The renal excretory responses to acute renal interstitial angiotensin (1–7) infusion in anaesthetised spontaneously hypertensive rats

Elaine F. Barry1 | Julie O’Neill2 | Mohammed H. Abdulla1 | Edward J. Johns1

Abstract
This study investigated the impact of intrarenal angiotensin 1–7 (Ang [1–7]) infusion on renal excretory function in a rat model of hypertension. Eleven-week-old spontaneously hypertensive rats (SHRs, n = 7) and Han Wistar controls (NCR, n = 7) were anaesthetised with sodium pentobarbital (60 mg/kg i.p.) and prepared for the measurement of mean arterial pressure (MAP) and left renal function during renal interstitial infusion of Ang (1–7) (50 ng/min). The kidneys were harvested, the renal cortex and medulla separated, prepared for measurement of Ang II and Ang (1–7) and Western blot determination of AT1 and Mas receptor protein expression. MAP, glomerular filtration rate (GFR), urine flow (UF) and absolute sodium excretion (UNaV) were 109 ± 16 mmHg, 4.4 ± 1.0 mL/min/kg, 102 ± 16 µL/min/kg and 16 ± 3 µmol/min/kg, respectively in the NCR and 172 ± 24 mmHg, 3.4 ± 0.7 mL/min/kg, 58 ± 30 µL/min/kg and 8.6 ± 4.8 µmol/min/kg respectively in the SHR. Ang (1–7) increased UF (31%), UNaV (50%) and fractional sodium excretion (FENa+) (22%) in the NCR group (all p < 0.05) but had no effect on GFR in either group. The magnitudes of the Ang (1–7)-induced increases in UF and UNaV were significantly blunted in the SHR group (model × drug p < 0.05). The renal cortical AT1: Mas receptor expression ratio was significantly higher in the SHR group (p < 0.05) but renal Ang II and Ang (1–7) levels were not statistically different between groups. The Ang (1–7)-induced increases in sodium and water excretion were impaired in the SHR group in the context of an unstimulated RAS. The decrease in responsiveness of the SHR kidney to Ang (1–7) appears to be associated with higher levels of AT1 receptor expression in the renal cortex.

Keywords
Ang (1–7), AT1 receptors, glomerular filtration rate, Mas receptor, spontaneously hypertensive rat
1 | INTRODUCTION

Inappropriate activation of the renin angiotensin system (RAS) contributes to the pathogenesis of hypertension through ACE/Angiotensin II/AT1 receptor mediated oxidative stress, inflammation, sodium and water retention and vasoconstriction. The traditional RAS pathway has been a frontline therapeutic target for the treatment of patients with hypertension. An increasing proportion of patients have been identified as becoming resistant to conventional anti-hypertensive drugs and the need for additional therapeutic targets prevails. More recently, it has emerged that a novel RAS pathway (ACE2/Angiotensin [1–7]/Mas receptor axis) exists and opposes the actions of angiotensin II (ang II) in the kidney by enhancing renal blood flow and inhibiting sodium and water retention. Indeed, the renal excretory and haemodynamic actions of angiotensin (1–7) (Ang [1–7]) have been reported by a number of investigators. It is well established that the inhibitory effect of Ang (1–7) on basal sodium and water reabsorption is mediated by Mas receptors. However, the factors that govern the interrelationship between renal Ang (1–7) and Ang II in determining the regulation of renal excretory and haemodynamic function remain unclear.

There is a consensus that the level of endogenous RAS activity could importantly influence the responsiveness of the kidney to exogenous Ang (1–7). We have previously demonstrated that the magnitude of Ang (1–7) induced natriuresis and diuresis was enhanced when the endogenous RAS was stimulated by a low sodium diet and diminished when the RAS was suppressed by a high sodium diet. These observations support those of Bürgelová and co-workers who demonstrated that the inhibitory effect of Ang (1–7) on renal sodium reabsorption was attenuated by acute intrarenal Mas receptor blockade when the RAS was either physiologically activated by a low sodium diet or patho-physiologically activated in a rat model of renovascular hypertension. Taken together, these data provide evidence that the renal excretory actions of both endogenous intrarenal Ang (1–7) and exogenous intrarenal Ang (1–7) infusion are enhanced during physiological and patho-physiological Ang II accumulation.

The impact of acute intrarenal Ang (1–7) infusion on renal haemodynamic and excretory function is less characterised in disease models that do not involve endogenous intrarenal RAS stimulation such as the spontaneously hypertensive rat (SHR). Indeed, the chronic systemic administration of Ang (1–7) to SHRs significantly reduced mean blood pressure but was without significant effect upon the excretion of sodium and water. However, given that glomerular filtration rate (GFR) was not measured in conjunction with the other variables, it was not clear whether a decrease in the filtered load of sodium accompanied the Ang (1–7) induced decrease in mean arterial pressure (MAP) in SHRs. This investigation examined whether the renal functional and haemodynamic actions of Ang (1–7) in the SHR with a normal RAS would be different from that in control normotensive rats. This was achieved by evaluating the direct effects of

Ang (1–7) on renal haemodynamic and excretory function in SHRs following its acute renal interstitial infusion.

2 | RESULTS

2.1 Mean arterial pressure

The first set of measurements evaluated the baseline levels of MAP in the NCR and SHR groups and how it was altered by the renal interstitial infusion of Ang (1–7). MAP in the SHR group was significantly (p < 0.001) higher at baseline than the NCR group (SHR vs. NCR, 172 ± 24 vs. 109 ± 16 mmHg). The renal interstitial infusion of Ang (1–7) had no statistically significant effect on MAP in either the NCR or SHR groups (Figure 1A).

2.2 Glomerular filtration rate

The impact of renal interstitial infusion of Ang (1–7) on GFR was determined by measuring the changes in the variable before and following infusion of the peptide. Baseline GFR in the SHR group was significantly (p < 0.05) less than the NCR group (SHR vs. NCR, 3.4 ± 0.7 vs. 4.4 ± 1.0 mL/min/kg). The renal interstitial infusion of Ang (1–7) had no statistically significant effect on GFR in either the SHR or NCR groups (5.2 ± 1.4 mL/min/kg; Interaction: Model × Drug p = 0.05) (Figure 1B).

2.3 Urine flow

The renal excretory responses to intrarenal interstitial Ang (1–7) were determined by measuring urine flow (UF) before and following infusion of the peptide. UF in the SHR group at baseline was significantly (p < 0.001) lower than the NCRs (SHR vs. NCR, 58 ± 30 vs. 102 ± 16 µL/min/kg). Renal interstitial infusion of Ang (1–7) significantly increased UF in both groups (SHR, to 69 ± 24; NCR, to 134 ± 17 µL/min/kg, both p < 0.05) but to a significantly greater degree in the NCR group (Interaction: Model × Drug p < 0.05) (Figure 1C).

2.4 Absolute sodium excretion

The potential natriuretic action of Ang (1–7) was determined by measuring sodium excretion while saline was infused and then following the infusion of saline plus Ang (1–7) into the renal interstitium. Absolute sodium excretion (UNaV) was significantly (p < 0.001) reduced in the SHRs at baseline than in the NCR group (SHR vs. NCR, 8.6 ± 4.8 vs. 16.2 ± 3.0 µmol/min/kg). Renal interstitial infusion of Ang (1–7) significantly increased UNaV in both groups (SHR, to 12.2 ± 4.0; NCR, to 23.7 ± 2.9 µmol/min/kg, both p < 0.05) but to a significantly greater degree in the NCR group (Interaction: Model × Drug p < 0.05) (Figure 1D).
Fractional sodium excretion

The changes in fractional sodium excretion (FENa+) caused by Ang (1–7) was determined by measuring the variable before and following the intrarenal infusion of the peptide. FENa+ at baseline was approximately 30% lower in the SHR group, but this was not statistically significant compared with the NCR group (SHR vs. NCR, 1.6 ± 0.8% vs. 2.3 ± 1.2%). The renal interstitial infusion of Ang (1–7) significantly elevated FENa+ in both groups (SHR, 2.5 ± 0.8%; NCR, 2.8 ± 1.2%, both p < 0.05) (Figure 1E).

Renal Ang II and Ang (1–7) concentrations

The renal concentration of Ang II and Ang (1–7) were measured in separate groups of rats which were anaesthetised, the kidneys removed and dissected into cortex and medulla to allow extraction of the peptides. Tissue concentrations of Ang II and Ang (1–7) in the renal cortex and medulla of the NCR and SHR groups are presented in Figure 2A-D. Renal cortical and medullary Ang II levels were comparable in the SHR (Cortex, 4.8 ± 2.2; Medulla, 3.9 ± 0.6) vs. NCR groups (Cortex, 4.8 ± 1.4; Medulla, 3.2 ± 0.9 pg Ang II/µg of protein). Similarly, the concentration of Ang (1–7) in the cortex and medulla of the SHR group (Cortex, 30.6 ± 17.6; Medulla, 14.8 ± 10.7 pg Ang (1–7)/µg of protein) was not statistically different from those of the NCR group (Cortex, 32.7 ± 22.2; Medulla, 14.1 ± 7.3 pg Ang (1–7)/µg of protein).

Renal AT1 receptor expression

Kidneys were removed from anaesthetised rats, separated into cortex and medulla, subjected to homogenisation followed by Western blot analysis to determine the level of protein expression. AT1
receptor expression, measured as arbitrary units (AU), was 2-fold greater in the renal cortices of the SHR group than the NCR group (0.96 ± 0.21 vs. 0.49 ± 0.16 AU, p < 0.05). AT1 receptor expression in the renal medulla was comparable in both SHR and NCR groups (0.81 ± 0.23 vs. 0.83 ± 0.13 AU, respectively) (Figure 3A-B).

2.8 | Renal Mas receptor expression

Renal cortical and medullary tissue was derived from separate groups of rats as indicated above (renal Ang II receptor expression) and Western blots run to measure Mas receptor expression. The Mas receptor appeared on the blot as a distinct band with a molecular weight of roughly 45 kDa. Mas receptor protein expression levels were comparable in the renal cortex and medulla of both the SHR and NCR groups (Cortex, 1.23 ± 0.26 vs. 1.36 ± 0.36 AU respectively; Medulla, 0.72 ± 0.23 vs. 0.62 ± 0.33 AU, respectively) (Figure 4A-B).

3 | DISCUSSION

The present study aimed to investigate the impact of acute renal interstitial Ang (1–7) infusion on renal haemodynamic and excretory function in an animal model of hypertension in which the intrarenal RAS was neither activated nor suppressed. The main novel finding was that the ability of Ang (1–7) to increase GFR and sodium and water excretions were significantly impaired in the SHRs. Importantly, these effects were observed in the absence of any change in MAP, reflecting a direct renal action of this peptide under normal conditions and in the context of hypertension. Moreover, the impaired natriuretic and diuretic actions of Ang (1–7) in the SHRs were associated with high levels of AT1 receptor expression in the renal cortex.

Previous studies from this laboratory have highlighted the extent to which alterations of the endogenous RAS can affect the actions of Ang (1–7) on renal function.8 The SHR model of hypertension was chosen as one which exhibits similar levels of endogenous RAS activity to that of NCR rats compared to other hypertensive models characterised by either an elevated or suppressed RAS.13,14 In this study, renal cortical and medullary Ang II and Ang (1–7) contents were similar in the SHR and NCR groups. These findings were comparable to those of Kohara et al.,15 who found that plasma levels of Ang II as well as renin and ACE, but not Ang (1–7), were similar in SHR and their normotensive controls. On the other hand, there is evidence that ACE2 gene expression and activity is reduced in the SHR compared to WKY control rats16 although renal Ang (1–7) concentrations were not measured in those studies. The lack of difference in Ang (1–7) concentration in renal cortex and medulla between the SHR and NCR groups, as reported in the present study, despite a potential suppression of ACE2 activity could indicate a compensatory increase in an ACE2 independent generation of Ang (1–7). Neutral endopeptidase, an alternative enzyme involved in the formation of Ang (1–7), has been reported to have higher mRNA and protein expression in both the renal cortex and medulla in the SHR compared to the WKY at 10 weeks of age.17

A comparison of AT1 protein expression in the medulla between the SHR and the NCR groups showed no significant difference. By contrast, the AT1 receptor protein expression in the cortex was significantly higher in the SHR compared with the NCR group. This was in agreement with a report that renal AT1 receptor density was elevated in the SHR compared with normotensive control rats.18 Data have been reported that the elevated renal cortical AT1 receptor expression level in the young SHR was enhanced by
FIGURE 3 Expression of the AT1 receptor in the renal cortex and medulla: A comparison of angiotensin II receptor 1 (AT1) expression in the renal cortex (A) and medulla (B) of normal control rats (NCR, n = 4) and spontaneously hypertensive rats (SHR, n = 5). * denotes p < 0.05 compares AT1 receptor expression levels in the renal cortex of NCR and SHR. Ponceau S stained membrane = loading control. BM = Biomarker lane. Inset, compares the relative AT1 receptor density (normalised to Ponceau S loading control)
a concurrent reduction in AT2 receptor expression levels. Greater concentrations of AT1 receptor in the renal cortex could contribute to the perturbed water and sodium excretion observed in the SHR. Activation of the AT1 receptor in vitro stimulated the sodium/hydrogen exchanger 3 (NHE3) and epithelial sodium channel (ENaC) which are the primary sodium transporters in the proximal tubule
and cortical collecting duct, respectively.\textsuperscript{20–23} The observed upregulation of AT1 receptor concentration in the renal cortex of the SHR could potentially contribute to the increased tubular sodium reabsorption through heightened NHE3 and ENaC activity. Furthermore, activation of the AT1 receptor has also been implicated in the down-regulation of ACE\textsuperscript{2}\textsuperscript{2}\textsuperscript{3}, which is involved with the generation of Ang (1–7). This in turn would prolong the half-life of Ang II, thereby potentiating its actions.

There was no significant difference in Mas receptor protein expression between the SHR and NCR groups in either the cortex or the medulla. This observation builds on earlier reports in which total renal Mas mRNA and protein expression was similar in SHR and WKY.\textsuperscript{25} This lack of change in Mas receptor protein expression in the cortex, despite an upregulation of the AT1 receptor, could indicate that these receptors are differentially regulated. Separate regulatory mechanisms of these two receptors would support the hypothesis that the balance between the Ang II/ACE/AT1 receptor axis and the Ang (1–7)/ACE2/Mas receptor axis determines the overall function of the RAS. Furthermore, the increase in renal cortical AT1 receptor expression in the SHR group, in the absence of changes either in renal cortical Mas receptor protein expression or renal Ang II/Ang (1–7) levels, associates with impaired sodium and water excretion in the present study. These observations suggest that the balance of receptor expression and possible activity is critical in the regulation of renal function as opposed to upstream effectors.

Acute intrarenal Ang (1–7) infusion had no significant effect on GFR in the NCR group. These observations were consistent with those of others using similar experimental conditions.\textsuperscript{5,9,26} On the other hand, there are reports that Ang (1–7) increased the diameter of isolated rabbit afferent arterioles following incubation with the peptide in vitro and increased renal blood flow and GFR when given intravenously in anaesthetised rats.\textsuperscript{27} It is possible that potential vasodilator actions were not observed in the current studies because of the somewhat higher dose of Ang (1–7) infused directly into the renal interstitium which may have resulted in non-specific binding to AT1 receptors, thus increasing efferent arteriolar tone. This view would be supported by others who observed decreases in renal plasma flow in a comparable experimental context.\textsuperscript{26,28} Ang (1–7) had distinct natriuretic and diuretic actions when infused into the renal cortical Mas receptor protein expression or renal Ang II/Ang (1–7) levels, associates with impaired sodium and water excretion in the present study. These observations suggest that the balance of receptor expression and possible activity is critical in the regulation of renal function as opposed to upstream effectors.

In conclusion, renal interstitial Ang (1–7) administration induced a diuretic and natriuretic response which was attenuated in the SHR. Moreover, the blunted natriuretic and diuretic actions of Ang (1–7) in the SHR group was associated with higher levels of renal cortical AT1 receptor expression. The present study examined the role of Ang (1–7) in the context of an unstimulated RAS and further studies should be directed towards examining the renal haemodynamic and excretory responses to Ang (1–7) in hypertensive models with different degrees of activation or suppression of the endogenous RAS. However, the present findings do point to Ang (1–7) administration as a potential therapeutic strategy for the treatment of hypertension and renal disease.

4 | MATERIAL AND METHODS

4.1 | Animals

Male Han Wistar rats (normal control rats, NCRs) and SHRs at 11 weeks of age were obtained from Harlan (Bicester) and maintained under a 12 h light-dark regimen at 20 ± 3°C in the Biological Services Unit, University College Cork. The animals had free access to standard rodent chow (Harlan-Teklad; Bicester) and tap water. Animals in each group were weight-matched for the purposes of comparative studies with animals from both groups generally weighing between 250 and 300 g. All experimental procedures were performed under the European Community Directive 86/609/EC (Licence code B100/4481) and were approved by the local Animal Experimentation Ethical Committee at University College Cork.

4.2 | Surgical protocol

Anaesthesia was induced in overnight fasted NCRs and SHRs using intraperitoneal sodium pentobarbital (60 mg/kg) and maintained using bolus intravenous doses (0.05 mL of 60 mg/kg every 20 min)
of the same anaesthetic. The trachea was cannulated (PP240, OD 2.42 mm; Smiths) using a small incision at the neck region to ensure a patent airway throughout the experiment. A cannula (PP50, OD 0.96 mm) was inserted into the right femoral vein to allow infusion of saline (3 mL/h of NaCl 9 g/L) and subsequently with fluorescein isothiocyanate (FITC) inulin in saline (3 mL/h of FITC Inulin, 2 g/L; Sigma Aldrich). The right femoral artery was cannulated (PP50) to permit the measurement of mean arterial pressure (MAP), heart rate (HR) and the collection of blood samples. The arterial cannula was attached to a blood pressure transducer (MLT844; ADInstuments Ltd), linked to a PowerLab system (ADInstuments Ltd), with the output signal processed using LabChart software to obtain an on-screen blood pressure trace which was saved for later analysis.

The kidney was exposed via a flank incision and prepared as previously described.8,9 A small cannula (PP10) was inserted approximately 4.5 mm into the rostral pole of the kidney to allow saline or Ang (1–7) to be infused at 1 mL/h and it was fixed in place using superglue. This method of intrarenal administration has been utilised before in this laboratory31,32 and the distribution of infusate validated using Lissamine Green dye infusion.9,35 A stabilisation period of 1.5 h was allowed following completion of the surgical procedures before commencement of the experimental protocols. At the end of each experiment the animals were killed using an overdose of sodium pentobarbital.

### 4.3 | Experimental protocol

The experimental protocol involved a series of four clearance periods each comprising a 20 min urine collection from the left ureter. Two clearances (C1 and C2) were taken prior to and two (C3 and C4) during the renal interstitial infusion of either saline (time control, Figure 5A) or Ang (1–7) (50 ng/min; Sigma Aldrich)5,26 (Figure 5B). A period of 50 min was allowed before initiating C3 and C4. Plasma was collected from arterial blood samples taken prior to C1, following C2 and following C4.

### 4.4 | Analytical techniques

Urine flow was determined gravimetrically while plasma and urine concentrations of sodium were estimated using flame photometry (Model 410C, Ciba; Corning). FITC inulin concentration in urine and plasma samples was measured by means of a fluorometric plate reader (Victor 2; Wallac) and used to calculate GFR. FENa+ was calculated using the formula:

\[
\text{FENa}^+ = \frac{\text{Sodium Clearance}}{\text{GFR}} \times 100
\]

Blood samples (0.4 mL) were obtained from the femoral artery and were immediately centrifuged at 16,000 g for 1 min. A volume of 75 μL of plasma was separated and stored at −20°C for further analysis and an equal volume of heparinised saline (0.2 mL heparin 5000 I.U./mL in 50 mL saline) was gently mixed with the remaining red cells and reinfused into the animal.

### 4.5 | Determination of AT1 and Mas receptor expression in the renal cortex and medulla

#### 4.5.1 | Tissue preparation

Rats from a separate cohort of NCR (n = 6) and SHR (n = 6) groups were anaesthetised with intraperitoneal sodium pentobarbital
(60 mg/kg). The left kidney was then exposed via a retroperitoneal incision, its renal artery and vein cleared of connective tissue, a suture tied around the renal blood vessels and the kidney excised. The kidney was placed on ice, the cortex and medulla dissected, the separated tissues were placed in labelled tubes and snap frozen in liquid nitrogen prior to storage at −80°C. This procedure was repeated for the right kidney and the animal was killed using an overdose of sodium pentobarbital. A protease inhibitor cocktail, 10 μL in 1 mL of homogenisation buffer (10 mmol/L Tris, 25 mM Sucrose; Sigma Aldrich), was added to the renal cortical and medullary tissues (0.1 g tissue; 1000 μL homogenisation buffer) which were then homogenised (Omni international; GLH). Total protein content of the tissues was determined using a bicinchoninic acid (BCA) assay (Bio-Rad).

4.5.2 | Western blotting protocol

Renal cortical and medullary protein extracts from the left kidneys of NCR (n = 4) and SHR (n = 5) were loaded into wells (30 μg protein/well) on a 5% stacking gel and separated on a 10% resolving gel according to their molecular weight using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad). After the proteins were separated by electrophoresis, they were transferred to a polyvinylidene difluoride (PVDF) membrane (pore size 0.45 μm; Merck Millipore) utilising the Semi-dry transfer method (Trans Blot SD; Bio-Rad). To evaluate the success of the transfer, the membrane was soaked in reversible Ponceau S stain (0.1% Ponceau S in 5% acetic acid; Sigma Aldrich Ireland Ltd) for 5 min. The membrane was washed in 5% milk made up in 1 x tris buffered saline Tween (TBST; Sigma Aldrich) for an hour to block the surface of the membrane and prevent non-specific binding of the antibody. This reduced the background interference when detecting the signal from the protein of interest. After the blocking step, the membrane was washed for 7 min with TBST. The membrane was incubated overnight with a primary antibody (anti-AT1R antibody; Alpha Diagnostics) at 4°C after which the membrane was washed, 4 x 7 min washes in 1 x TBST with gentle agitation, to remove any unbound primary antibody.

4.6 | Immunodetection, visualisation and quantification

The washed membrane was incubated with an anti-rabbit secondary antibody (1:2000 dilution in 5% milk in 1 x TBST; Sigma Aldrich) with gentle agitation at room temperature for 1 h. The amount of total protein and protein of interest in each sample was quantified using Image J software (Public domain, developed by National Institutes of Health, Bethesda, MD, USA). The expression of the protein of interest was normalised to total protein (Ponceau S Loading control) which was measured by densitometric analysis. Studies using Western blot analysis measured the relative expression of the AT1 receptor in the renal cortex and medulla of NCR and SHR groups. The AT1 receptor appeared on the blot as a distinct band at a molecular weight of roughly 52 kDa. When comparing expression levels of the AT1 receptor, the relative density of the AT1 band was normalised against the Ponceau S loading control and expressed as arbitrary units (AU).

4.7 | Determination of Ang II and Ang (1–7) concentrations in renal cortex and medulla

Homogenised renal cortical and medullary tissue samples were thawed on ice and re-centrifuged at 5000 g at 4°C for 5 min after which the resulting supernatants were stored in aliquots at −80°C. Ang (1–7) and Ang II ELISA kits (Cusabio Technology LLC) were utilised to measure Ang (1–7) and Ang II levels in the renal cortical and medullary tissues. Each ELISA used eight standards covering a range of concentrations of the protein of interest. Ang II standards ranged from 0 pg/mL to 300 pg/mL and Ang 1–7 standards ranged from 0 pg/mL to 1200 pg/mL. The 96 well assay plates, coated with either primary anti-Ang (1–7) or anti-Ang II antibody, were incubated at 37°C for 2 h with standards and samples (100 μL/well), in duplicate, to facilitate binding to the antibody. The bound protein of interest was then incubated at 37°C for 1 h with a second specific biotin conjugated detection antibody to create a sandwich (100 μL/well). After this incubation period, the wells were washed three times using 200 μL wash buffer/well. 100 μL of 1x Avidin conjugated Horseradish Peroxidase (HRP) was added to each well and incubated at 37°C for 1 h. Upon completion of incubation, the wells were washed again using 200 μL of buffer/well x3 times. The HRP converted the TMB substrate, which was then added, to a detectable form. Once a colour had developed, the reaction was stopped, the optical density of each well was measured at 450 nm and 570 nm with the SpectraMax M3 microplate reader and the optical density measurements processed using Softmax Pro 6 software (Molecular Devices).

4.8 | Statistical analysis

Renal functional data were analysed using a two-way repeated measures ANOVA with a Bonferroni post hoc test (Prism; GraphPad). Unpaired t-tests were used where appropriate to compare protein expression levels and intrarenal Ang II and Ang (1–7) levels between the NCR and SHR groups. All data were presented as mean ± SD and statistical significance was taken as p < 0.05.

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CONFLICT OF INTEREST
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTIONS
EB and EJ conceived and designed the experiments for this study. EB performed the experiments and collected the data. JON and MHA interpreted the data and drafted the manuscript. EB, JON, MHA and EJ revised the manuscript. EB, JON, MHA and EJ approved the final version of manuscript. All individuals who made contributions to this study are included.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author.

ORCID
Mohammed H. Abdulla https://orcid.org/0000-0001-5496-5017

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