Characterization of Renal Angiotensin-Converting Enzyme 2 in Diabetic Nephropathy

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Abstract—ACE2, initially cloned from a human heart, is a recently described homologue of angiotensin-converting enzyme (ACE) but contains only a single enzymatic site that catalyzes the cleavage of angiotensin I to angiotensin 1–9 [Ang(1–9)] and is not inhibited by classic ACE inhibitors. It also converts angiotensin II to Ang(1–7). Although the role of ACE2 in the regulation of the renin-angiotensin system is not known, the renin-angiotensin system has been implicated in the pathogenesis of diabetic complications and in particular in diabetic nephropathy. Therefore, the aim of this study was to assess the possible involvement of this new enzyme in the kidney from diabetic Sprague-Dawley rats to compare and contrast it to ACE. ACE2 and ACE gene and protein expression were measured in the kidney after 24 weeks of streptozocin diabetes. ACE2 and ACE mRNA levels were decreased in diabetic renal tubules by ~50% and were not influenced by ACE inhibitor treatment with ramipril. By immunostaining, both ACE2 and ACE protein were localized predominantly to renal tubules. In the diabetic kidney, there was reduced ACE2 protein expression that was prevented by ACE inhibitor therapy. The identification of ACE2 in the kidney, its modulation in diabetes, and the recent description that this enzyme plays a biological role in the generation and degradation of various angiotensin peptides provides a rationale to further explore the role of this enzyme in various pathophysiological states including diabetic complications. (Hypertension. 2003;41:392-397.)

Key Words: angiotensin-converting enzyme ■ diabetic nephropathy ■ angiotensin ■ diabetes mellitus

Angiotensin-converting enzyme (ACE) is a key enzyme in the renin-angiotensin system (RAS).¹ It contains 2 active domains and converts angiotensin I to angiotensin II, which is a potent vasoconstrictor, growth modulator, and proinflammatory peptide. In addition, this enzyme degrades bradykinin, a vasodilator.¹ A chemically related enzyme, ACE-related carboxypeptidase, also known as ACE2, has recently been cloned and identified by 2 different groups.²,³ ACE2 has 42% homology with ACE at the metalloprotease catalytic domain,²,³ but differs from ACE in having only one enzymatic site. In humans, ACE2 transcripts have been identified in the heart, kidney, and testis.²,³ It has been shown that recombinant ACE2 hydrolyses the carboxy terminal leucine from angiotensin I to generate angiotensin(1–9).²,³ ACE2 also has a high affinity for angiotensin II,⁴ resulting in its degradation to the vasodilator, angiotensin(1–7).² Furthermore, ACE2 is not inhibited by classic ACE inhibitors such as captopril and lisinopril.² A rat homologue of ACE2 has been cloned (GenBank No. AF291820) that allows exploration of this metalloprotease in rodents in normal and disease states such as diabetes, in which the RAS is considered to play a pivotal role in the development of complications.⁵

It is well known that the RAS has a range of hemodynamic and nonhemodynamic effects.⁶,⁷ All the previously reported components of the RAS system have been described and measured in the kidney, including the enzymes renin and ACE as well as the 2 angiotensin II receptor subtypes.⁵,⁸ In ACE2 mutant mice, changes in the angiotensin peptides A1 and AII have been described, implicating ACE2 as an important regulator of a number of angiotensins, including angiotensin I, angiotensin II, angiotensin(1–9), and angiotensin(1–7). ACE2 is also present in the kidney,²,³ but whether it is modulated, like ACE, in various disease states has not yet been examined.

The RAS in diabetes has been studied in detail including assessment of the various components of this pathway in the kidney.⁵,⁹,¹⁰ 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 hemodynamics¹¹ as well as structural changes in the diabetic kidney at both the glomerular and tubulointerstitial levels.¹¹,¹² Treatment with agents that interrupt the RAS such as ACE inhibitors and angiotensin II receptor antagonists have been shown to confer renoprotection in experimental and human diabetic nephropathy.¹³,¹⁴ To further characterize the RAS and to assess the possible involvement of this new component of the RAS, gene and protein expression and localization of
ACE2 have been examined in the kidney from Sprague-Dawley rats in the absence and presence of diabetes and compared with changes in the related enzyme, ACE. Furthermore, the effect of blocking the primary ACE with a specific ACE inhibitor, ramipril, on these parameters and in particular, ACE2 was studied.

Methods

Animal Model

Experimental diabetes was induced in 8-week-old male Sprague-Dawley rats weighing 200 g by intravenous injection of the β-cell toxin streptozocin (60 mg/kg) after an overnight fast. Only animals with plasma glucose concentrations >20 mmol/L 1 week after induction of diabetes were included in the study.15 Two to 4 units of Ultralente insulin (Ultratard HM, Novo-Nordisk) was administered daily to each diabetic animal (n=8) to promote well-being and improve survival. Body weight, albumin excretion rate,15 and glycated hemoglobin16 were measured after 24 weeks of diabetes. Sham-injected control animals (n=8) were followed concurrently. A separate group of diabetic animals (n=8) was treated with the ACE inhibitor ramipril at a dose of 3 mg/kg in drinking water. The dose of ramipril is based on previous studies by our group, confirming that this dose is associated with significant renoprotection.12,17 The 24-week time point was chosen because it has been shown previously that after this period of diabetes there is evidence of functional and structural injury in the kidney including albuminuria,15 glomerular ultrastructural changes,15 and tubulointerstitial injury.15 Throughout the study, animals were given access to food and water ad libitum. All animal procedures were in accordance with guidelines set by the Austin and Repatriation Medical Center Ethics Committee and the National Health and Medical Research Council of Australia.

Isolation of Total RNA and Synthesis of cDNA

Whole kidney was first minced and then sieved through graded sieves to yield glomerular and tubular fractions.15 Glomerular and tubular suspensions were homogenized using Ultra-Turrax (Janke & Kunkel IKA) in TRIzOL (Life Technologies Inc), and total RNA was isolated. cDNA was synthesized with a reverse transcriptase reaction carried out with the use of standard techniques (Superscript First Strand Synthesis System for RT-PCR, Life Technologies Inc) with random hexamers, dNTPs, and total RNA extracted from control and diabetic rat kidneys. An aliquot of the resulting single-strand cDNA was used in the real-time polymerase chain reaction (RT-PCR) experiments as described below. To assess genomic DNA contamination, controls without reverse transcriptase were included.

Real-Time PCR

RT-PCR is a fully quantitative method for the determination of amounts of mRNA.18 Briefly, gene-specific 5′-oligonucleotide corresponding to the rat ACE (5′-CACCGCAAGGGTCGCT), ACE 3′-oligonucleotide primer (5′-TCTTGGCATATTCTTGGAG), and ACE probe (FAM5′-CACAAGAATGCGCCTCTGGTACC TAMRA), for ACE gene specific 5′-oligonucleotide corresponding (5′-ACCTTCTTACATCAGCCCTACTG), an ACE 3′-oligonucleotide primer (5′-TGGCTTCATGACCCATCCAT), and ACE2 probe (FAM5′-ATGCTCCCTTGCCTATTGGCTGT TAMRA) were designed with the use of the software program Primer Express (PE, Applied Biosystems). The generation of amplification products was defined by the point during cycling when amplification of the PCR product is first detected.

The real-time PCR reaction took place with 500 nmol/L of forward and reverse primer and 50 nmol/L of FAM/TAMRA ACE/ACE2 probe and VIC/TAMRA 18S ribosomal probe, in 1X Taqman universal PCR master mix (PE Biosystems). Each sample was run and analyzed in triplicate. The samples from control kidney were then used as the calibrator with a given value of 1, and the diabetic groups were compared with this calibrator.19

In Situ Hybridization

ACE2 Riboprobe

The 2260-bp rat ACE2 cDNA sequence (coding for the open reading frame) was inserted in the sense direction into the BamHI and Xho I sites of the pGEM-7Zf (+/−) vector (Promega). The vector was digested with XbaI and transcribed with SP6 polymerase to provide the antisense ACE2 riboprobe.

The site-specific expression of ACE2 mRNA was determined by in situ hybridization, as previously described.20 In brief, 4-μm paraffin kidney sections were hybridized after digestion with Pronase E at 37°C. The hybridization buffer containing 2X 10^6 cpm/μL 32P-labeled riboprobe, 0.72 mg/mL yeast RNA, 50% deionized formamide, 100 mmol/L DTT, 10% dextran sulfate, 0.3 mol/L NaCl, 10 mmol/L Na,HPO4, 10 mmol/L Tris HCl (pH 7.5), 5 mmol/L EDTA (pH 8.0), 0.02% BSA, 0.02% Ficoll 400, and 0.02% polyvinyl pyrrolidone was added to each section and incubated at 60°C overnight. After stringent washing with 50% formamide, 2X SSC, at 55°C, the slides were air-dried and exposed to BioMax MR film (Kodak) for 3 to 5 days. Slides were coated in Amershalm LM-1 emulsion in a darkroom, then incubated at 4°C in a light-proof container with dessicant for a period of 2 to 4 weeks, according to the autoradiography results. The slides were developed with Kodak D19 developer for 4 minutes, 1% acetic acid for 1 minute, and Ilford Hypan fixative for 4 minutes, followed by rinsing in distilled water for 15 minutes. The sections were then fixed in 4% paraformaldehyde and treated with a progressive hematoyxin and eosin stain.

Western Blotting

Kidneys from control, diabetic, and diabetic plus ramipril animals were quickly removed and minced with a scalpel blade, resuspended in buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EGTA, 5 mmol/L MgCl2, and 0.02% NaN3, pH 7.4, containing 0.5 μg/mL pepstatin [Sigma], 0.25 μg/mL leupeptin [Sigma], 0.1 mg/mL benzamidine [Sigma], and 0.1 mg/mL bacitracin [Sigma]), homogenized at 13 000 rpm with the Ultra-Turrax (Janke and Kunkel IKA, Labortechnik), and centrifuged at 1000 g (4°C) for 30 minutes.

Sample (50 μg of total protein) was loaded and run on a 10% sodium dodecyl sulfate denaturing gel, and proteins were transblotted onto nitrocellulose filters (Hybond P, Amersham-Pharmacia Biotech) through the use of a transfer tank at 15 V for 30 minutes. At the end of the transfer, the filters were blocked with 10% nonfat skim milk powder in Tris-buffered saline and 0.1% Tween (TBS/Tween) for 1 hour at room temperature with gentle rocking. The primary antibody, ACE2 (1/5000 with 10% skim milk powder in TBS/Tween) was incubated overnight at room temperature. The following day, the membrane was washed thoroughly 3 times (10-minute washes) in wash solution (TBS/Tween). Positive bands were developed with the use of the Western Blotting Analysis system (Amersham-Pharmacia Biotech), in which HRP-labeled secondary anti-rabbit antibody was diluted in 1/1000 and incubated for 1 hour at room temperature. Exposed Biomax film of bands representing ACE2 protein were quantified on an Automated Imaging System (Imaging Research Inc).

Immunohistochemistry

Immunohistochemical staining for ACE2 (antibody kindly donated by Millennium Pharmaceuticales, Cambridge, Boston, Mass) and ACE protein (Chemicon, Temecula, Calif) were performed as outlined below. Four-micrometer paraffin serial sections were prepared from 4% paraformaldehyde-fixed, paraffin-embedded rat kidney. Sections were dewaxed and hydrated, then endogenous peroxidase was quenched for 20 minutes with 3% (vol/vol) hydrogen peroxide in PBS. The primary antibodies were applied at room temperature for 1 hour. Specific staining was detected with the standard ABC (avidin-biotin complex) method.21 Briefly, slides were incubated for 20 minutes with the secondary antibody (biotin-conjugated goat anti-rabbit IgG, DAKO) at a concentration of 1/250. The Vectastain ABC system (Vector Laboratories) was then applied for 20 minutes. After thorough washing, the final detection step was carried out with the use of 3,3′-diaminobenzidine (Sigma) as the chromogen. Sections were lightly counterstained with hematoxylin.
Results

Animal Characteristics
Diabetic rats had reduced weight gain, elevated plasma glucose and glycated hemoglobin levels, and increased urinary albumin excretion and blood pressure (Table). The ACE inhibitor ramipril did not affect glycemic control but was associated with significantly less albuminuria and lower blood pressure (Table).

ACE and ACE2 mRNA Expression
ACE2 mRNA was detected in the kidney and localized to both tubules and glomeruli. However, there was approximately a 100-fold greater expression of this gene in tubules than in glomeruli. In tubules from diabetic kidney there was a >50% reduction in tubular ACE2 mRNA levels. This reduction in gene expression of ACE2 was also observed in kidneys from diabetic rats treated with ramipril (Figure 1). There was modest gene expression of ACE2 in the glomerulus of controls, with no significant increase in the diabetic rat kidney.

A similar pattern of ACE gene expression in tubules was observed in the kidney. ACE gene expression was more abundant (at least 10-fold) than ACE2 gene expression. ACE mRNA levels were also reduced by ~60% in the tubules from the diabetic kidneys and by a similar amount in the kidneys from diabetic rats treated with ramipril (Figure 1).

Localization of ACE and ACE2
Immunohistochemistry was performed to localize both ACE2 and ACE in the kidney (Figures 4 and 5). As for gene expression, the major site of ACE2 protein expression was in renal tubules (Figure 4D), with only very occasional glomerular cells expressing this protein in control kidney (Figure 5D). In diabetes (Figure 4E), ACE2 was also predominantly expressed in renal tubules, albeit to a lesser extent than in kidneys from control (Figure 4D) and diabetic plus ramipril-treated groups (Figure 4F). Furthermore, a significant number of glomerular cells stained for this enzyme in the diabetic kidney (Figure 5E), a phenomenon not readily observed in glomeruli from control or diabetic plus ramipril-treated rats. These changes paralleled those seen with respect to the ACE2 observed in the gene expression studies (Figure 1). By contrast, in ramipril-treated rats, renal ACE2 protein expression was similar to that seen in control rats (Figures 3A and 3B).

Protein Levels of ACE2
Protein levels of ACE2 from whole kidney were quantified by Western blot analysis (Figure 3). ACE2 protein was decreased by ~30% in the diabetic kidney, consistent with the findings observed in the gene expression studies (Figure 1). By contrast, in ramipril-treated rats, renal ACE2 protein expression was similar to that seen in control rats (Figures 3A and 3B).

Figure 1. Quantification of ACE (A) and ACE2 (B) mRNA levels in kidneys from control (cnt, n=5), diabetic (diab, n=7), and diabetic plus ramipril (diab+ram, n=8) rats measured by RT-PCR. Data are shown as mean±SEM. Control values are arbitrarily standardized to 1. *P<0.05, **P<0.01 vs control.
mRNA findings. ACE was also primarily localized to renal tubules (Figure 4A), although this enzyme was also detected in glomerular cells.

Discussion

The present study has identified ACE2 predominantly in renal tubules and shown that its expression, similar to that seen with ACE, is reduced in experimental diabetic nephropathy. The pathophysiological role of this enzyme remains to be determined, but its localization primarily in the heart and kidney suggests an important role for ACE2 in cardiovascular and renal function.\(^2\)\(^3\) The importance of ACE2 has been further delineated with the recent demonstration that in the ACE2 knockout mouse there is a local increase in angiotensin II levels and evidence of cardiac dysfunction.\(^2^2\) ACE2 has been shown to be involved in the generation of A1–9 and A1–7 which, in contrast to angiotensin II, does not have vasoconstrictor effects but may have vasodilatory actions.\(^2\) Furthermore, as predicted, ACE2 knockout mice have increased organ levels of angiotensin I and II. With increasing evidence that the local RAS plays a prominent role in the progression of kidney disease,\(^2^5\) it is possible that the relative balance of vasoconstrictor and vasodilatory angiotensin peptides is important in the modulation of both hemodynamic and trophic effects of these peptides within the kidney.

ACE has been previously reported to have a widespread distribution that is more ubiquitous than ACE2.\(^2\) Nevertheless, within the kidney, ACE2 has a distribution similar to ACE, the major site of localization being renal tubules. With the use of lectin vulgaris staining (data not shown), which specifically identified proximal tubules, it could be clearly demonstrated that ACE and ACE2 were mainly but not exclusively localized to proximal tubules. There was evidence of both metalloproteases being present in distal tubules and to a much lesser extent in glomeruli, as assessed by both gene and protein expression. It is likely that the findings in the rodent can be extrapolated to humans, since in preliminary human studies we have identified ACE2 immunostaining in the human kidney (data not shown).

The importance of ACE2 both in normal physiology as well as in various pathophysiological states is unknown. The enzyme ACE plays a pivotal role in the conversion of angiotensin(1–10) (angiotensin I) to the vasoconstrictor angiotensin(1–8), (angiotensin II)\(^1\) (Figure 6).\(^2^4\)\(^2^5\) This enzyme also inactivates the vasodilator bradykinin by sequential cleavage of 2 C-terminal dipeptidases. By contrast, ACE2 appears to be involved in promotion of vasodilation for several reasons. First, this enzyme converts angiotensin I to angiotensin(1–9), which is subsequently subjected to enzymatic cleavage by ACE, resulting in the formation of angiotensin(1–7), a vasodilator\(^2\) (Figure 6). Second, angiotensin II may be converted to angiotensin(1–7) by ACE2 (Figure 6), resulting in this vasoconstrictor being cleaved to generate a vasodilatory angiotensin. Finally, ACE2 is also capable of cleaving other vasoactive peptides such as des-Arg bradykinin, neurotensin, and kinetensin.\(^2\) Indeed, recent studies have emphasized the affinity of ACE 2 to certain peptides and in particular to angiotensin II as well as unrelated peptides including apelin-13 and dynorphin angiotensin(1–13).\(^4\) The role of
angiotensin(1–7), which is generated by ACE2, has not been fully clarified but has been implicated in various disorders including hypertension. Furthermore, it has been suggested that angiotensin(1–7) opposes the actions of angiotensin II and that there is a specific angiotensin(1–7) receptor.26 In addition, other enzymes such as aminopeptidases and cathepsins have also been reported to be involved in the formation and degradation of the various angiotensin peptides.24,25

A reduction in renal ACE2 has previously been reported in 2 animal models of hypertension, the Sabra hypertensive rat and the spontaneously hypertensive rat.22 The present study extends this finding of renal ACE2 deficiency to the model of diabetes, even in the absence of systemic hypertension. It is not yet known if this reduction in ACE2 is of pathophysiological significance, but one could postulate that ACE2 deficiency leads to a local increase in tubular angiotensin II with consequent effects such as promotion of tubulointerstitial fibrosis. Indeed, our group has demonstrated local increases in angiotensin II, particularly in damaged tubules in various models of progressive renal disease,23 including diabetic nephropathy.27

In the present study only a moderate level of ACE2 was identified in the glomerulus. Interestingly, there appears to be an increase in glomerular ACE2 expression in the diabetic kidney, as assessed at the gene level by in situ hybridization (Figure 2) and at the protein level by immunohistochemistry (Figures 4 and 5). One must be cautious in interpreting these glomerular findings because the approaches used can only be described as semiquantitative. Similar to the apparent increase in ACE2 in the diabetic glomerulus, ACE protein was also observed to be increased in the diabetic kidney (Figures 5B and 5E). Indeed, this phenomenon of an increase in ACE in the glomerulus in association with a reduction in ACE in renal tubules in the diabetic kidney has been previously reported.5

In a previous study, in ACE2 knockout mice, the additional deletion of the ACE gene, producing double knockout mice, reversed the cardiac abnormalities observed in the single mutant ACE2 knockout mouse.22 To explore if ACE inhibition may influence ACE2 expression, additional diabetic animals treated with the ACE inhibitor ramipril were assessed. ACE inhibition appears to prevent the diabetes associated decreases in renal ACE2 protein. However, this phenomenon did not appear to be
mediated by an effect on ACE2 gene transcription, which remained unchanged with ramipril treatment. Although not directly addressed in this study, it is possible that ACE inhibition confers some of its renoprotective effects through modulation of ACE2-mediated phenomenon, as has been postulated for cardioprotection.22

A major focus of renoprotective treatment in diabetes involves blockade of the RAS by different approaches, including ACE inhibition and angiotensin II receptor antagonism.28 The identification of ACE2 in the kidney and modulation of this enzyme by diabetes at that site provide further complexity to the nature of the renal RAS. It is anticipated that this enzyme will be a target for the development of therapeutics, which could be relevant to progressive renal disorders such as diabetic nephropathy.

**Perspectives**

ACE2 is a newly described enzyme identified in both rodents and humans. It has a much more restricted distribution than ACE, being expressed mainly in the heart and kidney. The recent ACE2 knockout studies illustrate the potential physiological role of ACE2 in cardiac function. The present study identifies a pathological disorder, diabetes, which is associated with a reduction in renal ACE2 expression. Further elucidation of the impact of ACE2 on the generation of angiotensin peptides in renal and cardiovascular disorders is necessary.

**Acknowledgments**

This study was supported by grants from the Juvenile Diabetes Research Foundation and the National Health and Medical Research Council of Australia. The authors thank Millenium Pharmaceuticals, Cambridge, Mass, for provision of the antibody to ACE2 for immunohistochernical and Western blotting studies and the ACE2 cDNA for subsequent development of a riboprobes for in situ hybridization.

**References**

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Hypertension. 2003;41:392-397; originally published online February 24, 2003;
doi: 10.1161/01.HYP.0000060689.38912.CB
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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World Wide Web at:
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