Angiotensin I–Converting Enzyme Isoforms (High and Low Molecular Weight) in Urine of Premature and Full-Term Infants

Monica A. Hattori, Graziela L. Del Ben, Adriana K. Carmona, Dulce E. Casarini

Abstract—Angiotensin I–converting enzyme (ACE) isoforms in urine from healthy and mildly hypertensive untreated patients have been described in the literature. Healthy subjects have high- and low-molecular-weight ACEs (170 and 65 kDa), whereas mildly hypertensive untreated patients have only low-molecular-weight ACEs (90 and 65 kDa), both of which resemble ACE from the N-terminal domain. Previous studies have shown that ACE is regulated during development, and renal tubules of premature human infants are not completely mature, given that nephrogenesis is not complete until the 36th week of gestation. The aim of the present study was to purify and characterize ACE isoforms from urine of premature and full-term infants and to detect the presence of the N-domain form of ACE during prenatal development. Urine from premature and full-term infants was concentrated in an Amicon concentrator, dialyzed in the same equipment against 50 mmol/L Tris-HCl buffer (pH 8.0) that contained 150 mmol/L NaCl, and submitted to gel filtration on an AcA-34 column equilibrated with the buffer described above. Two peaks (P1 and P2 for premature infants; TP1 and TP2 for full-term infants) with ACE activity on hippuryl-His-Leu (Km, 3 mmol/L) were detected. All enzymes were Cl– dependent and inhibited by captopril and EDTA. The peptides angiotensin-(1-7) and N-acetyl-Ser-Asp-Lys-Pro, described as specific for N-domain ACE, were hydrolyzed by P2 and TP2, which suggests that both enzymes are N-domain ACE. In premature infants, P1 activity with hippuryl-His-Leu was 12-fold lower than P2 activity, but in full-term infants, the difference between TP1 and TP2 was 1.6-fold. Chromatography profiles of urine from premature infants were analyzed on days 1, 3, 7, 14, 21, and 30 after birth. The P1 of ACE was detected around the 21st and 30th days, whereas P2 was detected from day 1. These results suggest that ACE activity is related to renal development and that N-domain ACE as well as full-length ACE is present in urine from premature infants. This may indicate that healthy subjects produce and secrete the N-domain form of ACE even before term development.

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Key Words: infants, premature ▪ infants, full-term ▪ angiotensin-converting enzyme ▪ urine ▪ nephrogenesis

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richest source of ACE in human. The function of ACE in the kidney is related to the conversion of Ang I to Ang II in the glomerular and peritubular circulation. The proximal tubular cells express mRNA and protein for angiotensinogen, renin, and ACE. In addition to full-length ACE, N-domain ACE (such as the somatic enzyme) is also found in mesangial cells. ACE is present at high levels on the brush border of the proximal tubule as well as in the vascular endothelial cells. ACE in the brush border of the proximal tubule and glomerulus is probably involved in the local metabolism of peptides, especially in the production of Ang II in the tubule and tubular lumen, which is present here at high levels and is probably involved in sodium reabsorption.

Although extensive studies have been performed in cultured endothelial cells, the regulation of ACE synthesis in vivo is largely unknown. In the rat, ACE concentration in kidney microsomes increases during the first 2 weeks of life and then declines to reach low values at the end of the first month. Kidney development is incomplete at birth in rats, with maturation occurring during the third postnatal week after an increase in renal mass. Yosipiv et al reported that ACE activity is present in the developing kidney and that synthesis from precursor mRNA is the likely source of renal ACE activity.

Human plasma ACE levels are high during infancy and decrease to stable values during adult life. The reason for this fact is unknown, as is the ontogeny of ACE gene expression and tissue levels.

On the basis of the fact that the kidney reaches maturity at the 36th week of human gestation and that human urine and mesangial cells have N-domain ACE, the aim of the present study was to purify and characterize the ACEs from urine of premature and full-term infants to detect the presence of the N-domain form of ACE. We also studied the ACE isoform activity profile in postnatal evolution to verify whether nephrogenesis could influence the secretion of ACE.

Methods

Purification of ACE

Urine Collection
The parents of healthy, premature (n=15; 1500 g and gestational age of 30 weeks) and full-term infants (n=15) approved the protocol after reading about the project. The protocol was approved by the Ethics Committee on Human Experimentation (Hospital São Paulo/Universidade Federal de São Paulo). All urine was collected from children with normal renal function (assessed by creatinine clearance).

Urine Preparation
Urine was collected from premature and full-term infants, pooled, concentrated in an Amicon concentrator, and dialyzed in the same equipment against 50 mmol/L Tris/HCl buffer, pH 8.0, that containing 150 mmol/L NaCl. Urine from premature infants was also collected on days 1, 3, 7, 14, 21, and 30 after birth. Urine samples were treated as described above.

Gel-Filtration Chromatography on an AcA-34 Column

Concentrated fresh urine was submitted to gel filtration on an AcA-34 column (1.5×108 cm) equilibrated with 50 mmol/L Tris/HCl buffer, 150 mmol/L NaCl; pH 8.0. Fractions (2.0 mL) were collected at a flow rate of 20 mL/h. A, Premature infants; B, term infants; , ACE activity with HHL as substrate.

Protein Determination
Protein concentration was determined by the method of Bradford with bovine serum albumin as standard, except when absorbance at 280 nm was used for the chromatographic elution profile.

Enzymatic Activity Assay
ACE catalytic activity was determined fluorometrically by the method of Friedland and Silverstein. The enzyme was incubated with 200 μL of assay solution that included 5 mmol/L HHL in 100 mmol/L potassium buffer (pH 8.3) that contained 300 mmol/L NaCl and 10 mol/L ZnSO4 for 3 hours at 37°C. The enzymatic reaction was stopped by the addition of 1.5 mL of 0.28N NaOH, 100 μL of o-phthaldialdehyde (20 mg/mL) in methanol was added and the fluorescent reaction was stopped by the addition of 200 μL of 3N HCl. The product, L-His-Leu, was measured fluorometrically (360 nm excitation and 500 nm emission) by use of an Aminco fluorometer.

Characterization of ACE

Kinetic Parameters
Enzymatic activities of the different ACEs were determined as described above, by measurement of initial velocity of
product formation with HHL as substrate at concentrations that ranged from 0.25 to 5 mmol/L. Data were plotted according to the Lineweaver-Burk equation and analyzed by the Grafit program.23

Influence of Chloride on ACE Activity
The influence of chloride on ACE activity was determined with 5 mmol/L HHL as substrate in standard buffer, in which NaCl concentration ranged from 50 to 400 mmol/L. The activity was measured as described above.

Inhibitor Studies
Captopril and EDTA were used as inhibitors. Enzymes were preincubated with inhibitor for 30 minutes at 37°C before addition of the substrate (5 mmol/L HHL). Under these conditions, the assay was developed as described above. The inhibitor concentration in the final mixture was 2.5 µmol/L captopril and 10 µmol/L EDTA.

Determination of Optimum pH
Optimum pH was determined by use of 5 mmol/L HHL diluted in the following buffers that contained 300 mmol/L NaCl (in mmol/L): sodium acetate buffer 125 (pH 5.0 and 5.5), sodium phosphate buffer 125 (pH 6.0 and 6.5), Tris/HCl buffer 125 (pH 7.0, 7.5, 8.0, and 8.5), and glycine/NaOH buffer 125 (pH 9.0 and 10.0). Activity was measured as described above.

SDS-PAGE and Western Blot Analysis
PAGE in the presence of SDS was performed on a 7.5% slab gel according to the method of Laemmli24 with 10 µg of denatured and reduced protein. Proteins were stained with the BioRad Silver Stain Plus kit. Protein standards (all from BioRad) used were myosin (MW, 205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

Electrophoretic transfer was performed for 90 minutes at constant voltage (90 V) with a transfer membrane (polyvinylidene fluoride membrane micropore; BioRad) and transfer buffer (15 mmol/L Tris, 190 mmol/L glycine, and 0.1% SDS). The membrane was incubated in 0.1 mol/L PBS that contained 3 mg/mL BSA for 1 hour before overnight incubation at 4°C with the monoclonal antibodies 9B9, 5F1 (diluted 1:250; gift from Dr Sergei Danilov, University of Illinois of Chicago), and the polyclonal antibody Y1 against ACE (diluted 1:250; gift from Dr François Alhenc-Gelas, INSERM, Paris, France). Subsequent steps were performed with the biotin/streptavidin system (Amersham) as recommended by the manufacturer.

Hydrolysis of Ang-(1-7) and N-acetyl-Ser-Asp-Lys-Pro
The hydrolysis of Ang-(1-7) and N-acetyl-Ser-Asp-Lys-Pro peptides by the ACE enzymes was assayed by high-performance liquid chromatography. An aliquot of the samples was incubated with Ang-(1-7) (10 µg) in 100 mmol/L phosphate buffer (pH 7.5) that contained NaCl 150 mmol/L and N-acetyl-Ser-Asp-Lys-Pro (10 µg) in 50 mmol/L Tris/HCl buffer (pH 8.0) at a final volume of 500 µL for 18 hours to obtain total hydrolysis of peptides at 37°C. The reaction was stopped by the addition of 10 µL of 10% H₃PO₄. The hydrolysis products were separated by high-performance liquid chromatography by use of an Aquapore ODS 300 (7 µm; reverse-phase column) equilibrated with 0.1% phosphoric acid that contained 5% acetonitrile (vol/vol). Peptides were separated by initial isocratic elution for 5 minutes followed by a 20-minute linear gradient of 5% to 35% (vol/vol) acetonitrile in 0.1% phosphoric acid at 1.5 mL/min. Reaction products were detected by absorbance at 214 nm; AUFS, 0.02. Amounts of hydrolysis products formed were estimated from peak areas by comparison to known standards.

Results
Using gel-filtration chromatography on an ultrogel AcA-34 column, we separated 2 peaks of ACE activity from urine of

| TABLE 1. Purification of ACE Isoforms from Urine of Premature Infants |
|-------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Steps                   | Volume, mL       | Protein HHL mg/mL | Specific Activity mU/mL | Yield, % | Purification, fold |
|                        |                  |                  |                  |        |                   |
| Concentrated urine      | 2.0              | 1.23              | 2.46              | 1.77    | 3.54              | 1.44             | 100              | 1 |
| Gel filtration on AcA-34|                  |                  |                  |        |                   |
| P1                      | 15.0             | 0.013             | 0.195             | 0.062   | 0.93              | 4.77             | 7.93             | 3.31 |
| P2                      | 20.0             | 0.020             | 0.400             | 0.589   | 11.8              | 29.5             | 16.3             | 20.5 |

| TABLE 2. Purification of ACE Isoforms from Urine of Term Infants |
|-------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Steps                   | Volume, mL       | Protein HHL mg/mL | Specific Activity mU/mL | Yield, % | Purification, fold |
|                        |                  |                  |                  |        |                   |
| Concentrated urine      | 2.0              | 1.61              | 3.22              | 3.5     | 7                | 2.17             | 100              | 1 |
| Gel-filtration on AcA-34|                  |                  |                  |        |                   |
| TP1                     | 10               | 0.025             | 0.25              | 0.11    | 1.1              | 4.4              | 7.8              | 2.03 |
| TP2                     | 15               | 0.026             | 0.39              | 0.11    | 1.65             | 4.2              | 12               | 1.9 |
both premature and full-term infants; each group had HMW and LMW (N-domain ACE) forms (Figure 1). The enzymes from premature urine, denominated P1 and P2, were purified 3 and 20 times, respectively, with a specific activity of 4.8 and 29.5, with HHL as substrate. In urine from full-term infants, TP1 and TP2 were purified ∼2-fold, with specific activity of 4.4 and 4.2, respectively (Tables 1 and 2). The chromatographic profile of urine from premature infants from the 1st to 30th day demonstrated that peak 1 appears only around the 30th day after birth (Figure 2).

The enzymes (P1, P2, TP1, and TP2) were found to be chloride dependent by use of HHL as substrate. The highest activity was found in 300 mmol/L NaCl, and the optimum pH for all enzymes was 8.0 with the same substrate. Captopril inhibited P1 and P2 ACE activity from premature infants by 100% and 93%, respectively, and TP1 and TP2 ACE activities from term infants by 60%. The chelating agent, EDTA, inhibited 100% of P1, TP1, and TP2 activity and 92% of P2 activity.

K_m values for all enzymes (P1, P2, TP1, and TP2) with HHL were calculated in millimoles per liter, and molecular mass, as determined by SDS-PAGE, was 170 kDa for P1 and TP1 of the HMW enzyme and 65 kDa for P2 and TP2 of the LMW enzymes (Figure 3).

Western blot analysis was performed with polyclonal antibody Y1 and monoclonal antibodies 9B9 and 5F1 against ACE N-domain. The purified ACE N-domains (P2 and TP2) were recognized by 9B9 and 5F1, as was the recombinant N-domain ACE used as a positive control. The negative control, recombinant C-domain ACE, did not bind with those antibodies (Figure 4). All enzymes (P1, P2, TP1, TP2, recombinant N-domain, and recombinant C-domain) were recognized by polyclonal antibody Y1.

The results of hydrolysis of Ang-(1-7) and N-acetyl-Ser-Asp-Lys-Pro, described being as specific substrates for N-domain ACE by P2 and TP2 ACE are given in Tables 3 and 4. The amounts of Ang-(1-7) and N-acetyl-Ser-Asp-Lys-Pro cleaved by the 2 enzymes and the C-domain ACE differed greatly, which suggests that P2 and TP2 are N-domain ACE.

Discussion

The present article is the first to describe ACE in urine of premature and full-term infants. We detected HMW and LMW forms of ACE enzyme in the infant urines. The present profile was similar to that of urine from adult subjects, with activity on HHL of P1 from premature infants differing from the peak 1 from term infants (TP1) by ∼7-fold.
Molecular mass of 170 kDa for P1 and TP1 was similar to that of the somatic ACE forms, in which molecular mass ranges from 140 to 180 kDa.25–27 P2 and TP2 showed a molecular mass of 65 kDa, which was similar to the N-domain ACE (65 to 68 kDa) found in human urine5 and ileal fluid.28

All enzymes showed Cl$^-$ dependence similar to that of urinary ACE isoforms.5 HHL hydrolysis by ACEs (P1, P2, TP1, and TP2) was catalyzed more rapidly in the presence of 300 mmol/L Cl$^-$, which differs from renal ACE, which shows maximal activation at 800 mmol/L, and ileal ACE, which shows maximal activation at 10 mmol/L Cl$^-$.29–31

Optimum pH, with HHL as substrate, was 8.0 for P1, P2, TP1, and TP2 ACEs. In the literature, similar values are described for different ACEs with this substrate: pH 7.8 for ACE from human lung32 and pH 8.0 for urinary ACE from normal human subjects.4

$K_m$ values of enzymes P1, P2, TP1, and TP2 are calculated in millimoles per liter for HHL. In the literature, ACEs from human urine,4 lung,32 and kidney33 and from rabbit lung34 also have $K_m$ values calculated in millimoles per liter with HHL as substrate.

Captopril, a classical specific ACE inhibitor,33,35 inhibited P1, P2, TP1, and TP2 activities in a manner similar to the inhibition described in the literature for ACE from different sources.30,35 EDTA, the chelating agent, also inhibited all enzymatic activities.

Danilov et al36 described that monoclonal antibodies against ACE recognize at least 9 different epitopes, all located in the N-domain, and that monoclonal antibodies were not able to bind well to the C-domain. Urinary P2 and TP2 ACEs bound well to the monoclonal antibodies, as did N-domain ACE. We conclude that isoforms P2 and TP2 have characteristics similar to those of the N-domain and thus represent the active N-domain of the human ACE.

### TABLE 3. Results of Hydrolysis of Ang-(1–7)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Hydrolysis</th>
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<tr>
<td>Wild-type recombinant</td>
<td>80.0</td>
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<tr>
<td>C-domain recombinant</td>
<td>26.0</td>
</tr>
<tr>
<td>N-domain recombinant</td>
<td>80.0</td>
</tr>
<tr>
<td>P1</td>
<td>20.0</td>
</tr>
<tr>
<td>P2</td>
<td>100.0</td>
</tr>
<tr>
<td>TP1</td>
<td>28.0</td>
</tr>
<tr>
<td>TP2</td>
<td>68.1</td>
</tr>
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</table>

Final concentration of Ang-(1–7) was 10 μg. Results are given in percentage of hydrolysis of Ang-(1–7). Incubates are as described in Methods. Recombinant enzymes were used as assay control.
Ang-(1-7) is an active metabolite released by peptidases from Ang I or Ang II. Instead of being a vasoconstrictor, as is Ang II, Ang-(1-7) is a vasodilator on coronary arteries and exhibits potent natriuretic and diuretic actions in the kidney. In vitro, Ang-(1-7) is a substrate for ACE. Dental demonstrated that Ang-(1-7) is cleaved to Ang-I and His-Pro by N-domain ACE 100 times faster than by C-domain ACE. Ang-(1-7) appears to be a relatively specific substrate for this domain. The present study showed that P2 and TP2 were able to hydrolyze this specific substrate better than P1 and TP1. ACE has recently been demonstrated to be both in vitro and in vivo (see reference 44) in the hydrolysis of N-acetyl-Ser-Asp-Lys-Pro. The peptide N-acetyl-Ser-Asp-Lys-Pro is also highly specific for N-domain ACE. Our results demonstrate that P2 and TP2 were also able to hydrolyze this peptide with a similar percentage of hydrolysis of recombinant N-domain. On the basis of the hydrolysis of Ang-(1-7) and N-acetyl-Ser-Asp-Lys-Pro, both substrates having been described as specific for N-domain ACE, we suggest that the enzymes P2 and TP2 are N-domain ACEs.

Nephrogenesis and tubular function are known to be incomplete in premature infants. Renal embryological development begins during the first week of gestation and continues until around the 36th week of gestation. Functional capacity, although not mature, begins around the 6th week of gestation. Infants that are born premature have underdeveloped structures and decreased renal function. Comparing the chromatographic profiles of urine from premature and full-term infants, we detected low activity of HMW ACE (P1) compared with LMW ACE (P2) in urine from premature infants. When the chromatographic profile of urine from premature infants was analyzed on different days, we detected the HMW P1 form of ACE around the 21st and 30th days after birth, whereas the P2 form of ACE (N-terminal) was present from the first day after birth. On the basis of the fact that morphological and physiological development of the kidney is incomplete at birth, we suggest that ACE expression follows a tissue-specific pattern of evolution during postnatal development. Costerousse et al. reported that ACE concentration in the kidney microsomes followed the same pattern of evolution as the relative abundance of ACE mRNA, which increased during the first 2 weeks of life before declining and reaching a low value at the end of the first month. During the first 2 weeks after birth, a rapid increase in renal mass occurs that precedes the completion of renal morphogenesis in the third postnatal week. Our results demonstrated that until the third postnatal week, the level of HMW ACE (P1) was lower, perhaps because of brush-border differentiation and renal maturation. Further studies are necessary to understand this mechanism of enzyme production and liberation from the brush-border membrane of the kidney and also to determine whether posttranslational mechanisms could influence the release of ACE. In conclusion, our results suggest that, in addition to full-length ACE, N-domain ACE is also produced or secreted before birth, given that it is present at a premature age (day 1), and our results also suggest that ACE activity is related to renal development.

Acknowledgments
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References

<table>
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<th>Enzyme</th>
<th>% Hydrolysis</th>
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<td>N-domain recombinant</td>
<td>80.0</td>
</tr>
<tr>
<td>P1</td>
<td>20.0</td>
</tr>
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Final concentration of N-acetyl-Ser-Asp-Lys-Pro was 10 μg. Results are given as percentage of hydrolysis of N-acetyl-Ser-Asp-Lys-Pro. Incubates were made as described in Methods. Recombinant enzymes were used as assay control.

TABLE 4. Results of Hydrolysis of N-Acetyl-Ser-Asp-Lys-Pro

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