



Angiotensin-(1–7) infusion is associated with increased blood pressure and adverse cardiac remodelling in rats with subtotal nephrectomy

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A B S T R A C T

ACE (angiotensin-converting enzyme) 2 is expressed in the heart and kidney and metabolizes Ang (angiotensin) II to Ang-(1–7) a peptide that acts via the Ang-(1–7) or *mas* receptor. The aim of the present study was to assess the effect of Ang-(1–7) on blood pressure and cardiac remodelling in a rat model of renal mass ablation. Male SD (Sprague–Dawley) rats underwent STNx (subtotal nephrectomy) and were treated for 10 days with vehicle, the ACE inhibitor ramipril (oral $1 \text{ mg} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{day}^{-1}$) or Ang-(1–7) (subcutaneous $24 \mu\text{g} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{h}^{-1}$) (all $n = 15$ per group). A control group ($n = 10$) of sham-operated rats were also studied. STNx rats were hypertensive ($P < 0.01$) with renal impairment ($P < 0.001$), cardiac hypertrophy ($P < 0.001$) and fibrosis ($P < 0.05$), and increased cardiac ACE ($P < 0.001$) and ACE2 activity ($P < 0.05$). Ramipril reduced blood pressure ($P < 0.01$), improved cardiac hypertrophy ($P < 0.001$) and inhibited cardiac ACE ($P < 0.001$). By contrast, Ang-(1–7) infusion in STNx was associated with further increases in blood pressure ($P < 0.05$), cardiac hypertrophy ($P < 0.05$) and fibrosis ($P < 0.01$). Ang-(1–7) infusion also increased cardiac ACE activity ($P < 0.001$) and reduced cardiac ACE2 activity ($P < 0.05$) compared with STNx-vehicle rats. Our results add to the increasing evidence that Ang-(1–7) may have deleterious cardiovascular effects in kidney failure and highlight the need for further *in vivo* studies of the ACE2/Ang-(1–7)/*mas* receptor axis in kidney disease.

INTRODUCTION

Activation of the RAS (renin–angiotensin system) and generation of Ang (angiotensin) II is pivotal for the development and progression of renal disease [1]. Current treatment based on RAS blockade using ACE (angiotensin-converting enzyme) inhibitors and/or ARBs (angiotensin receptor blockers) has benefits, but cardiovascular disease remains the most frequent cause of death in patients with chronic kidney disease [2].

New components of the RAS, including ACE2 and Ang-(1–7), have been suggested to play a role in the cardiac and renal consequences of kidney disease [3,4]. Ang-(1–7) is a peptide with antifibrotic effects [5,6] produced from AngII by ACE2 [7–9]. As with ACE and the AT₁R (angiotensin type 1 receptor), both ACE2 and the Ang-(1–7) receptors or *mas* receptor [10] are highly expressed in the heart and kidney. There is now significant data to support the notion that the ACE2/Ang-(1–7)/*mas* receptor axis represents a

Key words: angiotensin peptide, cardiac fibrosis, kidney failure, renal mass reduction, renin–angiotensin system.

Abbreviations: ACE, angiotensin-converting enzyme; Ang, angiotensin; AT₁R etc., angiotensin type 1 receptor etc.; BNP, brain natriuretic peptide; EMT, epithelial-to-mesenchymal transition; LV, left ventricle; LVEDP, left ventricular end-diastolic pressure; KO, knockout; PRA, plasma renin activity; QFS, quenched fluorescent substrate; qRT, quantitative real-time; RAS, renin–angiotensin system; SD, Sprague–Dawley; STNx, subtotal nephrectomy; TGF- β 1, transforming growth factor- β 1; 2K1C, two-kidney/one-clip.

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counter-regulatory arm to the ACE/AngII/AT₁R axis that can protect injured tissues from the harmful effects of locally produced AngII [7,11,12].

To date, few studies have assessed the effect of kidney failure on the ACE2/Ang-(1-7)/mas receptor axis. We have reported that renal mass reduction produced by STNx (subtotal nephrectomy) [3,4] causes cardiac remodelling, characterized by hypertrophy and fibrosis, and marked increases in cardiac ACE2 activity [3]. Inhibition of cardiac ACE with ramipril lowered blood pressure and abrogated the cardiac changes. As the major role for ACE2 is to produce Ang-(1-7), these results suggested a cardioprotective role for ACE2 in renal failure, possibly through increased cardiac Ang-(1-7). Studies have shown that infusion of Ang-(1-7) has significant cardioprotective effects in various experimental models of heart disease. For example, Ang-(1-7) improves remodelling after myocardial infarction [13], improves recovery from ischaemia/reperfusion injury in diabetic animals [14] and reverses cardiac hypertrophy and fibrosis in experimental hypertension [15].

There is limited information on the effect of Ang-(1-7) in experimental models of renal disease, and the results are quite variable. Ang-(1-7) infusion accelerated renal damage in the streptozotocin-induced diabetic rat [16] and increased mesangial area in a mouse model of renal mass reduction induced by 5/6 STNx [17], but had no effect on renal function or hypertension in the 2K1C (two-kidney/one-clip) Goldblatt model [18]. In the only study, to date, on the cardiac effects of Ang-(1-7) in kidney failure, Ang-(1-7) reduced blood pressure and improved cardiac remodelling in a mouse model of renal mass reduction [19].

The aim of the present study was to examine the effect of renal mass reduction on the cardiac ACE2/Ang-(1-7)/mas receptor axis and assess the effect of Ang-(1-7) infusion on blood pressure, cardiac structure/function and plasma and cardiac tissue RAS components. We also compared the effect of Ang-(1-7) infusion with that of the ACE inhibitor ramipril.

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. Male SD (Sprague–Dawley) rats (200–250 g) were housed in a 12:12 h light/dark cycle, with *ad libitum* food containing 0.4–0.6% NaCl (Norco) and water. STNx ($n = 45$) or sham surgery ($n = 10$) was performed in rats by right nephrectomy and ligation of all but one of the extrarenal branches of the left renal

artery as described previously [3,4]. STNx rats were randomly allocated to 10-day treatment with vehicle (0.9% saline, $n = 15$), the ACE inhibitor ramipril (oral $1 \text{ mg} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{day}^{-1}$, $n = 15$) or Ang-(1-7) (subcutaneous $24 \mu\text{g} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{h}^{-1}$, $n = 15$) via osmotic mini-pump (model # 2002; Alzet). The dose and mode of delivery of Ang-(1-7) used is the same as previously published studies [13,15,20]. Sham-operated rats (control) received vehicle.

On day 9, rats were housed in metabolic cages, and 24-h water intake and urine volume measured and a urine sample collected for the measurement of creatinine and sodium. On day 10, rats were anaesthetized with an intraperitoneal sodium pentobarbitone (60 mg/kg of body weight), and cardiac haemodynamics were determined using a microtipped pressure transducer catheter (Millar, 1.5F) inserted into the left carotid artery and advanced into the LV (left ventricle). Data were stored and analysed using Millar conductance data acquisition and analysis software, and heart rate, systolic blood pressure, maximal rate of ventricular contraction ($+dP/dt$) and LVEDP (left ventricular end-diastolic pressure) were determined. Diastolic function was assessed by measuring the time constant of isovolumic relaxation (τ), which assess active relaxation with higher values of τ implying impaired relaxation [21].

Rats were then killed by a lethal dose of sodium pentobarbitone, decapitated and trunk blood collected into EDTA and lithium heparin tubes. The residual volume (approx. $20 \mu\text{l}$) in the pump was removed for analysis of Ang-(1-7) levels. The remnant kidney and heart were removed and weighed. The LV was transversely dissected into three pieces and one piece fixed in 4% paraformaldehyde and embedded in paraffin for histopathology. The remainder was snap frozen in isopentane and stored at -80°C for *in vitro* autoradiographic studies, ACE2 activity assay and RNA extraction.

In addition, to verify that infused Ang-(1-7) was detectable in the circulation, a separate group of control rats received vehicle (0.9% saline, $n = 5$) or Ang-(1-7) (subcutaneous $24 \mu\text{g} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{h}^{-1}$, $n = 10$) via osmotic mini-pump for 10 days, and rats were killed and plasma Ang-(1-7) levels measured.

Drugs

Sodium pentobarbitone was obtained from Boehringer Ingelheim, Ang-(1-7) was from Auspep, and ramipril was from Sanofi-Aventis.

Biochemical analysis

Urinary and plasma creatinine, urea and sodium were measured using an autoanalyser (Beckman Instruments).

Plasma ACE, Ang-(1-7), AngII and renin activity

Blood samples for measurement of angiotensin peptides were collected into tubes containing 20 μl /ml of blood of an inhibitor cocktail [50 mM EDTA, 0.2 M *N*-ethylmaleimide and 1–2 TIU (trypsin inhibitory units)/ml aprotinin made up in saline] and plasma was snap-frozen and stored at -80°C . The RIAs for AngII and Ang-(1-7) have been described previously [4,22], and the antibodies used for AngII and Ang-(1-7) were raised in rabbit and guinea pig respectively, and the specific radioisotopes, ^{125}I -AngII and ^{125}I -Ang-(1-7), were made by Prosearch. The intra- and inter-assay coefficients of variation were 7.6 and 8.3% for AngII, and 4.5 and 10% for Ang-(1-7). Plasma ACE activity was measured using a modification of a previously published method [23]. Briefly, 5 μl of plasma was incubated at 37°C with the ACE substrate hippuryl-His-Leu (1 mM) in a total volume of 50 μl of buffer (0.4 M sodium borate buffer and 0.3 M NaCl, pH 8.3) in the presence and absence of EDTA (10 μM) for 30 min. Following incubation, 120 μl of 0.3 M NaOH and 10 μl of *o*-phthalaldehyde (20 mg/ml in methanol) were added. After 10 min at room temperature (24°C), 20 μl of 3 M HCl was added to stop the reaction, the tubes were centrifuged at 30000 g for 5 min and the supernatants were transferred to a black 96-well microtitre plate. Fluorescence was measured using a FLUOstar Optima plate reader (BMG Labtech). The rate of substrate cleavage was determined by comparison with a standard curve of the product His-Leu and are expressed as nmol of substrate $\cdot\text{ml}^{-1}$ of plasma $\cdot\text{min}^{-1}$. PRA (plasma renin activity) was measured by RIA as described previously [24].

QFS (quenched fluorescent substrate) assay for plasma and cardiac ACE2 activity

We have developed an assay for ACE2 enzymatic activity in tissue and plasma validated using Western blotting and immunohistochemistry [25]. The left ventricular membranes were prepared as described previously [3,25], and 100 μg of protein was incubated in duplicate with an ACE2-specific QFS, (7-methoxycoumarin-4-yl)-acetyl-Ala-Pro-Lys(2,4-dinitrophenyl) (Auspep), 10 μM Z-Pro-prolinal (Auspep), with or without 100 μM EDTA in a total volume of 200 μl [3]. The rate of substrate cleavage was determined by comparison with a standard curve of the free fluorophore MCA (4-amino-methoxycoumarin; Sigma) and are expressed as nmol of substrate cleaved $\cdot\text{mg}^{-1}$ of protein $\cdot\text{h}^{-1}$. For plasma ACE2 activity, blood collected into heparinized tubes was centrifuged at 4°C and assayed using an ACE2-specific QFS. Results are expressed as nmol of substrate $\cdot\text{ml}^{-1}$ of plasma $\cdot\text{h}^{-1}$.

In vitro autoradiography for cardiac ACE binding

Cardiac ACE activity was assessed by *in vitro* autoradiography on LV sections (20 μm) in 10 rats/group using the specific radioligand ^{125}I -MK351A ($K_i = 30$ pmol/l) as described previously [3,25]. Quantification of ACE binding density in two LV sections from each animal was performed using a microcomputer imaging device (Imaging Research), which measures the relative absorbance of the radioactive labelling. Results are expressed as a percentage of binding in control rats, and reflect ACE activity in the tissue.

Determination of cardiac collagen

Cardiac (LV) paraffin sections 4- μm thick were deparaffinized, rehydrated and then stained with 0.1% Sirius Red (Polysciences) in saturated picric acid (Picrosirius Red) for 1 h, differentiated in 0.01% HCl for 30 s and rapidly dehydrated. Interstitial collagen volume fraction was determined by measuring the area of stained tissue within a given field, excluding vessels, artefacts, minor scars or incomplete tissue fields; 15–20 fields were analysed per animal in a blinded manner. To measure perivascular collagen, all arteries in the LV section were analysed, and the whole artery including the adventitia was selected for assessment. For both interstitial and perivascular collagen, the area stained was then calculated as a percentage of the total area within a given field [25,26].

qRT (quantitative real-time)-PCR

Total RNA was isolated from the LV ($n = 10$ /group) 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 [25]. All primers and probes for BNP (brain natriuretic peptide), ACE, ACE2, *mas* receptor AT₁Rs and AT₂Rs (angiotensin type 2 receptors) were designed using the software program Primer Express (PE Applied Biosystems), and sequences are shown in Supplementary Table S1 (at <http://www.clinsci.org/cs/120/cs1200335add.htm>). qRT-PCR was performed using the multiplex method, and 18S VIC was used as the endogenous control. A relative expression method was applied in the present study using the control group as the calibrator.

Statistical analysis

Values are presented as means \pm S.E.M. *P* values were calculated using an unpaired Student's *t* test when comparing the control with STNx, and ANOVA followed by *post hoc* least significant difference analysis when comparing STNx+vehicle with the treatment groups. For analysis of cardiac ACE binding, results were log-transformed to stabilize variance. Significant differences were obtained when $P < 0.05$.

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

Values are means \pm S.E.M. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with control vehicle; # $P < 0.05$ and ## $P < 0.01$ and ### $P < 0.001$ compared with vehicle-treated STNx.

Parameter	Control	STNx		
	Vehicle (n = 10)	Vehicle (n = 15)	Ramipril (1 mg · kg ⁻¹ of body weight · day ⁻¹) (n = 15)	Ang-(1-7) (24 µg · kg ⁻¹ of body weight · h ⁻¹) (n = 15)
Body weight (g)	267 \pm 6	224 \pm 10**	228 \pm 6	223 \pm 8
Renal parameter				
Left kidney weight (g)	1.08 \pm 0.04	1.13 \pm 0.04	1.07 \pm 0.04	1.19 \pm 0.04
Left kidney/body weight (g/100 g)	0.40 \pm 0.01	0.51 \pm 0.02***	0.47 \pm 0.01#	0.53 \pm 0.01
Water intake (ml · 100 g ⁻¹ · 24 h ⁻¹)	10.4 \pm 1.2	27.2 \pm 3.4***	25.0 \pm 1.8	30.8 \pm 3.1
Urine output (ml · 100 g ⁻¹ · 24 h ⁻¹)	3.8 \pm 0.6	16.4 \pm 2.8***	15.6 \pm 2.1	18.7 \pm 3.5
Urinary protein (mg/24 h)	9.5 \pm 1.3	15.8 \pm 1.8*	10.6 \pm 1.0#	12.5 \pm 1.1
Plasma creatinine (mmol/l)	0.014 \pm 0.004	0.040 \pm 0.004***	0.043 \pm 0.003	0.039 \pm 0.005
Plasma urea (mmol/l)	4.0 \pm 0.3	10.9 \pm 2.1***	11.1 \pm 0.7	9.2 \pm 0.6
Creatinine clearance (ml/min)	4.44 \pm 0.75	1.30 \pm 0.20***	1.16 \pm 0.12	1.25 \pm 0.20
Sodium excretion (mmol/24 h)	0.11 \pm 0.04	0.43 \pm 0.1**	0.37 \pm 0.07	0.47 \pm 0.11
Plasma RAS component				
PRA (ng · ml ⁻¹ · h ⁻¹)	19.5 \pm 1.8	38.7 \pm 5.3**	56.0 \pm 6.1##	26.5 \pm 2.3
ACE (nmol · ml ⁻¹ · min ⁻¹)	12.4 \pm 1.0	9.9 \pm 0.7*	0.2 \pm 0.03###	9.2 \pm 0.6
ACE2 (nmol · ml ⁻¹ · h ⁻¹)	14.4 \pm 1.2	13.8 \pm 0.9	10.7 \pm 0.7#	13.7 \pm 0.5
AngII (fmol/ml)	19.3 \pm 5.3	59.9 \pm 19.1*	88.4 \pm 9.9	39.5 \pm 12.5
Ang-(1-7) (fmol/ml)	327 \pm 71	561 \pm 138	1695 \pm 359#	491 \pm 126
Ang-(1-7)/AngII ratio	18.9 \pm 4.8	8.3 \pm 1.1*	12.1 \pm 1.1	11.6 \pm 2.0

RESULTS

STNx and renal function

The changes in physiological and biochemical parameters after STNx and the effect of intervention are shown (Table 1). Following STNx, rats had poor weight gain ($P < 0.01$) and hypertrophy of the remnant kidney ($P < 0.001$). Renal impairment was present as indicated by elevated plasma urea and creatinine ($P < 0.001$), reduced creatinine clearance and increased urinary protein ($P < 0.05$) compared with control rats. STNx rats had increased water intake and urine volume ($P < 0.001$) and increased sodium excretion ($P < 0.01$). Treatment with ramipril reduced renal hypertrophy and urinary protein ($P < 0.05$), whereas Ang-(1-7) did not affect any of the renal parameters assessed.

Plasma RAS components

The changes in the circulating components of the RAS after STNx and the effect of treatment are shown (Table 1). STNx led to a significant increase in PRA and AngII ($P < 0.01$ and $P < 0.05$ respectively), and a reduction in plasma ACE ($P < 0.05$), but no change in plasma ACE2 activity or plasma Ang-(1-7) compared with control rats. We also calculated the Ang-(1-7)/AngII ratio, which provides an index of the conversion of

AngII into Ang-(1-7); this ratio decreased significantly ($P < 0.05$) in the STNx rats.

Ramipril increased PRA ($P < 0.01$) and plasma Ang-(1-7) ($P < 0.05$), and reduced plasma ACE ($P < 0.001$) and ACE2 ($P < 0.05$). Ang-(1-7) infusion had no significant effect on plasma PRA, ACE, ACE2 or Ang-(1-7). At the end of the study, all pumps had a minimal residual volume, and the concentration of Ang-(1-7) in the residual volume was similar to that originally added to the pump [baseline Ang-(1-7) concentration, 8 mg/ml; post-infusion residual volume Ang-(1-7) concentration, 7.5 mg/ml], indicating a continued delivery of Ang-(1-7) over the course of the study.

In a separate 10-day study in rats without STNx, we showed infusion of Ang-(1-7) via a subcutaneous mini-pump was associated with a 2-fold increase in plasma Ang-(1-7) compared with rats that received vehicle [147 ± 24 fmol/ml in the vehicle group compared with 250 ± 27 fmol/ml in the Ang-(1-7)-treated group; $P < 0.05$; $n = 5-10$ per group]. There was no effect of Ang-(1-7) on blood pressure, heart weight or cardiac fibrosis (results not shown).

Blood pressure and cardiac function

The change in blood pressure and cardiac function after STNx is shown (Figure 1). Compared with control rats,

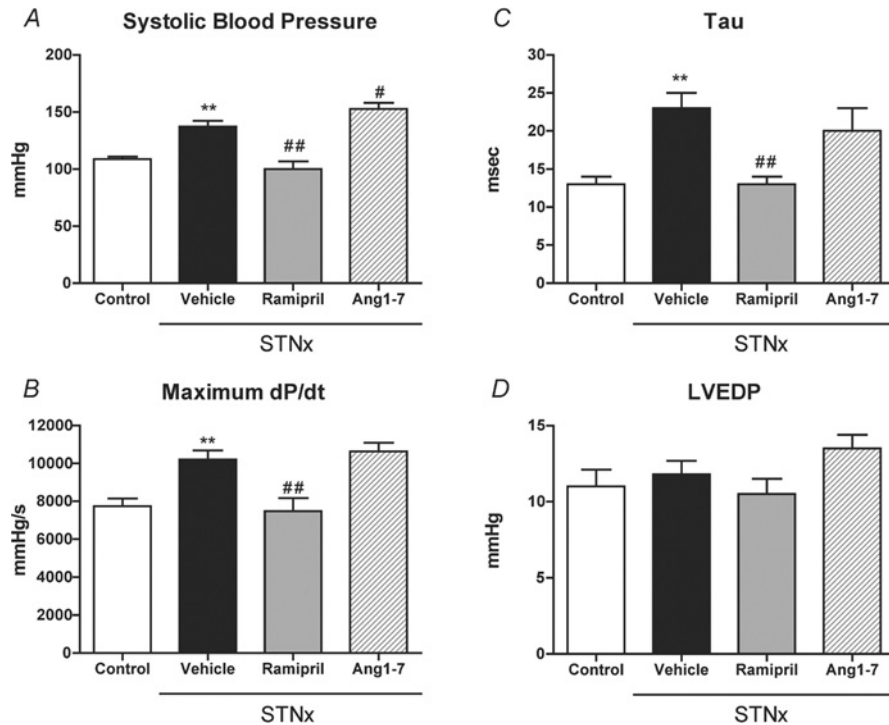


Figure 1 Systolic blood pressure (A), ventricular contractility (B), time constant of isovolumic relaxation (C) and LVEDP (D) in control and STNx [vehicle, ramipril and Ang-(1-7)] rats

Values are means \pm S.E.M. ($n = 10$ for the control group and $n = 15$ for the STNx groups). ** $P < 0.01$ compared with control; # $P < 0.05$ and ## $P < 0.01$ compared with vehicle-treated STNx.

STNx resulted in a significant increase in systolic blood pressure (Figure 1A; $P < 0.01$), associated with hypercontractility and increased systolic function (Figure 1B, max. dP/dt ; $P < 0.01$). Diastolic dysfunction was present with impaired active relaxation as shown by the significant increase in the time constant for isovolumic relaxation (Tau) (Figure 1C; $P < 0.01$). Elevated ventricular filling pressure is the main physiological consequence of diastolic dysfunction, but, at this early stage, there was no increase in STNx rats (Figure 1D). There was no effect of STNx on heart rate (results not shown).

Treatment with the ACE inhibitor ramipril had significant cardiovascular benefits, decreasing blood pressure ($P < 0.01$), returning the rate of systolic contraction to levels observed in control rats ($P < 0.01$) and improving diastolic function ($P < 0.01$). By contrast, Ang-(1-7) infusion was associated with adverse cardiovascular effects in STNx rats and significant increases in blood pressure (Figure 1A; $P < 0.05$) and left ventricular hypertrophy (Figure 2A; $P < 0.05$) over and above that in STNx rats treated with vehicle. Rats receiving Ang-(1-7) maintained the hypercontractile state (Figure 1B) and had diastolic dysfunction (Figure 1C) with a non-significant increase in LVEDP (Figure 1D). An indirect marker of cardiac function, cardiac BNP, was increased at the gene level in STNx rats and was only decreased with ramipril treatment (Table 2).

Cardiac hypertrophy, fibrosis and ACE/ACE2 activity

STNx was associated with marked cardiac (total, right and left ventricular) hypertrophy, as well as significant interstitial fibrosis (Figures 2A and 2B). The relative quantification of cardiac ACE and ACE2 after STNx and the effect of treatment are shown (Figure 2, right-hand panels). STNx increased both left ventricular ACE activity (Figure 2C, $P < 0.001$) and ACE2 activity (Figure 2D; $P < 0.05$).

Treatment with the ACE inhibitor ramipril led to a significant decrease in left ventricular hypertrophy (Figure 2A; $P < 0.001$) and inhibited cardiac ACE activity (Figure 2C; $P < 0.001$), with no change in cardiac ACE2 activity. The adverse effect of Ang-(1-7) infusion already noted on blood pressure was associated with further increases in left ventricular hypertrophy ($P < 0.05$) and cardiac fibrosis ($P < 0.01$). Ang-(1-7) infusion led to a 3-fold induction of cardiac ACE binding ($P < 0.001$) over and above that observed in STNx-vehicle rats and a significant reduction in ACE2 activity ($P < 0.05$).

Representative images of cardiac ACE binding using autoradiography and total collagen staining are shown (Figure 3); the degree of interstitial fibrosis follows the intensity of cardiac ACE binding.

The gene expression of cardiac ACE, ACE2 and the RAS receptors in STNx are shown (Table 2).

Table 2 Cardiac expression of RAS genes

Values are means \pm S.E.M. * $P < 0.01$ and ** $P < 0.05$ compared with control vehicle; # $P < 0.05$ and ### $P < 0.01$ compared with vehicle-treated STNx.

mRNA (arbitrary units)	Control	STNx		
	Vehicle ($n = 10$)	Vehicle ($n = 10$)	Ramipril ($1 \text{ mg} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{day}^{-1}$) ($n = 10$)	Ang-(1-7) ($24 \mu\text{g} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{h}^{-1}$) ($n = 10$)
BNP	1.00 ± 0.18	$2.14 \pm 0.23^{**}$	$1.38 \pm 0.14^{\#}$	1.91 ± 0.19
ACE	1.00 ± 0.13	0.87 ± 0.07	$0.66 \pm 0.05^{\#}$	0.84 ± 0.06
ACE2	1.00 ± 0.15	$1.65 \pm 0.19^*$	1.27 ± 0.16	1.54 ± 0.22
<i>mas</i> receptor	1.00 ± 0.06	$1.23 \pm 0.08^*$	$0.85 \pm 0.07^{##}$	$0.87 \pm 0.11^{\#}$
AT ₁ R	1.00 ± 0.09	$1.50 \pm 0.21^*$	1.13 ± 0.08	1.18 ± 0.11
AT ₂ R	1.00 ± 0.11	1.31 ± 0.22	0.84 ± 0.12	0.99 ± 0.19

Cardiac ACE2 was significantly elevated after STNx at the gene level ($P < 0.05$), as were *mas* receptor and AT₁R expression ($P < 0.05$), whereas ACE mRNA and expression of the AT₂R were unchanged. Ramipril reduced the gene expression of ACE and the *mas* receptor, whereas ACE2 mRNA was unchanged. Treatment with Ang-(1-7) significantly reduced *mas* receptor expression. AT₁R and AT₂R expression were unchanged in any treatment group.

DISCUSSION

The present study confirms and extends our previous work that an acute reduction in renal mass by STNx increases blood pressure and leads to cardiac remodelling with cardiac hypertrophy and fibrosis resulting in cardiac dysfunction. These changes are associated with activation of both the circulating RAS and the cardiac RAS and are ameliorated with ACE inhibition.

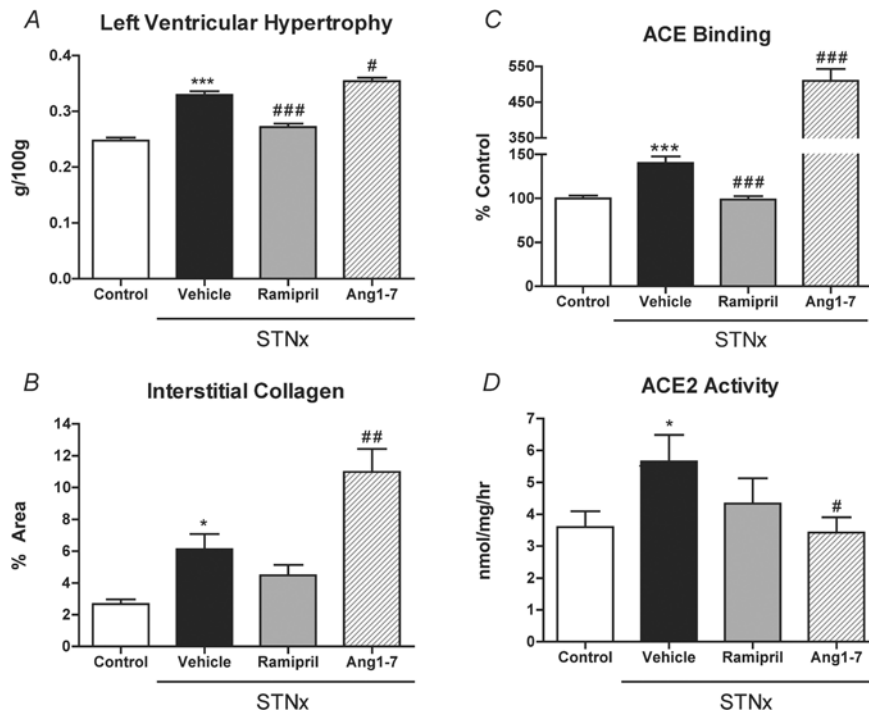


Figure 2 Left ventricular hypertrophy (A), interstitial collagen (B), ACE binding (C) and ACE2 activity (D) in control and STNx [vehicle, ramipril and Ang-(1-7)] rats

Values are means \pm S.E.M. ($n = 10$ for the control group and $n = 15$ for the STNx groups) * $P < 0.05$ and *** $P < 0.001$ compared with control; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ compared with vehicle-treated STNx.

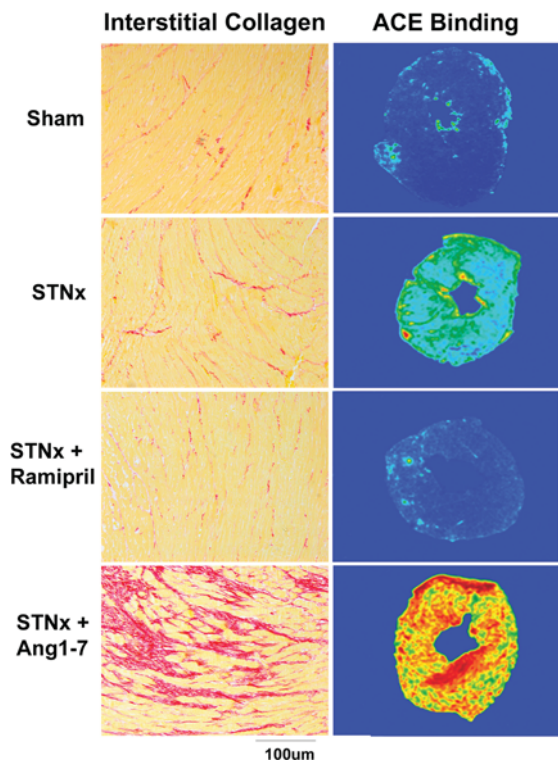


Figure 3 Representative photomicrographs of left ventricular total collagen content (red staining; left-hand panel) and representative macroscopic autoradiographs demonstrating ACE binding in the LV (right-hand panel)

The red colour denotes the highest density of radiolabelling, whereas the blue colour represents background labelling.

Contrary to our expectation that Ang-(1-7) infusion would alleviate the cardiac consequences of kidney failure, we found that infusion of Ang-(1-7) in STNx rats was associated with further increases in blood pressure and acceleration of cardiac hypertrophy and fibrosis; these changes occurred in association with a 3-fold increase in cardiac ACE. As ACE is responsible for the degradation of Ang-(1-7), increased ACE would negate any benefit of exogenously infused Ang-(1-7) and, indeed, we found no increase in circulating Ang-(1-7) following Ang-(1-7) infusion. In addition, Ang-(1-7) significantly decreased cardiac ACE2 activity, and the imbalance in cardiac ACE/ACE2 is likely to contribute to the adverse cardiac effects observed. Although we were unable to measure tissue angiotensin peptide levels due to a lack of available tissue, we can speculate that increased cardiac ACE will not only increase the degradation of cardiac Ang-(1-7), but also generate more of the profibrotic peptide AngII. Attenuated expression of cardiac ACE2 with Ang-(1-7) infusion will also favour accumulation of tissue AngII and reduce the endogenous generation of Ang-(1-7). The increase in cardiac fibrosis

observed would be consistent with this hypothesis. It is also known from studies in ACE2-KO (knockout) mice that a lack of ACE2 leads to increased cardiac AngII and impaired cardiac function, and that inhibition of cardiac ACE2 exacerbates cardiac hypertrophy and fibrosis [27].

By contrast, inhibition of ACE with ramipril reduced cardiac ACE and maintained levels of cardiac ACE2, leading to a more favourable ACE/ACE2 profile in the direction of decreased AngII and increased Ang-(1-7). The peptide Ang-(1-7) has a very short half-life (seconds) [28], due to degradation by ACE, and ACE inhibition increases circulating levels of Ang-(1-7).

There is limited information on the effect of Ang-(1-7) in the kidney, whether at the cellular level or in whole animals, and the results that are available are at variance with the effect of Ang-(1-7) on the heart [29–31]. Furthermore, *in vitro* studies have shown that Ang-(1-7) has opposing effects on different cell types in the kidney. In the proximal tubule, Ang-(1-7) displays growth inhibitory properties and antagonizes the effects of AngII [32], whereas, in mesangial cells, it stimulates growth through increases in the profibrotic cytokine TGF- β 1 (transforming growth factor- β 1), fibronectin and collagen IV [33]. More recently, Ang-(1-7) infusion has also been shown to increase relative mesangial area in a mouse model of renal mass reduction induced by 5/6 STNx [17]. The renal effects of Ang-(1-7) differ diametrically from its effects in cardiac fibroblasts, where it exerts potential antifibrotic and antitrophic effects [34]. With regard to the vasculature, *in vitro* results with Ang-(1-7) cannot always be validated in long-term *in vivo* studies; Ang-(1-7) attenuated AngII-induced vasoconstriction in renal vessels *in vitro*, but *in vivo*, it had no significant effect on renal vessels [35]. The effects of Ang-(1-7) in the vasculature may also vary according to prevailing blood pressure and the degree of RAS activation. It has been reported recently that Ang-(1-7) caused coronary constriction in spontaneously hypertensive rats (but not in Wistar rats) in an AT₂R-dependent manner [36].

In vivo studies in experimental renal disease that have examined the effect of Ang-(1-7) infusion on the kidney have shown varying results in renal disease. In streptozotocin-induced diabetic rats, a 6-week infusion of Ang-(1-7) (intravenous 25 $\mu\text{g} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{h}^{-1}$) accelerated renal damage and increased profibrotic TGF- β 1 mRNA and protein levels. Interestingly, the adverse effects of Ang-(1-7) were associated with increased renal ACE mRNA expression and down-regulation of renal ACE2 and mas receptor expression [16], results not dissimilar to our own, albeit in the heart. In the 2K1C Goldblatt hypertensive rat [18], neither of the two approaches used to increase Ang-(1-7) including a 12-week subcutaneous Ang-(1-7) infusion (50 $\mu\text{g}/\text{h}$) and transgenic rats expressing an Ang-(1-7) producing fusion protein modified the course of

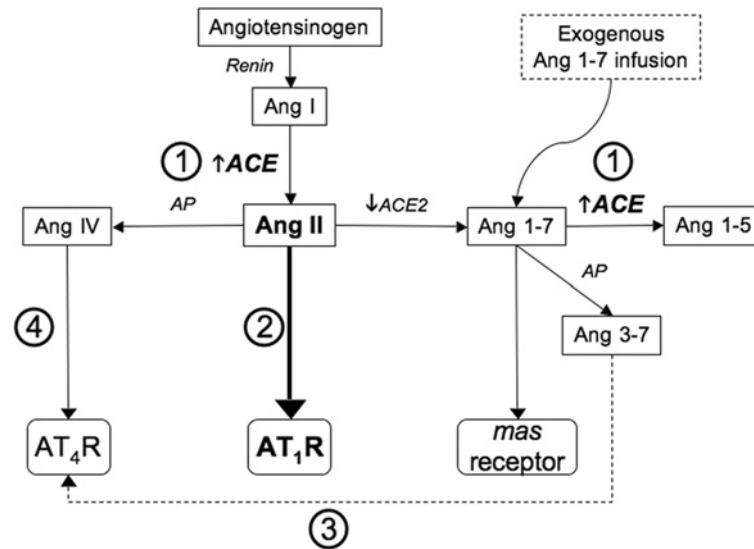


Figure 4 Schematic representation of possible mechanisms responsible for the adverse effects of Ang-(1–7) administration in STNx

Exogenous administration of Ang-(1–7) results in increased ACE activity (①), leading to increased levels of AngII, which bind to AT₁R (②) and activate the pressor arm of the RAS system. Furthermore, excess Ang-(1–7) can also be broken down to Ang-(3–7), a potent agonist of the AT₄R (③), which has been shown to contribute to cardiovascular disease via the activation of the NF- κ B (nuclear factor κ B) pathway. Activation of the AT₄R by AngIV, a metabolite of AngII may play a role (④). Finally, reduced renal excretion of angiotensin metabolites may also be contributing to the effects observed in the present study.

hypertension or altered renal function. One study in renal disease that was positive found that a 5-day infusion of Ang-(1–7) (subcutaneous $24 \mu\text{g} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{h}^{-1}$) improved glomerulosclerosis in a rat model of glomerulonephritis induced with the monoclonal anti-thy-1 antibody OX-7 [37], although two lower doses of Ang-(1–7) failed to show a benefit.

Ang-(1–7) is thought to mediate its effects via the *mas* receptor. In the present study, exogenous Ang-(1–7) infusion resulted in reduced expression of the cardiac *mas* receptor. As the *mas* receptor can hetero-oligomerize with the AT₁R to inhibit the effects of AngII [38], down-regulation of the *mas* receptor may increase the adverse effects of AngII and promote cardiac damage. The *mas* receptor mediated actions of Ang-(1–7) can also be influenced by AT₂R-related mechanisms, suggesting a complex interaction between these receptors and highlighting the molecular complexity of this system [39].

The importance of the *mas* receptor in the progression of renal disease is not clear-cut. For example, although *mas* receptor KO mice have glomerular hyperfiltration and renal fibrosis, suggesting a protective role for the *mas* receptor [40], others have shown that *mas* receptor KO mice actually have less renal damage after unilateral ureteral obstruction [31]. Our own studies have demonstrated involvement of the *mas* receptor in the transition of tubulo-epithelial cells into myofibroblasts [tubular EMT (epithelial-to-mesenchymal transition)], an important contributor to renal fibrosis [41]. These *in vitro*

studies demonstrated that AngII-induced EMT in a normal rat kidney cell line was exclusively dependent on the Ang-(1–7)/*mas* receptor pathway, with various profibrotic cytokines up-regulated with Ang-(1–7) and reversed with the *mas* receptor antagonist A779 [41]. These observations were supported further by *in vivo* data showing increased renal expression of various markers of EMT following infusion of Ang-(1–7) [41].

With regard to the heart, the results in experimental heart disease have uniformly shown a beneficial effect of infusion of Ang-(1–7) on the heart [13–15,42]. To date, there has been only one other study to assess the effect of Ang-(1–7) on the heart in renal disease. In a chronic mouse model of renal mass reduction induced by removal of both poles of the right kidney, followed a week later by removal of the left kidney, a 12-week infusion of Ang-(1–7) (subcutaneous $300 \mu\text{g} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{day}^{-1}$) lowered blood pressure and improved cardiac function and fibrosis [19]. The mechanism of the benefit is unclear as both Ang-(1–7) and hydralazine lowered blood pressure and improved renal function, but only Ang-(1–7) improved cardiac hypertrophy and fibrosis. The authors did not measure circulating or cardiac ACE, ACE2 or Ang-(1–7). Major differences between the two studies include the species used (rat compared with mouse), the method of induction and chronicity of kidney failure (acute compared with chronic) and the duration of Ang-(1–7) infusion (10 days compared with 12 weeks), while the dose and route

of administration of Ang-(1-7) was the same in both studies. It remains unknown whether the adverse effects of Ang-(1-7) that we have reported in an acute model of renal injury are also observed in chronic renal disease secondary to renal mass reduction in the rat, but such studies are important.

The potential mechanisms by which Ang-(1-7) infusion leads to an imbalance of the cardiac ACE/ACE2/*mas* receptor axis and acceleration of cardiac fibrosis and hypertrophy after STNx are unclear, and a potential schema is illustrated (Figure 4). The role of elevated cardiac ACE (Figure 4 ①) and potentially AngII (Figure 4 ②) have been discussed previously. However, it is also possible that renal failure results in an increase in Ang-(1-7) metabolites, which would normally be excreted by the kidney. Although the function of the angiotensin fragments remain to be fully elucidated, increasing evidence shows that they are biologically active [43]. Given the elevated levels of ACE, it is possible that Ang-(1-7) is being metabolized to Ang-(1-5) as reported previously [44]. Ang-(1-5) can then be further broken down to Ang-(3-5) or Ang-(1-4) [45]. Ang-(1-7) can also be directly converted into Ang-(3-7) by aminopeptidase [46] (Figure 4 ③). Ang-(3-7) can elevate blood pressure [43,47], by activating the AT₄R ([46,48], which signals through the NF- κ B (nuclear factor κ B) pathway to promote pro-inflammatory and pro-thrombotic effects [49]. As AngIV (a product of AngII) [49] can also activate the AT₄ R, angiotensin metabolites may contribute to the cardiac damage observed in this study (Figure 4 ④). To date, this remains speculative, and more studies examining these complex pathways are required.

In summary, we have shown that a 10-day infusion of Ang-(1-7) in rats with STNx is associated with deleterious effects on blood pressure and the heart, with increases in cardiac ACE, and decreases in cardiac ACE2 activity. As ACE2 both degrades AngII and generates Ang-(1-7), the resultant imbalance in the cardiac ACE/ACE2 axis favours the accumulation of cardiac AngII and accelerates cardiac fibrosis and hypertrophy. Our results add to the increasing evidence that Ang-(1-7) may have deleterious effects in kidney disease and highlight the need for further *in vivo* studies of the alternative arm of the RAS in this increasingly common condition. Future studies should investigate whether enhancement of cardiac or renal ACE2 activity may represent a better therapeutic strategy to address both the cardiac and renal consequences of kidney disease.

AUTHOR CONTRIBUTION

Louise Burrell conceived the experiments and co-wrote the paper; Elena Velkoska, Rachael Dean, Karen Griggs and Luke Burchill carried out the study; Elena Velkoska

and Rachael Dean analysed the data and co-wrote the paper.

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■ SUPPLEMENTARY ONLINE DATA

Angiotensin-(1–7) infusion is associated with increased blood pressure and adverse cardiac remodelling in rats with subtotal nephrectomy

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Table S1 qRT-PCR probes and primers

F, forward; FAM, 6-carboxyfluorescein; *masR*, *mas* receptor; R, reverse; TAMRA, 6-carboxytetramethylrhodamine.

Gene	Probe/primer	Sequence (5'→3')
ACE	Probe	FAM-CAACAAGACTGCCACCTGCTGGTCC-TAMRA
	F-Primer	CACCGCAAGGTCTGCTT
	R-Primer	CTGGCATAAGTTCTGTGAGGAA
ACE2	Probe	FAM-TTGTCTGCCACCCACA-TAMRA
	F-Primer	GCCAGGAGATGACCGGAAA
	R-Primer	CTGAAGTCTCCATGTCCAGATC
<i>masR</i>	Probe	FAM-CGGGATCCTCCTCTGG-TAMRA
	F-Primer	CATCTCTCCTCTCGGCTTTGTG
	R-Primer	CCTCATCCGGAAGCAAAGG
AT ₁ R	Probe	FAM-CATCGGCCAAAAGCCTGCGT-TAMRA
	F-Primer	CGGCCTTCGGATAACATG
	R-Primer	CCTGTCACTCCACCTCAAACA
AT ₂ R	Probe	FAM-CAACCTTCCTCTCTGGGCAACCTATTACTTTATA-TAMRA
	F-Primer	CAATCTGGCTGTGGCTGACTT
	R-Primer	TGCACATCACAGGTCAAAGA
BNP	Probe	6FAM-TCCTAGCCAGTCTCC-TAMRA
	F-Primer	GCCGCTGGGAGGTCCT
	R-Primer	AGCTTCTGCATCGTGGATTGT

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