

Association of somatic and N-domain angiotensin-converting enzymes from Wistar rat tissue with renal dysfunction in diabetes mellitus

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Abstract

Diabetes mellitus (DM) is characterised by alterations in the intrarenal renin-angiotensin system (RAS). Insulin treatment may reverse these changes by an unknown mechanism. We aimed to verify the association between somatic ACE with 136 kDa (sACE) and N-domain ACE with 69 kDa (nACE) from Wistar (W) rat tissue with DM. Three groups were studied: control (CT), insulin treated diabetic (DT) and untreated (D). ACE activity was determined using Hippuryl-His-Leu and Z-Phe-His-Leu as substrates. In D group, urine ACE activity increased for both substrates when compared with CT and DT, despite the decreased activity of renal tissues. Immunostaining of renal tissue demonstrated that ACE is more strongly expressed in the proximal-tubule of D than in the same nephron portion in the other groups. Angiotensin (Ang) 1-7 and Ang II are less expressed in DT group when compared with CT and D. Ang II levels decreased in the D and DT groups showed when compared to the control. Ang 1-7 was detected in all studied groups with low levels in DT. The modulation of angiotensin peptides suggests that sACE, nACE, ACE 2 and NEP could have important functions in renal RAS regulation through a counter-regulatory mechanism to protect the kidney in diabetes mellitus.

Introduction

Diabetes mellitus (DM) is a chronic disease caused by inhibition or deficiency in insulin production by the pancreas due to destruction of β -cells.¹ This disease can cause vascular complications such as hypertension, nephropathy and retinopathy,^{2,3} but little is known about how hyperglycaemia in diabetes can cause vascular damage.⁴ The prevalence of hypertension and other vascular problems increases in diabetic patients as a result of alterations in the renin angiotensin system (RAS).⁵

The RAS in diabetes has been studied in detail including assessment of many components of this system in the kidney.⁶⁻⁸ Furthermore, it has been postulated that in diabetes there is a role for the RAS in mediating many of the functional effects such as changes in intraglomerular

haemodynamics⁹ as well as structural changes in the diabetic kidney at both glomerular and tubulointerstitium levels.^{9,10}

There is strong evidence that angiotensin-converting enzyme (ACE) activity is increased in serum^{5,11-15} and tissues such as lung,^{11,12} ventricle,¹⁶ mesenteric artery,¹⁷ aorta and heart¹⁸ of STZ-induced diabetic rats. Insulin treatment could reverse some of these changes,¹⁹ but the mechanism by which insulin treatment affects ACE activity is still unknown.

ACE is a dipeptidyl carboxypeptidase which has two active sites, the C and N-domains and is involved in the metabolism of two major types of vasoactive peptides. It converts the inactive decapeptide angiotensin I (Ang I) to angiotensin II (Ang II) which is a potent vasoconstrictor and inactivates the vasodilators bradykinin and kallidin.^{20,21} There are two isoforms of ACE: a somatic isoform (150–180 kDa) which is particularly abundant in the endothelium,²² and a smaller germinal isoform (90–100 kDa) identical to the C-terminal portion of endothelial ACE except in the first 36 residues.²³ A soluble isoform of ACE which is derived from the membrane-bound isoform by the action of secretases is also present in serum²⁴ and other body fluids such as urine.²⁵⁻²⁷

More recently, an ACE-related carboxypeptidase (ACE 2) was identified. This enzyme has 42% homology with ACE at the metalloprotease catalytic domain^{28,29} but differs from it by having only one enzymatic site. ACE 2 activity may counterbalance Ang II promoting effects by preventing Ang II accumulation in tissues.³⁰⁻³² ACE 2 hydrolyses Ang I to Ang 1-9 and also Ang II to Ang 1-7. Ang 1-9 in turn can be converted to Ang 1-7 by neutral endopeptidase and prolyl endopeptidase.

Two ACE isoforms with molecular weight of 190 and 65 kDa (N-domain ACE [nACE]) in the urine of healthy subjects, and two isoforms of 90 and 65 kDa (both nACE) in the urine of hypertensive patients, was observed by Casarini *et al.*^{25,26} The same profile was obtained by Marques *et al.*³³ in

the urine of Wistar–Kyoto and Spontaneously Hypertensive rats (SHR), and by Ronchi *et al.*³⁴ in different tissues of Wistar and SHR. In tissues of Wistar rats the somatic ACE (sACE) presented a molecular mass of 136 kDa and nACE 69 kDa.³⁴ The nACE is able to hydrolyse Ang 1-7 which is described as a specific substrate for this enzyme.²⁶ The current study was aimed to associate the possible alterations in expression and/or enzymatic activity of ACE forms with 136 and 69 kDa from Wistar rat tissue with renal dysfunction in DM.

Methods

Wistar rats used in the present experiments were obtained from the colony of Centro de Desenvolvimento de Modelos Experimentais (CEDEME), Escola Paulista de Medicina/Universidade Federal de São Paulo, Brazil. The Ethic Committee on Animal Experimentation from Universidade Federal De São Paulo approved this project.

Animal model

Experiments were performed on 299–328 g male Wistar rats. Diabetes was induced by a single intravenous injection of streptozotocin (50 mg/Kg; Sigma, Chemical, St. Louis, MO). Before the induction of DM, rats were kept in metabolic cages with free access to tap water, and measurement of their arterial blood pressure (BP) and blood glucose levels were performed. Only animals with plasma glucose concentration > 250 mg/dL two days after induction of diabetes were included in the study. Rats were divided into three groups: 1) control (CT) rats, (n=4); 2) untreated diabetic rats (D) (n=3) and 3) insulin treated diabetic rats (DT) (n=4) which were treated daily with 1 unit of human NPH insulin (Biohulin, Brazil). Eight weeks into the study and two days before completion, the rats were transferred into metabolic cages, urine was collected and then the rats were sacrificed by decapitation. The kidney was removed, washed in saline phosphate buffer (PBS) and frozen at -70°C until analysis.

Measurement of the systolic blood pressure and body weight

Systolic blood pressure (SBP) and body weight were measured in all groups twice weekly, during the study. SBP was determined by tail-cuff method and the Ohaus balance (Union, N. J.) was used to determine the body weight.

Measurement of blood glucose and proteinuria

Blood glucose levels were measured weekly during the study, using the ADVANTAGE (Roche, Swiss) equipment. Proteinuria was determined using the 24-hour urine collected two days before the animals were sacrificed

using the Kit Sensiprot (Labtest Diagnostica, Brazil).

Protein determination

Protein concentration was determined by the Bradford³⁵ method (Bio Rad Protein Assay Kit, Bio Rad, USA) using bovine serum albumin as standard, except when absorbance at 280 nm was used for the chromatographic elution profile.

Urine preparation

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. Urine samples were submitted to centrifugation (3,000 rpm) and the supernatant was concentrated in Centricon (Millipore, USA) and dialysed 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.

Tissue preparation

Tissue was homogenised as described by Oliveira *et al.*³⁶ in 100 mmol/L phosphate buffer (PB), pH 7.2, containing 340 mmol/L sucrose, 300 mmol/L NaCl (1 g tissue: 10 mL buffer) and inhibitor PMSF (100 mmol/L). The homogenates were centrifuged at 3,000 rpm, at 40°C for 10 min and the supernatant was frozen at -70°C until experimental use.

Enzymatic activity assay

ACE catalytic activity was determined fluorimetrically as described by Friedland and Silverstein.³⁷ An aliquot of kidney homogenate (20 µL) and urine (50 µL) was incubated with a 200 µL assay solution containing 1 mmol/L Z-Phe-His-Leu (ZPhe-HL) or 5 mmol/L Hippuryl-His-Leu (HHL) in 100 mmol/L potassium phosphate buffer, pH 8.3, with 300 mmol/L NaCl and 0.1 mmol/L ZnSO₄, for 10 minutes at 37°C. The enzymatic reaction was stopped by the addition of 1.5 mL 280 mmol/L NaOH and incubated with 100 µL o-phthalaldehyde (20 mg/mL methanol) for 10 minutes. The fluorescent reaction was stopped by the addition of 200 µL 3N HCl. The liberated dipeptide HL was measured fluorimetrically (360 nm excitation and 500 nm emission) using a Hitachi fluorimeter (Hitachi, Japan). The standard curve was obtained using varying concentrations of L-HL in the blank reaction mixture and it showed a linear relation between relative fluorescence and HL concentration.

Western blotting analysis

Electrophoresis was performed on a 7.5% of slab gel in presence of SDS according to the Laemmli³⁸ method, using 100 µg denatured and

reduced protein. Electrotransference was performed for 42 minutes at constant voltage (40 V) using a nitrocellulose membrane (Hybond ECL, GE Healthcare, Sweden). The membrane was incubated in a 5% non-fat dry milk blocking solution for four hours before overnight incubation at room temperature (20°C) with monoclonal antibody 9B9 (Chemicon International, USA) (diluted 1:1,000). The subsequent steps were performed with the streptavidin/phosphatase alkaline system (GE Healthcare, Sweden) and the bands were revealed using substrates NBT/BCIP as recommended by the manufacturer (Bio Rad, USA).

Morphological analysis and immunohistochemistry

Renal rat tissues were fixed in 10% buffered formol. The samples were submitted to a conventional histology preparation and stained with haematoxylin-eosin (H&E), periodic acid – Schiff (PAS) and picro-sirius. The analysis of the histological and morphological parameters to detect the presence, or not, of glomerular sclerosis, alterations in basal membrane, tubule, mesangium and collagen were carried out by means of optical microscopy.

The tissues were immunostained by the peroxidase-antiperoxidase method with specific antibodies against ACE (Chemicon International, USA), Ang II and Ang 1-7, a generous gift from Dr Preenie Senanayake, Eye Cole Institute, Cleveland Clinic, Cleveland, Ohio, USA (1:100). Briefly, kidney was rinsed with 0.05 mol/L Tris-phosphate-saline buffer, pH 7.6. The micro-dissected segments were incubated with the primary antibody overnight at 22°C in a humidified chamber followed by 60 minutes of incubation at 22°C with the secondary antibody (1:20) and then the peroxidase-antiperoxidase complex (1:100) for 60 minutes at 22°C. The immunoperoxidase reaction was visualised by incubating the sections in 0.1% (wt/vol) 3,39-

diaminobenzidine and 0.03% (vol/vol) hydrogen peroxide. The immunostained samples were dehydrated and protected with coverslips and haematoxylin counterstaining. Controls for the immunostaining procedure were prepared by omission of the first antibody.

Angiotensin quantification by HPLC

Tissue was weighed and homogenised with 100 mmol/L PB, pH 7.2, containing 340 mmol/L sucrose, 300 mmol/L NaCl and a mix of proteases inhibitors ethylenediaminetetraacetic acid tripotassium salt dihydrate (25 mM), o-phenanthroline (0.44 mM), pepstatin A (0,12 mM) and 4-chloromercuribenzoic acid (1 mM). 20 mL of solvent was added to 1 g tissue followed by centrifugation at 15,000 rpm for 20 minutes. The samples were concentrated in C₁₈ Sep-Pak columns activated with methanol (5 mL), tetrahydrofuran (5 mL), hexane (5 mL), methanol (5 mL) and water (10 mL). The peptides were eluted with ethanol, acetic acid and water (90:4:6). The elutions were then lyophilised and resuspended in 500 µL of mobile phase A: 5% acetonitrile (50 mL) in 0.1% orthophosphoric acid (1 mL). The peptide was separated in a reverse-phase column Aquapore ODS 300 (250 x 4.6 mm), 7 µ, using the gradient 5–35% of mobile phase B: 95% acetonitrile in H₃PO₄ 0.1% in the flow 1.5 mL/minutes for 40 minutes in the Milton Roy System, constituted of two constaMetric 3000 pumps, a UV detector spectroMonitor 3100, a programmer GM 4000 and a mixer. Synthetic standards were used and peptide detection was carried out at 214 nm. The results were expressed in ng/mL and corrected by the weight of the tissue.

Statistical analysis

Data were presented as mean±SEM. Student *t*-test and one-way analysis of variance (ANOVA), with all pairwise comparisons followed by the Tukey test, were used in this study. A value of *p*<0.05 was considered statistically significant.

Table 1 Physiologic parameters of CT and D and DT groups.			
	CT	Diabetes rats	
		D	DT
Body weight (g)	338.9±15.6	315.4±16.2	365.4±13.6
Blood pressure (mmHg)	104.7±1.2	103.8±0.8	105.8±0.5
Blood glucose (mg/dL)	76.2±2.7	343.0±81.9*	56.0±14.0 [#]
Urine volume (mL/24 hours)	7.2±0.8	61.2±12.9*	12.5±0.8 [#]
Proteinuria (mg/24 hours)	8.1±0.9	26.4±1.5*	15.9±1.58**

Key: Control (CT), insulin-untreated (D) and treated (DT) STZ diabetes rats. Values are mean±SE. **p*<0.05: vs. CT, X±EP: [#] = *p*<0.05: vs. D.

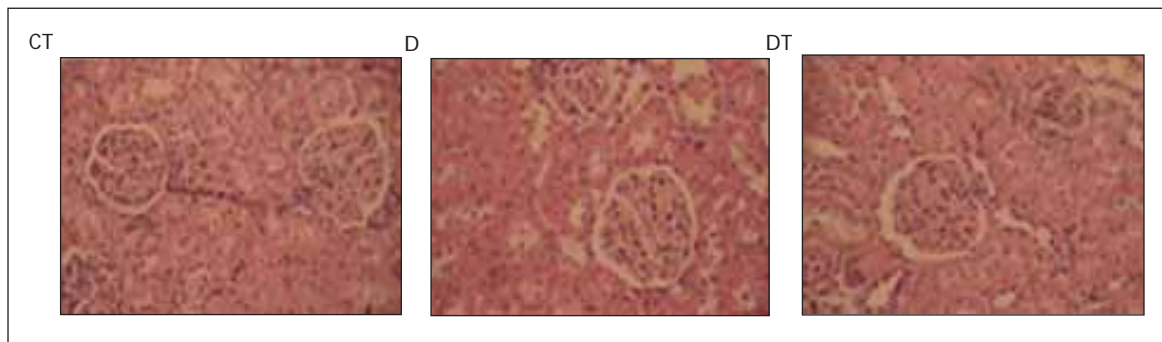


Figure 1
Morphological analysis of renal tissue using HE colouration for the different groups of Wistar rats studied: Control (CT); untreated diabetic rats (D); insulin-treated diabetic rats (DT). Magnification x 200.

Results

Physiologic parameters

Data on body weight, BP, blood glucose, urine volume and proteinuria in CT, D and DT rats are shown in table 1. Body weight of the D rats was reduced when compared to other groups. BP did not alter in any of the groups. D rats presented an increase (4-fold) in blood glucose levels and in urine volume. The proteinuria was significantly increased in both D and DT groups.

Morphological analysis

Morphological analysis of the rat kidney (figure 1) tissues from Wistar rats did not show any of the alterations normally described in diabetic nephropathy. We analysed also the basal membrane, tubules, mesangium using two different methods (data not shown) and did not detect modifications in renal histology in the D and DT groups.

ACE activity

ACE activity was increased significantly in the urine of the D group compared to the CT and DT rats for both substrates. ACE activity for the ZPhe-HL substrate in D increased when compared to CT and DT groups being 26.9 and 1.71-fold, respectively and for the HHL substrate 22.9 and 68.7-fold, respectively (figure 2).

Renal ACE activity was reduced slightly in the D group when compared to other groups for both substrates.

Expression of ACE protein in renal tissue

The expression of ACE protein was determined using Western blotting analysis. Proteins with molecular mass of ~136 and 69 kDa were expressed in renal tissue in CT, D and DT rats. The expression of 69 kDa isoform was reduced when compared to the 136 kDa isoform in renal tissue (figure 3a and b).

Localisation of ACE, Ang 1-7 and Ang II

Immunohistochemistry was performed to localise ACE, Ang 1-7 and Ang II in the kidney. The

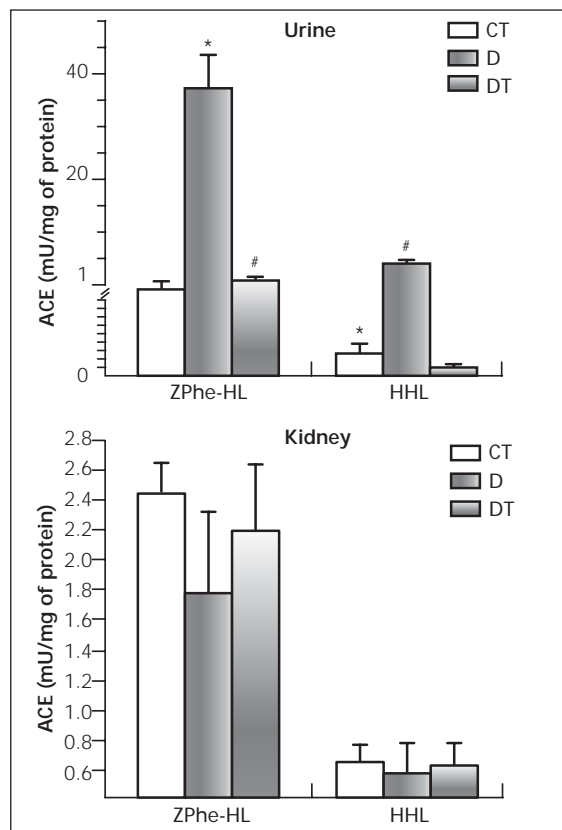


Figure 2
ACE activity (mU/mL) in urine and renal tissue of studied animals at the end of eight weeks of treatment. CT = control rats; D = untreated diabetic rats; DT = insulin-treated diabetic rats. Results are mean±SE. *p<0.05: vs. CT #p<0.05: vs. D rats.

highest expression of ACE was detected in the D groups (figure 4). There was a lower expression of Ang 1-7 in the D and DT groups. Ang II was strongly stained in the D when compared to CT and DT groups.

Angiotensin quantification

Ang 1-7, Ang II and Ang I concentration in total homogenates was determined by HPLC. No significant change in Ang 1-7 level was detected in renal tissue in any of the studied groups. In D and DT groups the levels of Ang II were

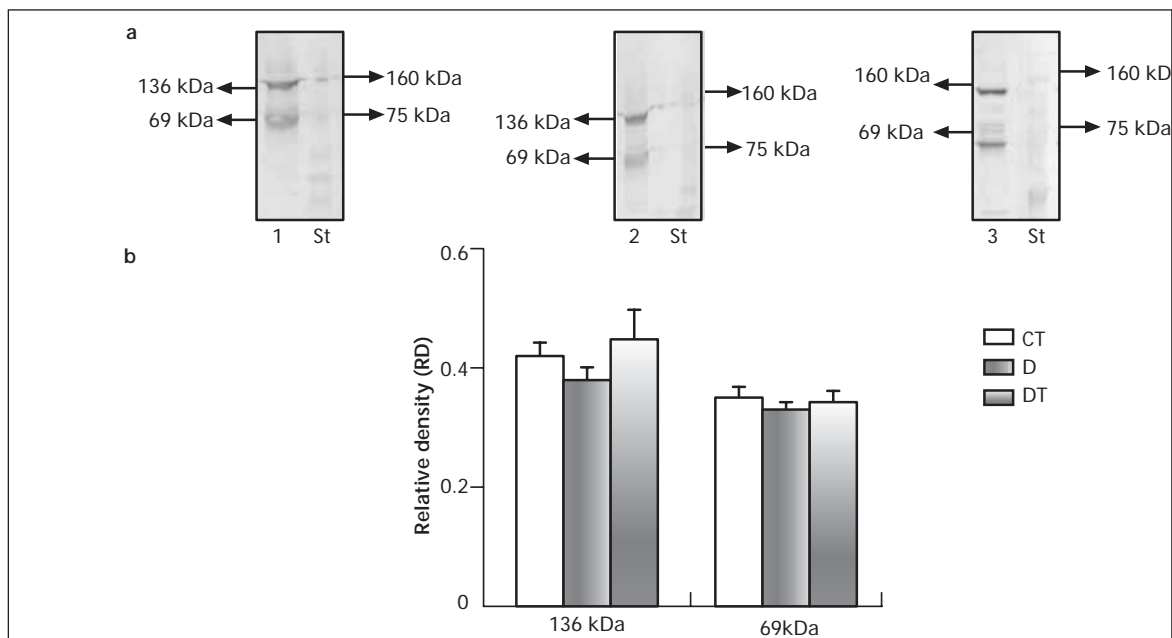


Figure 3
a: Western blotting analysis of ACE protein expression in renal tissue: 1 - control (CT); 2 - untreated diabetic (D); 3 - insulin-treated diabetic (DT) rats; standards (St) rainbow. Both isoforms were detected with the molecular weight of 136 and 69 kDa in all groups. **b:** Relative density of ACE protein isoforms in the different groups. Data is expressed as mean±SE.

decreased in the renal tissue when compared to CT. Ang I level did not alter in the different groups analysed (figure 5).

Discussion

DM is a serious risk factor for the development of renal and cardiovascular disease.³⁹ The RAS has been implicated in the pathophysiology of diabetic renal disease, based mainly upon the activity of ACE inhibitors (ACE-Is) and Ang II type I (AT₁) receptor blockers to reduce proteinuria and the progression of diabetic glomerulosclerosis.^{40,41}

ACE is widely distributed in tissues. The vasculature is one of the main locations of the enzyme, where activity of ACE promotes vascular responsiveness and cellular proliferation.^{42,43} There is evidence of a higher activity of this enzyme in serum and some tissues such as ventricle and lung^{11,12,16} in STZ-induced diabetic rats, but also a reduction of activity has been described in renal tissue.¹⁸

The objective of this study was to study the association of somatic and nACE protein expression and enzymatic activity in the renal tissue homogenate of Wistar rats, in which DM was induced by streptozotocin. We also aimed to analyse ACE activity in urine and quantify the Ang levels in tissues in the same model in association with DM.

In our study the body weight of the rats was reduced when we compared the CT to the DT

group (6.94% and 13.69%, respectively). The blood glucose was significantly higher in the D group (450%) when compared to the other groups, similar to data found by de Cavanagh *et al.*⁴⁴ The urine volume was 8.5-fold higher in the untreated diabetic rats than in the control group as shown by Erman *et al.*¹¹ Proteinuria is usually seen as a marker of glomerular damage. Many studies in different animal models suggest that the proteinuria may contribute to the glomerular and tubulointerstitial injury.⁴⁵ The analysis of urine 24 hours after induction of DM demonstrated an increase of proteinuria in the D and DT groups (26.4 and 15.7 mg/24 hours, respectively) when compared to control (8.1 mg/24 hours).¹¹

The morphologic study did not show any alteration in the renal tissue. We performed the morphological analysis 60 days after DM induction and probably this period of study was not sufficient to detect renal damage in Wistar rats. Siu *et al.* demonstrated that the number of podocytes per glomerular section from rats and mice with STZ-diabetes mellitus was reduced at 60 days only by 34%. Despite this early apparent loss of podocytes in the STZ diabetic rat they suggested that this model represents most animal models of diabetic complications in failing to develop classic changes of human diabetic nephropathy in the early stages of the development of diabetes.⁴⁶

Diabetes has been associated with the elevated activity of ACE in humans.⁴⁷ Mezzano *et al.*⁴⁸ described that in patients with diabetic

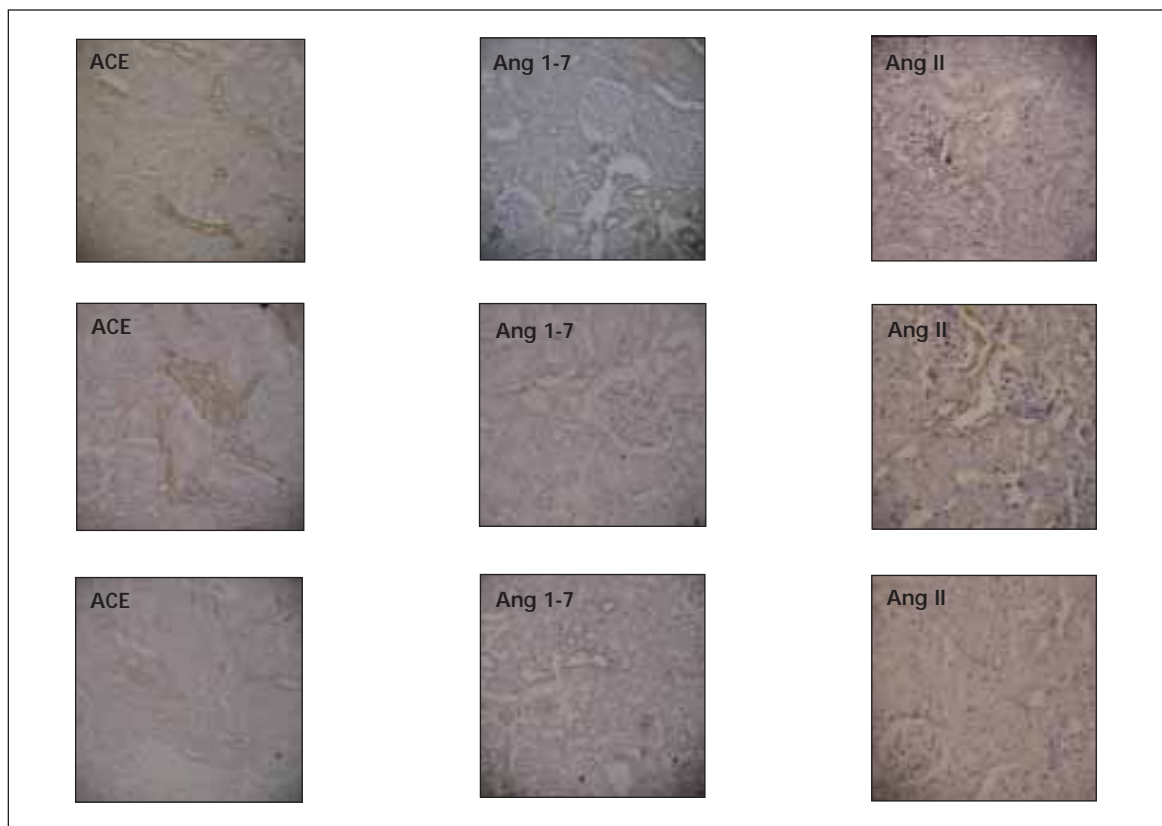


Figure 4
Localisation of ACE, Ang 1-7 and Ang II in the renal tissue using monoclonal antibody against ACE and polyclonal for Ang 1-7 and Ang II. Original magnification x 200.

nephropathy the components of RAS are modified in the renal compartments showing elevated local production of Ang II and activation of the tubule cells. These observations are in concordance with the results demonstrated by this study, where an increase of ACE activity was detected in the urine of the untreated diabetic rats when compared to other groups for both substrates (ZPhe-HL and HHL). The ZPhe-HL substrate is related to the N-domain activity of ACE while the HHL is described as a specific substrate for the C-domain portion. We suggest that nACE, despite its reduced expression, is more active in this model based on the high activity levels detected using ZPhe-HL. The decreased activity found for HHL could be attributed to sACE. The DT group showed a statistical significant reduction in urinary ACE activity when compared to D rats also for both substrates. This finding emphasises the potentially important role of renal ACE (somatic and N-domain) in the aetiology of the complications in diabetes.

Renal ACE activity using ZPhe-HL and HHL was decreased in the untreated diabetic rats when compared to other groups, which is consistent with previous studies^{8,18,49} where redistribution of ACE in the diabetic kidney has been shown indicating that ACE activity in the proximal

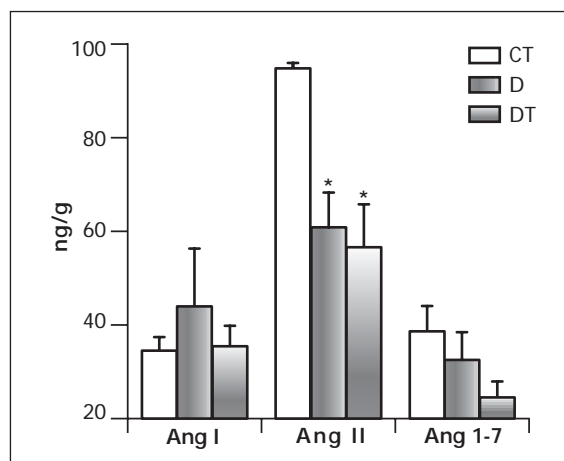


Figure 5
Qualification of Ang 1-7, Ang II and Ang I levels in kidney tissue from: CT = control (n=4); D = untreated diabetic (n+3); DT = insulin-treated diabetic (n=4) rats measured by HPLC. Data shown as mean±SE. *p<0.05: vs. CT.

tubule was reduced. Despite the reduction of ACE in renal tissue attributed to the proximal tubule, the increase of urinary ACE could be supported by the high ACE production in the collecting duct as described by Casarini *et al.*⁵⁰ in micropuncture studies, and by Quinto *et al.*⁵¹ in inner medullary connecting duct, suggesting a new site of ACE regulation in diabetes.

The molecular mass of ACE isoforms from renal tissues (136 and 69 kDa) of Wistar rats were similar to the recent findings described in healthy human urine,^{25,26} normotensive rat urine³³ and in different tissues of Wistar rats by Ronchi *et al.*³⁴ The protein expression of 136 and 69 kDa enzymes showed no statistically significant decrease in the renal tissue of D and DT groups when compared to the C which was similar to the results found for ACE activity. The isoform with a molecular weight of 90 kDa and considered a genetic marker of hypertension detected in SHR tissues was not expressed in the renal tissue of Wistar rats, confirming the results described by our group.³⁴

ACE was detected in complete kidney sections from untreated rats in the proximal tubule and glomerulus. In the same sections we detected Ang 1-7 and Ang II in untreated and treated animals confirming our previous results in the quantification of these peptides.

When we quantified the angiotensin peptides, Ang I was increased in renal tissue of D and DT groups indicating a probable activation of renin, similar to the data described in the same model by Anderson *et al.*⁸ Ang 1-7 levels are similar in all studied groups though being decreased non-significantly D and DT.

This finding could be supported by the lower levels of Ang II in the D and DT groups indicating the action of prolyl endopeptidase or ACE 2 upon this peptide to produce Ang 1-7 that could be hydrolysed by nACE that is activated in this model. The treatment of diabetic animals decreased production of the peptides when compared with D but did not normalise the levels to those of the control, suggesting that other mechanisms in diabetes need to be further studied. The presence of Ang 1-7 could protect the kidney against damage by maintaining adequate natriureses and diureses as described by Santos *et al.*⁵²

This is the first description of the presence of Ang 1-7 in the kidney in favouring ACE 2 or prolyl endopeptidase activation in STZ-induced diabetic rats. The increase in Ang I levels as a result of renin activation suggests an action of NEP upon this peptide, contributing also to the Ang 1-7 formation to counterbalance the action of Ang II to protect the organ against damage in DM. The presence of nACE with 69 kDa could contribute to the Ang 1-7 degradation to Ang 1-5.

Our observations are consistent with a modulation of angiotensin peptides and enzymes responsible for their production and degradation. The roles of this modulation represent new avenues for future investigation of the mechanisms involved in these pathways.

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