N-Domain Angiotensin I–Converting Enzyme With 80 kDa as a Possible Genetic Marker of Hypertension

Georgia D.M. Marques, Beata M.R. Quinto, Frida L. Plavinik, José E. Krieger, Odair Marson, Dulce E. Casarini

Abstract—We have previously described angiotensin I–converting enzyme (ACE) forms in urine of normotensive (190 and 65 kDa) and hypertensive patients (90 and 65 kDa, N-domain ACEs). Based on the results described above, experimental and genetic models of hypertension were investigated to distinguish hemodynamic and genetic influence on the generation of ACE profile in urine: Wistar-Kyoto and Brown Norway rats (WKY and BN), spontaneously and stroke-prone spontaneously hypertensive rats (SHR and SHR-SP), one kidney/one clip rats (1K1C), deoxycorticosterone acetate (DOCA) salt-treated and untreated rats, and enalapril-treated SHR (SHRen). Two peaks with ACE activity were separated from the urine of WKY and BN rats submitted to an AcA-44 column, WK-1/BN-1 (190 kDa), and WK-2/BN-2 (65 kDa), as described for urine of normotensive subjects. The same results were obtained for urine of 1K1C and DOCA salt-treated and untreated rats, analyzed to evaluate the influence of hemodynamic factors in the ACE profile in urine. The urine from SHR, SHR-SP, and SHRen presented 80 (S-1, SP-1, Sen-1) and 65 (S-2, SP-2, Sen-2) kDa ACE forms, differing from the urine profile of normotensive rats, but similar to that described for hypertensive patients. The presence of 80 kDa ACE in urine of SHR, SHR-SP, and SHRen and its absence in urine of experimental hypertensive rats (1K1C and DOCA salt) support the hypothesis that this enzyme could be a possible genetic marker of hypertension. Taken together, our results provide evidence that ACE forms with 90/80 kDa isolated from the urine of hypertensive subjects and genetic hypertensive animals behaves as a possible genetic marker of hypertension and not as a marker of high blood pressure. (Hypertension. 2003;42[part 2]:1111–1116.)

Key Words: angiotensin-converting enzyme ■ genetics ■ hypertension, genetic

Essential hypertension is a major public health issue and plays an important role in cardiovascular morbidity and mortality. It is not possible to consider that human hypertension depends on the expression of a single and major gene. Many studies have demonstrated that there are genetic basis and environmental factors implicated in the development of hypertension, and several different genes may be involved in its underlying cause.

Human essential hypertension is a classic example of a complex, multifactorial, and polygenic disease, with ≈30% to 50% of the variation in blood pressure between individuals being attributed to genetic factors. The angiotensinogen gene, genes encoding peptides and enzymes of the renin-angiotensin system, have been investigated as possible genes involved in blood pressure regulation. These studies suggested that human essential hypertension is inherited as a multifactorial trait, and several different genes may be involved in the regulation of blood pressure; alternatively, it may be a polygenic disease determined by the large effect of many genes and for which the final phenotypic value depends on the algebraic sum of genes that tend to increase and those that tend to prevent the increase of blood pressure.

The renin-angiotensin system (RAS) is one of the most important systems regulating blood pressure, as well as controlling fluid and electrolyte balance, having an important role in the pathogenesis of essential hypertension. This system consists of two key enzymes: renin, which initiates the enzymatic cascade by generating angiotensin (AI) from angiotensinogen, and angiotensin-converting enzyme (ACE), which catalyzes the conversion of AI to angiotensin II (AII) and inactivates the vasodepressor bradykinin (BK).

The fact that human essential hypertension is a complex disease with different pathogenic mechanisms, all of which lead to the final phenotype “high blood pressure,” is supported by studies on genetically hypertensive animals strains. To investigate the pathogenesis of experimental hypertension, the most used model is the spontaneously hypertensive rat (SHR) and its control, the normotensive Wistar-Kyoto rat (WKY). The hypertension found in SHR corresponds to

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essential hypertension in humans. It is associated with an increase in total and peripheral resistance with a normal cardiac output, extracellular fluid volume, and plasma sodium concentration. Heart, kidney, and brain may be affected with pathological complications caused by hypertension such as left ventricular hypertrophy, nephrosclerosis, and stroke. From 1991, the ACE gene locus was linked to blood pressure in SHR. Stroke-prone SHR (SHR-SP) have also been widely studied, and in this strain, cerebral lesions, a typical hypertensive complication in humans, is a well-known event.

The ACE could be associated with hypertension. The enzyme also called kininase II (dipeptidyl carboxypeptidase, EC 3.4.15.1) is a transmembrane ectopeptidase of vascular cells, also secreted as a soluble form in the plasma. It plays an important role in the regulation of blood pressure through its action on Al and BK. Two ACE forms have been described: a somatic form, with two homologous domains in the endothelium, and a testicular form (germinal ACE) in testis, shorter than the somatic ACE with only the C-domain.

In a previous studies, Casarini et al. described two ACE forms in human urine of normotensive subjects with molecular weights of 190 and 65 kDa in contrast to the urine of hypertensive patients that shown ACE forms of 90 kDa and 65 kDa. We also investigated the role of ACE form in animals. Urine collected from the WKY strain was analyzed and presented ACE forms with 190 and 65 kDa, similar to the profile found in normotensive subjects. Based on the results obtained with urine of human subjects (healthy and untreated patients) and urine of WKY, in the present work we used experimental models to distinguish genetic and hemodynamic influence on the generation of ACE profile in urine. ACE forms from urine of experimental and genetic animal models of hypertension were investigated.

**Methods**

**Rat Strains**

Male WKY, BN, SHR, and SHR-SP rats (n=5 for each group) were obtained from the Centro de Desenvolvimento de Modelos Experimentais (CEDEME) of EPM/UNIFESP. With the use of WKY animals, DOCA salt control (n=5) and DOCA salt (n=5) models were developed in the laboratory of Experimental Hypertension of Dr Oswaldo Kohlmann, Jr, Nephrology Division, EPM/UNIFESP. 1KIC (n=5) and SHR enalapril-treated (SHRen) (n=5), the experimental models, were prepared in the laboratory of Molecular Biology of Dr José Eduardo Krieger, INCOR, FMUSP, and the Brown-Norway rats were kindly provided by his laboratory. This study was realized under the approval of the Ethics Committee of the Federal University of São Paulo, São Paulo, Brazil.

**Hypertension Models**

**One-Kidney, One-Clip Renovascular Hypertension**

Male Wistar rats weighting 180 to 200 g had a silver clip with a gap size of 0.20 mm placed around the left renal artery and had the right kidney removed. Uninephrectomized sham-operated animals were prepared as control animals. Six weeks after the renal artery was clipped, the animals were put in metabolic cages.

**DOCA Salt Hypertension**

DOCA salt hypertension was induced in uninephrectomized Wistar-Kyoto rats weighting 290 to 320 g by weekly subcutaneous injections of deoxycorticosterone pivalate (20 mg/kg body wt) and 1% saline to drink instead of tap water. Control sham-treated rats were also uninephrectomized but were injected weekly with distilled water and drank tap water instead of 1% saline. The urine was collected at the sixth week of hypertension.

**SHR Enalapril Treatment**

SHR rats with systolic arterial pressure of 160 mm Hg were treated with 20 mg/kg per day enalapril by gavage during 2 weeks to achieve a blood pressure reduction. Untreated SHR served as a control animals.

**Partial Purification of Angiotensin-Converting Enzymes From Urine of BN, SHR, SHR-SP, DOCA Salt Control, DOCA Salt, 1KIC, and SHRen**

**Urine Preparation**

The urine was collected during a period of 24 hours from rats in metabolic cages and was processed individually. The volume of urine was measured and the pH was corrected to 8.0 with 1 mol/L Tris buffer. The urine was submitted to centrifugation (3000 rpm). The supernatant were concentrated in an Amicon concentrator and dialyzed in the same equipment against 50 mmol/L Tris-HCl, pH 8.0, containing 150 mmol/L NaCl, with the use of a 30-kDa molecular weight exclusion membrane.

**Gel Filtration on an AcA-44 Column**

The concentrated urine from the rats mentioned above (1.0 mL) were individually submitted to gel filtration on an AcA-44 column (1.6×80 cm; volume) previously calibrated with standard proteins (Sigma-Aldrich Co), equilibrated with 50 mmol/L Tris-HCl buffer, pH 8.0, containing 150 mmol/L NaCl. Fractions (2.0 mL) were collected at a flow rate of 20 mL/h. Protein concentration was monitored by absorbance at 280 nm, and ACE activity was measured with the use of Hippuryl-His-Leu (HHL) and Z-Phe-His-Leu (Z-PheHL).

**Enzymatic Activity Assay**

ACE catalytic activity was measured fluorometrically with 5 mmol/L HHL and 1 mmol/L Z-PheHL as substrates, as described by Friedland and Silverstein. The standard buffer assay was 100 mmol/L potassium phosphate buffer, pH 8.3, containing 300 mmol/L NaCl and 10 mmol/L ZnSO. The enzymes were incubated with 200 mL of assay solution containing 5 mmol/L HHL or 1 mmol/L Z-PheHL in the standard buffer for 18 hour at 37°C. The enzymatic reaction was stopped by the addition of 1.50 mL of 280 mmol/L NaOH and 100 mL of o-phthaldialdehyde (20 mg/mL) in methanol. Ten minutes later, the fluorescent reaction was stopped by the addition of 200 mL of 3N HCl. The product, L-His-Leu, was measured fluorometrically (365 nm excitation and 495 emission) with the use of an Aminco model J4–7461 fluorometer (American Instruments). The standard curve was obtained using varying concentrations of L-His-Leu in the blank reaction mixture. The standard curve obtained showed a linear relation between relative fluorescence and His-Leu concentration.

**Protein Determination**

Protein concentration was determined by the Bradford method, with bovine albumin used as standard (Bio Rad Protein Assay Kit, Bio Rad Laboratories), except when absorbance at 280 nm was used for the chromatographic elution profile.

**Characterization of ACE Forms From SHR Urine**

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis**

With the use of 50 mg of protein of S-1 and S-2 pooled fractions, SDS-PAGE was performed under dissociating conditions with di-thiothreitol on a 7.5% polyacrylamide slab gel by the method of Laemmli. Proteins were stained with Bio Rad Silver Stain Plus. The protein standards used were high-molecular-weight myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa),...
bovine albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), and low-molecular-weight bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20.1 kDa) (Sigma-Aldrich Co).

Effect of Inhibitors on Enzymatic Activity

The enzymes S-1 and S-2 were preincubated with the inhibitors for 30 minutes at 37°C, then HHL was added and the enzymatic activity was assayed as described above. The following compounds were tested as inhibitors: enalaprilat, captopril, and EDTA.

Effect of Cl⁻ on ACE Activity

The effect of NaCl influence on ACE activity was determined as described above, with 5 mmol/L HHL used as substrate in standard 100 mmol/L potassium phosphate buffer, pH 8.3, 10⁻⁵ mol/L ZnSO₄, with NaCl concentration ranging from 50 to 500 mmol/L.

Effect of Temperature on ACE Activity

The influence of temperature on ACE activity was examined; the enzymes were preincubated for 30 minutes at 4°C, 37°C, 56°C, and 95°C before the addition of the HHL substrate. ACE activity was measured as described above.

Identification of Peptide Bond Hydrolyzed in BK and AI Molecules and Hydrolysis of Angiotensin I and N-Acetyl-Ser Asp-Lys-Pro

The products derived from BK or AI after hydrolysis by the purified S-1 and S-2 ACE forms were submitted to high-performance liquid chromatography (HPLC), as described by Casarini et al.⁵ An aliquot of each enzyme was incubated separately with BK (20 μg) or AI (10 μg) or N-acetyl-Ser-Asp-Lys-Pro (20 μg) in 0.25 mmol/L sodium phosphate buffer, pH 7.5, for 3, 6, and 12 hours, at a final volume of 250 μL. The hydrolysis products were analyzed by reverse-phase HPLC, with the use of an Aquapore ODS 300 column, 7 μm, equilibrated with 0.1% phosphoric acid containing 5% acetonitrile (vol/vol). Peptides were separated by isocratic elution for 5 minutes followed by 20 minutes of linear gradient of 5 to 35% (vol/vol) acetonitrile in 0.1% phosphoric acid at 1.5 mL/min. Reactions products were detected by absorbance at 214 nm (AUF=0.02). The retention times of BK and BK fragments and AI, AII, and His-Leu used as standard were 2.24 minutes for Phe₁⁴⁴⁵Arg¹⁶, 11.16 minutes for Arg¹⁴⁴⁵-Pro¹⁷, 14.43 minutes for BK, 17.17 minutes for AI, 12.25 minutes for AI and 2.25 minutes for His¹⁴⁴⁵-Leu¹⁷. The retention time for Arg¹⁴⁴⁵ was 9.92 minutes. Peptide fragments were identified by elution position and quantified by integration area, with repeated injections of standard peptide solution used to correct for small differences in retention time (±7%) and peak height (±50%).

Western Blotting Analysis

SDS-PAGE was performed according to Laemmli²⁶ under reducing conditions. Samples were subjected to constant voltage or 50 minutes on minigels with a 3% stacking gel and 7.5% slab gel with a running buffer (15 mmol/L Tris, 190 mmol/L glycine, and 0.1% SDS). Electrophoretic transfer was performed for 2 hours with constant voltage (50 V), using a transfer membrane polyvinylidene fluoride membrane micropore (Bio Rad Laboratories) and the transfer buffer (15 mmol/L Tris, 190 mmol/L glycine, and 0.1% SDS). The membrane was incubated in 0.1 mol/L PBS containing 3 mg/ml bovine albumin for 30 minutes before overnight incubation at 4°C with antiserum against ACE antibody (1:250; polyclonal) prepared in the laboratory of Dr Carlos P. Vio from Universidad Catolica de Chile. The subsequent steps were carried out with the use of the biotin/streptavidin system (Amersham Biosciences), as recommended by the manufacturer. The Full Range Rainbow (Amersham Biosciences) was used as protein molecular weight markers.

Antiserum against rat urinary ACE was obtained with the use of conventional procedures by immunization in rabbits by intradermal injections of purified ACE (200 μg/animal) emulsified in complete Freund’s adjuvant, followed by booster injections every 3 to 4 weeks of urinary ACE (100 μg/animal) emulsified in incomplete Freund’s adjuvant. Blood was obtained before the immunization (preimmune serum) and during the immunization protocol in periodical bleeding to test the antibody titer. Purified ACE origin was prepared in our laboratory.

Phase Precipitation

This step was carried out by the procedure of Bordier.²⁹ Typically, 0.5 μM/mL of ACE was made up to 200 μL with 10 mmol/L Tris-HCl, pH 7.4, containing 150 mmol/L NaCl and 0.5% to 1.0% Triton X-114 at 0°C and incubated at 30°C for 3 minutes. The detergent-rich and detergent-poor phases were separated with a cushion of 0.06% (wt/vol) sucrose, 10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 0.06% Triton X-114 by centrifugation for 3 minutes at 300g and assayed in duplicate for enzyme activities by hydrolysis of HHL as described in the Methods section.
Amino-Terminal Sequence of Purified ACEs

Amino-terminal sequence of ACEs was deduced from amino acid sequencing (protein sequencer PPSQ-23, Shimadzu Corporation). The sequence was generously performed by Dr Isaura Yoshico Hirata from the Biophysics Division, UNIFESP, Brazil.

Results

Purification of ACEs

In a previous study we purified ACEs from urine of WKY rats named WK-1 (190 kDa) and WK-2 (65 kDa) (Figure 1).

An aliquot of 1.0 mL of concentrated and dialyzed urine from BN, SHR, SHR-SP, 1K1C, DOCA salt control, DOCA salt, SHRen were submitted individually to a gel filtration on an AcA-44 column, equilibrated, and eluted as described in the Methods section. Fractions of 2.0 mL were collected at a flow rate of 20 mL/h activity on HHL. Two peaks with ACE activity were separated from urine of SHR (S-1 and S-2), SHR-SP (SP-1 and SP-2), and SHRen (Sen–1 and Sen–2) with molecular weights of 190 kDa and 65 kDa, respectively. In contrast, the urine from BN (BN-1 and BN-2), 1K1C (C-1 and C-2), DOCA salt control (DC-1 and DC-2), and DOCA salt (D-1 and D-2) presented two peaks with molecular weights of 190 kDa and 65 kDa, similar to the ACE forms purified from urine of WKY rats.

We characterized two enzymes separated from the urine of SHR to compare with the WKY. The specific activity of pooled fractions from SHR rat urine named S-1 and S-2 was 2.45 mU/mg for each, and the enzymes were purified 16.3- and 14.5-fold, respectively.

Characterization of S-1 and S-2 ACE Forms From Urine of SHR

The enzymatic activities of ACE S-1 and S-2 were inhibited by ACE-specific competitive inhibitors (captopril and enalaprilat) and by the quelante agent EDTA in order of micromoles (Table 1). All ACE forms from urine of BN, SHR, SHR-SP, 1K1C, DOCA salt control, DOCA salt, SHRen also were inhibited by the competitive inhibitors captopril and enalaprilat in the same order.

SDS-PAGE

An aliquot (50 μg) of the enzymes S-1 and S-2 was submitted to SDS-PAGE. The molecular weight determined for S-1 and S-2 obtained from SHR rat urine was 80 kDa and 65 kDa, respectively. The ACEs analyzed under dissociation conditions were homogeneously purified (data not shown).

Identification of BK, AL, Ang 1–7, and N-Acetyl-Ser-Asp-Lys-Pro Hydrolysis Products

The enzymatic activities of purified ACEs (S-1 and S-2) were analyzed with the use of the best-known physiological sub-
strates, AI and BK. All enzymes were found to be able to convert AI to AII and to hydrolyze BK at the Pro 7–Phe 8 peptide bond liberating Arg 1–Pro 7 (graphic not shown). The percentage of BK and AI hydrolysis is shown in Table 2. The enzyme S-2 hydrolyzed Ang 1–7 and N-acetyl-Ser-Asp-Lys-Pro better than S-1 and similar to N-domain recombinant ACE (Table 2).

Western Blotting

The urinary ACE S-1 and S-2 were analyzed by Western blotting, with the use of the polyclonal antiserum raised against rat urinary ACE. As shown in Figure 6, antiserum recognizes both urinary ACEs.

Phase Separation of Urinary ACE in Triton X-114

Phase separation of urinary ACEs was performed to determine if the enzymes had or did not have transmembrane anchor. The results presented in Table 3 clearly show that all tested forms of ACE could be divided into two groups: Human plasma ACE, the secreted form of wild-type recombinant ACE, the S-1 and S-2 ACE forms, were partitioned predominantly into the aqueous phase, and the wild-type recombinant ACE was partitioned into the detergent phase. These results show that S-1 and S-2 forms of ACE in rat urine do not contain a transmembrane anchor, in contrast to the wild-type recombinant ACE form (Table 3).

S-1 and S-2 N-Terminal Sequence

The N-terminal sequence of purified S1 and S2 by Edman degradation is shown in Figure 7. A single sequence was obtained for both enzymes, and it is homologous with the N-terminal sequence of human, rattus norvegicus, and rabbit ACE, thus proving that S1 and S2 ACE contains the N-terminal portion of the molecule (Figure 7).

Discussion

On the basis of the knowledge that enzymes originate in the nephron, there are significant differences in their activities along the different segments of the nephron portions and in their relative mass. Although the proximal tubule constitutes ≈42% of the human kidney, where the high level of enzyme production and activities is in this tubular portion, we cannot exclude the participation of other nephron segments (distal and/or collector tubule) in the production of enzymes excreted in urine.30–32

Recently, many strains of genetic hypertensive rats were developed as a model of essential hypertension. The genetic models of hypertension usually evaluated are SHR, Dahl salt

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>µmol</th>
<th>% Inhibition</th>
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<tbody>
<tr>
<td>S-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enalapril</td>
<td>3.3</td>
<td>96</td>
</tr>
<tr>
<td>Captopril</td>
<td>2.0</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.33</td>
<td>100</td>
</tr>
<tr>
<td>S-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enalapril</td>
<td>1.67</td>
<td>59.7</td>
</tr>
<tr>
<td>Captopril</td>
<td>2.08</td>
<td>55</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.67</td>
<td>100</td>
</tr>
</tbody>
</table>

The enzymes S-1 and S-2 were preincubated with the inhibitors for 30 minutes at 37°C. Then Hippuryl-His-Leu was added and the enzymatic activity in duplicate was assayed as described in Methods. Results are given in percentage of inhibition.
sensitive, Milan hypertensive, Lyon hypertensive rats, and the spontaneously hypertensive mouse. In the present study, we purified the ACE forms from urine of normotensive rats (WKY and BN), hypertensive rats (SHR and SHR-SP), experimental hypertensive rats (1K1C and DOCA salt), and SHR treated with antihypertensive drug (SHR enalapril). We aimed to compare the chromatographic profiles to characterize the 80 kDa ACE form described as a possible marker of arterial hypertension.

In a previous study, we described WK-1 (190 kDa) and WK-2 ACE (65 kDa) forms in WKY urine, which presented a profile similar to that seen by Casarini et al in human urine from normal subjects. The urinary chromatographic profile described here for SHR, SHR-SP, and SHR treated with enalapril (S-1/SP-1/Sen-1, 80 kDa and S-2/SP-2/Sen-2, 65 kDa) differs from the one seen in WKY urine rats but were similar to the ACE forms found in urine of untreated hypertensive patients with 90 and 65 kDa. The differences found in molecular mass between ACE form from rat (80 kDa) and human urine (90 kDa) could be explained by glycosylation.

Both treated and untreated DOCA salt and 1K1C groups showed a chromatographic profile similar to that found in WKY and BN groups, being the molecular mass DC-1/D-1/C-1, 190 kDa, and DC-2/D-2/C-2, 65 kDa.

The molecular mass found for the ACE forms S-1/SP-1/Sen-1 (80 kDa), S-2/SP-2/Sen-2 (65 kDa) from urine of SHR, SHR-SP, SRHen rats; C-2 (65 kDa) from urine of 1K1C rats; DC-2/D2 (65 kDa) from urine of DOCA salt control and D-2 DOCA salt and BN-2 (65 kDa) from urine of BN rats was much lower than testicular ACE (90 to 100 kDa), which is heavily glycosylated, although they were similar to that of the nonglycosylated form of the single-domain tACE (76 to 84 kDa) and the N-domain ACE (65 to 68 kDa) found recently in human urine and ileal fluid. When we compared the forms from WKY rats and ACE BN-1, with 190 kDa and BN-2, with 65 kDa, the data indicated that different strains presented the same enzyme forms. At this time, we can conclude that the ACE form of 80 kDa is present only in the genetically hypertensive strains and may be a genetic marker of hypertension.

### Table 2. Hydrolysis of Al, BK, Ang1–7, and N-acetyl by S-1 and S-2 ACE

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>% Inactivation</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>N-acetyl</td>
<td>Ang1–7</td>
<td>BK</td>
</tr>
<tr>
<td>Wild-type recombinant ACE</td>
<td>35</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>N-domain recombinant ACE</td>
<td>80</td>
<td>80</td>
<td>85</td>
</tr>
<tr>
<td>C-domain recombinant ACE</td>
<td>40</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td>S-1</td>
<td>50</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>S-2</td>
<td>53</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The hydrolysis products were analyzed by HPLC as described in Methods. Results are given in percentage of hydrolysis of N-acetyl, Ang1–7, BK, and Al. Incubates are as described in Methods. Recombinant enzymes were used as assay control. N-acetyl indicates N-acetyl-Ser-Asp-Lys-Pro; Al, angiotensin I; BK, bradykinin; Ang1–7, angiotensin-(1–7).

### Table 3. Phase Separation Using HHL as Substrate

<table>
<thead>
<tr>
<th>Forms</th>
<th>ACE, %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Detergent Phase</td>
</tr>
<tr>
<td>S-1</td>
<td>7.0</td>
</tr>
<tr>
<td>S-2</td>
<td>13.0</td>
</tr>
<tr>
<td>Wild-type recombinant ACE</td>
<td>73.0</td>
</tr>
<tr>
<td>Secret recombinant ACE</td>
<td>28.0</td>
</tr>
<tr>
<td>Human plasma</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Phase precipitation is described in Methods. Results are given in percentage of ACE activity fractionated.

Figure 6. Western blotting analysis of purified WKY and SHR ACEs. Analysis was made with the use of polyclonal anti-ACE antibody. Arrows indicate bands recognized by the antibody. A, Lane 1, standards (Full Range Rainbow, Amersham Biosciences); lane 2, WK-1 ACE; lane 3, WK-2 ACE; lane 4, standards; B, lane 1, standard; lane 2, S-1 ACE; lane 3, standard; lane 4, S-2 ACE.

Figure 7. Amino-terminal sequence of S-1 and S-2 ACEs. Alignment with amino-terminal sequences of somatic human, rat, mouse, and bovine ACE. Bold amino acids indicate not conserved in respective species.
In the second step of this study, we characterized the ACE forms S-1 and S-2 from urine of SHR rats to compare with properties of ACE forms purified from urine of WKY rats. The enzymes ACE S-1 and S-2 presented a specific activity of 2.45 mU/mg and were purified 16.3-fold and 14.5-fold, respectively.

The optimum pH with HHL used as substrate was 8.5 for S-1 and 8.0 for S-2. Similar values were found for WK-1 and WK-2 ACE from WKY rats.21 for ACE of the microvillous membrane of pig kidney,40 for urinary ACE from normal subjects and from mesangial cell ACE forms19,40 with HHL used as substrate, and for human serum with Z-Pro-Phen-His-Leu.41 The Cl− was understood as an activator of ACE, since Skeggs et al42 described that its presence is necessary for the conversion of AI to All. With the use of recombinant mutated ACE having only a single functional active site or recombinant N- and C-fragments, the N-domain was activated by 20 mmol/L Cl−, whereas the C-domain reached optimal activity in >800 mmol/L Cl− when the substrate was HHL.43 We have shown in this study that the Cl− sensitivities of the S-1 and S-2 ACE forms reached optimal activity in >200 and 400 mmol/L Cl−, respectively, similar to that found for WK-1 and WK-2 ACEs.21

Both enzymes (S-1 and S-2 ACEs) were inhibited by the competitive inhibitors enalapril and captopril on the order of 2 μmol/L and also by 1.6 μmol/L of EDTA, as described in the literature for ACE purified from different sources.19,44,45 S-1 and S-2 ACE have a Km of 10−2 mol/L with HHL used as substrate, similar to the Km described in the literature for rat serum,46 human kidney,17 human urine,47,19 and mesangial cells.40

The activity of purified ACE from urine of SHR was maintained at temperatures <4°C and 37°C but was decreased at high temperatures, similar to the results described in the literature for Nishmura et al48 and Andrade et al.40

Al and BK, the physiologically important substrates of ACE, were hydrolyzed in a similar manner by ACE S-1 and S-2 and by all purified ACE forms in this study. All ACE forms isolated in this work were recognized by polyclonal antibody against rat urinary ACE. We have shown in this study that the Cl− sensitivities of the S-1 and S-2 ACE forms reached optimal activity in >200 and 400 mmol/L Cl−, respectively, similar to that found for WK-1 and WK-2 ACEs.21

Perspectives

In summary, based in the fact that the 80 kDa ACE could be a possible genetic marker of hypertension, it is necessary to
use controlled crosses of hypertensive and normotensive rat strains to study the relation between blood pressure and this physiological and biochemical phenotype. Because most genes influencing blood pressure are still unknown, linkage with markers will be necessary for studying the genetic component of hypertension.

Knowledge derived from this study may lead to a better understanding of hypertension in humans, opening the possibility for improved treatment and prevention.

Acknowledgments

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