Expression and localization of N-domain ANG I-converting enzymes in mesangial cells in culture from spontaneously hypertensive rats

Maria Claudina Camargo de Andrade,1 Giovana Seno Di Marco,1 Vicente de Paulo Castro Teixeira,1 Renato Arruda Mortara,2 Regiane Angélica Sabatini,3 João Bosco Pesquero,3 Miriam Aparecida Boim,1 Adriana Karaoğlanovic Carmona,3 Nestor Schor,1 and Dulce Elena Casarini1

1Disciplina de Nefrologia, Departamento de Medicina, 2Disciplina de Parasitologia, Departamento de Microbiologia, Imunologia, e Parasitologia, and 3Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, Brazil

MATERIALS AND METHODS

MC Culture

MC were cultured as described previously (27). Basically, macro-dissected cortex was subjected to serial sieving, and MC were obtained from collagenase treatment of isolated glomeruli to remove the epithelial cell component. The cells were plated on RPMI 1640 supplemented with 20% FBS, 50 U/ml penicillin, 2.6 mM HEPES, and 2 mM glutamine. The cultures were allowed to develop in a CO2

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incubator (5% CO₂-95% air) at 37°C. Before the experiments, the cells were incubated without FBS for 24 h. The medium and scraped cells were stored at −80°C. Cells from passages 3–4 were characterized according to the following criteria: 1) morphological aspect of stellate cells, 2) positive immunofluorescence stain of cellular fibronectin (clone FN-3E2, Sigma) and Thy 1.1 (Sigma), and 3) negative stain of cytokeratin (clone C-11, Sigma) and human von Willebrand factor.

**Real-Time PCR for ACE Gene Expression**

Total RNA was extracted from MC pellets using TRIzol reagent according to the manufacturer’s protocol, and its purity was evaluated by electrophoresis in 1% agarose gel. Contaminant genomic DNA in RNA samples was avoided by treatment for 1 h at 37°C with DNase I (1 U/2 μg RNA). To the same quantity of RNA, 20 U of RNase-OUT-RNase inhibitor and 3 mM MgCl₂ were added. After the incubation period, the samples were heated to 95°C and immediately chilled on ice for DNase I denaturation. Reverse transcription was performed using 2 μg of total pure RNA, 200 U of Maloney murine leukemia virus reverse transcriptase, 5 mM DTT, 50 ng of random hexamer primers, 1× PCR buffer, 0.5 mM dNTPs, and 3 mM MgCl₂. Reactions were subjected to the following protocol: 20°C for 10 min, 42°C for 45 min, 95°C for 50 min, and 4°C for 10 min. Resultant cDNA was then used for PCR as follows. Specific primers against rat ACE were synthesized: 5'-GAT CCC CTG ATA CTT GGT TC-3' (forward) and 5'-CTG CTA AGC AAC ATG AGC AG-3' (reverse) (Invitrogen, Carlsbad, CA). RT-PCR was carried out using a thermocycler (model 7700, Applied Biosystems, Foster City, CA). Cycling parameters were as follows: 4 min at 94°C and 27 cycles (50 s at 94°C, 50 s at 60°C, 1 min 20 s at 72°C, and 7 min at 72°C). The expected 1,060-bp products (according to various known ACE sequences) were resolved on a 1% agarose gel stained with ethidium bromide.

**Purification of ACEs From Medium and Cells**

**Gel filtration on AcA-34. MEDIUM.** The medium collected at passage 3 was concentrated in an Amicon concentrator. The concentrated medium (2.0 ml) was subjected to gel filtration on an AcA-34 column (1.5 × 100.8 cm, 178.0 ml) and equilibrated with 50 mM Tris·HCl buffer, pH 8.0, and 150 mM NaCl. Fractions (2.0 ml) were collected at a flow rate of 20 ml/h. Protein concentration was monitored by absorbance at 280 nm. Hippuryl-His-Leu (HHL) was used as substrate to measure ACE activity.

**MESANGIAL CELLS.** After extraction of ACE from MC collected at passage 3 in 4 ml of 50 mM Tris·HCl buffer, pH 8.0, containing 1% Triton X-114 and 500 μM phenylmethylsulfonyl fluoride by shaking for 1 h at 4°C, the sample was centrifuged at 3,000 rpm for 10 min at 4°C (14). The supernatant was concentrated in an Amicon concentrator, and 2.0 ml were subjected to chromatography.

**Affinity chromatography.** The effluent from gel filtration of the medium or MC on AcA-34 was applied separately to a lisinopril-Sepharose column (50) equilibrated with 10 mM HEPES buffer, pH 8.0, containing 800 mM NaCl and 10 μM ZnSO₄. The ACEs were eluted in 10 mM HEPES, pH 6.0, and 10 μM ZnSO₄. The optical density of each fraction was quantified at 280 nm, and HHL was used as substrate to determine ACE activity.

**Enzymatic activity assay.** ACE catalytic activity was measured fluorometrically using 5 mM HHL (26) as substrate. The standard buffer assay was 100 mM potassium phosphate, pH 8.3, containing 300 mM NaCl and 10⁻³ M ZnSO₄. Aliquots of the enzymes were incubated with 200 μl of assay solution containing 5 mM HHL in the standard buffer for 18 h at 37°C. The enzymatic reaction was halted by the addition of 1.5 ml of 280 mM NaOH. At 10 min after addition of 100 μl of o-phenyldihaldehyde (20 mg/ml) in methanol, the fluorescent reaction was stopped by the addition of 200 μl of 3 N HCl. The product, L-His-Leu, was measured fluorometrically (365 nm excitation and 495 nm emission) using a fluorometer (model F-2000, Hitachi).

**Protein determination.** Protein concentration was determined by the Bradford method (4) (Bio-Rad Laboratories, Hercules, CA), except when absorbance at 280 nm was used for the chromatographic elution profile. Samples containing Triton X-114 were assayed with the DC protein assay kit (Bio-Rad Laboratories).

**Characterization of ACEs**

**Effect of Cl⁻ and temperature on ACE activity.** The influence of Cl⁻ concentration (50–1,500 mM) and temperature on ACE activity was determined as described by Andrade et al. (2).

**pH profiles of ACE.** ACE activity at various pH values was measured at 37°C by the fluorometric assay described above, with HHL used as substrate, as described by Andrade et al. (2).

**Table 1. Purification of ACE from medium of SHR MC culture**

<table>
<thead>
<tr>
<th>Purification</th>
<th>Protein</th>
<th>HHL, mU/ml</th>
<th>Spec Act, mU/mg</th>
<th>Purification, times</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated medium</td>
<td>2</td>
<td>1.2</td>
<td>2.4</td>
<td>1.14</td>
<td>0.95</td>
</tr>
<tr>
<td>Gel filtration AcA-34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEm1</td>
<td>11</td>
<td>0.048</td>
<td>0.528</td>
<td>0.054</td>
<td>1.12</td>
</tr>
<tr>
<td>ACEm2</td>
<td>19</td>
<td>0.038</td>
<td>0.722</td>
<td>0.090</td>
<td>2.36</td>
</tr>
<tr>
<td>Lisinopril-Sepharose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEm1A</td>
<td>3</td>
<td>0.007</td>
<td>0.021</td>
<td>0.036</td>
<td>5.14</td>
</tr>
<tr>
<td>ACEm2A</td>
<td>3</td>
<td>0.009</td>
<td>0.027</td>
<td>0.031</td>
<td>3.44</td>
</tr>
</tbody>
</table>

HHL, Hippuryl-His-Leu; Spec Act, specific activity; ACE, angiotensin-converting enzyme; SHR, spontaneously hypertensive rat; MC, mesangial cell.
**Table 2. Purification of ACE from SHR MC**

<table>
<thead>
<tr>
<th>Protein</th>
<th>HHL, mU/mg</th>
<th>Spec Act, mU/mg</th>
<th>Purification, times</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACEInth1A</td>
<td>0.027</td>
<td>0.405</td>
<td>0.223</td>
<td>8.25</td>
</tr>
<tr>
<td>ACEInth2</td>
<td>0.029</td>
<td>0.696</td>
<td>0.100</td>
<td>3.44</td>
</tr>
<tr>
<td>ACEInth1</td>
<td>0.017</td>
<td>0.144</td>
<td>0.290</td>
<td>17.1</td>
</tr>
<tr>
<td>ACEInth2A</td>
<td>0.006</td>
<td>0.037</td>
<td>0.220</td>
<td>36.7</td>
</tr>
</tbody>
</table>

**Table 3. Inhibition of ACE activity from SHR MC**

<table>
<thead>
<tr>
<th></th>
<th>ACEem1A</th>
<th>ACEem2A</th>
<th>ACEInth1A</th>
<th>ACEInth2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Captopril (1 μM)</td>
<td>52</td>
<td>52</td>
<td>72</td>
<td>68.5</td>
</tr>
<tr>
<td>EDTA (2.5 μM)</td>
<td>65</td>
<td>81</td>
<td>45</td>
<td>57</td>
</tr>
<tr>
<td>ENalaprilat (2.5 μM)</td>
<td>60</td>
<td>72</td>
<td>92</td>
<td>98</td>
</tr>
</tbody>
</table>

Values are expressed as percent inhibition.
mU/mg, was applied to an AcA-34 column. The enzymatic activity was separated into two peaks: 90-kDa ACEInth1 (specific activity 8.25 mU/mg), which appeared near the void volume, and 65-kDa ACEInth2 (specific activity 3.44 mU/mg; Table 2).

Affinity chromatography column. The fractions corresponding to the peaks ACEm1 (0.054 mU/ml) and ACEm2 (0.090 mU/ml) from gel filtration of the medium were pooled separately, and each pool was applied to a lisinopril-Sepharose column. Only one peak with ACE activity was detected for ACEm1, and pooled fractions (ACEm1A) presented a specific activity of 5.14 mU/mg. For ACEm2, one peak with ACE activity was also obtained, ACEm2A (specific activity 3.44 mU/mg; Table 1). Purified pooled samples from the cell lysate, ACEInth1 (0.223 mU/ml) and ACEInth2 (0.100 mU/ml), from the first step were applied separately to a lisinopril-Sepharose column. One peak with ACE activity was detected for ACEInth1, and the pooled fractions (ACEInth1A) presented a specific activity of 36.7 mU/mg. When ACEInth2 was applied to the same column, one peak with ACE activity (ACEInth2A) was detected. The pooled fractions presented specific activity of 17.1 mU/mg (Table 2).

The purified enzymes from Wistar rat MC were prepared as previously described by Andrade et al. (2).

Characterization of ACE Activities

Effects of Cl⁻ and temperature on ACE activities. The NaCl concentration required for maximal activation was 500 mM for all purified ACEs (data not shown). The enzymatic activity of purified ACEs was maintained at 4–37°C (data not shown).

Optimum pH. The optimum pH determined for ACEm1A and ACEm2A was 8.0, and that for ACEInth1A and ACEInth2A was 7.5 (data not shown).

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![Fig. 2. SDS-PAGE (7.5%) analysis of reduced purified ACEs from SHR (A and B) and Wistar rat (C and D) MC. Ac: standard protein (lane 1) and 10 μg of ACEm1A (lane 2). Ab: 10 μg of ACEm2A (lane 1) and standard protein (lane 2). Bb: 10 μg of ACEInth1A (lane 1) and standard protein (lane 2). Ba: 10 μg of ACEInth2A (lane 1) and standard protein (lane 2). Ca: 10 μg of ACE1Aw (lane 1) and standard protein (lane 2). Db: 10 μg of ACE2Aw (lane 1) and standard protein (lane 2). Dc: 10 μg of ACEInt1Aw (lane 1) and standard protein (lane 2). Dd: 10 μg of ACEInt2Aw (lane 1) and standard protein (lane 2). Protein was stained with Bio-Rad silver.](image-url)
**K_m values, with HHL used as substrate.** K_m values determined for HHL hydrolysis were 2 mM for ACEm1A and ACEm2A from medium culture and 3 mM for ACEInth1A and ACEInth2A from MC.

**Inhibition of ACE activity.** The effects of captopril, enalaprilat, and EDTA inhibitors of ACE on HHL hydrolysis are presented in Table 3.

**SDS-PAGE.** The molecular mass of ACEm1A and ACEInth1A was 90 kDa and that of ACEm2A and ACEInth2A was 65 kDa. Figure 2 shows the molecular mass of purified ACE forms from medium (130 and 60 kDa) and intracellular compartment (130 and 68 kDa) of Wistar rat MC. All purified ACEs analyzed under dissociating conditions were homogeneous.

**Western blot analysis.** ACE isoforms were studied by Western blot analysis using the 2E2 antibody (Fig. 3). The enzymes purified from Wistar rat MC were also recognized by the same specific antibody for ACE (data not shown).

**Hydrolysis of BK, ANG I, and ANG-(1–7) and products of AcSDKP hydrolysis.** The enzymatic activities of purified ACEs were analyzed using the best-known physiological substrates ANG I and BK. All enzymes were capable of converting ANG I to ANG II and hydrolyzing BK at the Pro^7^-Phe^8^ peptide bond, liberating Arg^1^-Pro^2^ (Table 4). The results of

**Table 4. Substrate hydrolysis by ACEs from SHR and Wistar rat MC**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>SHR ACEm1A</th>
<th>SHR ACEm2A</th>
<th>SHR ACEInth1A</th>
<th>SHR ACEInth2A</th>
<th>Wistar ACE1A</th>
<th>Wistar ACE2A</th>
<th>Wistar ACEInt1A</th>
<th>Wistar ACEInt2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK</td>
<td>51</td>
<td>50</td>
<td>51</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>ANG I</td>
<td>43</td>
<td>45</td>
<td>43</td>
<td>45</td>
<td>40</td>
<td>45</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>ANG (1–7)</td>
<td>50</td>
<td>63</td>
<td>50</td>
<td>63</td>
<td>10</td>
<td>68.5</td>
<td>12</td>
<td>67.7</td>
</tr>
<tr>
<td>AcSDKP</td>
<td>49</td>
<td>47</td>
<td>49</td>
<td>47</td>
<td>15</td>
<td>45</td>
<td>10</td>
<td>50</td>
</tr>
</tbody>
</table>

Enzymes were incubated with 5 μg/ml substrate for 3 h at 37°C. Values are expressed as percent hydrolysis in relation to 0 min of reaction \((n = 2)\). Percent hydrolysis was calculated as IC_{50}. BK, bradykinin.
hydrolysis of ANG-(1–7) and AcSDKP, described as specific substrates for N-domain ACE by ACEm1A, ACEm2A, ACEInth1A, and ACEInth2A, are shown in Table 4.

Cell fractionation. Western blot analysis of nuclei, cytoplasm, and membrane isolated by cell fractionation using monoclonal antibody 9B9 against N-domain ACE showed predominant immunoreaction in the nuclei (Fig. 3).

Cell surface colocalization of ACE with ANG II and ANG-(1–7). To confirm the cell surface localization of N-domain ACEs from SHR MC, these cells were grown to confluence and probed with a monoclonal ACE antibody. Results of confocal microscopy are shown in Figs. 4 and 5. The nucleus from MC was localized with DAPI used as control. Wistar rat MC were analyzed under the same conditions used for controls (Figs. 6 and 7). SHR MC were incubated with staurosporine and analyzed by fluorescence microscopy. Our results demonstrate that the MC were not apoptotic. The same procedure was used for Wistar rat MC (data not shown).

Immunohistochemistry. We analyzed the SHR kidney section with antibodies against ACE, ANG II, and ANG-(1–7) using immunoperoxidase-antiperoxidase complex and immunofluorescence staining (Fig. 8). To demonstrate the presence of N-domain ACE in the cell nuclei, we also compared SHR MC with Madin-Darby canine kidney and LLC-PK1 cells, as negative and positive controls, respectively (Fig. 9).

ACEInth1A and ACEInth2A NH2-Terminal Sequence

The NH2-terminal sequence of purified ACEInth1A and ACEInth2A is shown in Fig. 10.

DISCUSSION

Andrade et al. (2) purified and characterized four enzymes from Wistar rat MC in culture: 130-kDa ACEInt1A and 68-kDa ACEInt2A from the intracellular compartment and 130-kDa ACE1A and 60-kDa ACE2A as secreted isoforms. On the basis of the results showing that SHR MC are able to express ACE mRNA and that the ACE profile (90 and 65 kDa) of hypertensive patients and SHR (8, 35) differs from that of healthy subjects (190 and 65 kDa) (7), the 90-kDa ACE being a possible genetic marker of hypertension, we decided to analyze the biochemical and enzymatic properties of the secreted and intracellular ACE isoforms from SHR MC in culture and compare them with those of MC enzymes from Wistar rats and also to locate the isoforms in those cells.

With use of gel filtration chromatography on an AcA-34 column followed by a lisinopril-Sepharose column, ACEm1A (5.4-fold) and ACEm2A (3.6-fold) were purified from the medium and ACEInt1A (21.7-fold) and ACEInt2A (10.1-fold) from the cell lysate of SHR MC in culture.

Molecular masses of 90 kDa for ACEm1A and ACEInth1A and 65 kDa for ACEm2A and ACEInth2A were similar to those previously published for the ACEs from urine of mildly hypertensive untreated patients (8) and SHR (35). Molecular masses of ACEm2A and ACEInth2A were similar to those described for the secreted and intracellular ACE isoforms purified from Wistar rat MC (2). The molecular masses of these enzymes were much lower than that of testicular ACE (90–100 kDa), which is heavily glycosylated, but were similar to those of the nonglycosylated form of the single-domain testicular ACE (76–84 kDa) (25, 36) and the N-domain ACEs (65–68 kDa) from human and rat urine (7, 8, 28, 35) and ileal fluid (13). When electroblotted to a nitrocellulose membrane, all enzymes purified from Wistar rat and SHR MC in culture reacted strongly with 2E2 antibody against ACE.

With HHL used as substrate, the optimum pH for ACEm1A, ACEm2A, ACEInth1A, and ACEInth2A was similar to that described for the purified ACEs from Wistar rat MC (2). Similar values were found in the literature for different ACEs: pH 8.4 for ACE from the microvillous membrane of pig kidney with HHL used as substrate (38), pH 7.5, 8.5, and 7.5 with Z-Phe-His-Leu, Z-Pro-Phe-His-Leu, and ANG I, respectively, used as substrates for ACE from human serum (41), and pH 8.0 with HHL used as substrate for urinary ACEs from normal subjects and premature and full-term infants (28).

The purified ACE activities from SHR MC were maintained at <4°C and 37°C but were decreased at high temperatures (50°C and 95°C). This profile was the same as that obtained by Andrade et al. (2). Similar results were described by Nishimura et al. (37), who studied the effect of preincubation at 50°C on the ACE activities isolated from human lung homogenate.

All enzymes from SHR MC showed Cl− dependence. The hydrolysis by ACEm1A, ACEm2A, ACEInth1A, and ACEInth2A was catalyzed more rapidly at 500 mM, in contrast to the ACE activities from Wistar rat MC (2), which showed maximal activation at 300 mM. These results are distinct from the data described for renal ACE, which showed maximal activation at 800 mM Cl− (49), and also from ileal ACE, with maximal activation at 10 mM Cl− (13). The alternative anion activation may have been introduced as a regulatory device; however, this is unlikely for conversion of ANG I to ANG II by endothelial ACE. Epithelial ACE at other locations (e.g., intestinal and renal tubular brush borders) is exposed to large ion fluxes and may be regulated in such a manner (18).

ACEs purified from SHR MC were inhibited by 1 μM captopril and enalaprilat, as were N- and C-domain recombinant ACE, ileal fluid ACE, and MC ACE from Wistar rats (2, 13). This inhibition of N-domain ACE suggests that these enzymes could have a special conformation with specific and different bonds with these inhibitors. The COOH-terminal sequence of the N-domain ACEs demonstrated that they are truncated somewhere between S482 and P637 (data not shown). This short amino acid sequence could be responsible for the conformational changes.
The $K_m$ obtained for purified ACEs from Wistar rat (2) and SHR MC was similar to that for ACEs from rabbit lung (30), rat serum (41), human kidney (45), and human urine (7, 8, 28) and wild-type recombinant ACE, with a $K_m$ of $10^{-3}$ M with HHL used as substrate (49). ANG I and BK are among the physiologically important substrates of ACE; conversion of ANG I and inactivation of BK may be relevant in explaining the changes in glomerular hemodynamics. Hydrolysis of the physiological ACE substrates ANG I and BK by purified ACEm1A, ACEm2A, ACEInt1A, and ACEInt2A was similar to hydrolysis of ACEs from Wistar rat MC (2).

ANG-(1–7) is an active metabolite released by peptidases from ANG I or ANG II. Enzymes necessary for the production and inactivation of ANG-(1–7) are abundant in the kidney and have been found in urine (10, 22). In contrast to ANG II, the cumulative effects of ANG-(1–7) suggest an antihypertensive role for the heptapeptide. ANG-(1–7) exhibits vasodepressor effects on the coronary and mesenteric vascular beds (16). The evidence that ANG-(1–7) can affect the function of the whole kidney was based on the observation that the heptapeptide had a positive effect on glomerular filtration rate in the absence of a change in renal vascular resistance and also increased water and electrolyte excretion (10). The pharmacological effects of this peptide may be attributed to indirect potentiation of the action of BK on its B2 receptor by binding to the active site of ACE (12). Deddish et al. (12) reported 100-times-faster cleavage of ANG-(1–7) to ANG-(1–5) and His-Pro by N-domain ACE than by C-domain ACE, showing that ANG-(1–7) is a relatively specific substrate of N-domain ACE. Our results demonstrate that ACEs purified from MC in culture from SHR hydrolyzed this substrate better than ACE1A and ACEInt1A from Wistar rat MC (2).

The tetrapeptide AcSDKP, also highly specific for N-domain ACE (40), was hydrolyzed better by ACEs purified from SHR MC and by ACE2A and ACEInt2A from MC of Wistar rats. This result was similar to that reported by Hattori et al. (28) and Casarini et al. (8). The hydrolysis of ANG-(1–7) and AcSDKP, specific substrates of the N-domain site, by the enzymes purified from SHR MC suggested that they are N-domain ACEs.

The NH2-terminal sequencing studies showed that ACEInt1A and ACEInt2A were similar in somatic rat, mouse, and human ACEs, proving that these enzymes contain the NH2-terminal portion of the molecule (45).

Immunofluorescence by confocal microscopy showed that ACEInt1A and ACEInt2A were similar in somatic rat, mouse, and human ACEs, proving that these enzymes contain the NH2-terminal portion of the molecule (45).

Immunofluorescence by confocal microscopy showed that N-domain ACE was expressed inside the nuclei of SHR MC, but not on the cell surface, and also that N-domain ACE was colocalized with ANG II and ANG-(1–7), the product and substrate for N-domain ACE. We also localized N-domain ACE inside the nuclei of Wistar rat MC and somatic ACE in the membrane, which distinguishes this profile from that of SHR MC. Danilov et al. (11) reported that the monoclonal

Fig. 6. Colocalization of ACE and ANG-(1–7) in Wistar rat MC. A: DAPI. B: localization of N-domain ACE using 9B9 monoclonal antibody. C: localization of ANG-(1–7). D: B and C superimposed to show colocalization of N-domain ACE and ANG-(1–7). Original magnification ×40.
antibody against ACE recognized at least nine different epitopes located in the N-domain and that monoclonal antibodies were not able to bind well to the C-domain portion of the ACE. After incubation of MC with staurosporine, we demonstrated that MC were not apoptotic and that there was no leakage of cytosolic protein into the nucleus. Comprehension of the physiological roles of these natural N-domain ACEs in the cell nuclei requires studies using potential specific inhibitors.

N-domain ACE was detected in glomerulus from Wistar rats and SHR by immunofluorescence of a whole kidney section. In the same sections from SHR, ANG-(1–7) was localized in tubule and glomerulus, but ANG II was found predominantly only in glomerulus from SHR. These results showed that both peptides and N-domain ACE were present in MC from whole kidney, supporting the theory that, in vivo, MC express the N-domain ACE. Recently, using immunostaining, Ferrario et al. (24) showed ANG-(1–7) in the kidney of five SHR (3 treated with vehicle and 2 exposed to omapatrilat). Intense ANG-(1–7) staining, more pronounced in omapatrilat-treated SHR, was found in renal proximal tubules throughout the outer and inner regions of the renal cortex and the thick ascending loop of Henle, whereas no ANG-(1–7)-positive immunostaining was found in glomerulus or distal tubules. These data differed from our results, which showed ANG-(1–7) in MC from SHR and Wistar rats. The presence of MC in the glomerulus supporting glomerular capillaries differs from the report by Ferrario et al. Immunofluorescence data demonstrated ANG-(1–7) production in glomerulus and MC, suggesting that this peptide may contribute to the regulation of renal function and blood pressure.

The results described here and in previous studies demonstrate that all components of the renin-angiotensin system are present in MC and that ANG II is produced by the same cells, predominantly in the nucleus, suggesting an intracrine action of renin, ACE, ANG II, and ANG-(1–7). This production occurs in the cells that have been serum deprived for 24 h, indicating the natural production of peptides (1, 2, 34, 48). As described by Chai and Danser (9), ANG synthesis at tissue sites is well established and depends largely, if not completely, on kidney-derived renin. There is still debate over the exact tissue site of ANG generation (extracellular fluid, cell surface, or intracellular compartment). Recently, it was reported that the actions of intracellular ANG II might be mediated by direct nuclear effects and/or interaction with nuclear AT1-like receptors (3, 25). ANG II accumulation inside the nucleus has been found in other tissues, including myocardium, brain, smooth muscle, and adrenal glands (3, 15, 19, 39). Van Kats et al. (47) demonstrated that local ANG II synthesis in adrenal and kidney occurs predominantly extracellularly and is followed by rapid receptor-mediated endocytosis, leading to high intracellular levels. Navar et al. (36) described elevations in intrarenal ANG.
II by several processes, leading to levels much greater than can be explained from the circulating levels. In ANG II-dependent hypertension, ANG II is internalized via an AT₁ receptor mechanism, but intrarenal production of ANG II is also sustained. Using radioligand binding on wild-type mouse kidneys, Santos et al. (42) demonstrated cell-specific binding of ANG-(1–7), and they suggested that the physiological effects of this peptide occur through an interaction with its own receptor.

Cell fractionation for the isolation of nuclei, cytoplasm, and membrane followed by Western blot analysis of each fraction showed the predominant immunoreaction in the nuclei of MC from Wistar and SHR when 9B9 antiserum against ACE was used, confirming the data obtained by confocal microscopy.

Therefore, we have described for the first time the localization of N-domain ACE inside the cell nuclei. Iborra et al. (29) detected nuclear sites of protein translation by labeling perme-
able to mammalian cells or purified nuclei with fluorescent lysine and also detected discrete sites of transcription and translation within the nuclei. In their study, the accumulation of nuclear fluorescence was time dependent and sensitive to inhibitors of protein synthesis (cycloheximide and puromycin).

The authors estimated that the nuclear fluorescence represents protein synthesis in the nucleus. However, evidence reported by Iborra et al. showed that it is necessary to verify whether these particles represent cytoplasmic contamination or are synthesized in the nuclei.

Although our results suggest for the first time the presence of N-domain ACE inside the cell nuclei of SHR and Wistar rat MC and colocalization of ACE with ANG II and ANG-(1–7), we need to elucidate the shedding, degradation, and cellular traffic of these N-domain ACE isoforms and clarify whether these enzymes originate from an alternative splicing of the ACE mRNA or from posttranslational modification.

The presence of ANG II in the cell nuclei could suggest an important role for this peptide in the regulation of gene expression, as described by Erdmann et al. (19) when they localized ANG II to the nuclei in cerebellar neurons and to the transcriptionally active euchromatin in endothelial and granule cells.

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GRANTS

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REFERENCES


Fig. 10. NH2-terminal sequences of ACEInth1A and ACEInth2A and alignment with NH2-terminal sequences of somatic rat, mouse, and human ACE.

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ACEInth1A Asp Pro Gly Leu Gln X Gly
ACEInth2A Asp Pro Gly Leu Gln
Rat ACE Asp Pro Gly Leu Gln Pro Gly
Mouse ACE Asp Pro Gly Leu Gln Pro Gly
Human ACE Asp Pro Gly Leu Gln Pro Gly


