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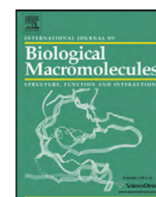

# Spectroscopic and structural analysis of somatic and N-domain angiotensin I-converting enzyme isoforms from mesangial cells from Wistar and spontaneously hypertensive rats

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Angiotensin I-converting enzyme (ACE) plays a key role in the renin-angiotesin aldosterone cascade. We analysed the secondary structure and structural organization of a purified 65 kDa N-domain ACE (nACE) from Wistar rat mesangial cells, a 90 kDa nACE from spontaneously hypertensive rats and a 130 kDa somatic ACE. The C-terminal alignment of the 65 kDa nACE with rat ACE revealed that the former was truncated at $Ser^{482}$, and the sequence of the 90 kDa nACE ended at $Pro^{629}$. Protein's secondary structure consisted predominantly of α-helices. The 90 and 65 kDa isoforms were the most stable in guanidine and at low pH, respectively. Enzymatic activity decreased with loss in secondary structure, except in the case of guanidine HCl where the 90 kDa fragment loses its secondary structure faster than its enzymatic activity. We identified and characterized the activity and stability of these isoforms and these findings would be helpful on the understanding of the role of nACE isoforms in hypertension.

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## 1. Introduction

Angiotensin I-converting enzyme (ACE, kininase II, E.C. 3.4.15.1) is an ectoenzyme that catalyzes the conversion of angiotensin I (AngI) to the vasoconstrictor angiotensin II (AngII) and the hydrolysis of bradykinin (BK) [1,2]. The enzyme is a widely distributed peptidase predominantly identified as a membrane-bound ectoenzyme in vascular endothelial cells, epithelial cells, and neuroepithelial cells. ACE isoforms are an evolutionarily conserved family of proteins. Somatic ACE (sACE) is a glycoprotein with a molecular mass of 130–190 kDa that consists of two homologous domains (N- and C-domains), each of which contains an active site with a conserved HEXXH zinc-binding motif. The two histidine zinc ligands are conserved in different tissues and organisms (Fig. 1A). Both domains have specific physiological roles and also display a certain degree of cooperativity [3–5]. The N-domain selectively hydrolyses angiotensin 1–7 [1,2,6–10] (Ang1–7) and AcSDKP a negative regulator of the recruitment of pluripotent hematopoietic stem cells into the cell cycle [6–13]. Male germinal cells also synthesize a shorter ACE form of 100–110 kDa that is essentially identical to the C-domain of somatic ACE [14,15]. Furthermore, a soluble form of ACE is also found in lymph, blood plasma, amniotic fluid, seminal plasma, and urine [9,16–20].

N-domain ACE (nACE) with a molecular mass of 65–108 kDa from ileal fluid and urine has been described previously [9,20,21]. A naturally occurring form of ACE comprising only N-domain was isolated from ileal fluid collected after surgery [21] and two ACE isoforms of 190 kDa (sACE) and 65 kDa (nACE) were purified from human urine of healthy subjects [9,20]. Furthermore, two N-domain ACE isoforms of 90 and 65 kDa that hydrolyze Ang1–7 and AcSDKP were detected in urine from mildly hypertensive untreated patients [9]. We previously purified and characterized sACE (130 kDa) and nACE (65 kDa) from mesangial cells (MC) of Wistar rats and two nuclear nACE (90 and 65 kDa) from SHRs

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**Fig. 1.** Schematic representation of primary structure of several members of the ACE protein family. (A) The locations of the active-site-zinc-binding motifs are indicated by HEXXH; transmembrane domains are in black. The sequence of tACE is identical to that of the C-domain of the sACE, except for its first 36 amino acids. Human tACE and sACE have the same carboxyl-terminal transmembrane and cytosolic sequence. Drosophila ACEs, AnCE and Acer all lack a membrane-anchoring sequence. Dimensions are not to scale. N, amino terminus; C, carboxyl terminus. Adapted from Riordan, 2003, Genome Biology, 4, 225 [39]. (B) The C-terminal alignment of 65 kDa N-domain ACE with rat ACE ended at Ser[482]. The same analysis for 90 kDa N-domain ACE evidenciated that the enzyme finished at Pro[629] amino acid after their alignment with rat ACE.

[12,13], of which the 90 kDa isoform has been linked with genetically caused hypertension [10]. Here we have analysed for the first time the secondary structure of purified somatic ACE and N-domain ACE fragments (90 and 65 kDa) from mesangial cells and also, we have identified and further characterized the activity and stability of these isoforms from rats to further our understanding of the role of N-domain isoforms in hypertension.

## 2. Materials and methods

### 2.1. Purification of ACE from medium and cells

All animal procedures and experiments were approved by the Institutional Ethics Committee.

ACE was purified from medium and cell lysate from primary MC cultures of Wistar rats and SHRs by gel filtration on AcA-34 and lisinopril-Sepharose chromatography as described previously [12,13]. The culture of mesangial cells was performed as described [22]. The cells from the 3rd and 4th passage number were characterized according to the following criteria: (1) morphology of stellate cells; (2) positive immunofluorescent staining for cellular fibronectin (clone FN-3E2, Sigma Co., USA) and Thy 1.1 (Sigma Co., USA); and (3) negative staining for cytokeratin (Clone C-11, Sigma Co., USA) and Human von Willebrand Factor.

### 2.2. Enzymatic activity assay

ACE catalytic activity was assayed using the substrate hippuryl-L-His-Leu (HHL) [23]. The derivatised product, L-His-Leu, was measured fluorimetrically (excitation at 365 nm and emission at 495 nm) using a Hitachi F-2000 fluorometer.

The enzymes were pre-incubated with different guanidine concentrations during 30 min, followed by the addition of Hip-His-Leu as substrate and incubation during 4 h at 37 °C. The reaction was carried out as described in the literature [23].

### 2.3. Protein determination

Protein concentration was determined by the Bradford method [24] (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the absorbance at 280 nm was used for chromatographic elution profiles. Samples containing Triton X-114 were assayed with the DC Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA).

### 2.4. C-terminal sequence of purified ACEs

The C-terminal amino acids of the 90 and 65 kDa mesangial cell nACE were determined after enzymatic hydrolysis with carboxypeptidases A and B. The ACE isoforms were incubated separately with carboxypeptidase A and B (Sigma Co., USA) in 10 mM Tris–HCl, pH 7.5 at 37 °C, and the enzymatic reaction was stopped after 0, 2.5, 5, 10, 15, 30, 60 and 90 min using 10% trifluoroacetic acid. Samples were evaporated to dryness under vacuum, resuspended in the mobile phase, and submitted for amino acid analysis. The amino acid sequences were analysed using the program BLAST (NCBI).

### 2.5. Circular dichroism spectroscopy

sACE and nACE (400 μg/mL) were dissolved in 50 mM Tris–HCl, pH 8.0 containing 150 mM NaCl for circular dichroism (CD) analysis. Far-ultraviolet CD spectra were obtained with a Jasco J-810

(Japan) spectropolarimeter using a 0.1 cm cell at 20 °C. Ellipticity was recorded from 200 to 260 nm and the scanning was repeated four times. Reference samples without protein were subtracted in all cases. Analysis of the α-helical content of the CD spectra was performed using the program CDNN [25]. ACE temperature-dependent far-ultraviolet CD spectra analysis was carried out in the same buffer as described above with a temperature range from 20 to 70 °C. The temperature was controlled using a Peltier-type temperature control system (TPC-423S/L, Jasco) and the heating rate used was 5 or 10 °C/min.

The pH effect on the structural integrity of ACE isoforms using CD analysis was investigated at three different pHs. The buffers used were: 50 mM Tris–HCl pH 9.0 and 50 mM sodium citrate pH 5.0 and pH 6.0.

### 2.6. Structural modeling

A model of rat nACE was generated by the ESyPred3D server (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/) [26] using testis ACE (PDB code 1O8A) as a model. This model was compared with the testis ACE structures [27,28] and the N-domain structure [29] and AnCE [30] using the program Coot [31]. Figures were generated with Pymol [32].

## 3. Results

We previously described the expression pattern and isolation of several forms of soluble ACE isoforms from Wistar (non-hypertensive) and spontaneously hypertensive rats. In order to characterize their structure and function further, we have purified and identified these isoforms by C-terminal sequencing.

### 3.1. ACE purification and activity

The concentrated lysate of MC from SHR and Wistar rat was purified by gel filtration followed by lisinopril-Sepharose chromatography as previously described [12,13]. The 90 and 65 kDa nACE forms had specific activities of 36.7 and 17.1 mU/mg, respectively. Somatic ACE from MC of Wistar rat was also purified and had a specific activity of 4.5 mU/mg. All purified ACEs analysed under dissociating conditions were homogeneous [12,13].

### 3.2. C-terminal sequence and modeling of ACE isoforms

The amino acid motifs at the C-termini of the N-domain constructs were determined by C-terminal sequencing (Fig. 1B). The C-terminal alignment of the 65 kDa nACE and the 90 kDa nACE with rat somatic ACE showed that they ended at $Ser^{482}$ and $Pro^{629}$, respectively. The 90 kDa nACE ending at $Pro^{629}$ corresponds to the entire N-domain as described by the crystal structure of the human N-domain [29]. The urinary 65 kDa N-domain is likely a limited proteolysis product of the native protein, therefore to visualize the structure of this N-domain fragment we created a model of the SHR 65 kDa nACE based on the human N-domain ACE structure.

The 65 kDa truncated form of mesangial cell ACE has 126 residues missing about 20% of the N-domain molecule, and one glycosylation site missing. This includes helices 21–27 compared to the human N-domain crystal structure. According to the model, this missing sequence, although mostly on the surface, also includes a helix that is threaded through the core of the molecule and forms part of the active site (Fig. 2). This deletion primarily affects the substrate or inhibitor binding of the S2 and S2′ residues of the active site. Helix 23 containing $Arg^{500}$, thought to be essential for chloride binding in the N-domain, is absent leaving, not only the chloride binding site, but also the active site exposed.

### 3.3. Stability of isoforms

In order to investigate the stability of these isoforms, CD spectra were collected for the 130 kDa sACE and the 90 and 65 kDa nACE (Fig. 3A) under conditions of varying pH, temperature and guanidine HCl. Under physiological conditions, the spectra for all three isoforms are very similar exhibiting ellipticity maxima at 222 and 208 nm, indicating a predominantly α-helical protein, as is observed in the crystal structure of the human enzyme [27]. The profile presented in Fig. 3A indicates the presence of some beta-sheets which was expected.

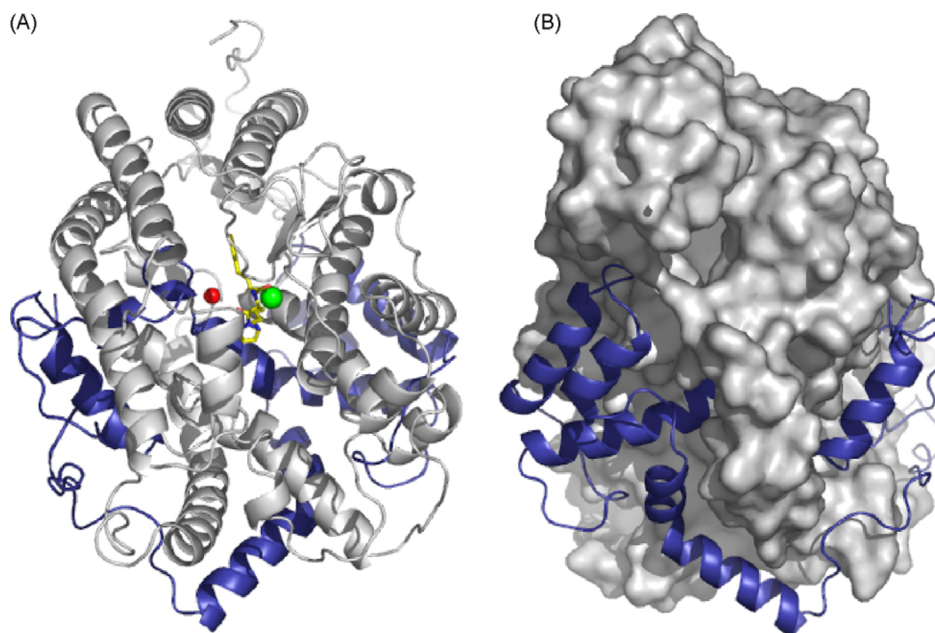


**Fig. 2.** Cartoon and surface representations of the modeled 65 kDa protein, viewed from the front of the molecule A, and the rear B. The region missing after truncation is shown in blue. (A) The inhibitor lisinopril modeled in the active site is shown yellow, zinc in green, and chloride in red.

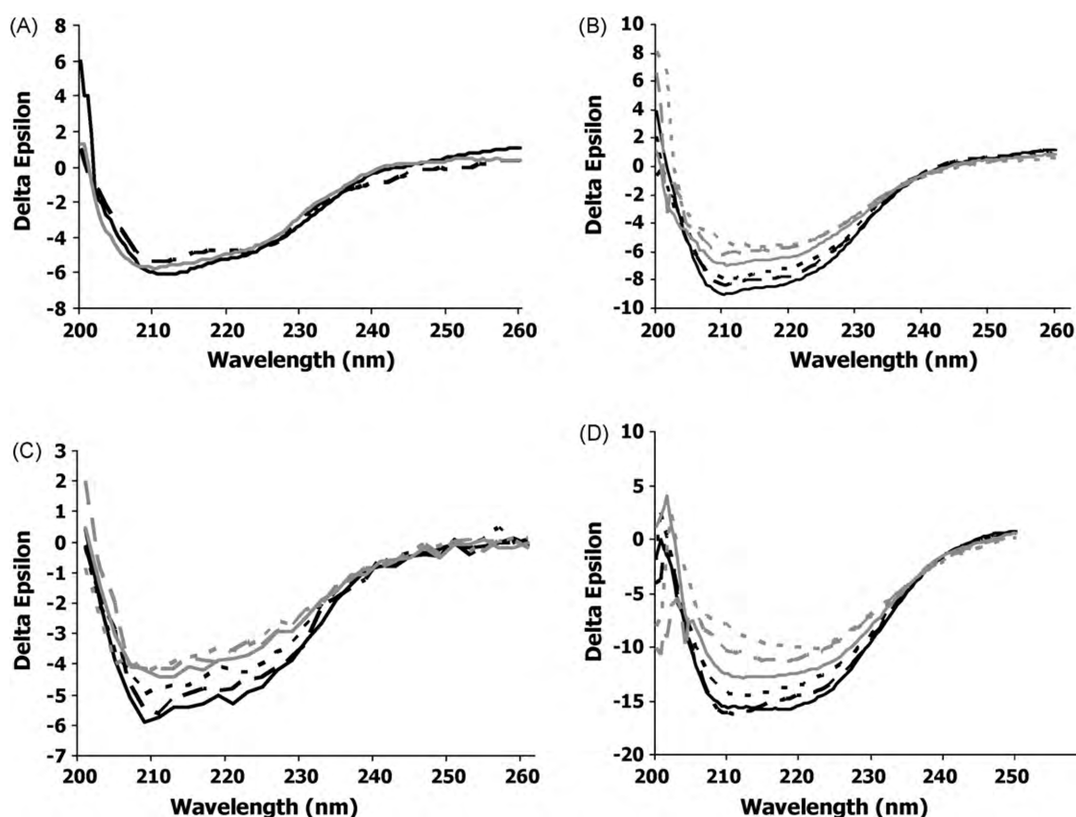


**Fig. 3.** Circular dichroism of ACEs. The CD spectra were measured from 260 to 200 nm using a 0.1 cm path length cell. (A) (–) 130 kDa sACE; (– –) 90 kDa nACE and (–) 65 kDa nACE. Temperature-dependent far-UV circular dichroism spectra were made with temperature range of 20–70 °C. The graphs represent experiments with the enzymes: (B) 130 kDa sACE, (C) 90 kDa and (D) 65 kDa nACEs. (—) 20 °C; (– –) 30 °C; (■ ■ ■) 40 °C; (—) 50 °C; (– –) 60 °C and (■ ■ ■) 70 °C. The loss of ellipticity as the temperature increases is indicative of loss of secondary structure. All of the experiments were made at buffer 50 mM Tris–HCl pH 8.0 and 150 mM NaCl

*3.3.1. Stability with low pH*

For all isoforms, on reduction of the pH to 5, a loss of helical structure was detected suggesting that the protein starts to unfold. This correlates with the previously observed reduction of enzymatic activity at low pH [12,13]. However, the 130 kDa sACE showed a 31.9% reduction in α-helices compared to 23% for 90 kDa nACE and 12.3% for 65 kDa nACE (Table 1) The random coil formation increased significantly by 20% and 33% for the 130 kDa sACE and 90 kDa nACE, respectively, but there was only a 5.4% increase in random coil formation for the 65 kDa nACE suggesting that the 65 kDa nACE is the most stable of these isoforms at low pH. The 90 kDa nACE also appeared to display a 12% increase in β-sheets with a concomitant decrease in pH. However, this apparent increase in the β-sheets could be related to the decrease of α-helices allowing an exposure of the β-structure.

*3.3.2. Stability with increase in temperature*

The thermal unfolding of the three ACE proteins was monitored by CD analysis (Fig. 3B–D). At temperatures ranging from 40 to 50 °C we detected significant alterations in the conformation of all enzymes with the loss of α-helix structure, which was confirmed by a loss of enzymatic activity, in agreement with work by Andrade et al. [12]. The 90 kDa fragment lost its structure quickest on thermal denaturation whereas the 65 kDa fragment was the most stable still retaining 96.9% of its helical structure at 50 °C.

*3.3.3. Stability in guanidine HCl*

The residual enzymatic activity after treatment with 0.5 M guanidine HCl was 8% for somatic ACE, 43% for the 90 kDa nACE, and 12% for and 65 kDa nACE (Fig. 4A). The 90 kDa nACE was only inactivated at 1.0 M guanidine suggesting that this form is more

**Table 1**
Percentage of secondary structure elements of ACEs enzymes in different pH. The used buffers were 50 mM Tris–HCl pH 8.0, Citrate sodium pH 5 and pH 6.0. Spectra were fitted using standard software (CDNN, available at http://bioinformatik.biochemetech.unihalle.de/cd_spec/cdnn).

| ACEs (kDa) | pH | α-Helix (%) | β-Sheets (%) | β-Turn (%) | Random coil (%) |
|---|---|---|---|---|---|
| 130 | 8 | 75.2 | 13.2 | 10.7 | 11.7 |
| | 6 | 45.8 | 11.5 | 13.5 | 31.9 |
| | 5 | 43.3 | 12.4 | 13.9 | 32.4 |
| 90 | 8 | 50.4 | 10.1 | 14.2 | 21.8 |
| | 6 | 33.5 | 16.7 | 16.2 | 34.4 |
| | 5 | 27.1 | 22.7 | 15.9 | 55.0 |
| 65 | 8 | 57.1 | 8.4 | 13.6 | 17.6 |
| | 6 | 30.0 | 18.4 | 18.8 | 23.1 |
| | 5 | 44.8 | 11.7 | 15.0 | 23.0 |

The values obtained by this software can have a deviation of between 5 and 10%.

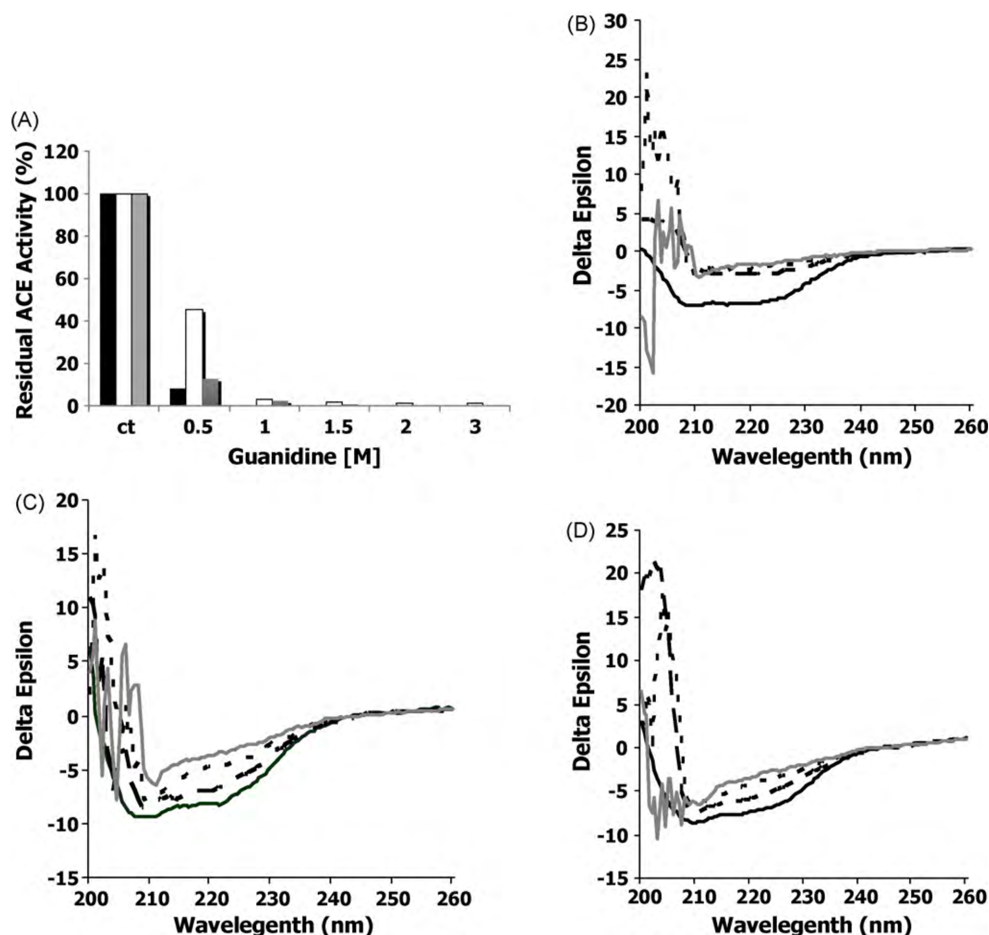


**Fig. 4.** ACEs activity and C D spectra in the presence of guanidine. (A) The activity detected in the guanidine studies demonstrated the recuperation of 8% and 12% of ACE activity for the somatic and nACE with 65 kDa at the guanidine concentration of 0.5 M. The 90 kDa nACE lost the activity only at the concentration of 1.0 M demonstrating that it is more resistant to the denaturation process. (■) 130 kDa sACE; (□) 90 kDa nACE and (■) 65 kDa nACE. The C D spectra of ACEs enzyme were represented in the three graphs: (B) 130 kDa sACE; (C) 90 kDa nACE and (D) 65 kDa nACE. These samples of ACEs at different guanidine concentrations were equilibrated for 1 h before CD experiments were performed. (–) control; (– –) 1 M guanidine; (■ ■ ■) 2 M guanidine and (–) 3 M guanidine.

resistant to guanidine. This reduction in activity for the 90 kDa nACE corresponded to a 60% decrease in α-helices at this concentration (Fig. 4B–D). Surprisingly, the 130 kDa ACE and 65 kDa nACE only showed a 50% decrease in α-helices in the presence of 2.0 M guanidine, suggesting that in this case the activity loss at low guanidine concentrations is not directly linked to loss of secondary structure and may be linked to small changes in tertiary structure instead.

## 4. Discussion

The occurrence of different isoforms of angiotensin-converting enzymes in both rats [12,13] and humans [21] is of functional interest as we have shown that the activity of these isoforms against the angiotensin I-like substrate Hip-His-Leu varies. We have shown in rats that the soluble N-domain fragment (65 kDa) observed in non-hypertensive rats has a specific activity approximately 30 times that of the full length soluble 2-domain ACE (130 kDa), whereas N-domain fragment (90 kDa) observed in spontaneously hypertensive rats (SHR) has a specific activity twice that of the 65 kDa fragment. This is particularly interesting as the 90 kDa fragment may be a genetic marker of hypertension in these rats [10]. The greater enzymatic activity of the 90 kDa fragment compared to the 65 kDa fragment is not surprising as modeling the structure of this fragment reveals that compared to the full length N-domain the 65 kDa fragment lacks several active-site residues. What is of more interest is why these N-domain isoforms have a higher specific activity towards the ACE substrate Hip-His-Leu compared to the fragment (130 kDa) containing both N- and C-domains, when hydrolysis of angiotensin I is predominantly hydrolyzed more efficiently by the C-domain [33]. The differences between somatic ACE and ACE fragments specific activity could be explained by the competition of the both active-sites N- and C-domain of somatic ACE for the same substrate (HHL and ZPheHL), differing from the N-domain fragment that has only one catalytic site, having for its action more disposable substrate. We cannot discard to explain also the difference between the N-domain and somatic ACE specific activity after purification, some loss at activity that always occurs due non ideal interaction with chromatographic materials or other small molecules in the solution. Despite the observation above, when the ratio ZPhe-His-Leu/Hip-His-Leu was analysed for N-domain fragment (65 kDa) and somatic ACE (130 kDa), the relation between these enzymes was 2:1, respectively, as expected for N-domain ACE [12]. The same ratio was observed when the 90 kDa was compared to somatic ACE.

To investigate these isoforms further we analysed their stability in different conditions of pH, temperature and guanidine HCl. ACE isoforms lost their secondary structure upon heating to 40–50 °C showing that these proteins are not highly thermostable, but there was more variation in their stability at low pH or in guanidine. At low pH the 65 kDa fragment appeared to retain the most of its secondary structure, whereas the 90 kDa fragment appeared to show some shift in secondary structure which hints at a complex response to changes in pH. These changes in CD spectra suggest that

there are minor structural rearrangements between neutral and acidic environments for all proteins. The 130 and 65 kDa forms lost their conformation and activity at lower guanidine concentration. The 90 kDa fragment also showed the greatest change in secondary structure upon denaturation with 1 M guanidine, although in this case it retained more of its activity for longer compared to the other two isoforms. This may be similar to creatine kinase which has been reported to retain partial enzymatic activity when denaturated or in an intermediately denatured state [34]. This ability to retain a higher level of activity against angiotensin I substrates and its possible potential to adapt to different conditions may be linked to its occurrence in hypertensive rats. In contrast, the presence of a partial N-domain in non-hypertensive rats may indicate that this degraded fragment is sufficient for the catalysis of substrates associated with the N-domain without such a high activity against angiotensin I substrates. The cause of this difference in the occurrence of these isoforms between non-hypertensive (with 130 and 65 kDa) and spontaneously hypertensive (90 and 65 kDa) rats is not clear, although it might be linked to the processing of the enzymes upon shedding from the cell membrane. Although not much is known about this process, it is possible that either the difference in glycosylation (the 65 kDa fragment lacks the C-terminal N-domain glycosylation site) and/or dimerization [35,36] may contribute to the complex nature of the activity and stability of these isoforms. Further work is needed to clarify the potential role of the 90 kDa fragment in hypertensive rats and how the variation in size and stability of naturally occurring N-domain isoforms contributes to their cleavage profile and functional role in general. Recent results published by our group indicated that the N-domain ACE has high affinity with angiotensin 1–7 a vasodilator peptide from angiotensin system that counter balances the actions of angiotensin II, thus collaborating to keep the hypertension panorama in the SHR animal [37]. In human studies, the presence of the 90 kDa N-domain ACE alone, or associated with a family history of hypertension, was correlated with hypertension and associated with endothelial disfunction. The presence of the 90 kDa N-domain ACE itself may have a negative impact on flow mediated dilatation stimulated by reactive hyperemia [38].

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