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Intrarenal Mas and AT1 receptors play a role in mediating the excretory actions of renal interstitial Angiotensin (1-7) infusion in anaesthetized rats.

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Introduction:

The natriuretic and haemodynamic actions of Ang (1-7) have been well studied by numerous investigators (Burgelova et al. 2005, Burgelova et al 2002, Dellipizzi et al. 1994, Hilchey and Bell-Quilley. 1995, Pinheiro et al. 2009). However, the factors that govern the interrelationship between renal Ang (1-7) and Ang II and the precise roles played by the various angiotensin receptors in determining the regulation of renal excretory and haemodynamic function have not been elucidated. In 2003, the G-protein coupled receptor Mas was identified as an endogenous receptor for Ang (1-7) (Santos et al 2003). More recently, a significantly lower UF and FENa was observed in euvolemic Mas deficient mice thereby indicating the important role played by this receptor in the regulation of basal sodium and water excretion (Pinheiro et al. 2009). Furthermore, Mas receptor mRNA has been detected in rat proximal tubular cells (Su et al. 2006) which further implicates its specific role in the transport of sodium and water at that site.

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We have previously demonstrated that the natriuretic and diuretic responses to acute renal interstitial Ang (1-7) infusion were enhanced when the RAS was stimulated by a low sodium diet and diminished when the RAS was suppressed by a high sodium diet (O Neill et al 2013). Though underlying mechanisms have yet to be fully elucidated, changes in the level of intra-renal Mas receptor expression may provide an explanation. Indeed, the chronic administration of a high sodium diet has been shown to decrease Mas receptor expression in the renal cortex of Obese Zucker rats (OZR) (Samuels et al 2012) and Spontaneously Hypertensive rats (SHR) (Vargaric et al 2010). On the other hand Samuels et al 2012 observed a tendency for Mas receptor protein expression to be upregulated in the renal cortex of lean zucker controls. However, to our knowledge the impact of dietary sodium manipulation on Mas receptor expression in normal Wistar rats has yet to be established. In the context of our previous observations it may well be that a low sodium diet increases renal Mas receptor expression, thereby increasing the responsiveness of the kidney to the exogenous infusion of Ang (1-7). The opposite might well be observed when rats are placed on a high sodium diet.

Alternatively, the situation may be more complex and the level of activation of receptors other than Mas may be indirectly involved in mediating the renal excretory actions of Ang (1-7). Indeed, past studies have demonstrated that the suppression of the RAS is characterised by a reduction in circulating (Douglas and Catt. 1976, Imig et al. 1999) and intra-renal (Fox et al. 1992, Ingert et al. 2002) levels of Ang II. In the context of our previous study (O'Neill et al. 2013), these observations suggest that the counter-regulatory capacity of Ang (1-7) might be lost when endogenous Ang II levels are diminished. Moreover, *invitro* studies in HEK cells provide evidence that Ang (1-7) can counter-regulate the actions of Ang II through a direct interaction with AT1 receptors (Galandrin et al 2016). Thus, it may well be that exogenous Ang (1-7) only has a natriuretic and diuretic action via the Mas receptor when Ang II/AT1 receptor induced reabsorption of sodium and water are at an

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appreciable/heightened level. Therefore, the question arises as to whether the magnitude of renal excretory and haemodynamic actions of Ang (1-7) could be modulated by the degree of Ang II induced AT1 receptor stimulation.

Thus the aim of the present study was twofold. Firstly, to examine how the intra-renal actions of Ang (1-7) might be modified following pharmacological inhibition of intra-renal AT1 and Mas receptors in the milieu of an unstimulated and stimulated RAS. Secondly, to determine whether the renal excretory and haemodynamic actions of Ang (1-7) during different levels of sodium intake were directly dependent on the level of activation of intra-renal Mas and AT1 receptors.

Materials and Methods:

Animals

Male Wistar rats (250g-350g) were obtained from Harlan (Bicester, UK) and maintained under a 12h light-dark regime at 20±3^{0}C in the Biological Services Unit, University College Cork. The animals were maintained on a normal sodium diet (Harlan-Teklad, Bicester, Oxon, UK: 0.3% Na^+); a high sodium diet (Lillico, Surrey, UK: 3% Na^+); or a low sodium diet (Lillico, Surrey, UK: 0.03% Na^+) for 2 weeks prior to experimentation. The diets only differed in terms of their NaCl content while the concentrations of all other food constituents such as vitamins, minerals, proteins, fats and carbohydrates were similar across all three diets. All experimental procedures were performed under the European Community Directive 86/609/EC and were approved by the local Animal Experimentation Ethical Committee at University College Cork.

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Surgical Protocol

Rats were fasted over-night but did have access to water. Anaesthesia was induced with 1 ml of chloralose-urethane (16.5 and 250 mg/ml, respectively) intraperitoneally and maintained using bolus intravenous (iv) doses of 0.05 ml every 30 min. A tracheostomy was carried out to ensure a patent airway. Cannulae were inserted into the right femoral vein, to facilitate the infusion of sustaining saline (3ml/h of NaCl 9g/L) and subsequently saline containing FITC inulin (3ml/h of FITC Inulin, 2g/L, Sigma-Aldrich, USA), and the right femoral artery to permit the measurement of mean arterial pressure (MAP), heart rate (HR) and the collection of blood samples. The kidney was exposed by a flank incision and prepared as previously described by Ahmeda and Johns 2012 and O Neill et al 2013). A small cannula was inserted approximately 4.5 mm into the rostral pole of the kidney to allow saline or Ang (1-7) to be infused at 1ml/h. Drugs infused into the kidney in the present study diffused in to both the cortex and the medulla (n=3). Lissamine Green validated this method of infusion. Staining was evident in both the cortex and medulla but the degree of staining appeared to be greater in the medulla. These observations are in agreement with previous reports (Ahmeda and Johns 2012). A 1.5 h post-surgical stabilisation period was given prior to the commencement of all experiments. At the end of each experiment animals were killed by an anaesthetic overdose.

Experimental Protocol 1

This experimental protocol was carried out in rats receiving either a normal or low sodium diet (Fig. 1(A)). A sequence of four 20 min clearance periods were taken, two clearances (C1 and C2) prior to and two (C3 and C4) during the renal interstitial infusion of Ang (1-7) (50 ng/min) (Sigma Aldrich,

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USA). A period of 45 min was given before initiating C3 and C4. Arterial blood samples were taken to acquire plasma samples prior to and following each pair of clearances. The dose of Ang (1-7) used in the present study was chosen on the basis of those utilized by others (Burgelova et al 2002).

Experimental Protocol 2

This experimental protocol was carried out in rats receiving either a normal or low sodium diet (Fig. 1(B)). A sequence of 6 X 20 min clearance periods were taken, two clearances (C1 and C2) during the renal interstitial infusion of saline vehicle, two clearance during the renal interstitial infusion of either the AT1 receptor antagonist, Losartan (17 µg/min) (Sigma Aldrich, USA) or the Mas receptor antagonist, A-779 (5-µg/min) (Sigma Aldrich, USA) (C3 and C4) and two clearances during the renal interstitial co infusion of Losartan or A-779 together with Ang (1-7) (C5 and C6). Losartan/ A-779 were infused for a period of 30 min prior to the commencement of C3 and C4. Losartan/A-779 +Ang (1-7) were co-infused for a period of 45 mins prior to the commencement of C5 and C6. Arterial blood samples were taken immediately prior to C1 and following C2 and immediately after C4 and C6. The doses of A-779 and Losartan used in the present study were chosen on the basis of those utilised by others (Burgelova et al. 2002, Li et al. 2006) (Fig 1(B)).

Control Protocol

The time control protocol was carried out in rats receiving either a normal (n=5) or low (n=4) sodium diet. 6X 20 min clearance periods were taken during the renal interstitial infusion of saline vehicle (Fig 1(C)).

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Summary of animal groups (renal functional studies)

Group 1: Normal Sodium Diet: Acute intra renal Ang (1-7) infusion (n=6)

Group 2: Low Sodium Diet: Acute intra renal Ang (1-7) infusion (n=8)

Group 3:Normal Sodium Diet: Acute intra renal Losartan infusion followed by co-infusion of Losartan with Ang (1-7) (n=6)

Group 4: Low Sodium Diet: Acute intra renal Losartan infusion followed by co-infusion of Losartan with Ang (1-7) (n=6)

Group 5: Normal Sodium Diet: Acute intra renal A-779 infusion followed by co-infusion of A-779 with Ang (1-7) (n=6)

Group 6: Low Sodium Diet: Acute intra renal A-779 infusion followed by co-infusion of A-779 with Ang (1-7) (n=6)

Group 7: Normal Sodium Diet: Acute intra renal saline infusion (time control) (n=5)

Group 8: Low Sodium Diet: Acute intra renal saline infusion (time control) (n=4)

Analytical Techniques

The sodium concentration of both plasma and urine samples was determined by flame photometry (Model 410C, Ciba, Corning, Halsted, Essex, UK.). GFR was calculated as the clearance of FITC inulin. FITC inulin concentration of the urine and plasma samples was determined using a fluorometric multi-label counter plate reader (Victor 2, Wallac, USA). Urine flow was determined gravimetrically. FENa was given by: ClNa (Sodium Clearance)/GFR X100). Blood samples (0.4 ml)

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were centrifuged at 16,000 g for 1 min, 75 µl of plasma was removed for analysis and heparinised (0.2 ml heparin 5,000 I.U./ml in 50 ml saline) saline of equal volume was added to the remaining red cells and immediately returned to the animal. A blood pressure transducer (Spectromed, Oxnard, CA, USA) and an amplifier (Grayden Electronics, Birmingham, UK) operated in conjunction with a computer (Power Macintosh 8600/250, Apple, USA) and appropriate software (Lab View 4, National Instruments, Austin, TX, USA) to monitor and record blood pressure and heart rate. Following the completion of the experiment the animal was killed using an anaesthetic overdose.

Renal Cortical and Medullary Mas receptor expression

Preparation of Renal cortex and medulla

After 2 weeks on a low, normal or high sodium diet both left and right kidneys were removed from anaesthetised rats, which were subsequently killed with an intravenous anaesthetic overdose. Kidneys were immediately put on ice, the renal cortex and medulla were dissected, flash frozen in liquid nitrogen and stored at -80 ^{0}C. Renal cortical and medullary tissues were homogenized using homogenization buffer (10 mM Tris, 25 mM Sucrose) (0.1 g tissue: 1000 µl buffer) and a tissue homogenizer (Omni international, GLH, Georgia, United States). The procedure was carried out on ice and a protease inhibitor cocktail (10 µl: 1 ml of Homogenization buffer) (Sigma Aldrich, USA) was added to the tissue prior to homogenisation. A bicinchoninic acid (BCA) assay (Biorad, California, USA) was then performed on the tissue to quantify total protein content.

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Western Blotting Protocol

Renal cortical protein extracts from rats on low (n=5), normal (n=4) and high (n=5) sodium diets and renal medullary protein extracts from rats on either a low (n=5) or high sodium diet (n=4) were loaded into wells (30 µg protein/well) on a 5 % stacking gel and were separated on a 10 % resolving gel according to their molecular weight using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Biorad, California, USA). Proteins were subsequently transferred from the gel on to polyvinylidene difluoride (PVDF) membrane (Merc, Millipore, Darmstadt, Germany) using the Semi-dry transfer method (Trans Blot SD, Biorad, California, USA). The efficiency of transfer was determined by applying a Ponceau S (Sigma Aldrich, USA) stain to the membrane following transfer. The membrane was blocked for approximately 1 hour in 5 % milk in 1X tris buffered saline (TBS) tween (Sigma Aldrich, USA) at room temperature. Finally, the membrane was incubated over-night with a primary antibody (AT11- S; Mas receptor antibody, 1:1500 dilution, Alomone Labs, Jerusalem, Israel: AAR-013) at 4 ^{0}C. The selectivity of the anti-Mas receptor antibody was tested by co-incubating the antibody with the control antigen (Fig. 12). The Mas receptor has a predicted molecular weight of approximately 45kDa.

Immunodetection, Visualization and Quantification

The membrane was washed thoroughly (4X 5 min washes in 1X TBS tween on an orbital shaker) prior to and following a 1-hour incubation period with an anti-rabbit secondary antibody (1:2000 dilution in 5% milk in 1X TBS tween, Sigma Aldrich, USA). The protein of interest was visualized using chemiluminescence and band intensities quantified using densitometry software (Image J: National Institute of Health, Bethesda Maryland, USA). To correct for any variations in protein loading and transfer, the band intensities of the proteins of interest were then normalized to the

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intensities of the Ponceau S staining proteins in the corresponding lanes on the transfer membrane which were also measured by densitometric analysis (Romero-Calvo et al 2012).

Renal Cortical and Medullary Ang (1-7) concentration

Preparation of Renal cortex and medulla:

Renal cortical and medullary samples from low high and normal sodium fed rats were weighed out, added to a protease inhibitor cocktail (2µl of stock) and homogenized as described above. However, the buffer used to homogenize these tissues was ice cold phosphate buffered saline (PBS) (0.02mol/litre, pH 7.4) and the tissue to buffer ratio was 1µg: 1µl, ie 200µg of tissue in 200µl of PBS. Following homogenization, the samples were sonicated on ice for 10 seconds to further disrupt cell membranes. Thereafter, the homogenates were centrifuged for 15 min at 1500g and the resulting supernatants were stored in aliquots at -80^{0}C.

Ang (1-7) ELISA:

An Ang (1-7) ELISA kit purchased from My Biosource, San Diego, USA was employed to measure Ang (1-7) levels in the renal cortex and medulla of rats receiving either a normal (n=5), high (n=5) or low sodium (n=5) diet.

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Statistical Analysis

Functional data was analysed using a 2X2 repeated measures ANOVA with a Fisher's LSD post-test (Prism, Graphpad. USA). Unpaired t-tests were employed where appropriate to compare protein expression levels between low, normal and high sodium groups (Prism, Graphpad, USA). One way ANOVA with Fishers LSD post –test was employed to analyse intra-renal Ang (1-7) levels across dietary groups. A one way repeated measures ANOVA was employed to analyse saline time control data. Statistical significance was taken when $P<0.05$. All data are presented in this study as mean±SD.

Results:

Intra-renal infusion of Antagonists

Immediately prior to their co-infusion with Ang (1-7), either Losartan or A-779 was solely infused and the individual impact of each antagonist upon haemodynamic and excretory function was measured and recorded in both normal and low sodium groups. This was carried out so as to establish the extent to which antagonists altered each parameter prior to their co-infusion with Ang (1-7) (Table 1).

Intra-renal Losartan infusion (Table 1)

The intra-renal infusion of Losartan reduced MAP by 8±5.5%in rats receiving a normal sodium diet and by 8±11% in rats receiving a low sodium diet (both $P<0.05$). GFR was not altered by the intra-renal infusion of Losartan in either group. On the other hand intra-renal AT1 receptor blockade increased UF by 1.7-fold in normal rats ($P<0.05$) and by 1.6-fold in low sodium fed rats. Intra-renal Losartan infusion had a natriuretic effect in both dietary groups; Intra-renal AT1 receptor blockade

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increased UNaV by 1.8-fold in rats receiving a normal sodium ($P<0.05$) diet and by 2-fold in rats receiving a low sodium diet. Intra-renal infusion of Losartan increased FENa by 1.7-fold in rats receiving a normal sodium diet ($P<0.05$) and by 2-fold in rats receiving a low sodium diet.

Intra-renal A-779 infusion (Table 1)

The intra-renal infusion of A-779 had no significant effect on MAP, UF, UNaV or FENa in either dietary group. However, intra-renal Mas receptor blockade did increase GFR by 27±35% in rats receiving a normal sodium diet ($p<0.05$) but was without significant effect on this parameter in rats receiving a low sodium diet.

Intra-renal Ang (1-7) infusion

The intra-renal infusion of Ang (1-7) caused small reductions in MAP of 4±5% in rats receiving a normal sodium diet ($P<0.05$) but was without significant effect on this parameter in rats receiving a low sodium diet (Fig. 2A+B). Intra-renal Ang (1-7) infusion had no effect on GFR in the normal sodium group but significantly increased this parameter in the low sodium group ($P<0.05$) (Fig. 3A+B) by approximately 37±21% Ang (1-7) infusion significantly increased UF by 2.2-fold in rats receiving a normal sodium diet and by 2.8-fold in rats receiving a low sodium diet (both $P<0.05$)(Fig. 4A+B). Ang (1-7) induced a robust natriuresis in both groups. Intra-renal Ang (1-7) infusion increased UNaV by 2.9-fold in the normal sodium rats ($P<0.05$) and by some 6.1-fold in the low sodium group ($P<0.05$)(Fig. 5A+B) while concomitantly there was a 2.7-fold increase in FENa in rats

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receiving a normal sodium diet and a 4-fold increase in this parameter in rats receiving a low sodium diet (both $P<0.05$)(Fig. 6A+B).

Intra-renal co-infusion of Ang (1-7) with Antagonists

Immediately after the infusion of either Losartan or A-779 alone, Ang (1-7) was co-infused with either antagonist and the impact on hemodynamic and excretory function was measured and recorded in both dietary groups. The hemodynamic and excretory responses to Ang (1-7) were compared in the absence and presence of each antagonist.

Intra-renal co-infusion of Ang (1-7) with Losartan/A-779

MAP (Fig. 2A+B) and GFR (Fig. 3A+B) during the intra-renal co-infusion of Losartan/A779 and Ang (1-7) were similar to that observed during the singular infusion of either Losartan/A-779 in either dietary group. Importantly, Ang (1-7) had no effect on GFR during either intra-renal AT1 or Mas receptor blockade in rats receiving a low sodium diet (interaction: $p=0.0892$)(Fig. 3B).

Ang (1-7) induced increases in UF were completely blocked in the presence of Losartan and A-779 in rats receiving a normal sodium diet (interaction: $P<0.01$) (Fig, 4A). In rats receiving a low sodium diet: Ang (1-7) only increased UF by 1.5 -fold in the presence of Losartan while Ang (1-7) induced increases in UF were blocked in the presence of A-779 (Fig. 4B).

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Ang (1-7) induced increases in UNaV were absent during intra-renal AT1 or Mas receptor inhibition (interaction: $P<0.001$) in rats receiving a normal sodium diet (Fig 5A). In the low sodium group, Ang (1-7) increased UNaV by only 2-fold during intrarenal AT1 receptor blockade in rats ($P<0.05$) but had no significant effect on UNaV during intrarenal Mas receptor blockade (Fig 5B).

Importantly, the magnitude of the Ang (1-7) induced increases in FENa was significantly abolished during intra-renal AT1 or Mas receptor inhibition in rats receiving normal or low sodium (Both: Interaction: $P<0.001$) (Fig. 6A+B).

Saline time control experiments

The continuous infusion of saline over the course of the protocol had no significant effect on any of the parameters measured in the present study (Table 2).

Ang (1-7) concentrations in the renal cortex and medulla of rats receiving either a low, normal or high sodium diet

Ang (1-7) concentrations were 2.6-fold higher in the renal cortex of animals on a low sodium diet relative to those on a normal sodium diet (Fig. 7). Conversely, the chronic administration of a high sodium diet for 2 weeks did not impact upon Ang (1-7) levels in the renal cortex (Fig. 7). Ang (1-7) levels in the renal medulla were not different across dietary groups (data not shown).

Mas receptor expression in the renal cortex of rats receiving either a low, normal or high sodium diet:

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Mas receptor expression in the renal cortex was not altered by the administration of a low sodium diet (Fig 8A). Mas receptor expression tended to be upregulated in the renal cortex of rats receiving a high sodium diet but this did not reach statistical significance (P=0.1338) (Fig 8B).

Mas receptor expression in the renal medulla of rats receiving either a low or high sodium diet

Mas (Fig. 9) receptor expression in the renal medulla of rats receiving a low or high sodium diet did not differ significantly.

Control Antigen: Mas receptor antibody

The band corresponding with the Mas receptor (approx 45kDa) did not appear when the anti-Mas receptor antibody was co- incubated with the control antigen (Figure 10) in the renal cortex of rats receiving either a low or high sodium diet, indicating the specificity of this antibody for the Mas receptor.

Discussion

This study aimed to determine if the differences in the excretory responses to Ang (1-7) previously observed by O Neill et al 2013 during dietary sodium manipulation could be related to changes in the regulation of renal Angiotensin receptors and/ the renal production of endogenous Ang (1-7). The first novel finding was that the enhanced urinary excretion of sodium observed in animals on a low sodium diet during intra-renal Ang (1-7) infusion was associated with heightened intra-renal Ang (1-7) levels

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at the outset of experimentation. This association strongly suggests that the natriuretic response to exogenous Ang (1-7) in the milieu of a stimulated RAS was heightened because the endogenous Ang (1-7) concentration in the kidney was higher under these conditions at baseline. Secondly, Ang (1-7) induced natriuresis and diuresis was inhibited during intrarenal AT1 receptor blockade and during intrarenal Mas receptor blockade. These findings demonstrate that both AT1 and Mas receptors mediate the renal excretory actions of Ang (1-7). The precise mechanism by which both receptors mediate these actions remains unclear and requires further investigation.

In agreement with past reports (Vallon et al 1998, Burgelova et al 2002), intra-renal A-779 administration inhibited Ang (1-7) induced natriuresis indicating that these actions are mediated by intrarenal Mas receptors. We additionally observed that the degree to which A-779 blocked the natriuretic and diuretic actions of Ang (1-7) was similar in both dietary groups. Moreover, renal Mas receptor protein expression was not affected by the low sodium diet. Together, these data indicate that manipulating dietary sodium in this way does not alter the sensitivity or expression of renal Mas receptors. In the context of our previous study (O Neill et al 2013), it is clear that the enhanced diuretic and natriuretic responses to Ang (1-7) observed in animals on a low sodium diet are not likely to be explained by alterations in the expression and/ activity of renal Mas receptors.

However, the stimulation of the RAS with a low sodium diet did impact upon intra-renal Ang (1-7) levels, which were markedly increased in the renal cortex. These observations to a degree support those of Hamming et al (2008) who demonstrated that feeding a low sodium diet reduced the ACE/ACE2 ratio in the rat kidney, which could theoretically potentiate intrarenal Ang (1-7) levels (ACE degrades Ang (1-7). Thus when ACE activity is reduced so too is the degradation of Ang (1-7))

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(Hamming et al 2008). Indeed, the present observations indicate that heightened Ang (1-7) levels in the renal cortex at the outset of experimentation were clearly associated with the large Ang (1-7) induced natriuresis observed in animals on a low sodium diet. Moreover, these associations are consistent with the fact that the majority of sodium transporters (NHE3, Na^+/K^+ATPase) (Lopez-Ordieres et al 1998, Castelo-Brancho et al 2013) inhibited by Ang (1-7) are located in the renal cortex.

We previously demonstrated that Ang (1-7) induced natriuresis and diuresis was diminished when the RAS was suppressed by a high sodium diet. Mechanisms underlying these observations are currently unclear. Thus, the approach taken in this study was to determine whether pharmacological inhibition of AT1 receptors might also diminish the natriuretic/diuretic actions of Ang (1-7). Intra-renal administration of the AT1 receptor antagonist Losartan caused a natriuresis and diuresis in both the normal and low sodium dietary groups (table 1). These data are consistent with the findings of others in dogs, rats and water loaded mice (Heller et al 1997, Wang et al 1997, Pinhero et al 2004). Importantly, when the intra-renal AT1 receptors were blocked, the natriuretic and diuretic actions of exogenously infused Ang (1-7) were diminished in rats receiving a low sodium diet and completely absent in the control animals, the latter being consistent with previous reports in anaesthetised dogs (Heller et al 2000). Thus, AT1 receptor blockade induced a diuresis and natriuresis, which was not further increased by the concomitant infusion of Ang (1-7). Furthermore, the blunted excretory actions of Ang (1-7) during intra-renal AT1 receptor blockade resemble our previous observations in rats fed a high sodium diet (O Neill et al 2013). These pharmacological findings suggest that intra-renal AT1 receptors importantly influence the excretory responses to Ang (1-7).

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The precise way in which AT1 receptors contribute to Ang (1-7) induced diuresis and natriuresis is unclear and beyond the scope of the present study. However, within the current literature there is evidence to suggest that Ang (1-7) and/or its receptor Mas can interact with the AT1 receptor and inhibit its activation either by acting directly at the AT1 receptor and acting as a natural biased agonist (Galandrin et al 2016) or via Mas/AT1 receptor dimerization (Kostensis et al 2005). In the context of the present study it may well be that AT1 receptors must be functional for mediating the natriuretic and diuretic effects of Ang (1-7).

Mas receptor protein expression tended to be upregulated in the renal cortex in rats on a high sodium diet in agreement with the findings of others in lean Zucker control animals (Samuels et al 2012). These data are not consistent with our previous functional data, which demonstrated that the natriuretic and diuretic actions of Ang (1-7) were diminished in rats receiving a high sodium diet (O Neill et al 2013). One must acknowledge here that the measurement of Mas receptor expression only provides information about changes in the relative abundance of Mas receptor protein in the tissue. However, it does not give insight into how many of these receptors were expressed on the cell membrane versus how many were internalized. Thus, one cannot rule out the possibility that the diminished response to renal interstitial Ang (1-7) infusion observed in our previous study in animals fed a high sodium diet (O Neill et al 2013) was due to a reduction in the sensitivity of tubular epithelial cells to Ang (1-7).

Ang (1-7) induced increases in GFR were absent in the presence of A-779 in the low sodium group indicating that Mas receptors play a role in mediating this vascular response in the milieu of a stimulated RAS. These observations are consistent with previous reports *in vivo* in rats (Burgelova et

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al 2005). The A-779 induced increases in GFR observed in the present study are in direct contrast to the findings of others in anesthetized rats (Vallon et al 1998, Burgelova et al 2002, Burgelova et al 2005) and are possibly confounded by the effect of tubuloglomerular feedback (TGF). Moreover, Tetzner et al 2016 recently demonstrated that the depressor effects of Ang (1-7) are also mediated by MrgD receptors. Thus, the observed increase in GFR in this study could also be due to Ang (1-7)/MrgD mediated vasodilation.

It is important to acknowledge that there are limitations to the experimental approach used in the present study. Possible over spill of Losartan in to the systemic circulation caused reductions in MAP which most likely masked the expected renal hemodynamic effects of Losartan (Wang et al 1997). Moreover, the dose of Losartan administered was not increased to offset increased intrarenal Ang II in the rats receiving a low sodium diet (Fox et al. 1992, Ingert et al. 2002, Sica et al 2005) Thus, Losartan induced increases in sodium and water excretion were of similar magnitude in both dietary groups. This also explains why Ang (1-7) induced diuresis and natriuresis were not diminished to a greater degree in the presence of Losartan in the low sodium group.

In conclusion, the main important finding of the present study was that Ang (1-7) induced natriuresis and diuresis was influenced by AT1 receptor functionality in the milieu of an unstimulated and stimulated RAS. Collectively these findings show that both intra-renal AT1 and Mas receptors play a role in mediating the natriuretic and diuretic actions of Ang (1-7). Future studies are needed to examine in more detail the relative contribution of each receptor subtype in mediating the intrarenal actions of Ang (1-7) in the kidney. This is particularly true given that MrgD receptors have now also been identified as Ang (1-7) receptors (Tetzner et al 2016). Gaining a deeper understanding of such

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interactions could help to promote the development of therapeutic agents for the treatment of hypertension and chronic kidney disease.

Competing Interests

There are no competing interests to declare

Author Contribution

JON collected, analysed and interpreted data. JON drafted paper. JON EJJ and VH revised paper.

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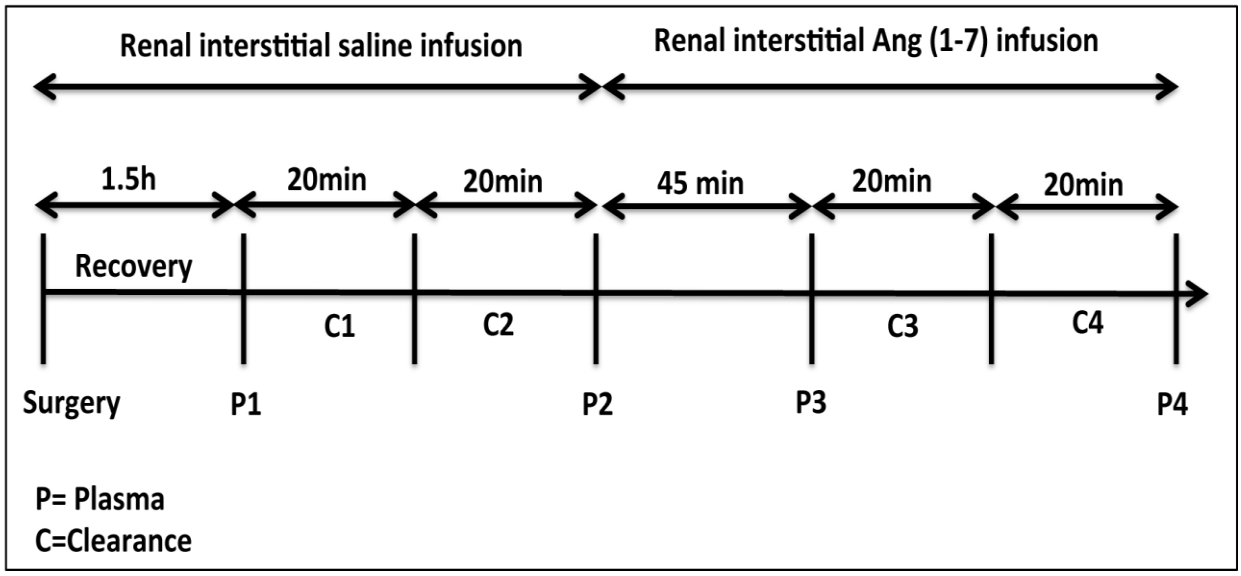
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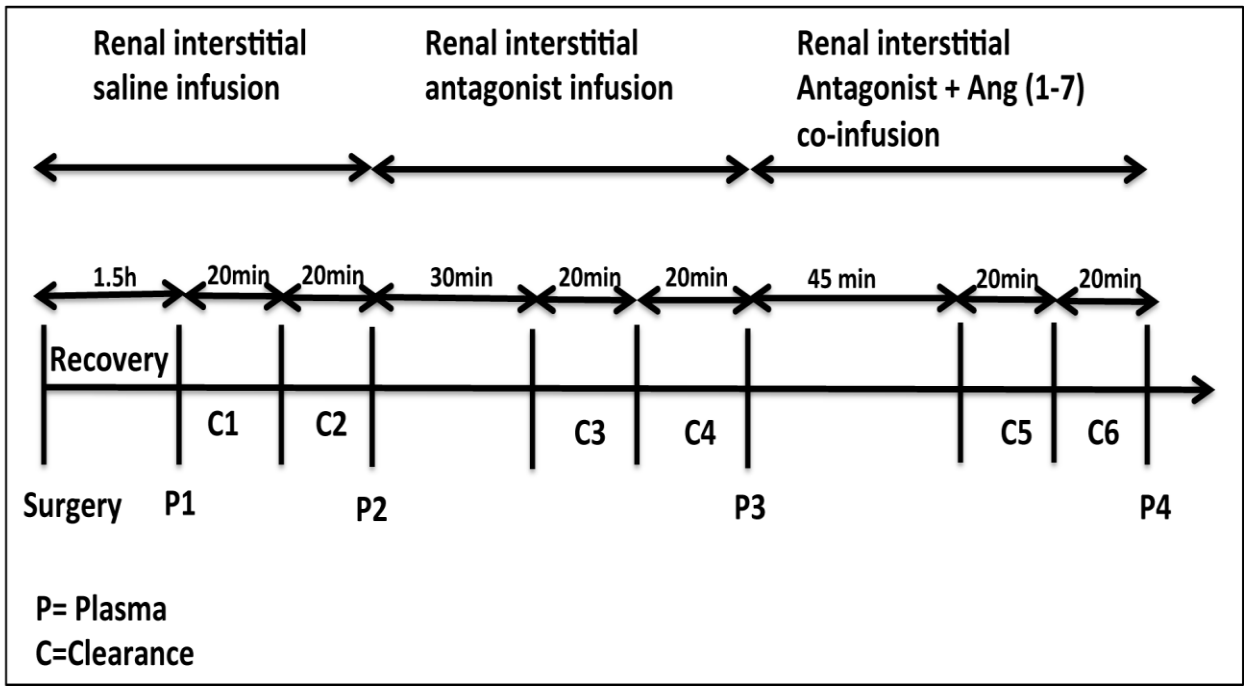
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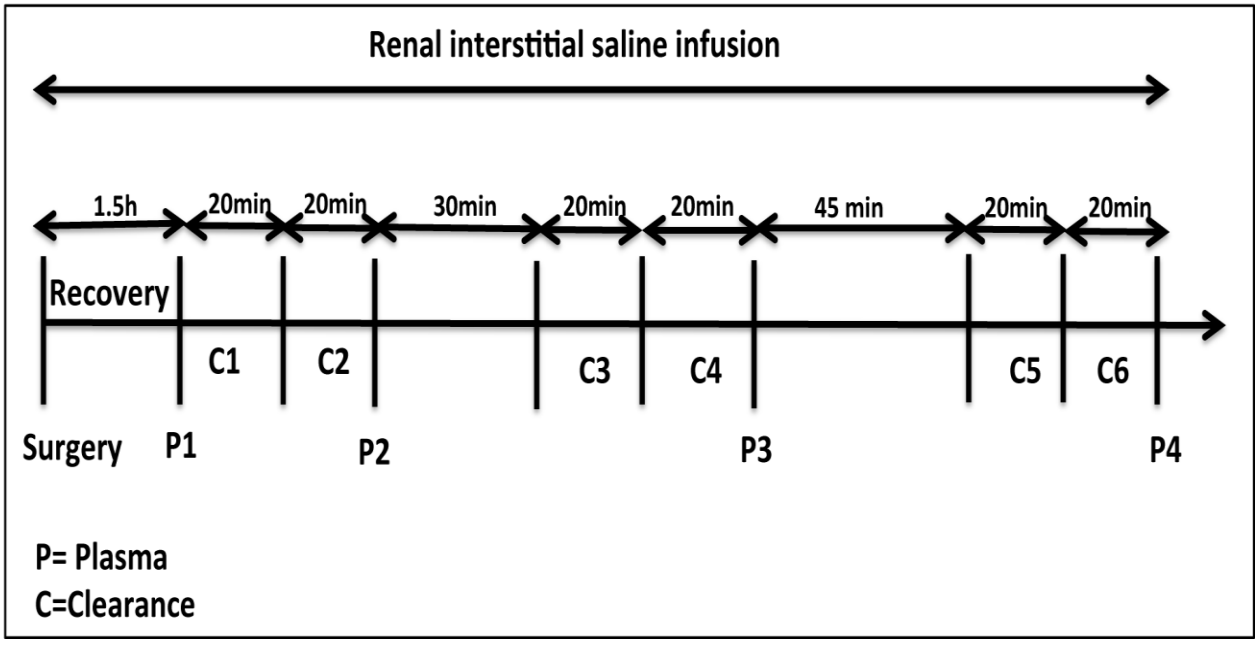
Figure 1 comprises timeline diagrams describing the experimental protocols employed in the present study. (A) Renal interstitial Ang (1-7) infusion. (B) Renal interstitial co- infusion of Ang (1-7)+Antagonist. (C) Renal interstitial infusion of saline (time control).



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Figure 2 describes the impact of renal interstitial Ang (1-7) infusion on MAP during renal AT1 (+ Losartan) or Mas (+A-779) receptor inhibition in rats receiving either (A) normal (NS) or (B) low (LS) sodium diet. * denotes P<0.05 versus corresponding baseline.

Figure 2(A)

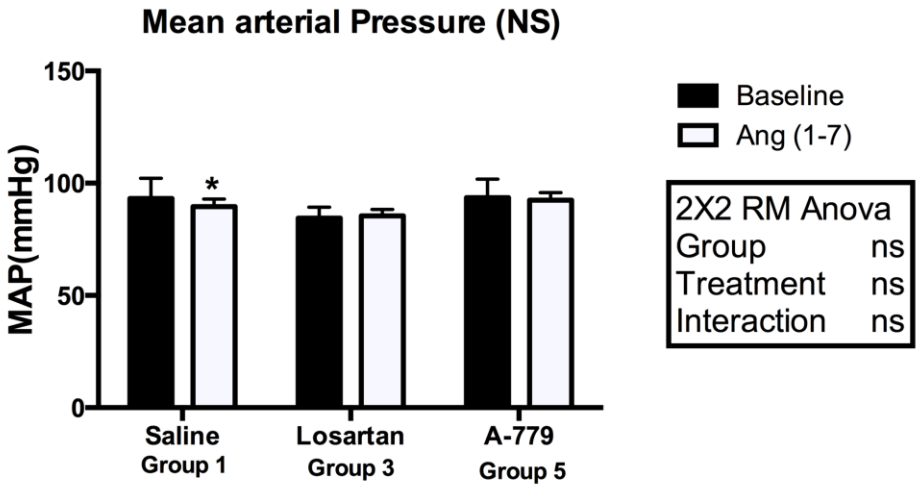
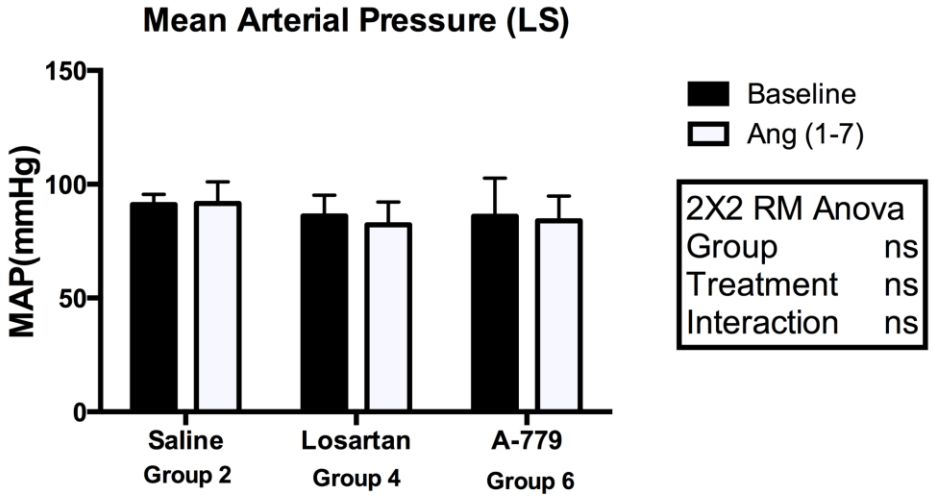


Figure 2(B)



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Figure 3 shows the effect of renal interstitial Ang (1-7) infusion on GFR during the blockade of AT1 (+Losartan) or Mas (+A-779) receptors in the kidney of animals receiving either (A) normal (NS) or (B) low sodium (LS) diet. * denotes P<0.05 versus corresponding baseline. #denotes P<0.05 versus corresponding control.

Figure 3(A)

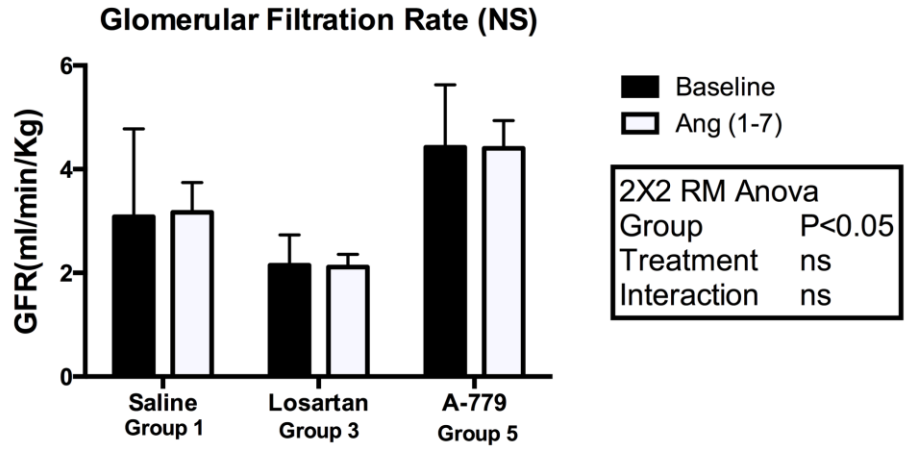
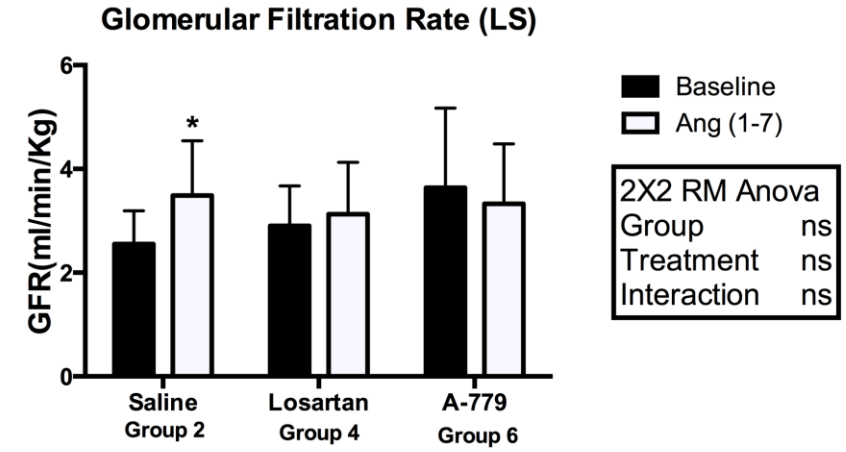


Figure 3(B)



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Figure 4 describes the effect of intra-renal AT1 or Mas receptor inhibition on Ang (1-7) induced diuresis in rats receiving either (A) normal (NS) or (B) low (LS) sodium diet. * denotes P<0.05 versus corresponding baseline.

Figure 4(A)

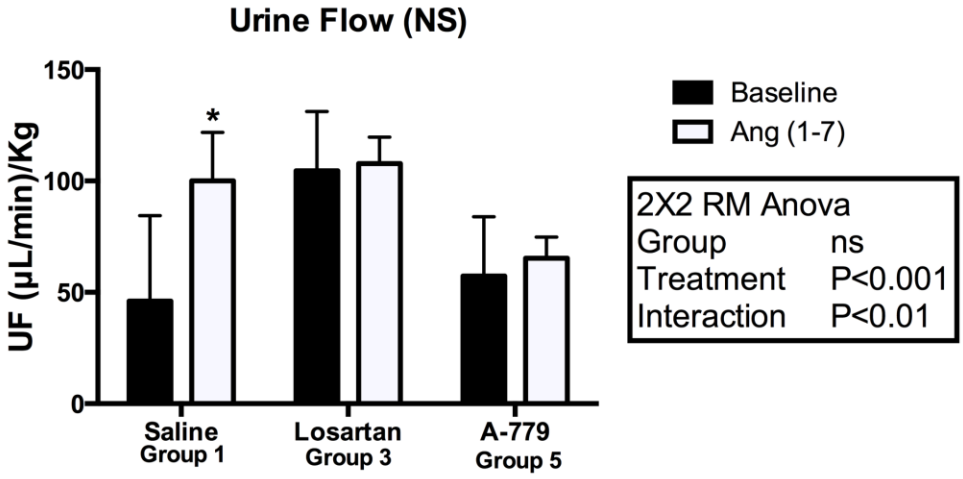
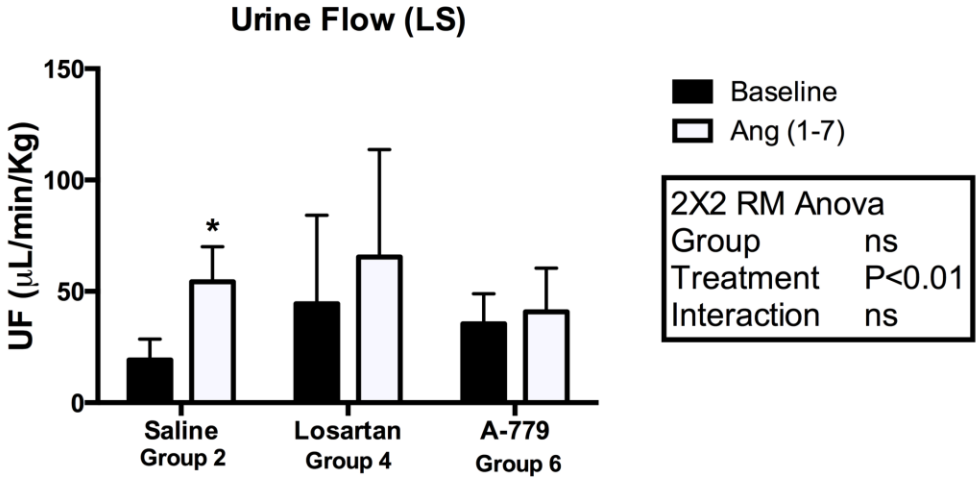


Figure 4(B)



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Figure 5 illustrates the effect of renal interstitial Ang (1-7) infusion on $U_{Na}V$ in the absence or presence of either Losartan or A-779 in rats receiving either (A) normal (NS) or (B) low (LS) sodium diet. * denotes P<0.05 versus corresponding baseline.

Figure 5(A)

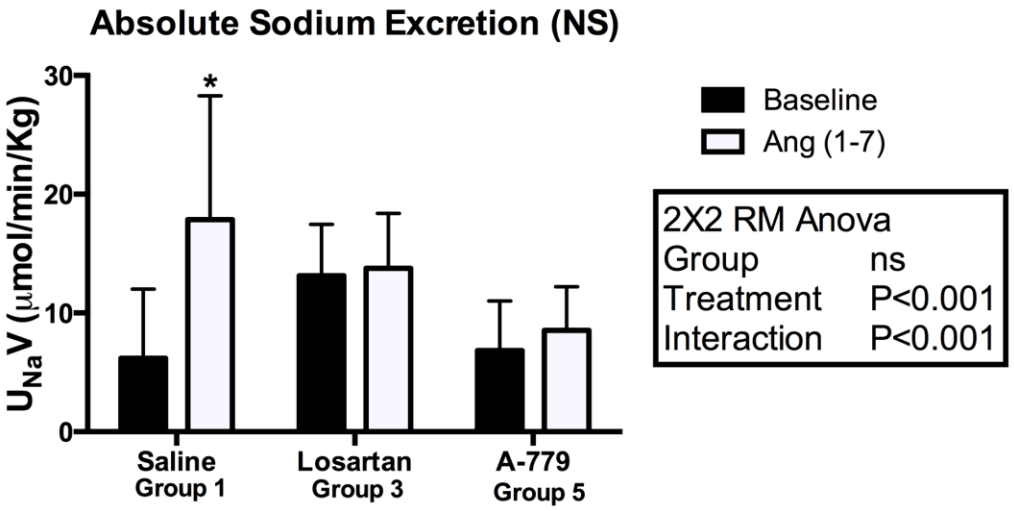
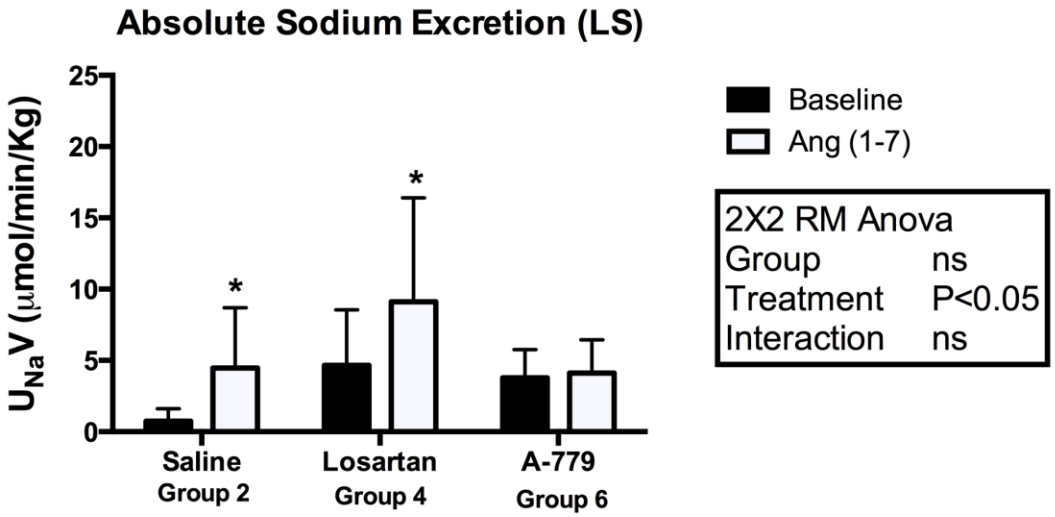


Figure 5(B)



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Figure 6 demonstrates the effect of intra-renal AT1 or Mas receptor inhibition on Ang (1-7) induced reductions in tubular sodium reabsorption in rats receiving either (A) normal (NS) or (B) low (LS) sodium diet. * denotes P<0.05 versus corresponding baseline. #denotes P<0.05 versus corresponding control

Figure 6(A)

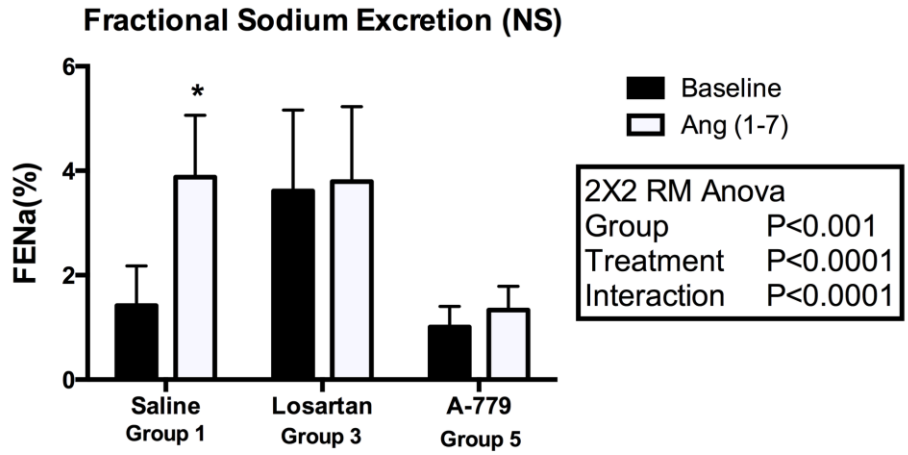
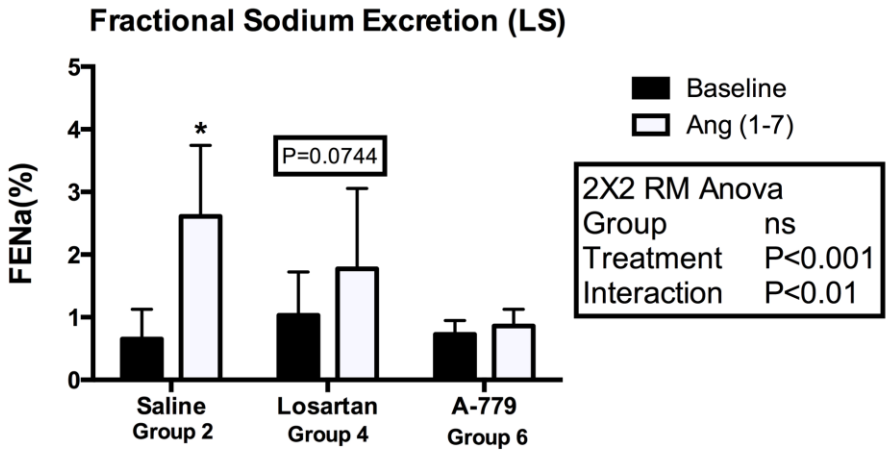


Figure 6(B)

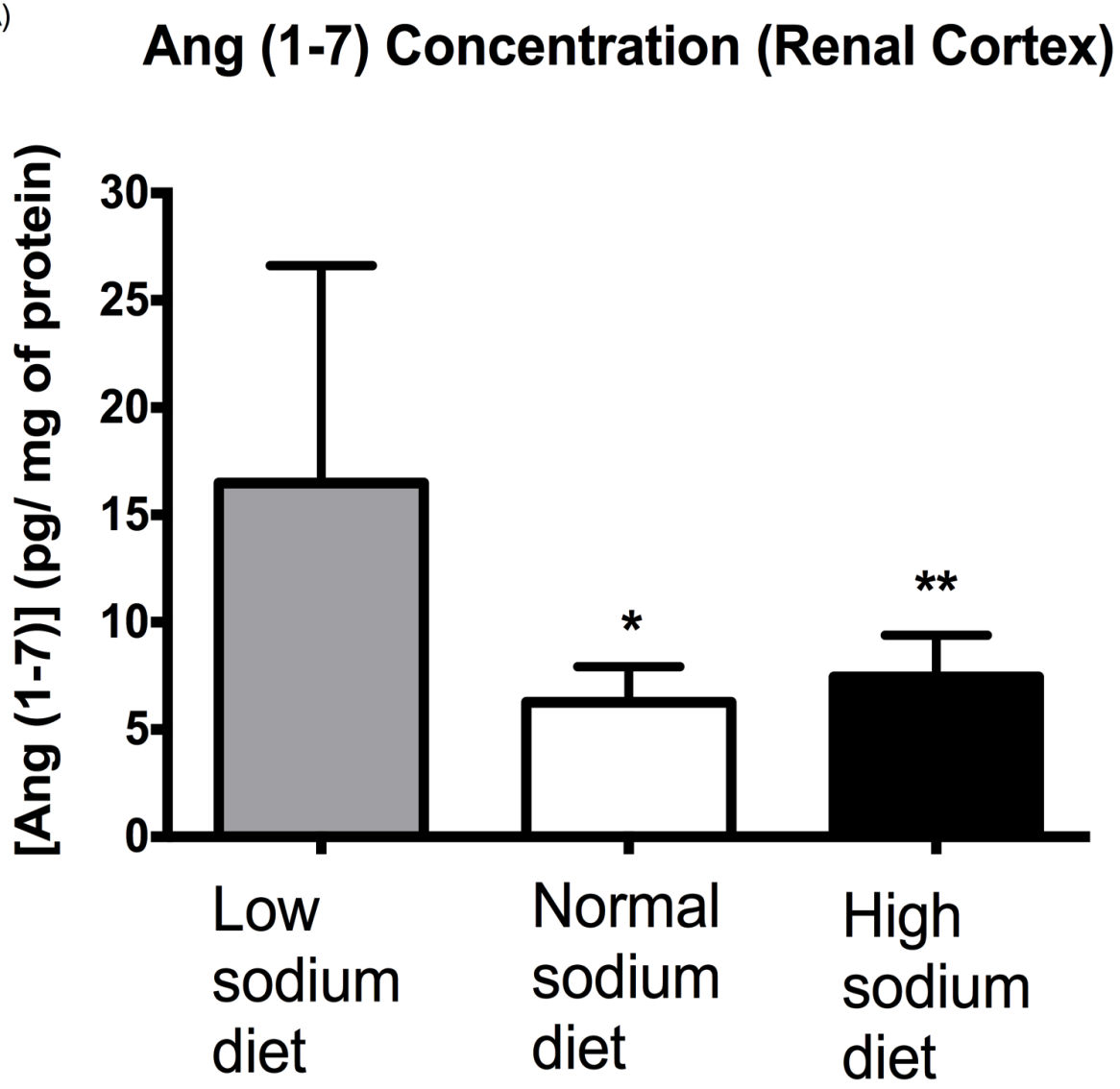


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Figure 7. A comparison of Ang (1-7) concentration in the renal cortex of rats receiving a low, normal or high sodium diet *=P<0.05 Comparing Ang (1-7) levels in the cortex of animals receiving a normal sodium diet versus a low sodium diet. **=P<0.05 Comparing Ang (1-7) levels in the renal cortex of animals receiving a high sodium diet versus a low sodium diet.

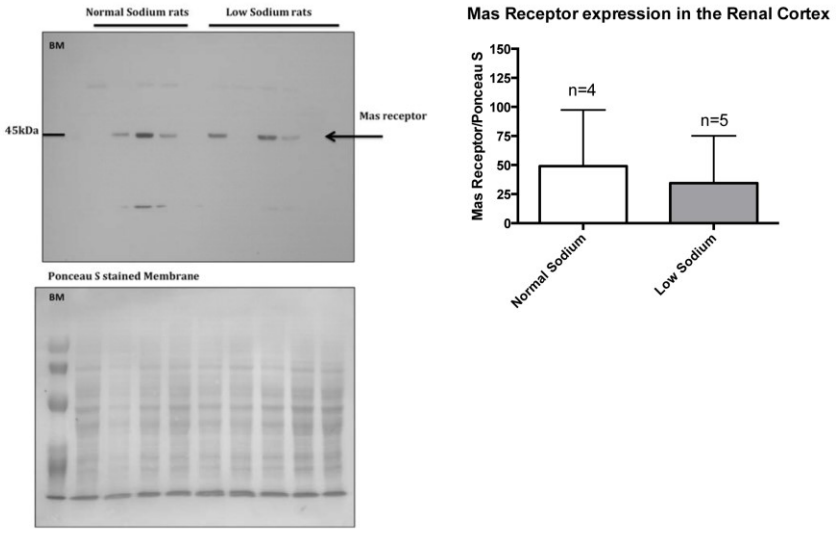
Figure 7(A)

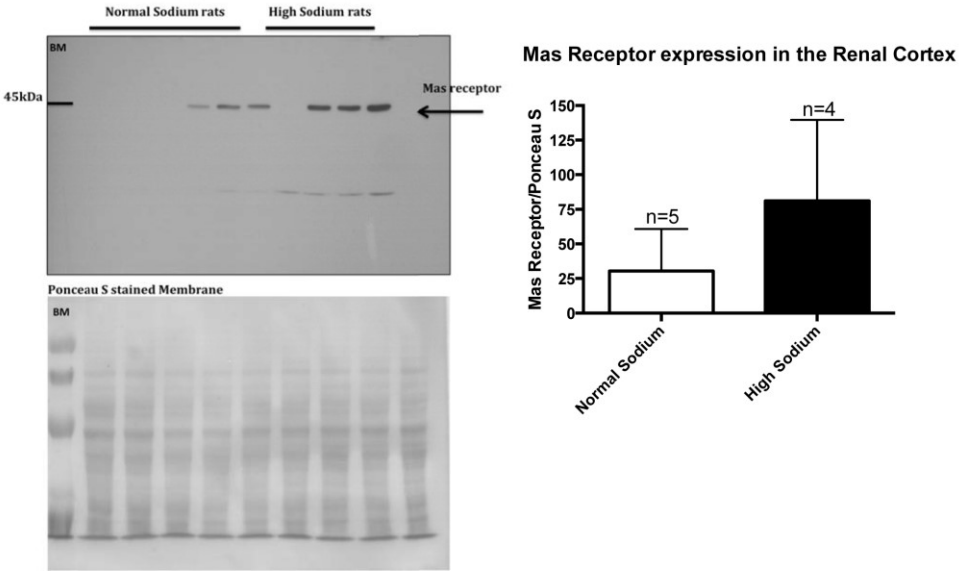


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Figure 8. A comparison of Mas receptor expression (band appearing at 45kDa) in the renal cortex of rats receiving a low (A), normal or high sodium diet (B). Ponceau S stained membrane= loading control. BM= Biomarker lane. Mas receptor expression was normalized against total protein, which was detected using Ponceau S. The n number refers to the number of animals used in each group.

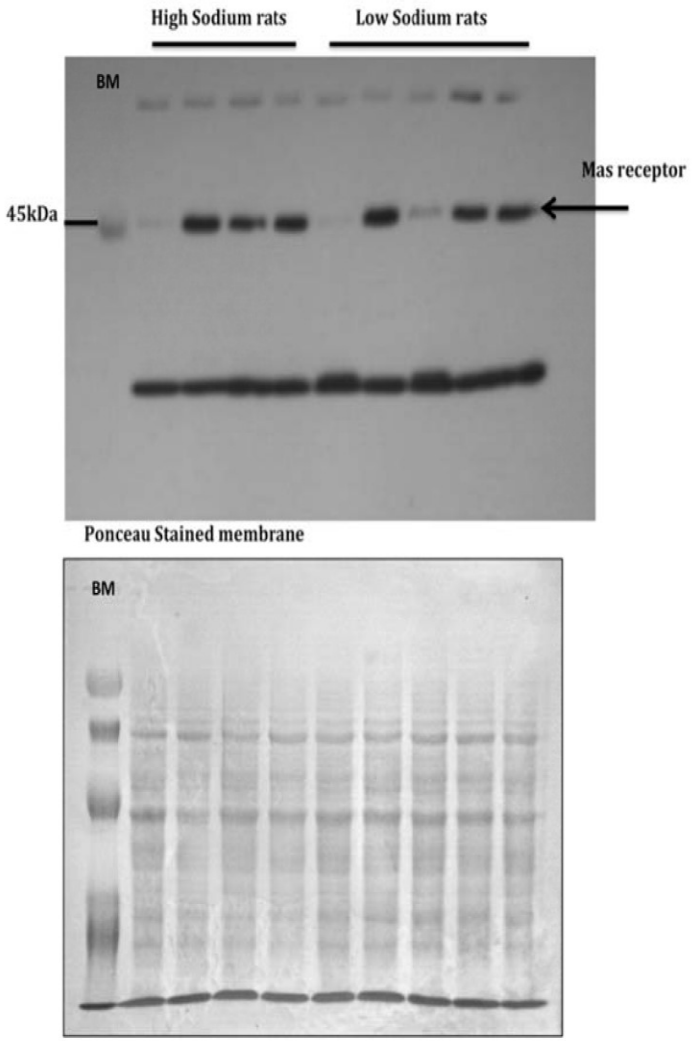


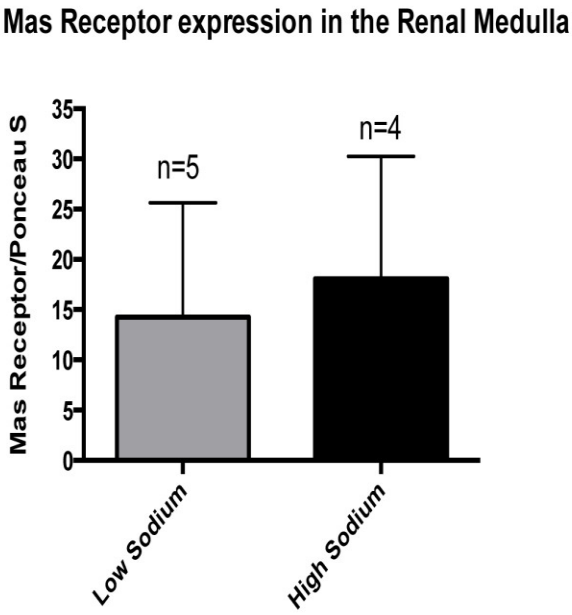


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Figure 9. A comparison of Mas receptor expression (band appearing at 45kDa) in the renal medulla of rats receiving a low or high sodium diet. Ponceau S stained membrane= loading control. BM= Biomarker lane. Mas receptor expression was normalized against total protein, which was detected using Ponceau S. The n number refers to the number of animals used in each group.

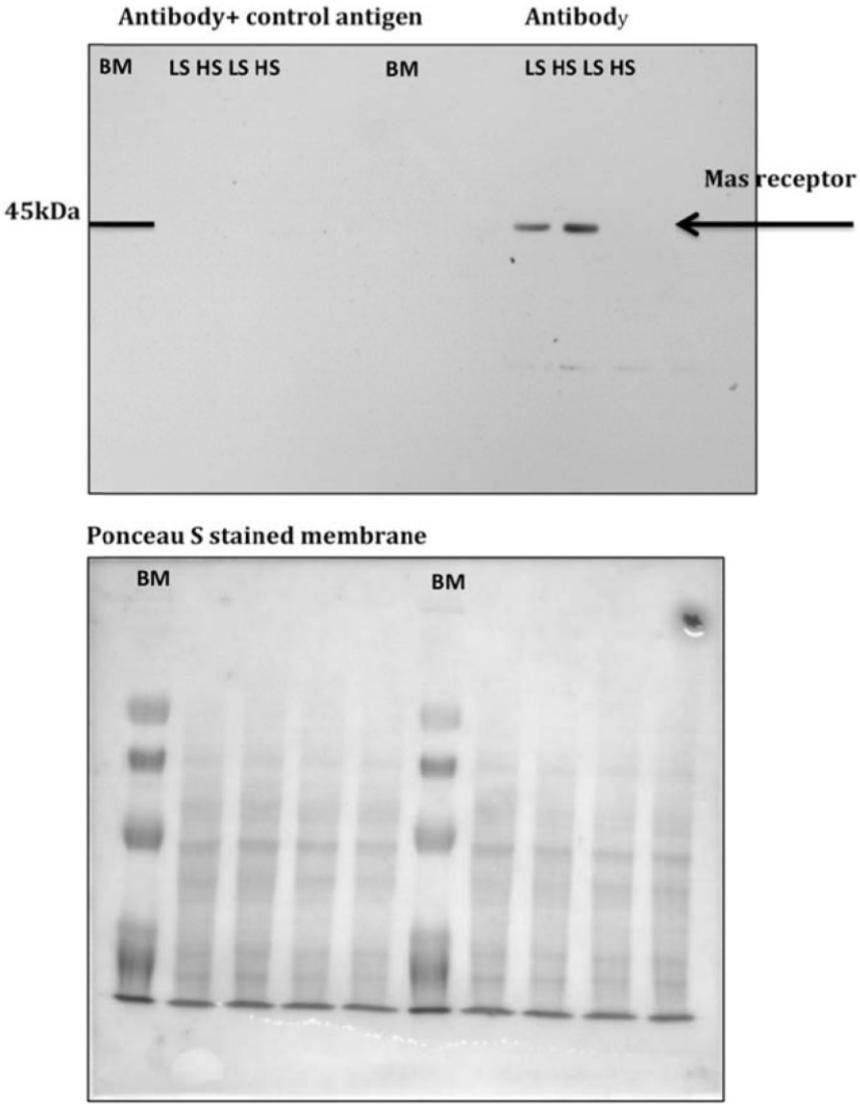




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Figure 10. A comparison of Mas receptor antibody binding in the renal cortex of either low or high sodium rats, +/- pre-incubation with the Mas receptor control antigen. LS = Low sodium group, HS= High sodium group, BM = Biomarker lane.



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Table 1. Hemodynamic and excretory effects of singular Losartan or A-779 infusion in rats receiving either a normal sodium (NS) or low sodium (LS) diet.

		MAP (mmHg)	GFR (ml/min/Kg)	UF (μL/min/Kg)	UNaV (μmol/min/Kg)	FENa (%)
NS: Group 2	Saline	91.62±6.68	2.12±0.55	58.40±33.29	6.92±3.40	1.88±0.84
	Losartan	84.39±4.98*	2.15±0.59	104.51±26.63*	13.13±4.32*	3.61±1.55*
	Losartan+Ang (1-7)	85.39±7.17	2.11±0.59	107.87±28.83**	13.75±4.63**	3.79±1.43**
LS: Group 5	Saline	94.44±10.64	2.85±0.95	28.03±18.29	2.37±1.66 (P=0.09)	0.52±0.28#
	Losartan	86.09±9.10*	2.90±0.77	44.46±39.75#	4.65±3.90#	1.03±0.69#
	Losartan+Ang (1-7)	82.16±10.03**	3.13±1.00#	65.48±48.21**#	9.10±7.31**	1.77±1.29**#
RM 2X2 ANOVA	***Treatment***	***P=0.0008***	***ns***	***P=0.0030***	***P=0.0004***	***P=0.0007***
	Group	***ns***	***P=0.0295***	***P=0.0125***	***P=0.0165***	***P=0.0024***

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	Interaction	***ns***	***ns***	***ns***	***ns***	***ns***
NS: Group 3	Saline	98.13±8.85	3.66±1.15	50.64±29.21	6.13±4.07	1.12±0.45
	A-779	93.58±8.24	4.42±1.20*	57.31±26.67	6.85±4.17	1.01±0.39
	A-779+Ang (1-7)	92.40±8.49	4.40±1.30	65.35±23.05**	8.54±3.67**~	1.33±0.45
LS: Group 6	Saline	88.02±16.51	3.09±1.23	27.46±7.83	3.10±1.59	0.76±0.46
	A-779	85.88±16.85	3.64±1.54	35.40±13.55	3.78±1.98	0.73±0.22
	A-779+Ang (1-7)	83.94±10.93	3.33±1.16	40.83±19.58**	4.11±2.33	0.86±27
RM 2X2 ANOVA	***Treatment***	***ns***	***P=0.0476***	***P=0.0065***	***P=0.0070***	***ns***
	Group	***ns***	***ns***	***P=0.0703***	***P=0.0697***	***P=0.0541***
	Interaction	***ns***	***ns***	***ns***	***ns***	***ns***

All data presented as Mean±SD * denotes P<0.05 Losartan/A-779 versus saline. ** denotes P<0.05 Losartan/A-779+Ang (1-7) versus saline # denotes P<0.05 versus corresponding control. ~ denotes P<0.05 A-779 + Ang (1-7) versus A-779

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Table 2. Saline time control data from rats receiving either a normal sodium (NS) or low sodium (LS) diet.

		MAP (mmHg)	GFR (ml/min/Kg)	UF (µL/min/K)	UNaV (µmol/min/Kg)	FENa (%)
NS: Group 7 (n=5)	Saline 1	94.90±16.27	3.02±1.54	37.13±26.28	8.70±7.25	2.21±1.92
	Saline 2	94.67±15.67	3.07±0.94	40.93±29.10	10.40±8.67	2.17±1.62
	Saline 3	94.56±15.81	3.18±1.75	42.23±28.17	10.81±8.39	2.74±2.15
ONE WAY RM ANOVA	***Treatment***	***ns***	***ns***	***ns***	***ns***	***ns***
	Group	***ns***	***ns***	***ns***	***ns***	***ns***
	Interaction	***ns***	***ns***	***ns***	***ns***	***ns***
LS: Group 8 (n=4)	Saline 1	83.80±11.39	3.90±0.48	64.09±34.11	7.84±3.99	1.23±0.41
	Saline 2	81.28±10.70	4.05±1.03	65.39±25.49	8.53±3.11	1.29±0.14
	Saline 3	78.55±10.92	3.54±0.71	60.20±10.43	8.05±1.94	1.40±0.21
ONE WAY RM ANOVA	***Treatment***	***ns***	***ns***	***ns***	***ns***	***ns***
	Group	***ns***	***ns***	***ns***	***ns***	***ns***
	Interaction	***ns***	***ns***	***ns***	***ns***	***ns***

All data presented as Mean±SD.

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