fluorescence relative to isotype control. D, Rate of ACE secretion. ACE activity in culture medium of immature DC and PHA/IL-2 activated PBL, from which cell pellets were used in part B. Rate of ACE cleavage was determined as ratio between ACE in culture medium and total amount of cell-associated ACE. Data are expressed as percentage from mean of normal individuals. Determination of ACE amount gave similar results.
significant maximum LOD score of 6.63 at a mutation with the hyper-ACE phenotype showed a highly coding sequence of the ACE gene. Linkage analysis of this mutation at position 3705 is the only mutation present in the coding region of the ACE gene in 3 affected individuals and 166 unrelated Dutch individuals with normal this mutation, and the mutation was not present in the family shown). In our study, a complete segregation of a mutation in the stalk region of ACE with the hyper-ACE phenotype was observed. All affected individuals were heterozygous for ACE protein (Figure 3) [nt and aa numbering according to Reference 5]. All affected individuals were tested. Fluorescently activated cell sorter (FACS) analysis of leukocytes and dendritic cells from hyper-ACE and normal individuals demonstrated that they express a similar amount of ACE on the cell surface (Figure 2C). Also, the total amount of cell-associated ACE (Figure 2B) and ACE mRNA (not shown) was identical. In contrast, in both the activated PBL and immature DC, a 5- to 6-fold increase in the amount of secreted ACE was observed (Figure 2D, \( P<0.01 \)). These findings implicate that the rate of ACE shedding by activated PBL and DC is affected in the hyper-ACE individuals.

Point Mutation in the ACE Gene Cosegregates With the Hyper-ACE Phenotype

Sequence analysis in 3 affected, unrelated, individuals yielded a C-T mutation at nucleotide position 3705, which leads to the replacement of a proline residue by a leucine at amino acid position 1199 in the stalk region of the mature ACE protein (Figure 3) [nt and aa numbering according to Reference 5]. All affected individuals were heterozygous for this mutation, and the mutation was not present in the family members and 166 unrelated Dutch individuals with normal ACE levels. Moreover, sequence analysis of the complete coding region of the ACE gene in 3 affected individuals revealed that besides known polymorphisms, the C-T mutation at position 3705 is the only mutation present in the coding sequence of the ACE gene. Linkage analysis of this mutation with the hyper-ACE phenotype showed a highly significant maximum LOD score of 6.63 at a \( \theta \) of 0.00. All affected individuals had an identical haplotype carrying the mutation with T at position 577 bp upstream and G at position 154 bp downstream. Finally, analysis of RNA isolated from the DC from affected individuals confirmed the mutation (not shown). The amino acid sequence of the stalk region is thought to be of minor importance. Recently, the peptide bond preceding the Arg-1204 site was shown to be the processing site in somatic ACE expressed in transfected Chinese Hamster Ovary (CHO) cells. Apparently, the mutation of Pro-1199 into Leu results in more efficient cleavage of somatic ACE. Proline residues allow \( cis\text{-}trans \) isomerization of the peptide bond, and leucine at this site may force the peptide chain into a conformation that is much more favorable to processing by the secretase.

At first sight, it may appear confusing that cells harboring the 1199 Pro/Leu alteration shed 5-fold the amount of ACE but maintain the same level of membrane-bound ACE without an increase in the mRNA level. The fact that cell-surface ACE is unaltered despite increased shedding is also found in cultured human umbilical vein endothelial cells, where shedding can be increased severalfold by repeated change of the culture medium (S.M. Danilov et al, unpublished observation, Moscow, 1990). Concerning the unaltered mRNA level, one must realize that the method we used may be unable to detect a subtle change of mRNA. Second, assuming that mRNA is not changed, there may be alteration in ACE trafficking/breakdown in cells carrying the mutation. Interestingly, CHO cells stably transfected with mutated ACE revealed a decreased amount of ACE in the lysosomal fraction relative to their wild-type counterpart. So, increased shedding may be compensated by decreased lysosomal breakdown of ACE.

Although it is thought that the balance between membrane-bound and solubilized proteins is physiologically important, for ACE this appears not to be true. In the presence of similar amounts of membrane-bound ACE, the higher extracellular concentration of ACE is of no clinical significance. The unaltered plasma renin levels indicate that this mutation has no consequences for the physiology of the renin-angiotensin system. The slightly increased plasma angiotensin II level in the hyper-ACE individuals can be explained by increased conversion during blood sampling as a consequence of the increased amount of ACE. In contrast to our hyper-ACE individuals, in subjects with the DD genotype, both membrane-bound and soluble ACE are elevated. This might explain that some authors have found that the DD genotype influences cardiovascular morbidity, whereas we do not find clinical effects in the hyper-ACE individuals.

In summary, we define a mutation in the ACE gene leading to an alteration in the juxtamembrane stalk region of the ACE protein. This mutation causes a dramatic increase in serum ACE levels found in familial elevation of ACE. This is the first mutation of the ACE gene to correlate with an increase of circulating ACE far higher than the upper limit of normal. Because no alteration of renin physiology nor of blood pressure or other clinical parameters was found, this finding sheds new light on the physiological role of circulating versus cell-bound ACE. Moreover, this experiment of nature pro-

Discussion

In our study, a complete segregation of a mutation in the stalk region of ACE with the hyper-ACE phenotype was observed. Our additional findings implicate that this ACE-Pro/Leu is more susceptible to the ACE secretase. Analysis of ACE secretion of cells transfected with the ACE cDNA encoding the Pro1199Leu mutation are in line with this conclusion. One determinant of ACE solubilization is the presence of the distal domain, since testicular ACE, lacking this domain, is cleaved much more efficiently than somatic ACE. The amino acid sequence of the stalk region is thought to be of minor importance. Recently, the peptide bond preceding the Arg-1204 site was shown to be the processing site in somatic ACE expressed in transfected Chinese Hamster Ovary (CHO) cells. Apparently, the mutation of Pro-1199 into Leu results in more efficient cleavage of somatic ACE. Proline residues allow \( cis\text{-}trans \) isomerization of the peptide bond, and leucine at this site may force the peptide chain into a conformation that is much more favorable to processing by the secretase.
vides further insight into the mechanism and physiology of the solubilization of ACE.

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References

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