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]:693-701.)

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,¹ and several different genes may be involved in its underlying cause.²

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.³ The angiotensinogen gene, genes encoding peptides and enzymes of the renin-angiotensin system,⁴–⁶ genes of B3 subunit of human G-protein,⁷ 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 of the algebraic sum of genes that tend to increase and those that tend to prevent the increase of blood pressure.⁸

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).⁹ This system is an obvious candidate as a potential pathogenic factor linked to hypertension.¹⁰

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...
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 A1 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.

Testicular shorter than the somatic ACE with only the C-domain. 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, 1K1C, 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 x 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 Friedeland and Silverstein. The standard buffer assay was 100 mmol/L potassium phosphate buffer, pH 8.3, containing 500 mmol/L NaCl and 10 mmol/L ZnSO₄. The enzymes were incubated with 200 μL 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 μL of o-phenthaldehde (20 mg/mL) in methanol. Ten minutes later, the fluorescent reaction was stopped by the addition of 200 μL of 3N HCl. The product, L-His-Leu, was measured fluorometrically (365 nm excitation and 495 emission) with the use of an Aminco model 14–746.1 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 μg 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 Laemmi. 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), and...
bovine albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), and low-molecular-weight bovine albumin (66 kDa), egg albumin (45 kDa), glyceroldehyde-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, captorpril, and EDTA.

K cat Determination
Kinetic parameters for the hydrolysis of HHL were obtained at 37°C, as described above. At least 6 different substrate concentrations over a wide range of 0.025 to 5 mmol/L were used. The kinetic data were analyzed with the use of the Grafit Program.27

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 mmol/L NaOH (pH 9.0), 125 mmol/L sodium phosphate (5.5–8.0), and 125 mmol/L Tris-HCl (8.0–pH<8.5), and 125 mmol/L glycine-NaOH (pH<9.0).

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−27 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.28 An aliquot of each enzyme was incubated separately with BK (20 µg) or AI (10 µg) in 50 mmol/L Tris-HCl buffer, pH 8.0, and with Ang1−10 (20 µg) or N-acetyl-Ser-Asp-Lys-Pro (20 µg) in 0.125 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, AI, and His-Leu used as standard were 2.24 minutes for Phe5−Arg8, 11.16 minutes for Arg3−Pro7, 14.43 minutes for BK, 17.17 minutes for AI, 12.25 minutes for AI and 2.25 minutes for His3−Leu6. The retention time for Ang1−7 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 (±6%).

Western Blotting Analysis
SDS-PAGE was performed according to Laemmli29 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 micro pore (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 bovine albumin for 30 minutes before overnight incubation at 4°C with antisem against ACE antibody (1:250; polyclonal) prepared in the laboratory of Dr Carlos P. Vio from Universidad Católica de Chile. The subsequent steps were carried out with the use of the biotin/streptavidin system (Amerelsham Biosciences), as recommended by the manufacturer. The Full Range Rainbow (Amerelsham 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/L 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 of hydrolysis of HHL as described in the Methods section.

Figure 1. Gel filtration of urine from WKY and BN: 1.0 mL of concentrated urine from WKY and BN was submitted separately to 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, (−) OD 280 nm; A, (■) WK-1 and (●) WK-2 activities on HHL; B, (■) BN-1 and (●) BN-2 activities on HHL.
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, and 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: (–) OD 280 nm; (●) S-1 and (●) S-2 activities on HHL; (●) SP-1 and (●) SP-2 activities on HHL.

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 enaprilat 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 urine rat was 80 kDa and 65 kDa, respectively. The ACEs analyzed under dissociation conditions were homogeneously purified (data not shown).

Km Determination, Effect of Cl–, pH profile, and Effect of Temperature on ACE Activity

The Km values determined for HHL hydrolysis were 6 mmol/L for S-1 and S-2. The enzymes S-1 and S-2 are Cl–-dependent, and the concentration of NaCl required for maximal activation was in the range of 200 and 400 mmol/L.

Optimum pH values determined for S-1 and S-2 enzymes were 8.0 and 8.5, respectively. The S-1 and S-2 ACE activities were maintained between 4°C and 37°C.

Identification of BK, AI, 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
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-H11001 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.19 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>
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<tr>
<th>Enzymes</th>
<th>N-acetyl</th>
<th>Ang1-7</th>
<th>BK</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type recombinant ACE</td>
<td>35</td>
<td>35</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>N-domain recombinant ACE</td>
<td>80</td>
<td>80</td>
<td>85</td>
<td>95</td>
</tr>
<tr>
<td>C-domain recombinant ACE</td>
<td>40</td>
<td>32</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>S-1</td>
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<td>95</td>
</tr>
<tr>
<td>S-2</td>
<td>53</td>
<td>100</td>
<td>100</td>
<td>98</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 AI. Incubates are as described in Methods. Recombinant enzymes were used as assay control. N-acetyl indicates N-acetyl-Ser-Asp-Lys-Pro; AI, angiotensin I; BK, bradykinin; Ang1-7, angiotensin (1–7).

<table>
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<td>S-2</td>
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<td>Wild-type recombinant ACE</td>
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<tr>
<td>Secret recombinant ACE</td>
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<td>Human plasma</td>
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<td>0.23</td>
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</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 specie.
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, for ACE of the microvilli membrane of pig kidney, for urinary ACE from normal subjects and from mesangial cell ACE forms with HHL used as substrate, and for human serum with Z-Pro-Phe-His-Leu. The Cl⁻ was understood as an activator of ACE, since Skeggs et al described that its presence is necessary for the conversion of AI to AII. 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. 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.

Both enzymes (S-1 and S-2 ACEs) were inhibited by the competitive inhibitors enalapril and captorpril 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. S-1 and S-2 ACE have a Kₘ of 10⁻³ mol/L with HHL used as substrate, similar to the Kₘ described in the literature for rat serum, human kidney, human urine, and mesangial cells.

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 by Nishmura et al and Andrade et al.

AI 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.

It is accepted that in addition to AI, other endogenous angiotensin peptides are biologically active, including AIII, AIV, and Ang⁻⁷. The heptapeptide Ang⁻⁷ can affect the function of the whole kidney, based on the observation that Ang⁻⁷ has a positive effect on glomerular filtration in the absence of a change in renal vascular resistance. It is also described that Ang⁻⁷ increases the excretion of water and electrolyte. Deddish et al reported that Ang⁻⁷ is cleaved to Ang⁻⁵ and His-Pro mainly by N-domain ACE, as it is hydrolyzed only very slowly by C-domain and somatic ACE; therefore, it was described as a relative substrate of N-domain ACE. The present results show that S-1 and S-2 ACE forms hydrolyzed this substrate better than wild-type recombinant ACE and C-domain recombinant ACE. Similar results were found for ACE purified from mesangial cells.

ACE plays a central role in blood pressure regulation by acting on two main physiological substrates, AI and BK. However, the physiological functions of ACE are not limited to it and to the cardiovascular role; it may be also involved in hematopoietic stem cell homeostasis by degrading N-acetyl-Ser-Asp-Lys-Pro, a natural circulating regulator hematopoietic stem cell proliferation. N-acetyl-Ser-Asp-Lys-Pro is hydrolyzed 50-fold faster by the N-domain active site compared with the C-domain and constitutes a natural specific substrate of the ACE N-domain. In our study, ACE S-1 and S-2 are able to hydrolyze the N-acetyl-Ser-Asp-Lys-Pro peptide as described in the literature for N-domain ACE. The observations described above favor a closer similarity of S-1 ACE and S-2 ACE activities to the N-domain form of ACE: the hydrolysis of Ang⁻¹⁻⁷ and N-acetyl-Ser-Asp-Lys-Pro.

With the use of a phase separation assay, we confirmed that S-1 and S-2 ACE forms do not contain segments corresponding to the transmembrane anchor, as described, for example, for plasma ACE.

The sequence obtained for both enzymes 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. However, S-2 presented the amino acid Lys in third position, differing from S-1 that has Pro.

Comparing the data obtained in this study for urine of SHR and SHR-SP animals with those found previously by Marques et al in urine of WKY and by Casarini et al in human urine, we suggest that both profiles are similar and that the 90 kDa/80 kDa form may be considered a marker of hypertension.

Comparing the chromatographic profiles of urine of BN and WKY (normotensive animals), 1K1C (experimental hypertension–Goldblatt), DOCA salt (control), DOCA salt (experimental hypertension) rats with that of the urine of SHR, SHR-SP, and SHR-treated with enalapril, we could certify that the basic difference in these profiles is the presence of the 80 kDa ACE form in the urine of genetically hypertensive rats.

The lack of the 80 kDa ACE form in 1K1C as well as in DOCA salt urine, both models representing hemodynamic changes of hypertension development, may suggest the hypothesis in which this enzyme is linked to a genetic factor. The 80 kDa ACE was detected in purified urine of SHR animals since the first week after birth (data not show). Thus, in rats with a genetic predisposition to hypertension, the detection of an 80 kDa ACE instead of the 190 kDa form suggest that this form is an earlier marker of hypertension.

Taken together, our results provide evidence that the ACE form with 90/80 kDa isolated from the urine of hypertensive subjects and genetic hypertensive animals behaves as a genetic marker of hypertension and not as a marker of high blood pressure. Published results describing an association between ACE genotype and blood pressure are as numerous as those that have not found such association.

It has been demonstrated that several hypertension-related phenotypes are abnormal before the development of high blood pressure. Further studies using genetic segregation are necessary to understand the ACE phenotype in urine and its cosegregation with blood pressure.

**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.

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