Renin–angiotensin system may trigger kidney damage in NOD mice

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Abstract
Diabetic nephropathy is a complication of diabetes and one of the main causes of end-stage renal disease. A possible causal link between renin–angiotensin aldosterone system (RAAS) and diabetes is widely recognized but the mechanisms by which the RAAS may lead to this complication remains unclear. The aim of this study was to evaluate angiotensin-I converting enzyme (ACE) activity and expression in numerous tissues, especially kidney, of non-obese diabetic mouse. Kidney, lung, pancreas, heart, liver and adrenal tissues from diabetic and control female NOD mice were homogenized for measurement of ACE activity, SDS-PAGE and Western blotting for ACE and ACE2, immunohistochemistry for ACE and angiotensins I, II and 1-7 and bradykinin quantification. ACE activity was higher in kidney, lung and adrenal tissue of diabetic mice compared with control mice. In pancreas, activity was decreased in the diabetic group. Western blotting analysis indicated that both groups presented ACE isoforms with molecular weights of 142 and 69 kDa and a decrease in ACE2 protein expression. Angiotensin concentrations were not altered within groups, although bradykinin levels were higher in diabetic mice. In pancreas, activity was decreased in the diabetic group. Western blotting analysis indicated that both groups presented ACE isoforms with molecular weights of 142 and 69 kDa and a decrease in ACE2 protein expression. Angiotensin concentrations were not altered within groups, although bradykinin levels were higher in diabetic mice. The immunohistochemical study in kidney showed an increase in tubular ACE expression. Our results show that the RAAS is affected by diabetes and the elevated ACE/ACE2 ratio may contribute to renal damage.

Introduction
Hypertension is frequently associated with diabetes mellitus, especially type II, increasing the incidence of vascular complications. In addition, about 40% of type I diabetic patients may develop diabetic nephropathy which can lead, in advanced stages, to renal failure. This complication can be divided into five stages, and during the first three it has already been shown that diabetic nephropathy can be at least partly reversed using angiotensin-I converting enzyme inhibitors (ACEI).1 The etiology of diabetic nephropathy is poorly understood, but family studies have suggested a genetic predisposition. The connection between diabetic nephropathy and angiotensin-I-converting enzyme (ACE) indicates that a high constitutive level of ACE is deleterious to the kidney in the setting of chronic hyperglycemia.2,3

ACE is a dipeptidase capable of hydrolyzing the carboxyl end of the decapeptide Angiotensin-I (AI), thus releasing the vasopressive octapeptide angiotensin-II and the dipeptide histidyl-leucine. Angiotensin-II plays an important role in the renin-angiotensin aldosterone system (RAAS) of blood pressure. Moreover, ACE inactivates bradykinin (BK) and angiotensin(1-7) vasodilator peptides.4 ACE presents two distinct catalytic domains, called N- and C-terminus.5 Both sites hydrolyze AI; however, the N-domain has two specific physiological substratum, Ang(1-7) and N-acetyl-Seryl-Aspartyl-Lysyl-Proline (AcSDKP).6-8 ACE has been purified and characterized from a wide range of tissues and species, and has already been described in different isoforms: somatic ACE, containing both N and C catalytic domains, weighs between 190 kDa and 140 kDa and has a wide tissue distribution; testicular ACE, found in male germinal cells, contains the C domain and has a molecular weight of between 100 kDa and 110 kDa;9,10 and a soluble form of ACE has been described in lymph, blood plasma, amniotic fluid, seminal plasma and urine.11-16

In addition to the RAAS, the kallikrein/kinin system (KKS) represents an important regulator of cardiocirculatory function. Kinins are potent vasodilator peptides formed by the action of

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enzymes known as kallikreins acting on substrates called kininogens. The role of the KKS and RAAS in cardiocirculatory control can be better understood if we analyze the interaction between Ang(1-7) and BK in many tissues and animal models. In experiments with canine coronary arteries and mesenteric arteries of rats, it was observed that Ang(1-7) potentiates the vasodilatory effects of BK, and this effect is also observed in human vessels.

Casarini et al. described 190 kDa and 65 kDa ACE isoforms in human urine from healthy subjects. They also analyzed the urine from mildly hypertensive untreated patients and separated 90 kDa and 65 kDa isoforms of the N-domain ACE. These results, combined with data from Marques et al. who used genetically and induced hypertensive rat strains, suggest that the 90 kDa N-domain isoform may function as a genetic marker of hypertension.

The main objective of our study was to evaluate ACE activity and expression in several tissues, especially kidney, of the non-obese diabetic mouse, and to evaluate the role of the RAAS in the setting of diabetes complications.

**Materials and methods**

**Blood glucose levels measurement**

Ten-week-old mice had their blood glucose levels determined weekly using the ACCU-CHECK Sensor® (Roche). Blood samples from the caudal vein were obtained and used for glucose level determination. Female NOD mice were considered diabetic when a glucose level higher than 250 mg/dl was observed (group D-NOD). Mice from the same offspring that did not develop diabetes were used as controls (CT-NOD). Mice were 20–24 weeks old when the experiments were performed.

**Mouse tissues samples**

Assays were performed using kidney, lung, heart, liver, adrenal and pancreas samples. Animals were decapitated and tissues were rapidly harvested, rinsed and homogenized in 0.4 M borate buffer (BB), pH 7.2, containing 0.34 M sucrose and 0.9 M NaCl. Tissue homogenate was centrifuged at 3000 rpm for 10 min and the supernatant was frozen at -70°C (1 g tissue: 10 ml buffer).

**Protein concentration**

Protein concentration was determined by Bradford method using serum albumin as the standard. In order to perform this assay, samples from all tissues, except adrenal, were diluted tenfold.

**ACE activity**

ACE activity in mouse tissues was determined as described by Oliveira et al. Tissue homogenates (10 μl) were incubated with 500 μl of assay buffer containing 5 mM of hippuryl-His-Leu in 0.4 M sodium BB. The reaction was stopped by the addition of 1.0 mL of 0.34 N NaOH. The product, His-Leu, was measured fluorimetrically at 365 nm excitation and 500 nm emission with a spectrophotometer as follows. Samples were incubated with 100 μl of o-phtaldialdehyde (20 mg/ml) in methanol for 10 min followed by acidification by the addition of 200 μl 3 N HCl and centrifugation at 3000 rpm for 10 min. In order to correct the intrinsic fluorescence of the tissues, blank samples were prepared adding tissue homogenate after NaOH.

**Western blotting**

SDS-PAGE (7.5%) was developed with the equivalent of 50 μg of protein, which was lyophilized and dissolved in 30 μl of sample buffer. The following steps were performed according to the method described by Laemmli et al. Samples were reduced with a solution of 2-mercaptoethanol (20 mg/ml) and heated up in a dry bath at 95°C for 5 min before separation through electrophoresis in polyacrylamide gel 7.5%. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (Hybond GE Healthcare) which was then incubated with a blocking solution (5% of non-fat dry milk in TBST) for 4 h before incubation overnight with specific monoclonal antibody against ACE (9B9, Sigma) and ACE2 (RD Biosystems) followed by membrane incubation with secondary antibody (1:2000) for 1 h with streptavidin/alkaline phosphatase (1:2500). Color development was performed using NBT-BCIP complex (BioRad) according to manufacturer’s recommendations.
Immunohistochemistry and morphology

Kidney sections were incubated overnight at 22°C with the primary antibody (anti-ACE – H-170, Santa Cruz Biotechnology), followed by incubation with the unlabeled secondary antibody, PAP complex, and diaminobenzidine/hydrogen peroxide for color development, yielding a brown color. Incubation in 3% hydrogen peroxide/methanol for 20 min eliminated any remaining peroxidase activity. The tissue sections were observed and photographed using a Nikon Optiphot microscope with a Nikon Microflex UFX IIA photographic system (Nikon Corp., Tokyo, Japan). In addition, kidney slices were also stained using hematoxylin–eosin in order to investigate the presence of renal lesions related to diabetes.

Angiotensin and bradykinin quantification by high-performance liquid chromatography

Kidney was weighed and homogenized in 100 mM sodium phosphate buffer pH 7.2, 240 mM sucrose and 300 mM NaCl, containing a mix of proteases inhibitors (EDTA potassium (25 mM), o-phenanthroline (0.44 mM), pepstatin A (0.12 mM) and 4-chloromercuribenzoic acid (1 M)). A volume of 20 ml of solvent was added to 1 g tissue, followed by centrifugation (15,000 rpm, 20 min). Supernatants, provided from wash steps, were submitted to Sep-Pak Cartridge columns (Waters, USA). Samples were concentrated in C18 Sep-Pak columns, activated in methanol (5 ml), tetrahydrofuran (5 ml), hexane (5 ml), methanol (5 ml) and water (10 ml) (extraction of angiotensins), and acetonitrile 90% (3 ml), water (5 ml) and 5% acetonitrile in H3PO4 0.1% (mobile phase A, 5 ml) (BK extraction). After sample introduction, columns were washed with water (10 ml) and peptides of interest were eluted with ethanol, acetic acid and water (90:5:6) for angiotensins and 35% acetonitrile in H3PO4 0.1% for BK. The eluted fractions were lyophilized and resuspended in 500 µl mobile phase A: 5% ACN in 0.1% orthophosphoric acid (1 ml), in a final volume of 1 ml, and filtered with 0.22 µm membrane before high-performance liquid chromatography (HPLC). Peptides was separated by HPLC (Shimadzu System, Japan) in reverse phase column Aquapore ODS 300 (250 mm × 4.6 mm), 7 µm, using a linear gradient from 5% to 35% of mobile phase B (95% acetonitrile in H3PO4 0.1%), at a flow rate of 1.5 ml/min for 40 min. The HPLC column was calibrated using synthetic standards and peptide detection was analyzed with absorbance at 214 nm. Results were expressed as nmol/g of tissue.

Statistical analysis

Results are expressed as means ± SE. The data were compared by unpaired Student’s t-test. Differences were considered statistically significant when p<0.05.

Results and discussion

In the kidney, ACE activity (Figure 1) and expression (Figure 4) were found to be significantly higher in D-NOD as compared with the CT-NOD group. Moreover, we also showed increased local expression of ACE in the cortical region (proximal tubules) of kidneys from D-NOD mice, (Figure 7b) with no changes in local expression of AI or AII (Figure 7c, 7d, 7e and 7f). It is interesting to note that this result is consistent with previous data from our group that showed increased ACE activity in cell lysate of mesangial cells from diabetic mice (data not shown). Huang et al. reported that diabetes increases ACE activity in mice bearing one, two or three copies of the ACE gene, which is associated with increased urinary albumin excretion, in line with our results. On the other hand, Wysocki et al. showed decreased activity, protein and mRNA
ACE expression in renal cortex extracts from diabetic, db/db or streptozotocin-treated mice, and we believe that this difference could be attributed to numerous factors such as the animal model that was used and the duration of diabetes. Considering data from the literature and our results, we suggest that high levels of ACE activity and/or expression, caused by decrease in the clearance of ACE, could be one of the mechanisms responsible for the initiation of events leading to diabetic nephropathy.\(^5\)

Hematoxylin–eosin staining revealed no structural damage in kidney slices of the D-NOD group, when compared with the CT-NOD group (Figures 6a and 6b). This result is consistent with the literature, showing that NOD mice present mild morphological changes\(^32\) and therefore represent one of the best mouse models to study the very early stages of diabetic nephropathy.\(^5\)

In a previous study from our group in the streptozotocin diabetes model, we demonstrated an increase in the concentration of AI and a slight reduction in AII in kidney homogenates.\(^34\) However, in the present study no difference between groups was observed regarding angiotensin peptides concentration (Figure 2). This response seems to be related to the small effect that increased ACE activity exerts upon its products, and is in accordance with previous data presented by Huang et al.\(^3,35,36\)

![Figure 4](image_url)

**Figure 4**
Western blotting analysis of ACE protein expression in heart (H), lung (Lg), kidney (K), pancreas (Pa), adrenal (Ad) and liver (Lv) homogenates of CT-NOD (A) and D-NOD (B) (Sd = molecular weight standard). Two ACE isoforms were detected in all tissues from both groups weighing 142 kDa and 69 kDa. Graphs present the relative density (RD) of D-NOD/RD of the CT-NOD group for both forms of ACE.
The relatively recent description of ACE2 brought important new data and perspectives to the understanding of RAAS regulation. The role of ACE2 in the context of diabetes has been widely explored in the past few years, and different studies have shown that decreased ACE2 expression was associated with increased albuminuria, and pharmacological inhibition of the enzyme increased urinary albumin excretion three to fourfold in diabetic mouse models. Although some studies describe an increase in ACE2 mRNA levels and protein expression in diabetes, Reich et al. described a decrease in the same parameters in renal biopsies of patients with kidney disease due to diabetes. Similarly, our results showed a decrease in ACE2 protein expression in the kidney from D-NOD mice (Figure 5). Taken together with the increased ACE activity and expression that we found in the kidney, we can suggest that an imbalance between ACE and ACE2 could represent an important pathological mechanism in the setting of diabetic nephropathy.

A provocative finding in our study was that BK levels were increased in the D-NOD group (Figure 3), despite the increased activity and expression of ACE which degrades this peptide. Campbell et al. also found an increased amount of BK in renal tissue from STZ-treated rats, with no change on the ratio BK(1-7)/BK(1-9). This finding shows an existing balance between formation and degradation of BK, and suggests that the enhanced BK concentration would be caused by increased activity of kallikrein rather than by changes in kinins metabolism by NEP or ACE.

Looking at the other tissues studied, we found significantly increased activity of ACE in the lung and adrenal tissue in the D-NOD group. The same group also exhibited a significant decrease in ACE activity in the pancreas, while heart and liver presented no major change. In lung tissue, literature has described the increase of ACE activity in diabetic animals, again in STZ model of diabetes. Pueyo et al. demonstrated a higher level of ACE mRNA and increased shedding of ACE from the pulmonary bed in diabetic animals, correlated to increased activity in plasma, suggesting that this would be due to increased expression and release of the enzyme.
In adrenal tissue, Fredersdorf et al. demonstrated increased levels of aldosterone in diabetic animals. These results could be related to augmented activity of ACE both in kidney and adrenal tissue, which could enhance the amount of AII with stimulation of the production of aldosterone. However, it is important to emphasize that the size of the gland represents a limiting factor for assessing the concentration of angiotensin peptides present in the tissue.

Through western blotting it was also possible to investigate whether the 90 kDa N-domain ACE was present in tissues from the NOD mice, more...
specifically in kidney homogenate. Two isoforms of ACE were detected: the somatic ACE (sACE) at 142 kDa and a 69 kDa N-domain ACE (nACE), corroborating the results presented by Ronchi et al. The absence of the 90 kDa ACE isoform in both groups of NOD mice reinforces the specificity of this biomarker for hypertension once this mouse strain is not described as hypertensive. In fact, our D-NOD group presented a significant decrease in mean arterial blood pressure (data not shown), and this effect seems to be due to osmotic diuresis caused by hyperglycemia.

The combined effects of increased ACE activity and expression and decreased ACE2 expression in kidney may be associated with the modulation of the RAAS in this mouse model. Based on our findings and previous studies, we may assume that also in the NOD mouse model the higher ACE/ACE2 ratio in kidneys may contribute to renal injury leading to overt nephropathy. Taken together, our results show the role of RAAS in kidney damage in NOD mice, and represent a promising field of study on this model which is still poorly explored in the setting of diabetes complications.

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Conflict of interest

None declared.

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