Reduction in renal ACE2 expression in subtotal nephrectomy in rats is ameliorated with ACE inhibition

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A B S T R A C T

Alterations within the RAS (renin–angiotensin system) are pivotal for the development of renal disease. ACE2 (angiotensin-converting enzyme 2) is expressed in the kidney and converts the vasoconstrictor AngII (angiotensin II) into Ang-(1–7), a peptide with vasodilatory and anti-fibrotic actions. Although the expression of ACE2 in the diabetic kidney has been well studied, little is known about its expression in non-diabetic renal disease. In the present study, we assessed ACE2 in rats with acute kidney injury induced by STNx (subtotal nephrectomy). STNx and Control rats received vehicle or ramipril ($1 \, \mathrm{mg} \cdot \mathrm{kg}^{-1}$ of body weight $\cdot \, \mathrm{day}^{-1}$), and renal ACE, ACE2 and *mas* receptor gene and protein expression were measured 10 days later. STNx rats were characterized by polyuria, proteinuria, hypertension and elevated plasma ACE2 activity (all $P < 0.01$) and plasma Ang-(1–7) ($P < 0.05$) compared with Control rats. There was increased cortical ACE binding and medullary *mas* receptor expression ($P < 0.05$), but reduced cortical and medullary ACE2 activity in the remnant kidney ($P < 0.05$ and $P < 0.001$ respectively) compared with Control rats. In STNx rats, ramipril reduced blood pressure ($P < 0.01$), polyuria ($P < 0.05$) and plasma ACE2 ($P < 0.01$), increased plasma Ang-(1–7) ($P < 0.001$), and inhibited renal ACE ($P < 0.001$). Ramipril increased both cortical and medullary ACE2 activity ($P < 0.01$), but reduced medullary *mas* receptor expression ($P < 0.05$). In conclusion, our results show that ACE2 activity is reduced in kidney injury and that ACE inhibition produced beneficial effects in association with increased renal ACE2 activity. As ACE2 both degrades AngII and generates the vasodilator Ang-(1–7), a decrease in renal ACE2 activity, as observed in the present study, has the potential to contribute to the progression of kidney disease.

INTRODUCTION

Alterations within the RAS (renin–angiotensin system) are pivotal for the development and progression of renal disease [1,2]. ACE2 (angiotensin-converting enzyme 2) [3–5] is a recently discovered monocarboxypeptidase that regulates RAS through degradation of the vasoconstrictor and pro-fibrotic peptide AngII (angiotensin II) into Ang-(1–7) [angiotensin-(1–7)], a peptide reported to have vasodilatory and anti-fibrotic actions [6,7] via actions at

the Ang-(1–7) or *mas* receptor [8]. In normal physiology, ACE2 is highly expressed in the kidney and has been localized to the glomerulus, where it is expressed in podocytes and mesangial cells [9,10], and to the proximal tubules [11]. ACE2 is also expressed in the medulla in collecting ducts and the vasa rectae [12].

Previous studies have suggested that the expression of ACE2 may be an important determinant of injury [11,13,14], and the therapeutic potential of increased ACE2 activity has now been recognized [15,16]. Certainly, in renal disease, ACE2 may have renoprotective effects, as when ACE2 is deleted or inhibited renal pathology ensues, presumably because of the removal of a degradative pathway for AngII. For example, ACE2-knockout mice develop glomerulosclerosis, which can be prevented by angiotensin receptor blockade [17], and pharmacological inhibition of ACE2 in the spontaneously diabetic (*db/db*) mouse accelerates albuminuria and glomerulosclerosis [9] and causes glomerular injury in STZ (streptozotocin)-induced diabetes [18]. In addition, ACE2-knockout mice crossed with a model of Type 1 diabetes also had accelerated kidney injury that was ameliorated by an ARB (angiotensin receptor blocker) [19].

As both diabetic and non-diabetic renal disease are associated with an overactive intrarenal RAS [1], we were interested in the potential role of ACE2 in non-diabetic renal disease such as that induced by STNx (subtotal nephrectomy), especially as significant increases in renal ACE and AngII occur after renal mass reduction [20–22]. In this model, nephrons are lost through surgical ablation and the remaining nephrons undergo physiological changes, resulting in hypertrophy and hyperfiltration to compensate for the nephron loss. Early after surgery [22,23], rats develop hypertension, polyuria and proteinuria, whilst, over more prolonged time periods, glomerulosclerosis and tubular atrophy further accelerate nephron loss, and this leads to chronic renal disease associated with a decrease in creatinine clearance and tubulointerstitial fibrosis [20–22].

To characterize further the RAS in the kidney and assess the potential involvement of ACE2 in the pathophysiology that occurs after STNx, we examined gene and protein expression as well as localization of ACE2 in the kidney from Sprague–Dawley rats without or with STNx, and compared this with changes in the related enzyme ACE. In addition, the effect of blocking ACE using the ACEI (ACE inhibitor) ramipril on renal ACE, ACE2 and the *mas* receptor gene and protein expression in Control and STNx rats was assessed.

Part of this work was presented at the High Blood Pressure Research Council of Australia Annual Scientific meeting, held in Melbourne on 4–5 December 2008, and subsequently published in abstract form [23a].

MATERIALS AND METHODS

Experimental protocol

Experimental procedures were performed in accordance with the National Health and Medical Research Council of Australia guidelines for animal experimentation, and were approved by the Animal Ethics Committee, Austin Health. Rats were housed in a 12 h/12 h light/dark cycle, with *ad libitum* food containing 0.4–0.6 % NaCl (Norco) and water. STNx ($n = 21$) or sham surgery (Controls; $n = 21$) was performed in female Sprague–Dawley rats (body weight, 200–250 g), as described previously [23]. In brief, rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg/kg of body weight; Boehringer Ingelheim) and STNx was performed by right nephrectomy, followed by infarction of approximately two-thirds of the left kidney with selective ligation of all but one of the extrarenal branches of the left renal artery. Rats were randomly allocated to a vehicle-treated group ($n = 10$ for Control; $n = 9$ for STNx) or an ACEI-treated group ($n = 11$ for Control; $n = 12$ for STNx) for 10 days. The ACEI ramipril was administered by daily gavage at a dose of 1 mg/kg of body weight. On day 9, rats were housed in metabolic cages for 24 h to measure water intake and urine output, and to collect samples of urine for biochemical analysis and protein analysis. On day 10, rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg/kg of body weight), and SBP [systolic BP (blood pressure)] was measured via carotid artery cannulation. Rats were then killed by a lethal dose of sodium pentobarbitone. The remnant kidney was removed and weighed. A portion of the kidney was fixed in 4 % paraformaldehyde and embedded in paraffin for histopathology. The remainder of the kidney was snap-frozen in 2-methylbutane and stored at $-80\,°C$ for the *in vitro* autoradiographic studies, ACE2 activity assay and RNA extraction.

Biochemical analysis

Plasma and urinary urea and creatinine concentrations were measured using an autoanalyser (Beckman Instruments). Urinary protein was determined using the BCA (bicinchoninic acid) method with a commercially available BCA Protein Assay kit (Pierce).

Plasma Ang-(1–7) RIA

Blood samples for the determination of Ang-(1–7) were collected into tubes containing 20 μl of an inhibitor cocktail [50 mol/l Na_2EDTA, 0.2 mol/l N-ethylmaleimide and 1–2 TIU (trypsin inhibitor units)/ml aprotinin made up in saline] per ml of blood. Plasma was snap-frozen and stored at $-80\,°C$. Ang-(1–7) was measured by RIA, as described previously [24], using an Ang-(1–7) antibody raised in guinea-pig and ^{125}I-Ang-(1–7) (Prosearch). The intra- and inter-assay coefficients of variation for the assay were 4.5 and 10 % respectively.

QRT–PCR (quantitative real-time–PCR)

Kidneys were dissected into cortex and medulla (Control kidneys; $n = 7$/group) or cortex, medulla and scar (STNx remnant kidney; $n = 9$–10/group). Total RNA was isolated from the dissected kidneys using the RNeasy kit method (Qiagen). cDNA was synthesized with a reverse transcriptase reaction using standard techniques (Superscript II kit; Life Technologies) as described previously [11,13]. Primers and probes were designed using the Primer Express software program (PE Applied Biosystems) with the following sequences: rat ACE 5′ oligonucleotide primer, 5′-CACCGGCAAGGTCTGCTT-3′; ACE 3′ oligonucleotide primer, 5′-CTTGGCATAGTTTCGT-GAGGAA-3′; and ACE probe FAM (6-carboxyflu-orescin)-5′-CAACAAGACTGCCACCTGCTGGTC-C-3′-TAMRA (6-carboxytetramethylrhodamine); ACE2 5′ oligonucleotide primer, 5′-GCCAGGAGAT-GACCGGAAA-3′; ACE2 3′ oligonucleotide primer, 5′-CTGAAGTCTCCATGTCCCAGATC-3′, and ACE2 probe, FAM-5′-TTGTCTGCCACCCCACA-3′-TAM-RA; *mas* receptor 5′ oligonucleotide primer, 5′-CATC-TCTCCTCTCGGCTTTGTG-3′; *mas* receptor 3′ oligo-nucleotide primer, 5′-CCTCATCCGGAAGCAAA-GG-3′; and *mas* receptor probe, FAM-5′-CGGGATC-CTCCTCTGG-3′-TAMRA. QRT–PCR was carried out using a multiplex method with VIC (6-carboxy-rhodamine)-labelled 18S as the endogenous control. A relative expression method was applied in the present study using the vehicle-treated control group as the calibrator and all groups were compared with the calibrator group, which was given a value of 1.

Kidney histopathology

To assess glomerulosclerosis and tubulointerstitial injury, renal sections were stained with haematoxylin and eosin and examined in a blinded fashion under high power on a light microscope (magnification ×400). A total of 30 glomeruli and 20 fields of tubulointerstitial area were graded using a semi-quantitative method [20].

Renal ACE activity

Renal ACE ($n = 5$/group) was assessed on renal sections (20 μm) using quantitative *in vitro* autoradiography and the specific radioligand ^{125}I-MK351A ($K_i = 30$ pmol/l) as reported previously [25]. Quantification of two sections from each rat was performed using a microcomputer-imaging device (Imaging Research), and results are expressed as a percentage of the binding in control rats.

QFS (quenched fluorescent substrate) assay of renal and plasma ACE2 activity

We have developed an assay for ACE2 enzymatic activity in tissue and plasma which has been validated using Western blotting [11,26] and immunohistochemistry [13].

To measure renal ACE2 activity, kidneys were dissected into cortex and medulla (Control kidneys) or cortex, medulla and scar (STNx remnant kidney). The tissue was homogenized in ice-cold TBS [Tris-buffered saline; 25 mmol/l Tris/HCl (pH 7.4) and 125 mmol/l NaCl], and the homogenates were pelleted by ultracentrifugation (45 000 rev./min at 4 °C for 1 h in a TLA 100.2 rotor; Beckman). Prior to the ACE2 activity assay, an aliquot of each sample was diluted 10-fold in ACE2 assay buffer [100 mmol/l Tris/HCl (pH 6.5) and 1 mol/l NaCl]. For plasma ACE2 activity, blood collected into heparinized tubes was centrifuged at 4 °C and assayed using an ACE2-specific QFS [(7-methoxycoumarin-4-yl)-acetyl-Ala-Pro-Lys (2, 4-dintirophenyl); Auspep] [23].

Expression of ACE2 and *mas* receptor protein by immunohistochemistry

Immunohistochemical staining for ACE2 (polyclonal antibody T17 diluted 1:100; Santa Cruz Biotechnology) was performed in rat kidney sections as described previously [13]. *Mas* receptor immunohistochemistry was performed using a polyclonal *mas* proto-oncogene antibody (diluted 1:5; Novus Biologicals). Sections (4 μm) were rehydrated, blocked with 3 % (v/v) H_2O_2 and 10 % (v/v) normal goat serum before incubation with primary antibody at 4 °C overnight. The secondary antibody was goat anti-rabbit at a dilution of 1:400. Antibody labelling was visualized using an avidin–biotin complex (ABC kit; Vector Laboratories) and DAB (diaminobenzidine) before counterstaining with haematoxylin. The negative control for ACE2 and *mas* receptor immunostaining was carried out by omission of the primary antibody. Images were acquired on an Olympus BX50 microscope (objective lens, ×20/0.50), using a Leica DFC480 camera and Leica IM50Image Manager.

Statistical analysis

Values are shown as means ± S.E.M. Data were analysed using a Student's *t* test for comparison between the vehicle- and ramipril-treated Control, vehicle-treated Control and STNx, and between STNx and STNx with ramipril using the GraphPad PRISM program (GraphPad Software). A P value < 0.05 was considered statistically significant.

RESULTS

STNx model

Table 1 shows the changes in physiological and biochemical parameters after STNx and the effect of ACE inhibition with ramipril. Following STNx, rats had poor weight gain ($P < 0.05$), and developed increased SBP ($P < 0.01$) and hypertrophy of the remnant kidney ($P < 0.001$) compared with vehicle-treated Control rats.

Table 1   End-organ weight, physiological parameters, and plasma and urine biochemistry

$*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with control vehicle; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ and $\dagger\dagger\dagger P < 0.001$ compared with the appropriate vehicle-treated rats. Ramipril was administered at a dose of 1 mg · kg^{-1} of body weight · day^{-1}.

Characteristic	Control		STNx	
	Vehicle ($n = 10$)	Ramipril ($n = 11$)	Vehicle ($n = 9$)	Ramipril ($n = 12$)
Body weight (g)	218 ± 2	213 ± 4	$203 \pm 5*$	$219 \pm 3\dagger$
SBP (mmHg)	131 ± 5	$111 \pm 3\dagger\dagger$	$179 \pm 9**$	$115 \pm 6\dagger\dagger$
Renal parameter				
Left kidney weight (g)	0.765 ± 0.015	0.808 ± 0.017	$1.027 \pm 0.028***$	1.085 ± 0.023
Left kidney/body weight (g/100 g)	0.351 ± 0.008	0.379 ± 0.008	$0.506 \pm 0.014***$	0.499 ± 0.009
Water intake (ml · 100 g^{-1} · 24 h^{-1})	13 ± 1	16 ± 1	$26 \pm 3***$	30 ± 3
Urine volume (ml · 100 g^{-1} · 24 h^{-1})	5 ± 1	6 ± 1	$19 \pm 5***$	$11 \pm 2\dagger$
Urinary protein (mg/24 h)	22 ± 2	$36 \pm 4\dagger\dagger$	$37 \pm 4**$	38 ± 3
Creatinine clearance (ml/min)	1.3 ± 0.2	1.4 ± 0.2	1.5 ± 0.2	1.3 ± 0.1
Plasma parameter				
Plasma creatinine (μmol/l)	36 ± 4	42 ± 5	33 ± 3	41 ± 3
Plasma urea (mmol/l)	7 ± 1	9 ± 1	6 ± 1	8 ± 1
Plasma ACE2 activity (nmol · ml^{-1} · h^{-1})	5.5 ± 0.4	5.7 ± 0.5	$8.0 \pm 0.7**$	$4.4 \pm 0.5\dagger\dagger$
Plasma Ang-(1–7) (fmol/ml)	142 ± 14	$1862 \pm 162\dagger\dagger$	$248 \pm 41*$	$1340 \pm 191\dagger\dagger\dagger$
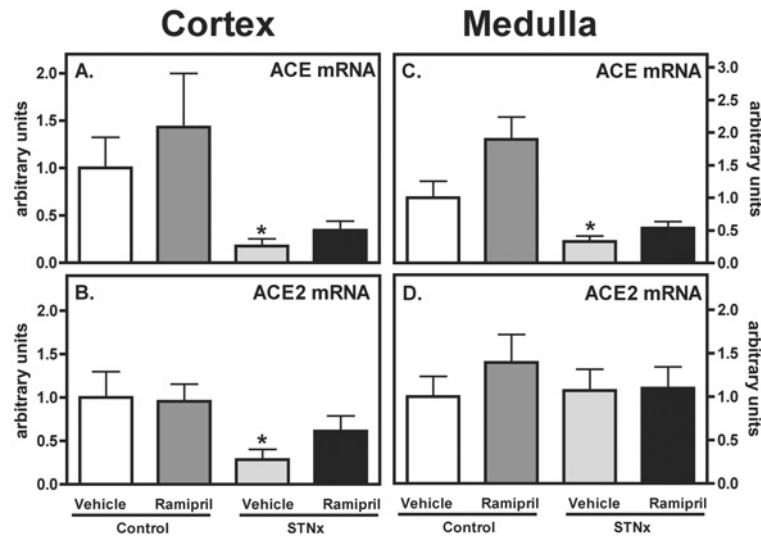
Figure 1   Relative quantification of cortical (A and B) and medullary (C and D) ACE and ACE2 mRNA using QRT–PCR in control or STNx rats with vehicle or ramipril treatment

$*P < 0.05$ compared with Control ($n = 7$–10/group).

STNx rats had increased urine volume ($P < 0.001$) and proteinuria ($P < 0.01$), with no change in plasma urea, plasma creatinine or creatinine clearance compared with Controls (Table 1). At this early stage of renal injury, there was no evidence of tubulointerstitial fibrosis or glomerulosclerosis in the remnant kidney of the STNx rats (results not shown), although more sophisticated techniques may detect more subtle injury.

Ramipril improved weight gain in STNx rats ($P < 0.05$), but did not affect kidney hypertrophy (Table 1). ACE inhibition significantly decreased SBP in both Control and STNx rats ($P < 0.01$), and reduced urine volume in STNx rats ($P < 0.05$) but not in Control rats. Ramipril had no effect on proteinuria in the STNx rats and caused proteinuria in the Control rats. This effect of ACE inhibition can also occur clinically early after commencing treatment, but does not persist long-term [27].

STNx increased plasma ACE2 activity and plasma Ang-(1–7) ($P < 0.01$ and $P < 0.05$ respectively; Table 1) compared with Control rats. Ramipril was associated with a decrease in plasma ACE2 activity in STNx rats

Table 2 ACE, ACE2 and *mas* receptor mRNA and ACE2 activity in scar tissue of the remnant kidney

Ramipril was administered at a dose of 1 mg · kg^{-1} of body weight · day^{-1}.

	STNx	
Measurement	Vehicle ($n = 7$)	Ramipril ($n = 10$)
ACE mRNA (arbitrary units)	1.00 ± 0.31	1.09 ± 0.22
ACE2 mRNA (arbitrary units)	1.00 ± 0.23	2.92 ± 1.01
mas receptor mRNA (arbitrary units)	1.00 ± 0.19	0.79 ± 0.15
ACE2 activity (nmol · mg^{-1} of protein · h^{-1})	6.0 ± 1.2	7.8 ± 1.2

($P < 0.01$), but plasma ACE2 activity was unchanged in Control rats. Ramipril treatment significantly increased plasma Ang-(1–7) in both the Control and STNx rats ($P < 0.01$ and $P < 0.001$ respectively; Table 1).

ACE and ACE2 mRNA expression

Figure 1 shows the relative quantification of renal ACE and ACE2 mRNA in the renal cortex and medulla in Control and STNx rats. In the remnant kidney after STNx, there was a reduction in ACE mRNA expression in the cortex and medulla (Figures 1A and 1C) and a decrease in ACE2 mRNA expression in the cortex but not medulla (Figures 1B and 1D) compared with Control kidneys.

Ramipril had no effect on ACE or ACE2 mRNA expression in either STNx or Control kidneys (Figure 1). No significant changes in ACE or ACE2 mRNA in scar tissue were observed (Table 2).

Renal ACE and ACE2 protein

Figure 2 shows an *in vitro* autoradiograph of ACE radiolabelling in Control kidneys and STNx remnant kidneys. In normal kidneys, ACE was localized to the proximal tubules in the inner cortex, as reported previously [28], with lower ACE binding detectable in the medulla. After STNx, ACE activity increased significantly in the inner cortex ($P < 0.001$). Ramipril reduced ACE binding in both Control and STNx rats ($P < 0.001$; Figure 2B).

Figure 3 shows ACE2 activity measured in kidney homogenates. In Control kidney, ACE2 activity was detectable in both the renal cortex and medulla. Interestingly, after STNx, ACE2 activity significantly decreased in the cortex ($P < 0.05$) and medulla ($P < 0.001$) compared with vehicle-treated Control rats (Figures 3A and 3B). Ramipril had no effect on renal ACE2 activity in Control rats, but was associated with a significant increase in ACE2 activity in both the cortex and medulla of STNx kidneys ($P < 0.01$). No significant changes in ACE2 activity were observed in the renal scar tissue (Table 2).
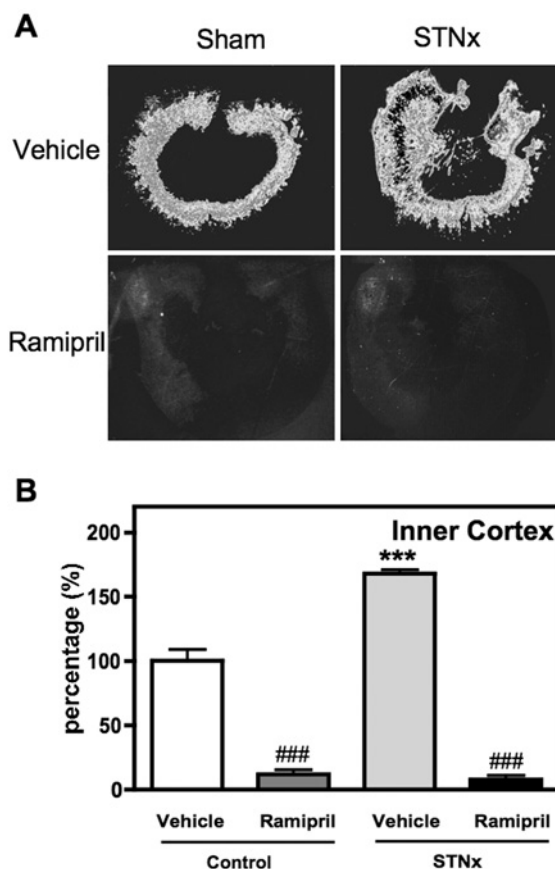

Figure 2 Localization and quantification of ACE radiolabelling in the rat kidney in Control or STNx rats with vehicle or ramipril treatment

(A) *In vitro* autoradiograph showing ACE radiolabelling in the rat kidney. The white colour denotes the highest density of labelling, whereas the black colour represents background labelling. Sections from Control (Sham) rat kidney and STNx kidney without and with ramipril treatment are shown. In the Control kidney, there is abundant ACE radiolabelling of the inner cortex and outer stripe of the outer medulla. STNx increases ACE radiolabelling, which was reduced by ramipril. (B) Relative quantification of ACE radiolabelling in the inner cortex of the rat kidney expressed as a percentage of that in Control rats. ***$P < 0.001$ compared with Control vehicle $^{\#\#\#}P < 0.001$ compared with appropriate vehicle-treated rats ($n = 5$/group).

Localization of ACE2 protein in the renal cortex and medulla

The renal cortex comprises both glomeruli and tubules, although the glomeruli comprise only approx. 5% of the cortex; the medulla includes collecting ducts and the vasa recta. Figure 4 shows the qualitative immunohistochemical localization of ACE2 in the kidney. ACE2 protein was present in the cortex, predominantly, but not exclusively, localized to proximal and distal tubules, and, in the medulla, ACE2 mainly localized to the collecting ducts (Figure 4). Both cortical (Figures 4A–4D) and medullary (Figures 4F–4I) ACE2 immunostaining decreased after STNx, and
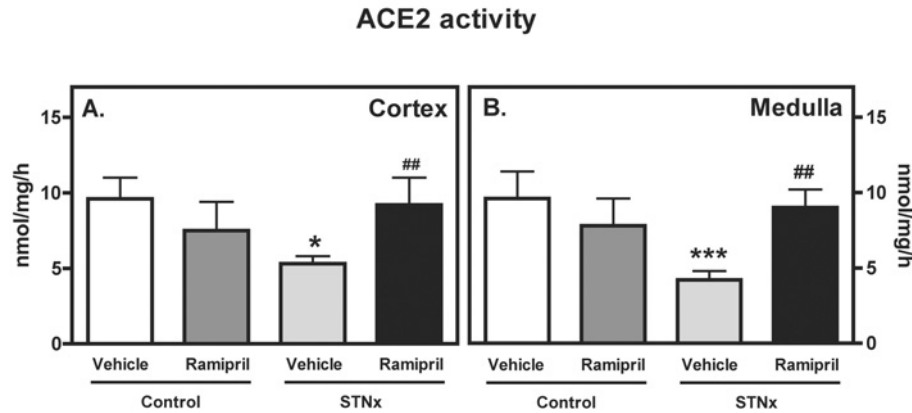
ACE2 activity

Figure 3   ACE2 activity in the cortex (A) and medulla (B) of rat kidney in Control or STNx rats with vehicle or ramipril treatment

$*P < 0.05$ and $***P < 0.001$ compared with Control vehicle $\#\#P < 0.01$ compared with STNx vehicle ($n = 7$–10/group).

were qualitatively increased by ramipril treatment. The changes in staining intensity observed with ACE2 immunohistochemistry were consistent with the measurement of renal ACE2 activity as shown in Figure 3.

mas receptor mRNA expression and localization in the renal cortex and medulla

Figure 5 (upper panel) shows the relative quantification of *mas* receptor mRNA in the renal cortex and medulla of Control and STNx rats. In the remnant kidney after STNx, *mas* receptor mRNA expression increased in the renal medulla ($P < 0.05$), but not in the cortex compared with Control. Ramipril led to reduced medullary *mas* receptor expression ($P < 0.05$) with no changes in renal cortex or scar tissue (Table 2).

Using qualitative immunohistochemistry (Figure 5, lower panel), *mas* receptor protein was localized predominantly to the basal and apical surfaces of distal tubules in the cortex (Figures 5A–5D). Medullary *mas* receptor protein was localized to the thin limb of Henle and to cells between the tubules and capillaries, likely to be renomedullary interstitial cells (Figure 5F–5I), although further studies are needed to confirm this. There were no qualitative changes in *mas* receptor protein expression in the STNx or ramipril-treated animals.

DISCUSSION

The major findings of the present study are that an acute reduction in renal mass by STNx increases circulating levels of the enzyme ACE2 and leads to a 50% decrease in renal ACE2 enzymatic activity, which is accompanied by a significant increase in renal ACE activity of the same order. After administration of ramipril to STNx rats, the renal expression of ACE changed in the opposite

direction to ACE2, so that as ACE decreased ACE2 activity was enhanced. Our results are consistent with the notion that ACE2 may be playing a protective role in the diseased kidney. As ACE2 both degrades AngII and generates the vasodilator and anti-fibrotic peptide Ang-(1–7), a decrease in renal ACE2 activity, as observed in the present study, has the potential to contribute to the progression of renal injury. Support for this hypothesis comes from a recent study in the *mas* receptor-knockout mouse, where a lack of Ang-(1–7) action at the renal level caused hyperfiltration, proteinuria and a tendency toward glomerulosclerosis [29].

At this early stage (10 days post-STNx), there was no evidence of glomerular or tubulointerstitial fibrosis or a decline in renal function, but rats were hypertensive with polyuria and proteinuria. Ramipril lowered BP in both Control and STNx rats and increased circulating levels of Ang-(1–7) approx. 80-fold, which may contribute to the antihypertensive effects observed. The elevated levels of plasma Ang-(1–7) observed following ACE inhibition are due to the fact that ACE is responsible for the breakdown of Ang-(1–7), and the results are consistent with findings from others studies that also report significant increases in plasma Ang-(1–7) after ACE inhibition [30,31]. Ramipril not only reduced BP, but also led to a significant decrease in urine volume in STNx but not in Control rats. Interestingly, it was only after STNx that changes in renal ACE2 activity with ramipril were observed.

To date the effects of RAS blockade on ACE2 gene expression in the kidney have not been well studied, but the results of the present study are in agreement with our previous work in STZ-induced diabetes in the rat [11]. In that study, renal pathology was associated with low renal tubular ACE2 protein expression, and ACE inhibition improved pathology in association with the restoration of renal ACE2 levels; however, the changes in renal ACE2 protein measured by Western blotting were not
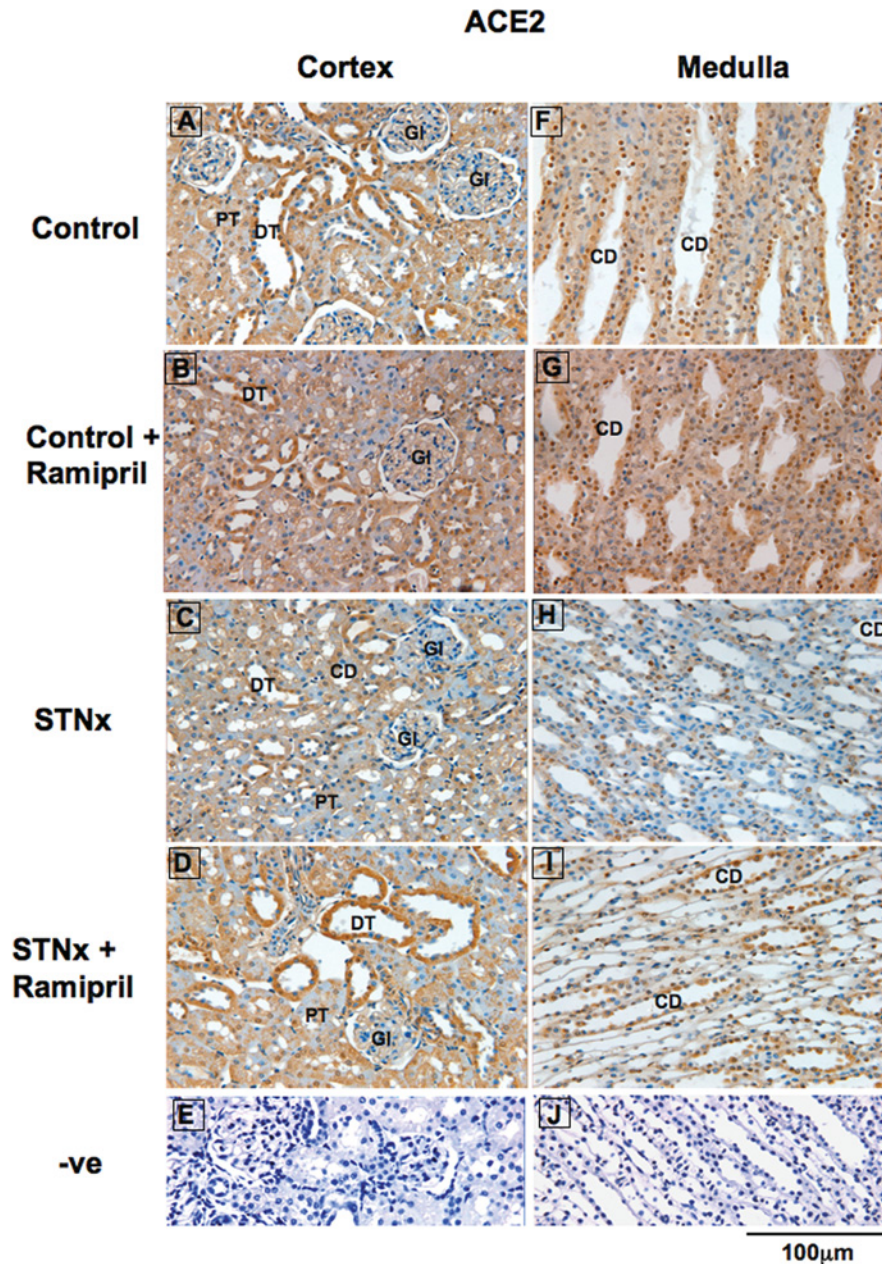
Figure 4   Light microscopic micrographs showing ACE2 immunohistochemical labelling (brown staining) of the rat kidney cortex (A–E) and medulla (F–J) in Control or STNx rats with vehicle or ramipril treatment

Micrographs show staining of the tubules in the cortex from vehicle- and ramipril-treated Control (A and B), STNx (C) and ramipril-treated STNx (D) kidneys. Medullary collecting ducts (CD) also stain ACE2 in vehicle- and ramipril-treated Control (F and G), STNx (H) and ramipril-treated STNx (I) kidneys. Immunohistochemical negative (-ve) controls are shown in (E) and (J). DT, distal tubule; Gl, glomeruli; PT, proximal tubules.

reflected by changes in ACE2 mRNA [11]. In the present study, ACE2 protein was measured using a specific ACE2 catalytic activity assay as well as immunostaining, and again no correlation between renal ACE2 activity and ACE2 gene expression was found. Discrepancies between ACE2 mRNA and protein/activity in the renal cortex have also been reported in the spontaneously diabetic mouse (*db/db*) and in STZ diabetic mice [32,33].

The relative effects of RAS blockade on ACE2 expression are also inconsistent. One group reports that, in the renal vasculature of mice, both ACE2 mRNA and protein expression increased after administration of the ARB telmisartan [10], but others have shown no effect of ACE inhibition or angiotensin receptor blockade on ACE2 mRNA in the kidney of normal rats [30]. The results suggest that the regulation of ACE2
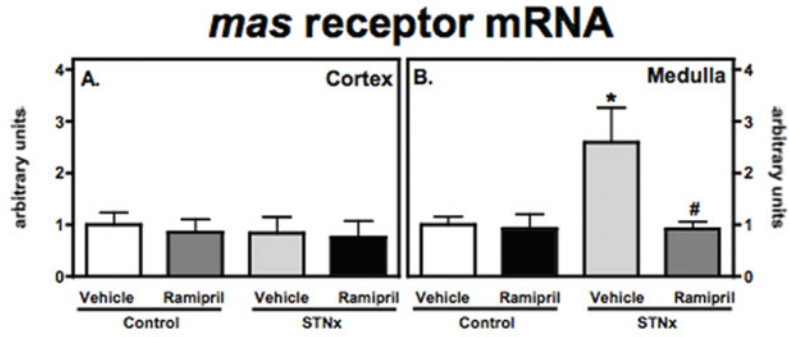
mas receptor mRNA

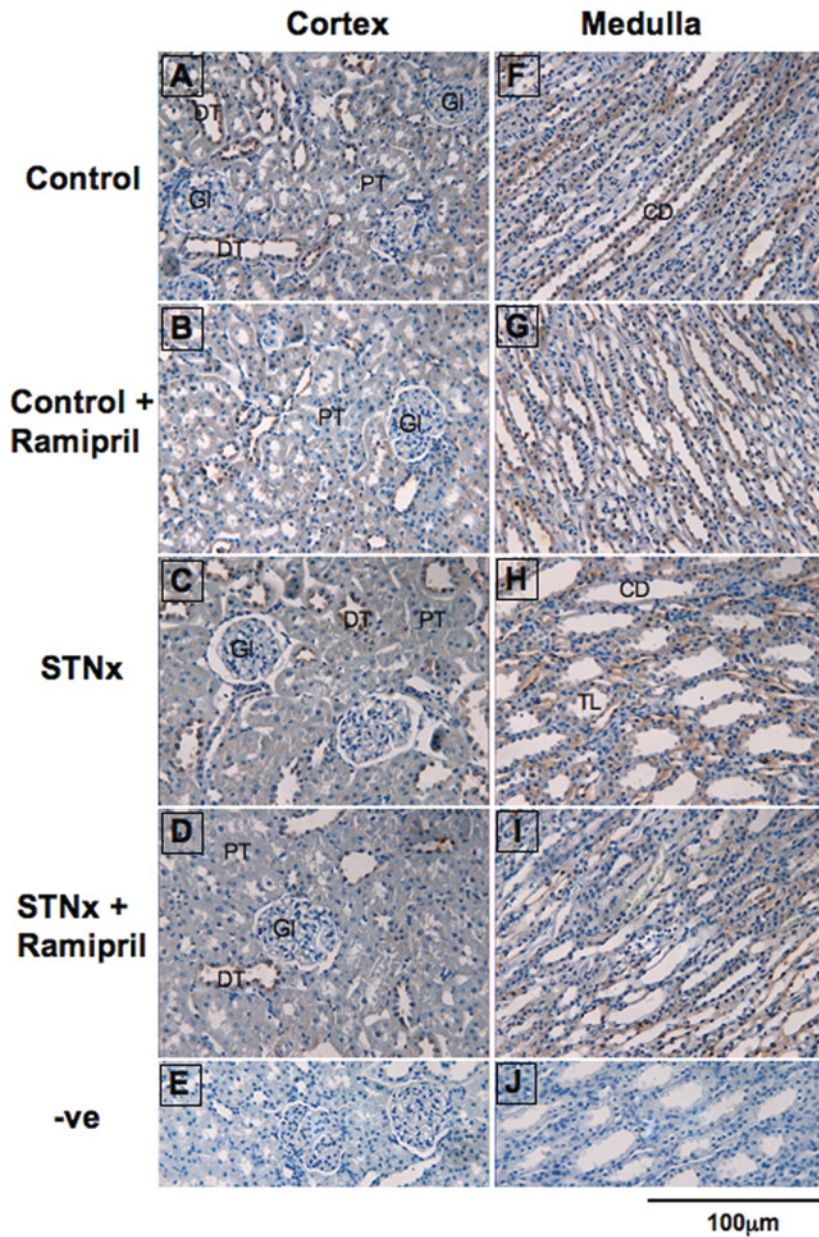## *mas* receptor immunohistochemistry

Figure 5 For legend see facing page

may vary according to cell type, as well as the disease and the prevailing BP. It also appears that, in some circumstances, ACE2 is altered at a post-transcriptional level. Taken together, the findings highlight the need for studies to assess not only ACE2 mRNA, but also ACE2 activity/protein.

A strength of the present study was the examination of the expression of ACE2 at the gene and protein level in both the cortex and medulla of the kidney. The cortex comprises both glomeruli and proximal tubules, but, as the glomeruli make up only 5% of cortical mass, changes in cortical ACE2 reflect changes occurring in the proximal tubule. In general, most groups use the cortex for RNA and protein preparations [32,33] or have sieved the preparations to separate glomeruli and tubules [11]. However, ACE2 is also present in the renal medulla [12], which is a structure that plays an important role in maintaining body fluid and electrolyte balance, and BP homoeostasis through the actions of vasoactive hormones to influence medullary/papillary blood flow and urinary water and sodium excretion [34–36].

The regulation of ACE2 in the medulla in renal disease has not been assessed previously. We found that ACE2 was localized to the medullary collecting ducts, where its role to generate Ang-(1–7) may in turn regulate water transport through an interaction with the Ang-(1–7) or *mas* receptor [37]. It is known that Ang-(1–7) exerts anti-diuretic effects [38,39], and Ang-(1–7) receptor blockade results in diuresis and natriuresis [40]. Moreover, in transgenic rats overexpressing Ang-(1–7), urinary flow is decreased [41]. As mentioned above, we have observed a reduction in ACE2 activity in the medulla in STNx rats, which also had polyuria. Although we were not able to measure renal Ang-(1–7) peptide levels, the reduction in medullary ACE2 might be expected to decrease renal Ang-(1–7) and increase urine flow. The finding of an increased expression of the *mas* receptor in the renal medulla in STNx would be consistent with this speculation, as would the observation that restoration of renal medullary ACE2 activity and *mas* receptor expression following ACE inhibition reduced urine volume.

Our results also highlight that tissue-specific changes in ACE2 occur in renal disease. The reduction in renal ACE2 activity in STNx contrasts with our previous work that demonstrated increased cardiac ACE2 activity in STNx [23]. Other investigators have observed a similar discordance in other experimental models; in the *db/db* mouse, ACE2 activity was increased in the kidney cortex but not the heart [32]. It is likely that the expression of ACE2 may be specific for each model as well as each organ, and importantly may be dependent on the time point studied. In this model of renal injury, the STNx rats had polyuria and proteinuria, but at this early hyperfiltering stage rats were able to maintain normal creatinine clearance. In long-term studies in STNx, rats develop worsening renal function as well as histological changes in glomerulosclerosis and tubulointerstitial fibrosis. Future studies should investigate whether the renal changes in ACE2 and the *mas* receptor observed 10 days after renal injury are maintained over the long-term and, indeed, contribute to the development of complications and the progression of disease.

The present study also highlights the problem of interpreting plasma ACE2 levels in the context of changes in ACE2 at the tissue level. The major contribution to plasma ACE2 levels is from shedding of the enzyme from the major sites of ACE2 expression, the heart and kidney [42]. We have shown previously that both plasma and cardiac ACE2 are elevated following STNx [23] and, in the present study, we report increased plasma ACE2 activity in the context of decreased renal ACE2. Taken together, our results suggests that circulating ACE2 levels are indicative of cardiac rather than renal ACE2 levels in STNx. Support for this comes from the results of the ACEI treatment arm; as ACE2 activity is not inhibited by ACEIs [42], any change in plasma ACE2 activity is likely to reflect changes at the tissue level. The finding that ramipril treatment was associated with a reduction in both plasma and cardiac ACE2 levels in STNx [23], but an increase in renal ACE2 activity, would support this.

It is currently unclear how the perturbations in tissue and circulating levels of ACE2 observed in an animal model of renal disease relate to the clinical setting. Several studies have assessed ACE2 in human renal disease, with inconsistent results. One study has shown that ACE2 immunostaining was increased in the glomeruli and peritubular capillaries in a variety of renal diseases in humans [43]. More recently, however, a significant

Figure 5   Quantification and localization of the *mas* receptor in the cortex and medulla in Control or STNx rats with vehicle or ramipril treatment

Upper panels, relative quantification of cortical (A) and medullary (B) *mas* receptor mRNA using QRT–PCR in Control or STNx rats with vehicle or ramipril treatment. $*P < 0.05$ compared with Control; $^{\#}P < 0.05$ compared with STNx ($n = 7$–10/group). Lower panels, light microscopic micrographs showing *mas* receptor immunohistochemical labelling (brown staining) in the rat kidney cortex (A–E) and medulla (F–J). Micrographs show staining of apical and basal membranes of distal tubules (DT) from Control (A), ramipril-treated Control (B), STNx (C) and ramipril-treated STNx (D) kidneys. Collecting ducts (CD), medullary thin limbs of Henle (TL) and cells between the tubules also stain for the *mas* receptor in Control (F), ramipril-treated Control (G), STNx (H) and ramipril-treated STNx (I) kidney medulla. Gl, glomeruli; PT, proximal tubules. Immunohistochemical negative (-ve) controls are shown in (E) and (J).

decrease in ACE2 gene and protein expression, and an increase in ACE mRNA, were reported in cortical tubules from diabetic patients, but not in those with either focal glomerulosclerosis or chronic allograft nephropathy [14]. Mizuri et al. [44] also showed decreased ACE2 and increased ACE expression in the tubulointerstitium and glomeruli of patients with overt diabetic nephropathy. Although these results may suggest that only diabetic kidney disease is associated with a reduction in ACE2 gene and protein expression, other investigators have found that ACE2 expression is similar in renal biopsies from patients with diabetes, primary glomerular disease, nephrosclerosis and lupus [45].

In conclusion, the emerging evidence suggests that the carboxypeptidase ACE2 serves as a negative regulator of RAS and acts to counterbalance the actions of ACE by regulating tissue AngII levels. As the injury in renal disease is modulated, in part, by enhanced ACE activity leading to increased AngII levels, a reduction in renal ACE2 activity and, hence, the loss of a pathway for AngII peptide degradation may contribute to the progression of renal injury. The lack of generation of a potentially renoprotective peptide, Ang-(1–7), may also contribute to ongoing renal damage. Strategies that amplify renal ACE2 expression or synthesis may be a useful therapeutic option in kidney disease, and the recent description of small-molecule ACE2 activators [46] will allow this approach to be tested, at least in experimental models.

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