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.
Rotterdam.

heparin for additional in vitro analysis of isolated blood cells. Selected individuals 300 mL of blood was drawn in tubes containing
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(No. 6, Figure 1) an echocardiography (Diasonics Vingmed Ultra-
sured in plasma by a radioimmunoassay kit (DPC). In one family
are marked (#).

cate probands; shaded symbol, subject who died. Seven individ-
5'
2900) were reviewed, and 4 unrelated patients
lines. All plasma ACE activities determined in the last 5 years in the
procedures followed were in accordance with institutional guide-
the probands) were given an interview and physical examination. An
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More pronounced elevation of serum ACE activity is found
in granulomatous disorders such as sarcoidosis and in some other
diseases. 15 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. 16, 17 In both families, the distribution of high
serum ACE levels (hyper-ACE) among the family members
suggested autosomal dominant inheritance. No known dis-
ease 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 guide-
lines. 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 >80 U/L. In addition, 4
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 (Dinamap, 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 glu-
cose), and thyroid-stimulating hormone. Blood samples for renin,
angiotensinogen, aldosterone, and angiotensin I and II determination
were taken and processed as described. 18, 19 Aldosterone was mea-
sured in plasma by a radioimmunoassay kit (DPC). In one family
(No. 6, Figure 1) an echocardiography (Diasonnics 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 den-
ritic cells (DC) were generated from 4 individuals with elevated
ACE and 3 nonaffected family members (subjects indicated in Figure
1) as described. 20 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(F(ab)2 (Zymed) as the secondary antibody
duly 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. 21 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. 22, 23 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. 24, 25

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 (Eu-
rogenatec), with the use of a forward primer ATGTTGAGCTACT-
TCAAGCCGGC and a reverse primer GTTAAGACCCAAAAAGCT-
GGAGGT, resulting in a 141-bp fragment. This fragment was
digested with Acyl (Boehringer Mannheim). The C-to-T mutation at
position 3705 eliminates this Acyl 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. 26 A PCR (forward primer CAGCCTT-
GACTGGCATATT, reverse CAGTGTTCCTCCACCTCACT) spanning 1508 bp around the mutation and the two flanking
polymorphisms was performed. The wild-type amplimer was dig-
gested by Acyl 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
(SnaPshot, Applied Biosystems). The 3′ marker was characterized
by digestion with BplI (New England Biolabs).

Statistical Evaluation

Linkage

We calculated 2-point LOD scores by using the subroutine MLINK
of the LINKAGE program (version 5.1). 27 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 hyper-ACE in each of the 8 families participating in the study. The Table summarizes the relevant clinical and laboratory data in these family members. 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 mAbs (not shown). Cell-surface ACE expression was indicated as mean fluorescence relative to isotype control. D, Rate of ACE secretion. ACE activity24 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.

![Comparison of Subjects With Elevated and Normal ACE Levels](image-url)
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 revealed that besides known polymorphisms, the C-T mutation in the DC from affected individuals confirmed the mutation (not shown). This binding pattern, as assessed by immunoprecipitation, is very sensitive even to subtle changes in the conformation of ACE molecule.

In Figure 2, cells obtained from 4 affected and 3 nonaffected 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 θ 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).

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-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-
vides further insight into the mechanism and physiology of the solubilization of ACE.

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References


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