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## Research Article

# Renal functional effects of the highly selective AT$_2$R agonist, β-Pro$^7$ Ang III, in normotensive rats

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Recently, we designed a group of peptides by sequential substitution of the naturally occurring α-amino acid throughout the Ang III peptide sequence with the corresponding β-amino acid. β-Amino acid substitution at the proline residue of Ang III (β-Pro$^7$-Ang III) resulted in a highly selective AT$_2$R ligand, demonstrating remarkable selectivity for the AT$_2$R in both binding and functional studies. To provide additional functional evidence for the suitability of β-Pro$^7$ Ang III as a novel AT$_2$R agonist, we tested effects of acute systemic administration of β-Pro$^7$-Ang III on renal hemodynamic and excretory function in anesthetized normotensive male and female rats. We also compared the natriuretic effects of acute intrarenal administration of native Ang III and β-Pro$^7$-Ang III in the presence of systemic AT$_1$R blockade in anesthetized female rats to allow for the differentiation of systemic versus direct intrarenal natriuretic actions of β-Pro$^7$-Ang III. In both male and female rats, acute systemic administration of β-Pro$^7$-Ang III elicited renal vasodilatation and natriuresis. Notably, greater renal vasodilatory effects were observed in female versus male rats at the highest dose of β-Pro$^7$-Ang III administered. Moreover, intra-renal administration of β-Pro$^7$-Ang III produced significant natriuretic effects in female rats and, like Ang III, evoked AT$_2$R translocation to the apical plasma membrane in renal proximal tubular cells. Taken together, our findings support the use of β-Pro$^7$-Ang III as a novel AT$_2$R agonist and experimental tool for exploring AT$_2$R function and its potential as a therapeutic target. Furthermore, our findings provide further evidence of a sex-specific influence of AT$_2$R stimulation on renal function.

## Introduction

The renin–angiotensin system (RAS) plays an integral role in the long-term regulation of extracellular fluid homeostasis, peripheral vascular resistance and arterial blood pressure. The principle effector peptide of the RAS, angiotensin II (Ang II), acts via two main receptor subtypes: angiotensin type 1 (AT$_1$R) and type 2 (AT$_2$R) receptors. It has long been recognized that all of the classical effects induced by Ang II, including vasoconstriction, anti-natriuresis, cellular growth and proliferation, result from stimulation of the AT$_1$R under physiological and pathological conditions. Conversely, significant evidence exists that AT$_2$R activation opposes AT$_1$R responses by evoking vasodilation, natriuresis, apoptosis and anti-inflammatory effects [1,2]. In addition to Ang II, other biologically active angiotensin metabolites can directly oppose the classical actions of Ang II via their interaction with the AT$_2$R, including the Ang II heptapeptide metabolite des-aspartyl$^1$-Ang II (Ang III) [1,2]. In fact, overwhelming evidence now exists that Ang III is the predominant endogenous ligand for AT$_2$R-mediated natriuresis by inhibition of sodium reabsorption in the renal proximal tubule [3–6]. Moreover, Ang III shows greater AT$_2$R:AT$_1$R selectivity than Ang II [7,8].

From our evolving understanding of this protective Ang II/Ang III-AT$_2$R arm of the RAS, it has become clear that the AT$_2$Rs may be an innovative therapeutic target for improving cardiovascular and renal function, which may contribute to reducing arterial pressure and associated cardiovascular and renal risk. However, research elucidating the therapeutic potential of the AT$_2$R has been hindered by a lack of selective, metabolically stable and readily available ligands that can be employed to discriminate and activate AT$_2$R function. Most recently, the non-peptide AT$_2$R agonist, Compound 21, which was modeled on the C-terminal pentapeptide structure of Ang II [9], has been adopted by many investigators to demonstrate the functional effects of selective AT$_2$R stimulation. Certainly, Compound 21 shows a high degree of selectivity for the AT$_2$R [7,9]; however some off-target effects have been reported, at least at high concentrations [10].

To that end, β-amino acid containing peptides show great potential as peptidomimetics, attributable to their increased stability to proteolytic degradation, high specificity, and relative ease of synthesis [11]. As such, there is obvious merit in performing modifications to RAS effector peptides to enhance their functional properties. We recently produced a new library of β-substituted Ang peptide analogues using native Ang III as the template [8]. These peptides have individual natural α-amino acids within the Ang III replaced by modified β-amino acids to provide unique agonists with distinct AT$_2$R selectivity. We demonstrated the selective nature of these newly developed agonists in competition binding studies using stably transfected AT$_1$R and AT$_2$R HEK-293 cells. Notably, the novel analogue β-Pro$^7$-Ang III was the most selective AT$_2$R ligand tested. It demonstrated little affinity for the AT$_1$R but high AT$_2$R affinity, resulting in >20,000-fold greater selectivity for the AT$_2$R than the AT$_1$R [8]. This selectivity for the AT$_2$R over the AT$_1$R is similar to the AT$_2$R agonist, CGP42112. However, CGP42112 also exhibits AT$_1$R binding at high concentrations [8]. Furthermore, β-Pro$^7$-Ang III demonstrated AT$_2$R agonistic effects in vascular reactivity studies. β-Pro$^7$-Ang III evoked AT$_2$R-mediated vasorelaxation in mouse aortic rings of a similar magnitude to CGP42112 [8]. In addition, under conditions of low level AT$_1$R blockade, β-Pro$^7$-Ang III reduced blood pressure acutely in conscious spontaneously hypertensive rats by ∼30 mmHg in an AT$_2$R antagonist-sensitive manner [8].

To provide further functional evidence *in vivo* for the potential use of β-Pro$^7$-Ang III as a novel AT$_2$R agonist, in the present study we tested the ability of acute systemic administration of β-Pro$^7$-Ang III to modulate renal hemodynamic and excretory function in normotensive male and female rats. Furthermore, in a second cohort of normotensive female rats, we also compared the natriuretic effects of acute intrarenal administration of native Ang III and β-Pro$^7$-Ang III in the presence of systemic AT$_1$R blockade. These two approaches together allowed differentiation of systemic versus direct intrarenal natriuretic actions of AT$_2$R activation.

# Methods
## Protocol 1: Acute renal function studies following intravenous β-Pro$^7$ Ang III

Ten-week-old male ($N = 28$) and female ($N = 31$) Sprague-Dawley rats were obtained from the Animal Resources Centre (Perth, WA, Australia). Animals were maintained on a 12-h light/dark cycle and were fed a normal sodium diet (0.25% sodium chloride; Specialty Feeds) and received water ad libitum. Experiments were approved by the Monash University Animal Research Platform Animal Ethics Committee (Approval number SOBSA/P/2010/09) and were performed at Monash University in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Experiments were performed in anesthetized rats (150 mg/kg Inactin; thiobutabarbital; Sigma Aldrich). which were randomly assigned to the following experimental groups: (i) male control ($N = 8$); (ii) male β-Pro$^7$-Ang III-treated ($N = 11$); (iii) male β-Pro$^7$-Ang III + PD123319 (PD)-treated ($N = 9$); (iv) female control ($N = 10$); (v) female β-Pro$^7$-Ang III-treated ($N = 13$); and (vi) female β-Pro$^7$-Ang III + PD-treated ($N = 8$). The rats were surgically prepared, as described previously [12]. Briefly, a tracheostomy was performed to facilitate breathing. A cather was then inserted into the left jugular vein for intravenous infusion of replacement fluids (2% BSA; Sigma Aldrich) and tritiated inulin ([$^3$H]-inulin; Sigma Aldrich). Furthermore, a catheter was inserted into the left carotid artery for measurement of MAP. Experiments were initiated at the same time each day to prevent any diurnal variation in blood pressure. The left kidney was then exposed and denervated and a catheter was placed in the left ureter for collection of urine. A transit-time ultrasound flow probe (0.7VB; Transonic Systems) was also placed around the renal artery for measurement of renal blood flow (RBF).

Upon completion of surgery, the selective non-peptide AT$_2$R antagonist, PD-123319 (PD; 1 mg/kg bolus plus 1 mg/kg/h; Sigma Aldrich) or vehicle (0.9% saline; 1 ml bolus plus 1 ml/h) was administered intravenously for the duration of the experiment. After 30 min, intravenous infusion of constant vehicle or graded β-Pro$^7$-Ang III (0, 7.5, 15.0 and 30.0 pmol/kg/min for 15 min at each dose) began. The doses chosen were similar to the lowest dose of

$\beta$-Pro$^7$-Ang III that we reported to evoke vasodepressor effects *in vivo* when given by systemic administration [8]. At each dose, following a 10-min equilibration period, RBF measurements were obtained for 5 min. Urine was collected during the baseline and 30 pmol/kg/min $\beta$-Pro$^7$-Ang III collection periods and corresponding arterial blood samples were taken from the left carotid artery at the period ends. Urinary and plasma sodium concentrations were measured as previously described [13] and fractional sodium excretion was calculated. Glomerular filtration rate (GFR) was estimated based on [$^3$H]-inulin clearance. At the completion of the experiment, the left kidney was removed and weighed.

## Protocol 2: Acute renal function studies following intrarenal Ang III or $\beta$-Pro$^7$ Ang III

The experiments were conducted on 12-week-old female Sprague-Dawley rats (Harlan; $N = 32$) housed in a vivarium under controlled conditions (temperature $21 \pm 1^\circ$C; humidity $60 \pm 10$%; light 8:00–20:00) and fed a normal sodium diet (0.30% Na$^+$). All experimental protocols were approved by the Animal Care and Use Committee at the University of Virginia (Approval # A3245-01) and performed at the University of Virginia in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

For all studies in this protocol, a 24-h osmotic mini-pump (Alzet Model 2001D) infusing candesartan (CAND; 0.01 mg/kg/min; AstraZeneca) subcutaneously was inserted 24 h prior to experimentation in order to block systemic AT$_1$Rs. While the rats were under short-term anesthesia with ketamine (100 mg/ml) and xylazine (20 mg/ml) via an intra-peritoneal (IP) injection, the pumps were implanted in the interscapular region using sterile technique. On the day of experimentation, the rats were anesthetized with pentobarbital (Nembutal; 50 mg/ml; given 5 mg/100 g body weight) via an IP injection and a tracheostomy was performed using polyethylene tubing (PE-240) to assist respiration. Direct cannulation of the right internal jugular vein using PE-10 tubing provided intravenous access through which vehicle 5% dextrose in water (D$_5$W) was infused at 20 μl/min. Direct cannulation of the right carotid artery with PE-10 tubing provided arterial access for monitoring MAP. Following a midline laparotomy, both ureters were cannulated (PE-10 tubing) to collect urine for the quantification of urinary Na$^+$ excretion (U$_{Na}$V).

For renal cortical interstitial infusion, an open bore micro-infusion catheter (PE-10) was inserted under the renal capsule into the cortex of each kidney to ensure renal interstitial (RI) infusion of vehicle D$_5$W or pharmacological agent at 2.5 μl/min with a syringe pump (Harvard; model 55-222). When more than one agent was simultaneously infused, separate interstitial catheters were employed. Vetbond tissue adhesive (3M Animal Care Products) was added to secure the catheter(s) and prevent interstitial pressure loss in the kidney. MAP was measured by the direct intra-carotid method (see above) with the use of a digital BP analyzer (Micromed, Inc). MAP was recorded every 5 min and averaged for all periods. Experiments were initiated at the same time each day (10:00 AM) to prevent any diurnal variation in blood pressure. Urinary Na$^+$ concentrations were measured using a flame photometer (Instrumentation Laboratory-943).

For the acute intrarenal Ang III or $\beta$-Pro$^7$-Ang III infusion in the presence of systemic AT$_1$R blockade, all studies involved a 2-kidney model where the right kidney served as a time control receiving RI infusion of vehicle D$_5$W while the left kidney received RI infusions of pharmacological agents. Following a 1-h equilibration period in which vehicle D$_5$W was infused into the RI space of each kidney, the following groups of rats were studied: (i) Ang III ($n = 8$): right kidney received RI infusion of D$_5$W for five 30 min periods and the left kidney received RI infusion of Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg/min; each dose for 30 min; Bachem) following a 30 min RI infusion of D$_5$W, based on previous studies [3–5]. (ii) Ang III $\pm$ PD ($n = 6$): right kidney received RI infusion of D$_5$W for five 30 min periods and the left kidney received RI infusion of Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg/min; each dose for 30 min) + PD (10 μg/kg/min; Parke-Davis) following a 30 min RI infusion of D$_5$W. (iii) $\beta$-Pro$^7$-Ang III ($n = 5$): right kidney received RI infusion of D$_5$W for five 30 min periods and the left kidney received RI infusion of $\beta$-Pro$^7$-Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg/min; each dose for 30 min) following a 30 min RI infusion of D$_5$W. (iv) $\beta$-Pro$^7$-Ang III $\pm$ PD ($n = 7$): right kidney received RI infusion of D$_5$W for five 30 min periods and the left kidney received RI infusion of $\beta$-Pro$^7$-Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg/min; each dose for 30 min) following a 30 min RI infusion of D$_5$W. (v) PD alone ($n = 6$): right kidney received RI infusion of D$_5$W for five 30 min periods and the left kidney received RI infusion of PD (10 μg/kg/min) for four 30 min periods following a 30 min RI infusion of D$_5$W. U$_{Na}$V and MAP were measured for each period. At the completion of each experiment, rats were killed with Euthansol solution IP.

## Protocol 3: Renal proximal tubule cell apical membrane and total cortical homogenate AT$_2$R protein expression following intrarenal Ang III or β-Pro[7] Ang III

### Total cortical cell membrane preparation and Western blot analysis

This was prepared from 12 additional treated animals, as described below: Slices of kidney cortex (approximately 100 mg per kidney) were homogenized (Polytron setting 6, 3 × 5 s pulses) in detergent free homogenization buffer (10 mM Tris, 200 mM sucrose, 1 mM EDTA, pH 7.4) with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) and then centrifuged at 900 × **g** for 10 min at 4°C to remove cellular debris. The supernatant was removed and used for total protein quantification with a bicinchoninic acid (BCA) assay (Pierce). During the tissue preparation, equal samples of each treatment group were processed at the same time. Sodium dodecylsulfate (SDS) samples were prepared, separated by SDS-PAGE (10% Tris-HCl polyacrylamide gels; 40 μg of protein loaded per lane), and transferred onto a nitrocellulose membrane by electroblotting. Membranes were blocked in 5% milk in TBS with 0.1% Tween-20 (TBST[1]) for 2 h at 4°C and then incubated overnight at 4°C with AT$_2$R antibody (Millipore; Cat # AB15554; 1:500) in 5% milk TBST[1]. After three washes in TBST[1], membranes were incubated with HRP-conjugated anti-rabbit secondary antibody (GE Healthcare; Cat # NA934V; 1:2,500) in 5% milk TBST[1] for 2 h at room temperature (RT). After three washes in TBST[1], signals were detected using chemiluminescence with the ChemiDoc MP Imaging System, and band densities were measured with ImageJ software (NIH). The membranes were then stripped for 15 min (Restore Western blot stripping buffer; Thermo Scientific) and blocked in 5% milk TBST[1] for 1 h at 4°C. The membranes were then incubated with β-tubulin (Millipore; Cat # 05-661; 1:5,000) in 5% milk TBST[1] for 2 h at RT. After three washes in TBST[1], membranes were incubated with HRP-conjugated anti-mouse secondary antibody (GE Healthcare; NA931V; 1:2500) in 5% milk TBST[1] for 2 h at RT followed by the remaining Western procedure as stated above. All signals were normalized to β-tubulin expression.

### Renal proximal tubule cell (RPTC) apical membrane isolation and Western blot analysis

One milligram of total protein was resuspended in 10 ml detergent-free homogenization buffer and incubated with 20 μg of biotinylated Lotus tetragonolobus agglutinin (LTA) lectin (Vector Laboratories; Cat # B-1325) on a 360° rocker for 2 h at RT. A 50% vol/vol slurry (20 μl) of Ultralink Neutravidin beads (Pierce) was then added and incubated on a 360° rocker for 30 min at RT. The beads were then pelleted and thoroughly washed with PBS using a microcentrifuge spin cup filter. The LTA affinity-attached membranes were eluted by incubating the beads in the spin cup filter with 200 μl of 2× sample buffer heated to 70°C for 10 min. During the immunoprecipitation procedure, equal samples of each treatment group were processed at the same time. The samples then underwent the Western blot procedure as described above. The membranes were incubated with AT$_2$R antibody (1:500) in 5% milk TBST[1] followed by HRP-conjugated anti-rabbit secondary antibody as mentioned previously. The membranes were then stripped for 15 min, blocked in 5% milk TBST[1] for 1 h at 4°C, and then incubated with the villin (Santa Cruz; Cat # sc-7672; 1:500) in 5% milk TBST[1] for 2 h at RT. The remaining Western procedure was followed as stated above using the HRP-conjugated anti-goat secondary antibody in 5% milk TBST[1] (Invitrogen; Cat # A16005; 1:2500) for 2 h at RT. All signals were normalized to villin expression, which is a RPTC brush border membrane marker.

### *In Vivo* kidney perfusion and fixation procedure

At the conclusion of the experiment following the final RI infusion dose of either Ang III or β-Pro[7] Ang III (28.0 nmol/kg/min), the rat heart left ventricular cavity was cannulated and the rat was perfused with 40 ml of cold 4% sucrose in PBS followed by 40 ml of cold 4% paraformaldehyde (PFA) in PBS. The kidneys were sliced in half and placed in 4% PFA for 2 h at RT. The slices were rinsed 3× in PBS, immersed in 100 mM Tris-HCl for 30 min, and then rinsed 3× in PBS before being stored in 30% sucrose in PBS overnight at 4°C. The next day, the kidney slices were embedded in Tissue Tek OCT Compound in Cryomold vinyl specimen molds, placed at −20°C until frozen, and then stored at −80°C until processing. Cryostat thin sections (5–8 μm) were placed on Probe On Plus positively charged microscope slides through the University of Virginia Research Histology Core and immediately stained.

### Confocal immunofluorescence microscopy

After the kidney sections had been spotted onto slides and washed with TBS, they were permeabilized with 0.2% Triton-X in TBS in TBS for 10 min. The sections were washed in TBS with 0.02% Tween-20 (TBST[2]) and then blocked in 1% milk TBST[2] for 1 h at RT. The kidney sections were incubated with the primary antibodies AT$_2$R (1:100) and adaptor protein-2 (AP-2; Santa Cruz; Cat # sc-17771; 1:100) in 1% milk TBST[2] overnight at 4°C. After washing with

**Table 1 Baseline levels of mean arterial pressure (MAP), renal blood flow (RBF), glomerular filtration rate (GFR), urine flow (UF), and urinary sodium excretion ($U_{Na}V$)**

| Group | MAP (mmHg) | RBF (ml/min/g) | GFR (ml/min/g) | UF (μmol/min/g) | $U_{Na}V$ (μmol/min/g) |
|---|---|---|---|---|---|
| Male vehicle | $119 \pm 4$ | $3.6 \pm 0.2$ | $1.4 \pm 0.2$ | $5.3 \pm 1$ | $0.3 \pm 0.09$ |
| Male β-Pro7-Ang III | $109 \pm 4$ | $3.7 \pm 0.2$ | $1.3 \pm 0.1$ | $8.1 \pm 2$ | $0.6 \pm 0.2$ |
| Male β-Pro7-Ang III + PD | $113 \pm 4$ | $3.4 \pm 0.3$ | $1.3 \pm 0.2$ | $4.2 \pm 1$ | $0.2 \pm 0.09$ |
| Female vehicle | $121 \pm 3$ | $3.7 \pm 0.4$ | $1.5 \pm 0.08$ | $5.7 \pm 0.4$ | $0.4 \pm 0.06$ |
| Female β-Pro7-Ang III | $117 \pm 3$ | $3.9 \pm 0.2$ | $1.6 \pm 0.05$ | $9.0 \pm 2.4$ | $0.7 \pm 0.2$ |
| Female β-Pro7-Ang III + PD | $118 \pm 3$ | $3.4 \pm 0.4$ | $1.6 \pm 0.1$ | $6.3 \pm 1.9$ | $0.5 \pm 0.1$ |

Data are presented as mean $\pm$ SEM and were analyzed using an ANOVA with Tukey's post-hoc tests. All RBF, GFR, UF, and $U_{Na}V$ values are expressed per gram of left wet kidney weight. For MAP and RBF, $N = 8$–$13$ per group. For GFR, UF and $U_{Na}V$, $N = 6$–$9$ per group. PD = PD123319.

TBST[2], Alexa 647 conjugated donkey anti-rabbit, Alexa 488 conjugated donkey anti-mouse secondary antibodies (Invitrogen; Cat # A31573 and A21202; 1:500), and Texas-Red phalloidin (Invitrogen; Cat # A12381; 1:200) were added in 1% milk TBST[2] for 1 h at RT. Following secondary antibody incubation, the slices were washed in TBST[2], Fluoromount G (Southern Biotech) was applied, and the specimens were covered with a glass coverslip. Stained tubules were photographed under epifluorescence illumination using an automated Olympus IX81 spinning disk confocal microscope using a 60 X plan apo 5 water immersion objective with a numeric aperature of 1.2. The microscope was controlled using Slidebook 5.5 software (3i, Denver CO) and 5 micron thick *z*-stack images were captured using a Hamamatsu EMCCD camera at 0.25 micron intervals and deconvolved using the autoquant spinning disk deconvolution module. The RPTCs that were analyzed were selected based upon first looking at the AP-2 and phalloidin staining alone in order to determine quality RPTCs for further analysis. After the RPTCs were selected, $AT_2Rs$ were examined. Calculation of immunoreactive $AT_2R$ within the RPTC brush border was conducted using F-actin staining with Alexa 594 phalloidin to create the mask and measuring $AT_2R$ fluorescence within that region. Quantifications were performed on six RPTCs with four measurements per cell from a rat and averaged.

At the conclusion of studies in protocol 2, kidneys from Ang III and β-Pro7-Ang III treatment groups were harvested for Western blot analysis ($N = 5$ for each group) and a representative rat for each group was also processed for confocal microscopy.

## Statistical analysis

All data are expressed as mean $\pm$ SEM. In the acute intravenous renal function studies, baseline values of MAP, RBF, GFR, urine flow (UF), and $U_{Na}V$ were analyzed using an analysis of variance (ANOVA) with Tukey's *post-hoc* tests. MAP, RBF and renal vascular resistance (RVR) data were analyzed using repeated-measures ANOVA with Sidak's *post-hoc* tests while renal excretory function data were analyzed using a two-way ANOVA. Comparisons were made between the control and β-Pro7-Ang III-treated groups and the β-Pro7-Ang III-treated and β-Pro7-Ang III + PD treated groups for both sexes, and between the male and female β-Pro7-Ang III-treated groups where appropriate. For the acute intrarenal studies, data were analyzed by two-way ANOVA followed by multiple comparisons testing with the Tukey *post-hoc* analysis. For confocal immunofluorescence and Western blot analysis measurements, data were analyzed by one-way ANOVA followed by multiple comparisons with Tukey *post-hoc* analysis. *P* values $\leq 0.05$ were considered statistically significant.

# Results
## Renal response to acute intravenous β-Pro7-Ang III administration

We examined the renal hemodynamic and excretory responses to acute intravenous administration of β-Pro7-Ang III in anesthetized male and female normotensive rats. Baseline values of MAP were similar between the male and female treatment groups (Table 1). Furthermore, MAP remained near baseline levels throughout the duration of the experiment in all of the male and female treatment groups (Figure 1A,D).

In males, RBF was similar between the three treatment groups at baseline (Table 1). Moreover, there was no significant change in RBF from baseline level in response to vehicle infusion. Conversely, RBF increased in response to β-Pro7-Ang III infusion in a dose-dependent manner ($P_{Group} < 0.001$ and $P_{Dose} = 0.05$ as compared with the male vehicle-treated group) (Figure 1B). At the highest dose of β-Pro7-Ang III administered, RBF was increased by $12.7 \pm 2.3\%$ in males as compared with baseline ($P < 0.001$). A reduction in renal vascular resistance (RVR)
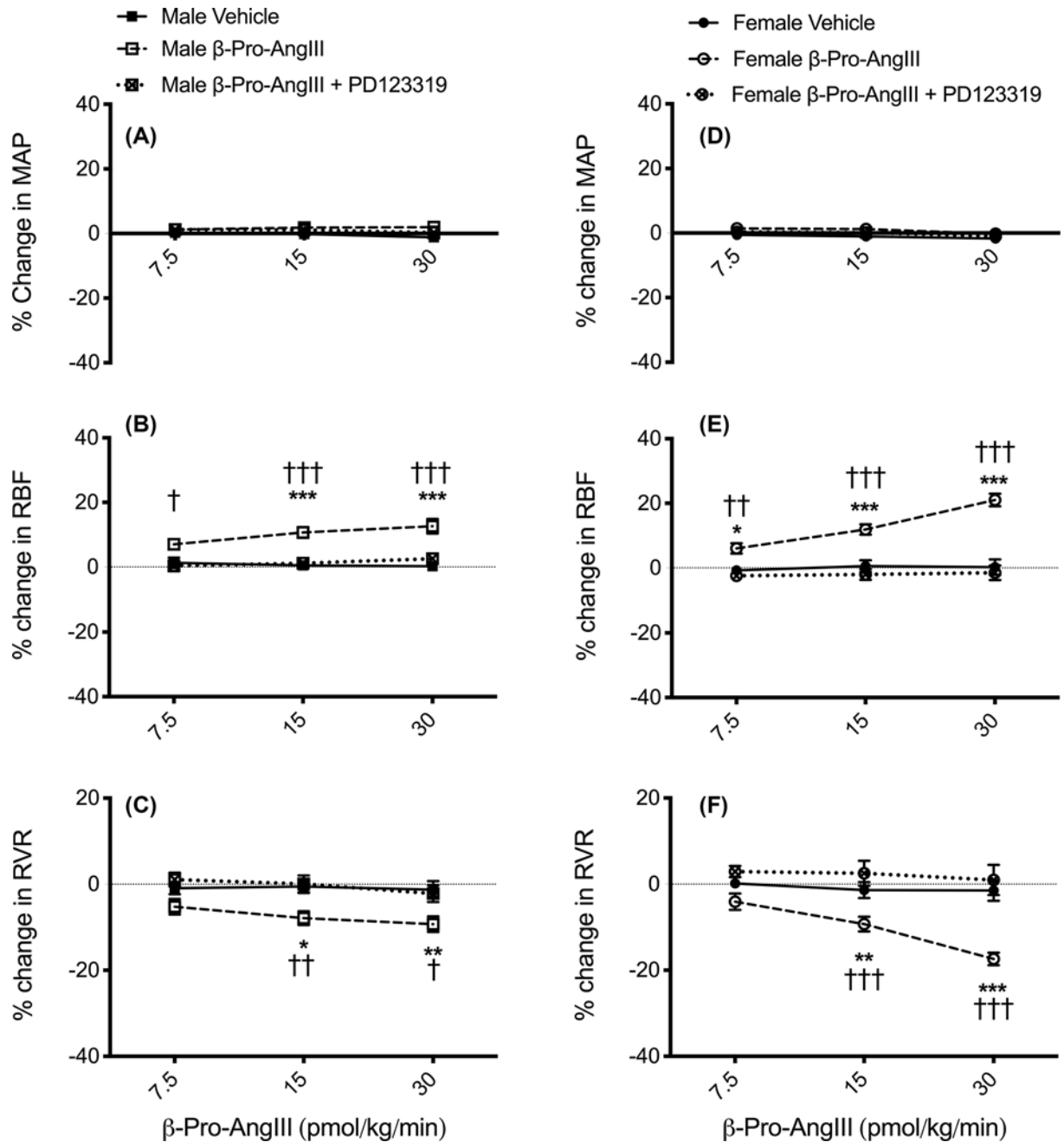
**Figure 1. Renal vasodilator effects of intravenous infusions of β-Pro7-Ang III**

Percent changes from baseline for mean arterial pressure (MAP), renal blood flow (RBF), and renal vascular resistance (RVR) in male (**A–C**) and female (**D–F**) rats in response to intravenous infusion of β-Pro$^7$-Ang III (7.5, 15, and 30.0 pmol/kg/min). Data are presented as mean $\pm$ SEM and were analyzed using repeated-measures ANOVA with Sidak's post-hoc tests. *$P < 0.05$, ***$P < 0.001$ vs vehicle-treated rats; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ vs β-Pro$^7$-Ang III + PD-treated rats; $n = 8$–13 per group. RBF values are expressed per gram of left wet kidney weight.

was also observed in the male β-Pro$^7$-Ang III-treated group as compared with the male vehicle-treated group ($P_{\text{Group}} < 0.01$) (Figure 1C). However, the RVR response to β-Pro$^7$-Ang III was not dose-dependent relative to the male vehicle-treated group levels ($P_{\text{Dose}} = 0.1$). The renal vasodilatory effects of β-Pro$^7$-Ang III were abolished by co-administration of the AT$_2$R antagonist, PD ($P_{\text{Group}} = 0.001$) (Figure 1B,C).
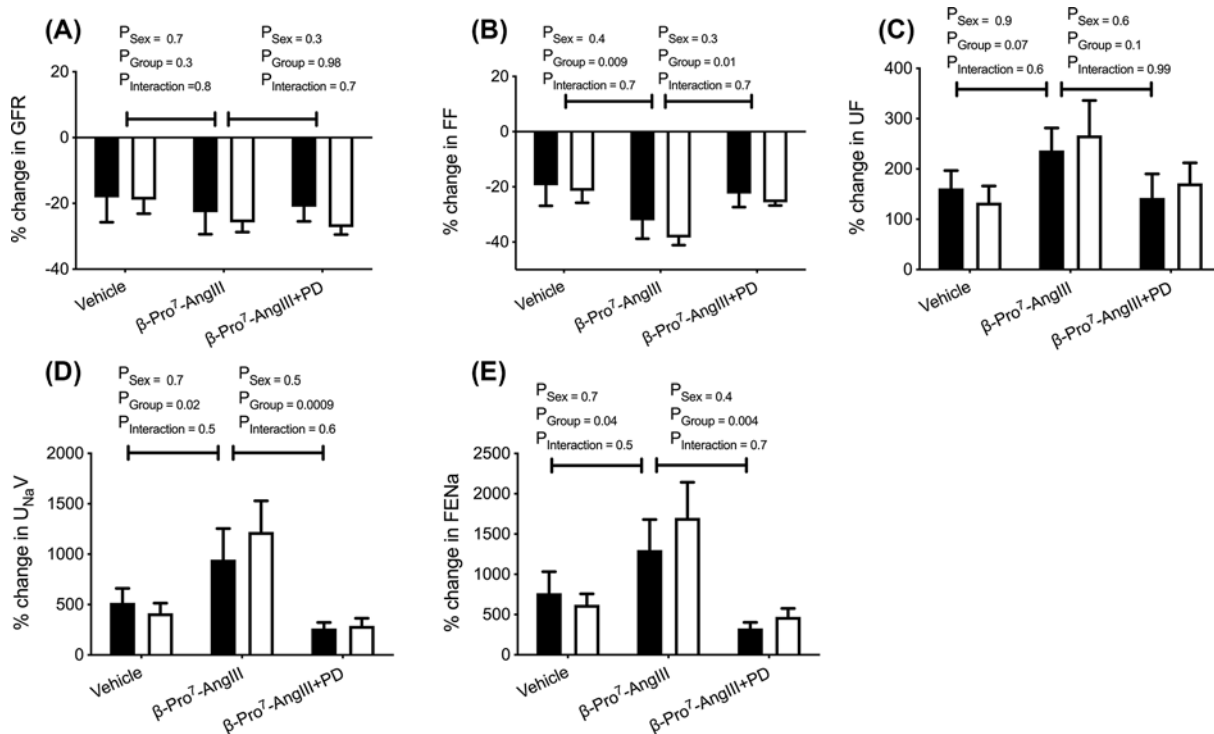
**Figure 2. Effects of intravenous infusions of β-Pro $^7$ -Ang III on sodium excretion**

Percent changes from baseline for (**A**) glomerular filtration rate (GFR), (**B**) filtration fraction (FF), (**C**) urine flow rate (UF), (**D**) sodium excretion ($U_{Na}V$), and (**E**) fractional sodium excretion (FENa) in male (closed bars) and female (open bars) rats in response to intravenous infusion of β-Pro$^7$-Ang III (30.0 pmol/kg/min). Data are presented as mean ± SEM and were analyzed using a two-way ANOVA using the factors sex ($P_{Sex}$), group ($P_{Group}$), and the interaction between sex and group ($P_{Interaction}$). $n$ = 6–9 per group.

Similarly, RBF did not differ significantly between the female treatment groups at baseline (Table 1), and remained near baseline levels in response to vehicle treatment (Figure 1D). However, a significant and dose-dependent increase in RBF was seen in response to β-Pro$^7$-Ang III infusion in female rats as compared with their vehicle-treated counterparts ($P_{Group} < 0.001$ and $P_{Dose} < 0.001$) (Figure 1E). At the highest dose of β-Pro$^7$-Ang III administered, RBF was increased by 21.0 ± 1.9% in females, versus baseline ($P < 0.001$). The renal vasodilatory response to β-Pro$^7$-Ang III in females was also reflected by a dose-dependent reduction in RVR, as compared with the female vehicle-treated group ($P_{Group} < 0.001$ and $P_{Dose} < 0.001$) (Figure 1F). Each of these effects in females was abolished by co-infusion of PD ($P_{Group} < 0.001$) (Figure 1E,F). Notably, the magnitude of the effects of β-Pro$^7$-Ang III on RBF and RVR was greater in females as compared with males at the highest dose of β-Pro$^7$-Ang III administered ($P < 0.01$).

Renal excretory function was examined during the baseline and 30 pmol/kg/min β-Pro$^7$-Ang III treatment periods in a subset of rats. GFR, UF, and $U_{Na}V$ were similar between the male and female treatment groups at baseline (Table 1). In response to vehicle treatment in both male and female rats, there was a time-dependent reduction in GFR. A similar reduction in GFR was observed in response to β-Pro$^7$-Ang III in both sexes ($P_{Group} = 0.3$) (Figure 2A). In turn, due to the observed changes in RBF and GFR, a greater reduction in filtration fraction was observed in response to β-Pro$^7$-Ang III as compared with vehicle treatment ($P_{Group} = 0.009$) (Figure 2B). This effect was not dependent on sex ($P_{Interaction} = 0.7$) and was abolished by AT$_2$R blockade with PD ($P_{Group} = 0.01$).

In both sexes, a time-dependent increase in UF, $U_{Na}V$, and fractional sodium excretion (FENa) was observed in response to vehicle treatment (Figure 2C–E). $U_{Na}V$ and FENa were also increased in response to β-Pro$^7$-Ang III in males and females, but to significantly greater extent as compared with the vehicle-treated rats ($P_{Group} = 0.02$ for $U_{Na}V$ and $P_{Group} = 0.04$ for FENa). There was also a trend for a significant increase in UF in the β-Pro$^7$-Ang III-treated rats as compared with the vehicle-treated rats ($P_{Group} = 0.07$). Each of these responses to β-Pro$^7$-Ang III was similar between the sexes ($P_{Interaction} = 0.6$ for UF and $P_{Interaction} = 0.5$ for $U_{Na}V$ and FENa) and was abolished in both males and females by co-infusion of β-Pro$^7$-Ang III and PD (Figure 2C–E).
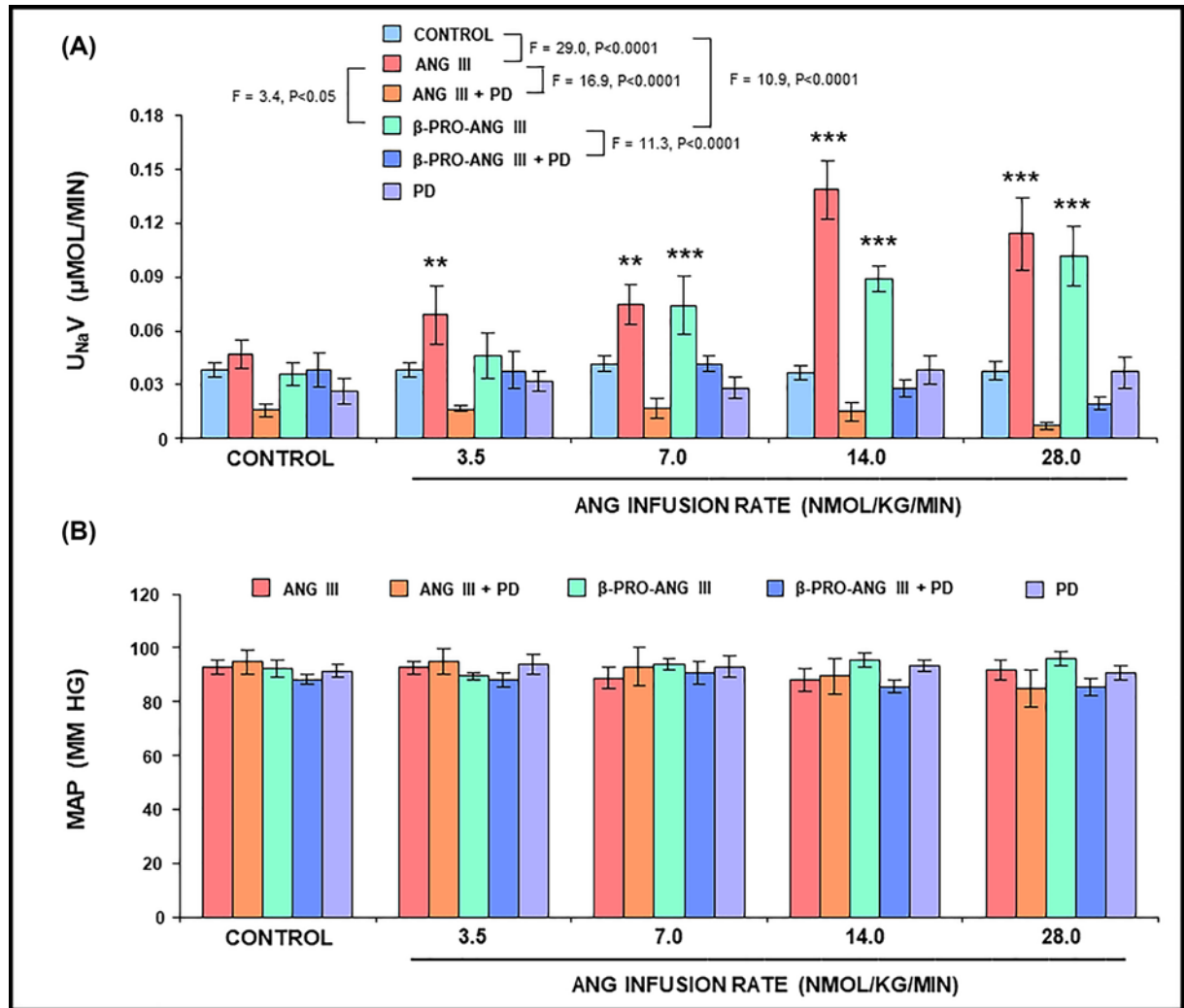
**Figure 3. Effects of renal interstitial infusions of β-Pro[7]-Ang III or Ang III on sodium excretion**

(**A**) Urine sodium excretion ($U_{Na}V$) under the following conditions: (▢) collective right kidneys renal interstitial (RI) control infusion ($N = 28$), (▢) left kidney RI Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg/min) infusion ($N = 8$), (▢) left kidney RI Ang III + PD (10 μg/kg/min) infusion ($N = 6$), (▢) left kidney RI β-Pro[7]-Ang III (3.5, 7.0, 14.0, and 28.0 nmol/kg/min) infusion ($N = 5$), (▢) left kidney RI β-Pro[7]-Ang III + PD infusion ($N = 7$), and (▢) left kidney RI PD infusion all in the presence of systemic $AT_1R$ blockade. (**B**) Mean arterial pressure (MAP) under conditions described in panel (A). Data represent mean ± SEM. **$P < 0.01$ and ***$P < 0.001$ from own respective control period.

## Renal response to acute intrarenal Ang III or β-Pro[7]-Ang III administration during systemic $AT_1R$ blockade

As shown in Figure 3A, RI infusion of Ang III increased $U_{Na}V$ throughout the range of infusion rates beginning at 3.5 nmol/kg/min ($P < 0.01$) and to a peak at 14 nmol.kg/min ($P < 0.001$). Overall, Ang III increased $U_{Na}V$ by 2.8-fold ($F = 29.0$; $P < 0.0001$) from baseline and time-control values. Concurrent intrarenal administration of $AT_2R$ antagonist PD abolished the increase in $U_{Na}V$ engendered by Ang III ($F = 16.9$; $P < 0.0001$). $U_{Na}V$ was lower in the Ang III + PD group compared with time control values ($P = 0.031$, $F = 2.51$). RI administration of β-Pro[7]-Ang III at the same molar concentrations as for Ang III also increased $U_{Na}V$ throughout the infusion periods ($F = 10.9$; $P < 0.0001$); this natriuretic response was also abolished by concurrent administration of PD ($F = 11.3$; $P < 0.0001$). The natriuretic response to intrarenal Ang III was significantly greater than to β-Pro[7]-Ang III ($F = 3.4$; $P < 0.05$). Intrarenal PD alone did not alter $U_{Na}V$ ($P = NS$). In response to β-Pro[7]-Ang III. UV increased from $1.05 \pm 0.19$ μl/min to $1.43 \pm$

0.27, 2.27 $\pm$ 0.67, 3.36 $\pm$ 0.74, and 3.76 $\pm$ 1.04 μl/min ($P = 0.035$; $F = 3.19$). As shown in Figure 3B, there was no change in MAP in response to any of the infused agents ($P = $ NS).

## Effects of Ang III and β-Pro$^7$-Ang III on RPTC apical plasma membrane AT$_2$R density

To determine whether AT$_2$R activation with β-Pro$^7$-Ang III induces similar receptor translocation to the apical plasma membranes of RPTCs as Ang III, we employed confocal immunofluorescence microscopy and lectin pull-down of RPTC apical plasma membranes followed by immunoblotting. Figure 4A–F illustrates the subcellular distribution of AT$_2$Rs in a representative set of RPTCs after intrarenal right kidney vehicle (VEH), left kidney Ang III, or left kidney β-Pro$^7$-Ang III infusion as determined by confocal immunofluorescence microscopy. Figure 4A,B shows the RPTC distribution of phalloidin (red) marking the apical plasma membrane and adaptor protein-2 (AP-2) (blue) marking the subapical region, respectively. Figure 4C demonstrates the subcellular distribution of AT$_2$Rs (green) using an antibody (Millipore; Cat # AB1554) specific for AT$_2$Rs as previously demonstrated by immunoblotting AT$_2$R-null mouse adrenal glands that normally have a high degree of AT$_2$R expression. Figure 4D,E at higher magnification of the area depicted in the respective box of Figure 4D shows merged phalloidin, AP-2, and AT$_2$R images. Figure 4F depicts a higher power image of only AT$_2$R staining in the brush border area, thus representing AT$_2$R in the apical plasma membrane. This panel demonstrates substantially higher staining in the apical plasma membrane in response to both Ang III and β-Pro$^7$-Ang III ($P < 0.001$ for both) quantitated in Figure 4G. As further corroborated by lectin pulldown of apical membranes followed by immunoblotting (Figure 4H), both Ang III and β-Pro$^7$-Ang III increased apical plasma membrane AT$_2$R density ($P < 0.01$ and $P < 0.001$, respectively) without changing total cortical homogenate AT$_2$R protein expression (Figure 4I). The AT$_2$R trafficking response to β-Pro$^7$-Ang III was slightly higher, but not significantly different from Ang III.

## Discussion

The present study demonstrates the ability of acute administration of the newly developed AT$_2$R selective peptidomimetic ligand, β-Pro$^7$-Ang III, to induce renal vasodilatory and natriuretic effects in normotensive rats. Notably, we showed that AT$_2$R activation with β-Pro$^7$-Ang III can promote significant natriuretic effects whether administered systemically or directly into the kidney. Moreover, the observed responses to β-Pro$^7$-Ang III administration were abolished by AT$_2$R blockade with PD confirming the AT$_2$R-mediated nature of these effects. It was also observed that systemic administration of β-Pro$^7$-Ang III induced greater renal vasodilatory effects in female versus male rats at the highest dose of β-Pro$^7$-Ang III examined. These findings, therefore, support the use of β-Pro$^7$-Ang III as a novel AT$_2$R agonist and research tool for exploring AT$_2$R function and the viability of this receptor as a therapeutic target, particularly since we have previously reported that this peptide did not exhibit off-target functional AT$_1$R-mediated effects [8], unlike Ang III [8] or Compound 21 [14]. Moreover, our findings provide further evidence of a sex-specific influence of AT$_2$R stimulation on renal function.

In the present study, we provided functional evidence for the ability of β-Pro$^7$-Ang III to evoke AT$_2$R-mediated effects in the kidney, equivalent to that previously observed in response to the most widely studied AT$_2$R agonist, Compound 21 [12]. First, we examined the RBF response to a graded systemic infusion of β-Pro$^7$-Ang III. In both male and female rats, β-Pro$^7$-Ang III significantly increased RBF, in doses that had no effect on arterial blood pressure. This renal vasodilatory response was also reflected by a significant reduction in RVR. Moreover, these renal vasodilatory effects were abolished in the presence of combined β-Pro$^7$-Ang III and PD, supporting the role of the AT$_2$R in these responses. As such, β-Pro$^7$-Ang III-mediated vasodilator effects were also observed in conscious spontaneously hypertensive rats at doses similar to the highest dose employed in the current study, as well as 10-fold higher [8].

Systemic administration of β-Pro$^7$-Ang III also modulated renal excretory function. At the highest dose of β-Pro$^7$-Ang III administered, an increase in U$_{Na}$V in both male and female rats was observed, which was also PD-sensitive. In addition, a trend for an increase in UF in response to β-Pro$^7$ Ang III administration was noted. These observations were seen in the absence of any significant change in GFR. Given the major increase in RBF in response to β-Pro$^7$-Ang III administration, a significant reduction in filtration fraction was, therefore, observed. It is also likely that similar dilatory effects on both preglomerular and postglomerular arterioles resulted in increased RBF whilst GFR remained unchanged. Notably, similar findings were reported previously in response to a graded intravenous infusion of the non-peptide AT$_2$R agonist, Compound 21, under similar experimental conditions [12]. These findings, therefore, suggest that the AT$_2$R-mediated natriuresis (as induced by β-Pro$^7$-Ang III and Compound

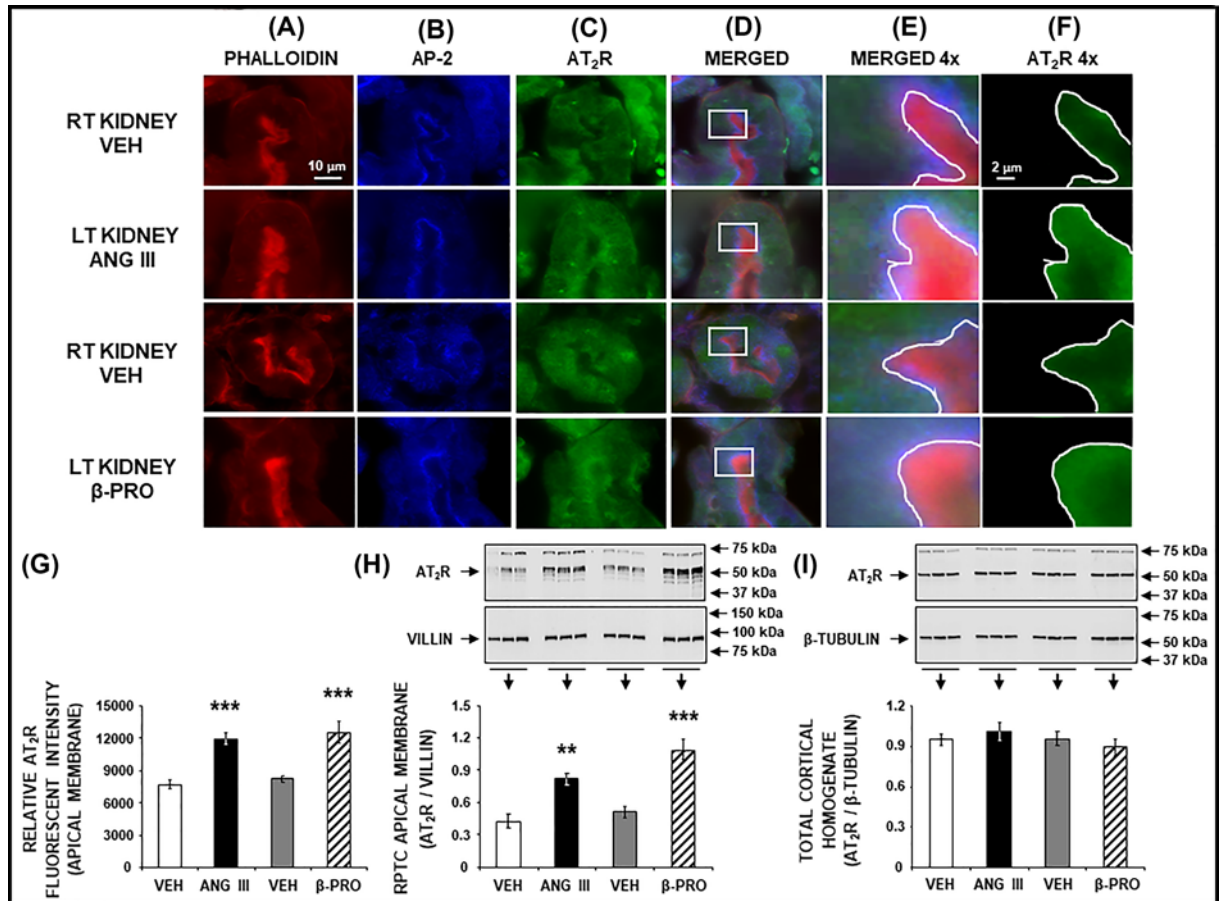**Figure 4. Effects of intrarenal infusions of β-Pro$^7$-Ang III or Ang III on subcellular distribution and receptor translocation of AT$_2$R in RPTCs**

Confocal micrographs (600× magnification) showing AT$_2$R localization in renal proximal tubule cell (RPTC) thin sections (5–8 μm) after renal interstitial (RI) infusion of right kidney vehicle (VEH), left kidney angiotensin III (Ang III), right kidney VEH, and left kidney β-Pro$^7$-Ang III (β-Pro). As indicated, rows of images show a representative set of RPTCs from (top-to-bottom) from right kidney VEH, left kidney Ang III, right kidney VEH, and left kidney β-Pro treatment groups. As indicated, columns (left-to-right) depict brush border membrane staining with phalloidin (**A**), subapical area staining with adaptor protein-2 (AP-2) (**B**), AT$_2$R staining (**C**), merged image (**D**), enlarged merged image (4×) of the square section in (**D** and **E**) and enlarged image with only AT$_2$R staining of the brush border membrane area quantified for AT$_2$R (**F**). The encircled areas in (E and F) encompass brush border apical membranes quantified for AT$_2$R intensity. Scale bars in the first and sixth columns represent 10 and 2 μm, respectively. (**G**) Quantification of RPTC apical membrane AT$_2$R fluorescence intensity performed on six RPTCs with four measurements per cell from one rat for right kidney right kidney VEH (□), left kidney Ang III (■), right kidney VEH (▨), and left kidney β-Pro (▨) treatments. Panels (**H** and **I**) depict Western blot analysis of AT$_2$R in RPTC apical membranes and total cortical homogenate, respectively, after the same treatments ($N = 5$ for each condition). RPTC apical membrane signals were normalized to villin, a brush border apical membrane marker. Total cortical homogenates were normalized to β-tubulin. Data represent mean $\pm$ 1 SE. **$P < 0.01$ and ***$P < 0.001$ from respective control kidney (right kidney)

21) is associated with changes in renal tubular sodium handling, rather than simply by changes in renal hemodynamic function, as we have explored in detail previously [12].

The findings of the present study also provide further evidence in support of a sex-specific role for the AT$_2$R in the regulation of renal function since renal the vasodilator response to β-Pro$^7$-Ang III was greater in females than males at the highest dose of β-Pro$^7$-Ang III examined. Moreover, the reduction in RVR in response to β-Pro$^7$-Ang III was dose-dependent in female rats but not male rats. However, the increases in U$_{Na}$V observed in both males and females in response to β-Pro$^7$-Ang III were not dependent on sex. These findings also corroborate our previous findings using the non-peptide AT$_2$R agonist, Compound 21 [12], and substantiate our previous reports, and that of others, that the

role of the $AT_2R$ in the renal vasculature is enhanced in females [13,15,16]. As we have discussed in detail elsewhere [1,17,18], the sex-specific role of the $AT_2R$ is likely attributable to direct effects of gonadal hormones and genes encoded on the sex chromosomes. In this regard, it is well established that estrogen modulates components of the RAS, shifting the balance toward the protective $AT_2R$ arm in females. Moreover, it has also been demonstrated that the RAS is differentially regulated by sex chromosomes. Genes encoding the $AT_2R$, in addition to other RAS components including angiotensin converting enzyme 2, are located on the X chromosome. In comparison, testosterone and the Y chromosome shift the balance of the RAS toward the $AT_1R$ arm of the RAS [18].

To provide further insight into the renal-specific effects of $AT_2R$ stimulation with β-Pro$^7$-Ang III, we also compared the acute natriuretic response to a graded intra-renal infusion of Ang III and β-Pro$^7$ Ang III against a background of systemic $AT_1R$ blockade as we have routinely done in previous studies [3–6,8]. Certainly, it is well established that the $AT_2R$ is highly expressed within the adult kidney, particularly in the proximal tubule [19,20]. Moreover, it was first demonstrated by Carey and colleagues that $AT_2Rs$ inhibit sodium reabsorption within the kidney and that Ang III, as opposed to Ang II, is the predominant endogenous agonist for $AT_2R$-mediated natriuresis [3–6]. However, it was identified that the natriuretic actions of intrarenal Ang III were only evident against a background of systemic $AT_1R$ blockade, unless Ang III metabolism was inhibited using an aminopeptidase N inhibitor [6]. Consequently, in the present study we examined the impact of intrarenal Ang III and β-Pro$^7$-Ang III administration during concurrent $AT_1R$ blockade, although this was not performed in males. In agreement with previous findings, Ang III promoted natriuresis at each dose administered. Moreover, intrarenal administration of β-Pro$^7$-Ang III, at the same molar concentrations as for Ang III, produced significant natriuretic effects, albeit to a significantly lesser extent than Ang III. Furthermore, the natriuresis induced by both Ang III and β-Pro$^7$-Ang III was seen in the absence of any change in arterial pressure, and was abolished by direct intrarenal infusion of the $AT_2R$ antagonist, PD. While these experiments were conducted during $AT_1R$ blockade (to minimize potential $AT_1R$ stimulation by Ang III), β-Pro$^7$-Ang III did evoke vasodilator and natriuretic effects in the first experimental protocol in the absence of $AT_1R$ blockade.

Finally, we demonstrated that $AT_2R$ activation with β-Pro$^7$-Ang III evoked $AT_2R$ translocation from intracellular sites to the apical plasma membranes of RPTCs. $AT_2R$ trafficking to apical plasma membranes almost invariably accompanies natriuresis in response to intrarenal $AT_2R$ activation [21–23]. Recent studies have demonstrated that $AT_2R$ trafficking to apical plasma membranes is mediated through protein phosphatase 2A pathway and is likely a mechanism for sustaining $AT_2R$ responses to prolonged agonist stimulation [24,25]. This trafficking mechanism is effective in making $AT_2Rs$ available on the apical plasma membrane because, at least in RPTCs, the receptors do not internalize due to their inability to bind β-arrestin [26,27]. Indeed, in the present study β-Pro$^7$-Ang III-induced $AT_2R$ trafficking to RPTC apical plasma membrane was similar to that of Ang III or to effects evoked by Compound 21 in analogous studies suggesting that this is a class effect of $AT_2R$ agonists [21–23].

Collectively, these observations also support the notion that the natriuretic response to systemic administration of β-Pro$^7$-Ang III is likely mediated by renal $AT_2R$ activation, given that both systemic and intrarenal β-Pro$^7$-Ang III treatment produced significant natriuretic effects in the two experimental models. The $AT_2R$-mediated natriuretic effects were of a similar magnitude between the two models although higher doses of peptides were administered directly into the kidney, which reflects the fact that Ang peptide levels are often up to 1000-fold higher in kidney than plasma [28–30]. The effects of β-Pro$^7$-Ang III were also blocked by PD, which has been the standard $AT_2R$ antagonist used in this field, although it is worth noting that PD was recently reported to inhibit the MasR and Mas-related G-protein coupled receptor type D (MrgD), both of which are stimulated by Ang(1–7) [31]. We have previously reported that β-Pro$^7$-Ang III was not inhibited by the MasR antagonist A-779 [8]. However, a limitation of the present study was that we did not test β-Pro$^7$-Ang III against the putative MrgD antagonist, D-Pro$^7$ Ang(1–7) [31]. At present, there are no validated MrgD-binding assays to determine any potential binding at this site for β-Pro$^7$-Ang III or indeed for any other $AT_2R$ ligands, including Compound 21. Therefore, while it cannot be stated unequivocally that β-Pro$^7$-Ang III or even Compound 21 do not bind to other alternative sites, we are confident that β-Pro$^7$-Ang III, at least, is a highly selective $AT_2R$ agonist since we have shown it has >20,000-fold $AT_2R$ selectivity over $AT_1Rs$. In addition, the vasodilator effects of this peptide were absent in vascular tissue obtained from $AT_2R$ knock out mice and were blocked by PD but not by the MasR antagonist A-779 [8].

In conclusion, the present study provides functional evidence to support the β-amino acid substitution approach to design and synthesize novel selective $AT_2R$ peptide ligands from Ang III. Importantly, we showed that our *in vitro* evidence of selectivity of β-Pro$^7$-Ang III for the $AT_2R$ [8] translated into functional agonist activity in the kidney, and that these effects were similar to those of Ang III with respect to natriuresis and $AT_2R$ trafficking in RPTCs, in agreement with equi-effective *in vivo* vascular effects reported previously for these two peptides [8]. Moreover, the fact that β-Pro$^7$-Ang III and Compound 21 evoked similar systemic and renal vascular effects, together with natriuretic effects [8,12,14] suggests that these are 'class' effects due to $AT_2R$ activation. β-Pro$^7$-Ang III, therefore, represents

a novel $AT_2R$-selective peptidomimetic ligand and is an ideal candidate for future investigation into the functional relevance of the $AT_2R$ as a therapeutic target. Moreover, targeting the $AT_2R$ may be of greater cardiovascular benefit in females.

## Clinical perspectives

- We recently developed a highly selective $AT_2R$ peptide ligand, $\beta$-$Pro^7$-Ang III, using the $\beta$-amino acid substitution approach. The present study was undertaken to provide further functional evidence *in vivo* for the suitability of $\beta$-$Pro^7$-Ang III as a novel research tool for probing $AT_2R$ function and the therapeutic potential of the $AT_2R$.

- Acute administration of $\beta$-$Pro^7$-Ang III induced renal vasodilatory and natriuretic effects in both male and female normotensive rats; similar to that previously reported in response to the widely studied $AT_2R$ agonist, Compound 21. $AT_2R$ activation with $\beta$-$Pro^7$-Ang III promoted significant natriuretic effects whether administered systemically or directly into the kidney that were associated with $AT_2R$ translocation to the apical plasma membranes of RPTCs. The renal vasodilatory effects of $\beta$-$Pro^7$-Ang III were greater in female versus male rats at the highest dose of $\beta$-$Pro^7$-Ang III examined.

- Our findings provide supportive evidence for the use of $\beta$-$Pro^7$-Ang III as a novel $AT_2R$ agonist and research tool for exploring $AT_2R$ function and the viability of this receptor as a therapeutic target. Our findings also provide further supportive evidence of a sex-specific influence of pharmacological $AT_2R$ stimulation on renal function, warranting further studies into the sex-dependent cardioprotective effects of $AT_2R$ agonist therapy.

## Abbreviations
Ang III, angiotensin III; ANOVA, analysis of variance; $AT_1R$, angiotensin type 1 receptor; $AT_2R$, angiotensin type 2 receptor; CAND, candesartan; FENa, fractional sodium excretion; GFR, glomerular filtration rate; IP, intra-peritoneal; MAP, mean arterial pressure; PD, PD123319; RAS, renin–angiotensin system; RBF, renal blood flow; RI, renal interstitial; RPTC, renal proximal tubule cell; RT, room temperature; RVR, renal vascular resistance; UF, urine flow rate; $U_{Na}V$, urinary sodium excretion.

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