ERRATA

p xv, line 1: "[^{125}I]" for "[^{123}I"]

p 7, line 24: "diacyglycerol" for "dicylglycerol"

p 20, line 14: "inactivate" for "inactivatie"

p 55, line 18: "10 mm" for "10 m"

p 74, line 20: "telemetered" for "telemetrised"

p 91, line 12: space between "shown" and "to"

p 113, line 3: delete "and"

p 121, line 3: "produced" for "produces"

p 123, Reference 8: "(1-7)" for "(1ñ7)"

ADDENDUM

Examiner 1

Comment: Unfortunately, out of 6 copies of thesis, page 30 was inadvertently missing in one copy only, and this copy was sent to an Examiner. This page has now been inserted into appropriate section.

Comment: In Chapter 3, [^{125}I] Ang II was in fact used as the radioligand, and not [^{125}I] Sar^{1}Ile^{8} Ang II, as inferred by the Examiner.

Comment: Chapter 4, entitled "Differential mechanism of Ang (1-7)-mediated vasodepressor effect in adult aged rats", has now been accepted for publication in the International Journal of Hypertension.

Examiner 2:

Comment: While the $AT_{1b}R$ isoform is expressed in rodents, this isoform only gains prominence if the dominant $AT_{1a}R$ isoform is deleted, which was not the case for the studies performed in this thesis. Moreover, previous studies have revealed that binding profiles of $AT_{1a}R$ and $AT_{1b}R$ are not pharmacologically different [1-3].

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CARDIOVASCULAR STUDIES OF AT_2 RECEPTOR IN VARIOUS PATHOPHYSIOLOGICAL SETTINGS

A THESIS SUBMITTED TO

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DOCTOR OF PHILOSOPHY

By

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Summary

The renin angiotensin system (RAS) plays an important role in blood pressure regulation and its importance in cardiovascular disease has been well researched. Hypertension is a major risk factor for the development of cardiovascular disease and the RAS is a chief regulator of arterial pressure. The main hormone of RAS, the octapeptide angiotensin II (Ang II), exerts its actions through stimulation of two major receptor subtypes, the angiotensin type-1 receptor (AT_1R) and the angiotensin type-2 receptor (AT_2R). The focus of current literature has been on the activation of the Ang II-AT_1R axis and the unfavourable effects in cardiovascular pathology. However, shorter peptide fragments of Ang II are thought to have biological activity in their own right and elicit effects that oppose those mediated by Ang II by stimulating other RAS receptors such as AT_2R. Given that hypertension is well established in aged populations, it is of particular interest to determine role of the AT_2R in this setting. Additionally, it has been hypothesized that females have a greater vasodilator component of the RAS than males, which may contribute to sex differences in the rates of cardiovascular disease. Therefore, aspects of this thesis explored the function of AT_2R stimulation with particular attention directed towards the influence of age and sex.

In Chapter 2, HEK-293 cells stably transfected with either the AT_1R or the AT_2R were used to perform a systematic analysis of binding affinities of all the major Ang peptides. Additionally, the novel AT_2R agonist, Compound 21, as well as the *Mas*R agonist and antagonist, AVE0991 and A779 respectively, were tested for their ability to bind to the AT_1R or the AT_2R. Candesartan, CGP42214 and PD123319 were used as reference compounds. Binding studies using [^{125}I]-Sar^{1}Ile^{8}Ang II in AT_1R transfected HEK-293 cells revealed only Ang II, Ang III and candesartan to have high affinity for AT_1R. In AT_2R transfected HEK-293 cells,

competition for [^{125}I]-Sar^{1}Ile^{8}Ang II binding was observed for all ligands except candesartan, AVE0991 and A-779; the latter 2 compounds having negligible affinity at either AT_1R or AT_2R. The rank order of affinity of ligands at AT_2R was CGP42112 > Ang II ≥ Ang III > Compound 21 ≥ PD123319 >> Ang IV > Ang 1-7. Of note, while Ang IV and Ang (1-7) exhibited only modest affinity at AT_2R compared to that of Ang II, these two Ang peptides, together with Ang III, showed substantial AT_2R selectivity over AT_1R.

In Chapter 3, AT_1R and AT_2R selectivity as well functional vascular effects of a series of novel ligands, designed as potential AT_2R agonists, were tested. Binding experiments performed in HEK-293 cells transfected with either AT_1R or AT_2R revealed that the relatively simple method of β-substitution performed at position 4 (β-Tyr Ang II) or position 5 (β-Ile Ang II) of the native Ang II sequence yielded ligands that were approximately 1000-fold more selective at AT_2R compared to AT_1R. Both ligands evoked vasorelaxation in precontracted aortic rings that was sensitive to AT_2R and nitric oxide (NO) inhibition. However, only β-5 Ile Ang II acutely lowered blood pressure in conscious spontaneously hypertensive rats (against a background of AT_1R blockade). Therefore, a single β-amino acid substitution resulted in a ligand that exhibited vasorelaxation and vasodepressor properties via stimulation of the AT_2R, as these effects were blocked by the AT_2R antagonist, PD123319.

In Chapter 4, the effect of aging on Ang (1-7)-mediated vasodepressor effects and vascular receptor localization was determined. Previous research indicated that (Ang (1-7)) evoked opposing effects to the actions of Ang II, acting via MasR and/or AT_2R sites. Based on binding results from Chapter 2 and previous laboratory data, blood pressure was measured in conscious adult (~17 weeks) and aged (~19 months) normotensive rats. During AT_1R blockade, Ang (1-7) reduced

blood pressure in adult normotensive rats via AT_2R. However, in aged rats, the depressor effect of Ang (1-7) was maintained but also inhibited by either AT_2R blockade or *Mas*R blockade. At the same time, AT_2R, *Mas*R and ACE2 immunoreactivity was markedly elevated in aortic sections from aged animals. These results indicate that the Ang (1-7)-mediated depressor effect was preserved in aged animals and involved both AT_2R and *Mas*R, whereas Ang (1-7) effects were mediated exclusively via stimulation of AT_2R in adult rats.

Chapters 5 and 6 examined the effect of both chronic and acute treatment with the AT_2R selective nonpeptide agonist, Compound 21. In Chapter 5, chronic treatment with Compound 21 failed to lower blood pressure in normotensive female mice or rats. However, in Chapter 6, the effect of acute administration of Compound 21 was examined in an hypertensive model in which β-5 Ile Ang II had already been shown to exert a vasodepressor effect (Chapter 3). In this case, against a background of AT_1R blockade, Compound 21 reduced blood pressure in adult hypertensive rats, an effect that was reversed by AT_2R blockade. Additionally, Compound 21 evoked vasorelaxation in both mouse and rat vessels via AT_2R stimulation. The development of Compound 21 has resulted in a useful tool for further investigation of AT_2R function but this thesis has also highlighted the inability of this compound to lower blood pressure under chronic conditions.

In conclusion, this thesis provides evidence for the hypothesis that shorter Ang peptide fragments may act as endogenous AT_2R ligands. Taken collectively, these results emphasise the importance of understanding the complex relationships that exist within the RAS. In particular, this thesis highlights that the AT_2R axis as well as the Ang (1-7)/*Mas*R axis (at least in aging) may act as counter-regulatory mechanisms to the Ang II/AT_1R axis. Moreover, the development of additional AT_2R ligands utilising relatively simple synthetic methods as shown in this thesis

is warranted as these receptors are potential therapeutic targets for the treatment of hypertension and related cardiovascular remodelling.

General Declaration

Monash University

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made.
I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.
This thesis includes 3 original papers published in peer reviewed journals and 2 unpublished publications. The core theme of the thesis is mechanisms of AT_2 receptor-mediated cardiovascular function, the influence of age and gender. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Pharmacology under the supervision of Professor Robert Widdop and Dr Emma Jones.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 1 to 7, my contribution to the work involved the following:

Chapter 1: General Introduction was written by myself with editorial assistance from Professor Robert Widdop and Dr Emma Jones.

Chapter 2: I completed all of the experiments and data analysis. I also wrote the manuscript with editorial assistance from co-authors.

Chapter 3: I completed the *in vivo* experiments and radioligand binding data analysis. I also contributed to the manuscript write up with editorial assistance from co-authors.

Chapter 4: I completed all of the experiments and data analysis. I also wrote the manuscript with editorial assistance from co-authors.

Chapter 5: I completed all of the experiments and data analysis, except organ bath experiments (performed by Ms Iresha Welungoda). I also wrote the manuscript with editorial assistance from Professor Robert Widdop and Dr Emma Jones.

Chapter 6: I completed all of the experiments and data analysis. I also wrote the manuscript with editorial assistance from co-authors.

Chapter 7: General discussion was written by myself with editorial assistance from Professor Robert Widdop and Dr Emma Jones.

Thesis Chapter	Publication Title	Publication Status	Nature and extent of candidates contribution
2	Relative affinity of angiotensin peptides and novel ligands at AT_1 and AT_2 receptors	Accepted	80%
3	A single β-amino acid substitution to Angiotensin II confers AT_2R selectivity and vascular function	Published	50%
4	Differential mechanism of Ang (1-7) -mediated vasodepressor effect in adult aged rats	Submitted	80%
6	Stimulation of angiotensin AT_2 receptors by the non-peptide agonist, Compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats	Published	80%

Signed: ……

Date: ……

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It truly is an extraordinary feeling to be writing this section of my thesis, as it means I have come to an end of a journey that has been for most part an unbelievable experience. It is a great pleasure to thank those who made this thesis possible and I must begin by thanking my co-supervisors Associate Professor Robert Widdop and Dr Emma Jones. Thank you both for taking me on as your honours student and providing me with the opportunity to further my studies as your PhD student. Rob, your guidance and encouragement along the way have been greatly appreciated. You knowledge, intellectual insight and your tireless commitment to both teaching and research never ceased to amaze me. Thank you for believing in me. I have gained so much, both professionally and personally, by having you as my mentor, and I cannot thank you enough for this opportunity. To Emma, thank you for all your help over the last few years, for sharing your surgical skills with me, for your patience, and your friendship. Thank you for being the first to read my drafts (I know that couldn't have been easy), and for always being available when frustrations took hold of me. Thank you both - I am forever grateful.

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This work is dedicated to my parents, without you I wouldn't have been able to achieve this.

Publications

JOURNAL ARTICLES

Bosnyak S, Welungoda IK, Hallberg A, Alterman M, Widdop RE, Jones ES. Stimulation of angiotensin AT(2) receptors by the non-peptide agonist, Compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats. *British Journal of Pharmacology.* (2010) **159**, 709-716

Jones ES, Del Borgo MP, Kirsch JF, Clayton D, **Bosnyak S**, Welungoda I, Hausler N, Unabia S, Perlmutter P, Thomas WG, Aguilar MI, and Widdop RE. A Single beta-Amino Acid Substitution to Angiotensin II Confers AT(2) Receptor Selectivity and Vascular Function. *Hypertension*. (2011) **57,** 570-576.

Bosnyak S, Jones ES, Christopoulos A, Aguilar MI, Thomas WG, Widdop RE. Relative affinity of angiotensin peptides and novel ligands at AT1 and AT2 receptors. *Clinical Science. (4 May 2011)***. (Epub ahead of print)**

PUBLISHED ABSTRACTS

Bosnyak S, Jones ES, Widdop RE (2007) Vasodepressor effect of Ang-(1-7) is via stimulation of AT_2R and is preserved in aged WKY rats. *2nd International Conference in Frontiers in Vascular Medicine, 103, Poster presentation.* (pg. 103)

Bosnyak S, Jones ES, Widdop RE (2007) Vasodepressor effect of Ang-(1-7) is via stimulation of AT_2R and is preserved in aged WKY rats. *Proceedings of the 29th Annual Scientific Meeting of the High Blood Pressure Research Council of Australia, Poster 32*. (abstract 32)

Bosnyak S, Hallberg A, Jones ES, Widdop RE. (2008) Vasodepressor effect of Compound 21 is via stimulation of AT_2R in conscious SHR. *Proceedings of the 30th Annual Scientific Meeting of the High Blood Pressure Research Council of Australia, Poster 144*. (abstract 144)

Bosnyak S, Jones ES, Vinh A, Widdop Re (2008) Vasodepressor effect if Ang-(1-7) is preserved in aged rats. *Proceedings of the 18th Scientific Meeting of the European-*

Society-of-Hypertension/22nd Scientific Meeting of the International-Society-of-Hypertension, Poster 20/MON/47 (pg. S300)

Bosnyak S, Jones ES, Widdop Re (2009) Vasodepressor effect of Ang-(1-7) is preserved in aged rats. *The RAS Club: An Expanding System, Oral Presentation.* (abstract 10)

List of abbreviation

[^{123}I]	Iodine-125
A-779	[D-Alanine7]-Angiotensin (1-7) (Asp-Arg-Val-Tyr-Ile-His-D-Ala)
ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme 2
ADH	Antidiuretic hormone
Ang I	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)
Ang II	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)
Ang III	Angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Phe)
Ang IV	Angiotensin IV (Val-Tyr-Ile-His-Pro-Phe)
Ang (1-7)	Angiotensin (1-7) (Asp-Arg-Val-Tyr-Ile-His-Pro)
Ang (1-9)	Angiotensin (1-9) (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His)
Ang (1-12)	Angiotensin (1-12) (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Tyr)
ANOVA	Analysis of variance
AP-N	Aminopeptidase N
AT_1R	Angiotensin type 1 receptor
$AT_{1A}R$	Angiotensin type 1A receptor
$AT_{1B}R$	Angiotensin type 1B receptor
AT_2R	Angiotensin type 2 receptor
AT_4R	Angiotensin type 4 receptor
AVE0991	5-formyl-4-methoxy-2-phenyl-1-[[4-[2-ethyl-aminocarbonylsulfonamido-5-isobutyl-3-thenyl1]-phenyl]-methyl]-imidiazole
β−Asp Ang II	βAsp-Arg-Val-Tyr-Ile-His-Pro-Phe
β−Ile Ang II	Asp-Arg-Val-Tyr-βIle-His-Pro-Phe
β−Tyr Ang II	Asp-Arg-Val-βTyr-Ile-His-Pro-Phe
B_2R	Bradykinin type 2 receptor
bpm	beats per minute
BK	Bradykinin
BSA	Bovine serum albumin
BP	Blood pressure
Ca^{2+}	Calcium ion
cGMP	cyclic guanosine 3′,5′-monophosphate
CGP42112	Nicotinoyl-Tyr-Lys(Z-Arg)-His-Pro-Ile
CHO	Chinese hamster ovary
cpm	counts per minute
CVD	Cardiovascular disease
DAG	(1,2)-diacylglycerol
DMEM	Dulbecco's modified essential medium
DS rat	Dahl salt rat
eNOS	Endothelial nitric oxide synthase

ERK	Extracellular signal regulated kinases
FBS	Foetal bovine serum
G418	Gentamicin sulphate 418
GPCR	G protein coupled receptors
H_2O_2	Hydrogen peroxide
HEK-293	Human embryonic kidney cells – 293
HEPES	N-(2-hydroxyethyl)piperazine-N′(2-ethanesulphonic acid)
HR	Heart rate
IC_{50}	Half maximal inhibitory concentration
ICAM	Intracellular adhesion molecule
IP_3	Inositol triphosphate
IRAP	Insulin regulated aminopeptidase
IV	Intra venous
K_i	Dissociation constant
JAK	Janus family kinase
KO	Knock out
L-NAME	N^G-nitro-L-arginine methyl ester
MAP	Mean arterial pressure
NOS	Nitric oxide synthase
NO	Nitric oxide
NaOH	Sodium hydroxide
NEP	Neutral endopeptidase 24.11
NADPH	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide
NaCl	Sodium chloride
mRNA	Messenger ribonucleic acid
mmHg	Millimetres of mercury
*Mas*R	mas receptor
MAPK	Mitogen activated protein kinase
PAI-1	Plasminogen activator inhibitor-1
PD123319	(s)-1-[[4-(dimethylamino)-3-methyl-phenyl]methyl]-5-(dipheylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazol[4,5-c]-pyridine-6-carboxylic acid
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
PKC	Protein kinase C
PLA_2	Phospholipase A2
PLC	Phospholipase C
PP_2A	Serine/Threonine phosphatase
PRR	Pro(renin) receptor
PTPase	protein tyrosine phosphatases
RAS	Renin angiotensin system
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SD rat	Sprague Dawley rat
SHP-1	SH2 domain-containing phosphatase 1

SHR	Spontaneously hypertensive rat
SHRSP	Spontaneously hypertensive stroke prone rat
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor - β
U46619	[1,5,5-hydroxy-11α,9α-(epoxymethano)prosta-5Z, 13E-dienoic acid]
VSMC	Vascular smooth muscle cell
WT	Wild type

Chapter 1:

General Introduction

1.1 RENIN-ANGIOTENSIN SYSTEM

The renin-angiotensin system (RAS) is considered one of the most important regulatory systems for cardiovascular and renal homeostasis [2]. The RAS exerts its effects mainly by influencing vascular tone, fluid and electrolyte balance as well as the sympathetic nervous system [3]. Furthermore, the influence of the RAS cascade is involved in long-term effects on cardiovascular (CV) structure such as cellular proliferation, differentiation, regeneration and apoptosis [4].

Traditionally the RAS has been considered a circulating system. This view proposes that renin, an aspartic protease that is synthesized in the juxtaglomerular cells of the kidney, acts on angiotensinogen, a protein synthesized principally in the liver but also locally, to produce the inactive decapeptide, angiotensin I (Ang I) [3, 5]. This is the rate-limiting step of the RAS, with release of renin controlled by a variety of stimuli such as increased sympathetic stimulation, a fall in renal perfusion pressure as well as circulating prostaglandins [6]. The renin gene encodes a 401 amino acid residue protein, prorenin, which is synthesized in the juxtaglomerular cells of the afferent arteriole in the kidney [2]. Renin is known to exist in two forms, mature renin which actively cleaves angiotensinogen, and the proenzyme, prorenin. Interestingly, prorenin is synthesized in a limited number of tissues and lacks enzymatic activity. However, it has been suggested that prorenin represents up to 90% of total plasma renin in normal physiological conditions while in certain pathological conditions it can circulate at 100-fold higher concentrations than mature renin [7].

The main effector of the RAS, the octapeptide angiotensin II (Ang II), mediates most of the biological actions of this system, and is cleaved from Ang I by angiotensin-converting enzyme (ACE) [8]. ACE is a glycoprotein with a

molecular mass of 180 kDa and two active sites [2], and exists in plasma as the circulating hormone, but it is also present in many tissues such as the heart, brain, blood vessels, adrenal glands, kidney, liver and reproductive organs [9]. In 2000, a homologue of ACE, ACE2, was discovered [10-12], thus expanding our view of the RAS (Figure 1.1). ACE2 is a zinc metalloprotease consisting of 805 amino acids with significant homology to ACE [2]. Comparable to ACE, ACE2 has a wide distribution throughout the body, and is particularly expressed in endothelial and vascular smooth muscle cells of the heart, kidney and testis [11]. Interestingly, although ACE2 shares 56% of the homology with the N-terminal domain of ACE [11] it is not sensitive to ACE inhibitors and does not metabolise bradykinin (BK) [12]. ACE 2 has been shown to cleave Ang I to produce Angiotensin (1-9) (Ang (1–9)), which may then be converted to the hexapeptide angiotensin (1-7) (Ang (1-7)), by ACE. However, ACE2 may also directly metabolise Ang II to produce Ang (1–7), which occurs at a faster rate than the formation of Ang (1-9) [2, 10, 13]. It is interesting to note that ACE2 expression is upregulated in pathological conditions such as heart failure [12], and is also upregulated in heart of the humans treated with angiotensin receptor blockers (ARBs) [14], suggesting an important role for this enzyme in cardiovascular disease states.

More recently, a novel peptide belonging to the RAS family has been identified as the dodecapeptide, Angiotensin (1-12) (Ang (1-12)) [15]. Ang (1-12) has been detected in many tissues including kidney, heart and brain and in these locations this peptide was present in levels higher than Ang I or Ang II [15]. These observations suggest that Ang (1-12) may be an additional player in the RAS, and may in fact be an alternative endogenous substrate for the formation of biologically active angiotensins. Indeed, recently it was revealed that enzymes

ACE, neutral endopeptidase 24.11 (NEP) and chymases, catalyse the conversion of Ang (1-12) to produce biologically active Ang II and Ang (1-7) [16].

Interestingly, recent studies have documented Ang (1-12) expression in the heart and kidney of both Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR), and this endogenous Ang (1-12) expression was significantly higher in SHR compared to WKY rats [17]. Additionally, it was documented that formation of Ang I, Ang II and Ang (1-7) occurred via cleavage of Ang (1-12) via a non-renin pathway in isolated hearts from both normotensive and hypertensive rats [18], and formation of Ang II via a renin-independent-Ang (1-12) pathway was also observed in anephric rats [18]. Thus although in its infancy, the investigation of this peptide is ongoing and seems to be of interest in the context of cardiovascular disease.

1.2 ANGIOTENSIN RECEPTORS

Ang II is the main effector of the RAS and plays an important role in cardiovascular function by influencing vascular tone, structure, fluid and electrolyte balance. Ang II has a very short plasma life in the circulation of ~30 seconds, while in the tissue, half-life may be as long as 15 to 30 minutes [19]. Most of the well-known biological actions of Ang II are mediated by a heterogeneous population of membrane-bound Ang II receptors, located on target cells, to activate multiple intracellular transduction pathways [3, 20].

Ang II binds with high affinity to two main receptor subtypes, Ang II receptor subtype 1 (AT_1R), and type 2 (AT_2R) [21, 22], although most of the established cardiovascular functions of Ang II, such as vasoconstriction, water and salt retention, aldosterone synthesis, cellular growth and remodeling are mediated via the Ang II-AT_1R interaction [23]. On the other hand, AT_2R stimulation is

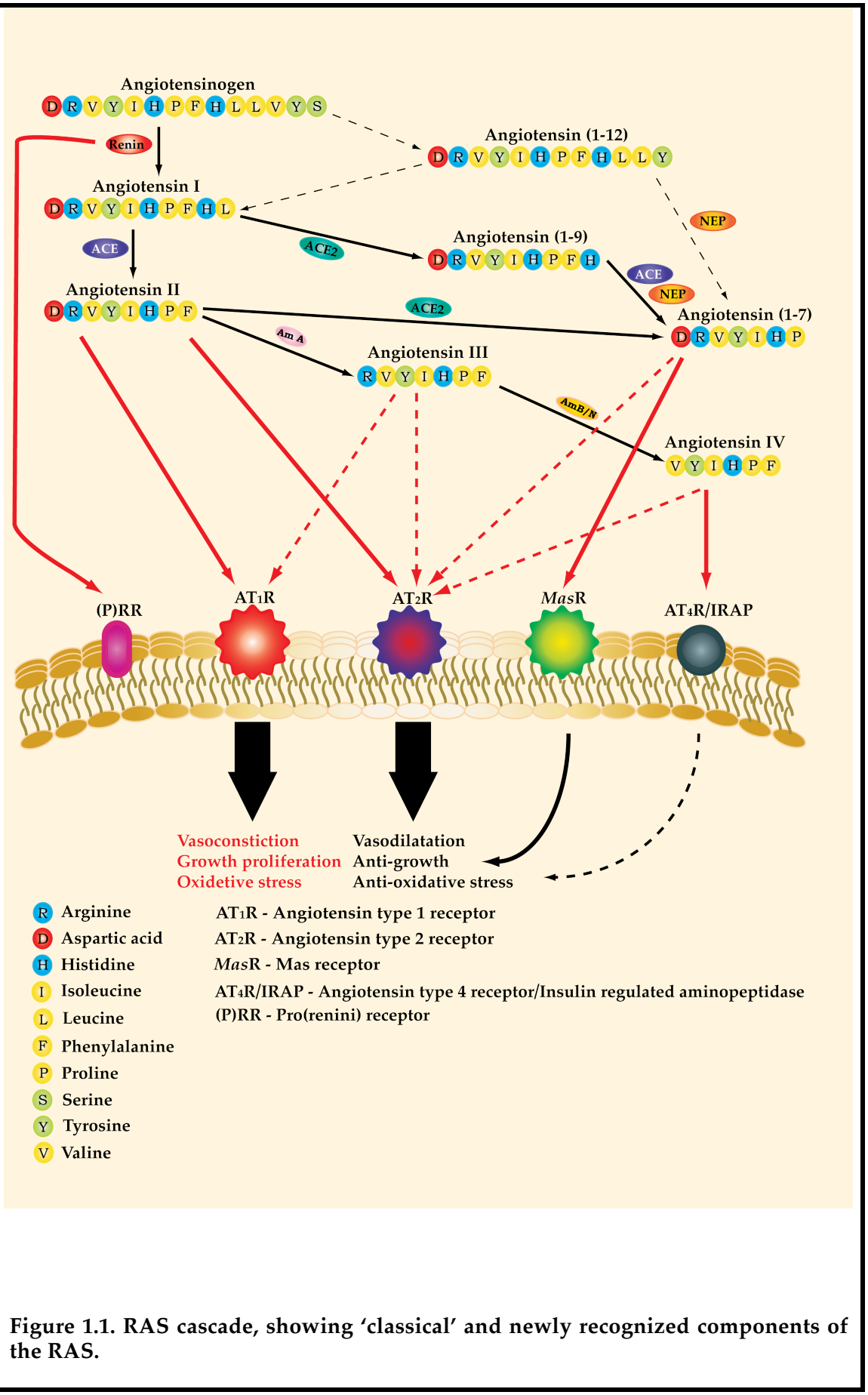

Figure 1.1. RAS cascade, showing 'classical' and newly recognized components of the RAS.

believed to oppose AT_1R-mediated effects, by promoting cardiovascular protective processes such as vasodilation [24, 25], anti-remodeling [26] and potential anti-atherosclerotic effects [27]. Ang II has similar affinity for both AT_1R and AT_2R, while other synthetic compounds such as candesartan, CGP42112, PD123319 and Compound 21, display high affinity for only one of the two receptor subtypes. For example, the AT_1R antagonists, including losartan, candesartan and irbesartan, selectively bind to the AT_1R with high affinity [3, 21], but have little affinity for AT_2R. On the other hand, ligands such as PD123319 (antagonist), or CGP42112 and Compound 21 (agonists), have been shown to be highly selective for the AT_2R [28-30].

Other binding sites have been categorized which bind specific angiotensin peptides, and exert biological effects in their own right. In this context, the Ang (1-7) receptor, also known as the *Mas* receptor (*Mas*R), preferentially binds Ang (1-7) [31], whilst insulin-regulated aminopeptidase (IRAP), preferentially binds angiotensin IV (Ang IV) [32].

Recently, renin has also been shown to have its own specific binding site identified as the specific renin/prorenin-receptor, (pro)renin receptor (PRR) [33]. The actions of renin/prorenin have stimulated considerable interest following the discovery that increased renin/prorenin at the cell surface can contribute to greater Ang I and Ang II formation in the immediate vicinity of Ang receptors and thus create a favourable environment for Ang II binding. In addition, signalling mechanisms have also been reported. For example, binding of renin and prorenin to PRR results in phosphorylation of the mitogen activated protein kinases (MAPKs) and extracellular signal-related kinases 1/2 (ERK 1/2) leading to the up-regulation of the pro-fibrotic genes such as transforming growth factor β1 (TGF-β1), plasminogen activator inhibitor-1 (PAI-1), collagens and fibronectin

[34, 35]. Indeed, PRR has been suggested to contribute to the pathogenesis of diabetes and hypertension, as its inhibition prevented the development of diabetic nephropathy in streptozotocin-induced rats [36], and also decreased perivascular fibrosis and left ventricular hypertrophy in spontaneously hypertensive stroke prone (SHRSP) rats [37]. Furthermore, overexpression of PRR in rats led to an increase in systolic blood pressure and heart rate, and this effect seemed to worsen with increased age [38]. Although exact mechanism by which these effects occur are still under investigation, it is becoming more clear that they are not simply due to the increased synthesis of Ang II, but are linked to altered PRR-mediated cardiovascular and renal functions [39].

1.2.1 AT_1 RECEPTOR

1.2.1.1 STRUCTURE, FUNCTION AND SIGNALLING

The AT_1R belongs to the seven-transmembrane class of G-protein coupled receptors (GPCR) superfamily [40]. The AT_1R gene product consists of 359 amino acids, has a molecular mass of 41 kDa, and in humans, the gene coding for the AT_1R is located on chromosome 3 [3, 20]. In rodents, two highly homologous subtypes of AT_1R have been identified, known as $AT_{1A}R$ and $AT_{1B}R$. Both isoforms share 94% sequence homology, and differ in only 22 of 359 amino acids [41]. In humans, only one subtype of the AT_1R has been identified and is expressed ubiquitously in all organs of cardiovascular relevance, including heart, kidney, lung, vascular smooth muscle, brain and adrenal glands [42].

AT_1R signalling involves multiple pathways predominantly activating phospholipase C (PLC), leading to phosphatidylinositol hydrolysis, formation of inositol triphosphate (IP_3) and dicylglycerol accumulation (DAG). The formation of IP_3 results in the release of intracellular calcium (Ca^{2+}), while DAG activates

the Na^+/H^+ exchanger via protein kinase C (PKC). Activation of the Na^+/H^+ via PKC results in cellular alkanisation, as well as phosphorylation of the target proteins resulting in the activation of physiological processes [3]. Therefore, a rise in intracellular Ca^{2+} levels together with a decrease in pH will initiate rapid cellular contraction [40, 43].

Other signaling pathways triggered by Ang II stimulation of the AT_1R include activation of MAPK, such as ERK1/2, Janus activated kinase-1 (JAK-1) and activators of transcription (STAT) pathways [23]. These pathways are responsible for gene transcription and stimulation of the early growth response genes, c-fos and c-jun, and therefore play an important role in the control of the Ang II-mediated cellular proliferation and differentiation [23, 44].

Additionally, Ang II-mediated stimulation of the AT_1R may result in the formation of reactive oxygen species (ROS), contributing to vasopressor activity and cardiovascular remodeling. In many situations, AT_1R stimulation causes the activation of the NADH/NADPH oxidase, and increased expression of many inflammatory mediators such as intracellular adhesion molecule (ICAM-1) and macrophage infiltration [45, 46]. Subsequent production of superoxide (O_2^-) and H_2O_2 activates MAPK, tyrosine kinase and tyrosine phosphatase pathways, which are involved in vascular contraction, cell growth and inflammation [47]. In addition, the AT_1R is also known to decrease endothelial nitric oxide synthase expression (eNOS), which again can potentiate vascular dysfunction and hypertension [45, 46]. In this context, both ACE inhibitors and AT_1R antagonists have been shown to decrease ROS production and oxidative stress by inhibiting Ang II-mediated NADPH oxidase activation via AT_1R [48, 49].

Thus, most of the known biological and pathological actions of Ang II are mediated through activation of the AT_1R [23]. These actions include vasoconstriction, stimulation of adrenal aldosterone and anti-diuretic hormone

(ADH) secretion, the induction of thirst, enhancement of renal tubular Na^+ absorption, activation of the sympathetic nervous system, increased cardiac contractility, stimulation of NADPH oxidase and stimulation of cell proliferation [21, 23, 29, 43, 50], with many of these contributing to the development of cardiovascular diseases.

1.2.1.2 AT_1 RECEPTOR SELECTIVE LIGANDS

There are many AT_1R antagonists that are divided into different categories according to their structure. These categories include biphenyl tetrazoles (candesartan, losartan, EXP3172 and irbesartan); tetrazoles (eprosartan and telmisartan) and the nonheterocyclics (valsartan) [51]. Furthermore, these AT_1R antagonists also vary in pharmacodynamic and pharmacokinetic properties, including their affinity for the AT_1R. For example in Chinese hamster ovary (CHO) cells expressing AT_1R, the relative order of the binding affinity of the various AT_1R antagonists was found to be: candesartan > irbesartan/valsartan > EXP31274/telmisartan > taosartan > losartan > eprosartan [52]. The AT_1R antagonists mostly bind within the transmembrane regions to inhibit AT_1R function [51].

1.2.2 AT_2 RECEPTOR

1.2.2.1 STRUCTURE, FUNCTION AND SIGNALING

In contrast to the well-established cardiovascular effects mediated by the AT_1R, the physiological role of the AT_2R remains controversial. The AT_2R is highly expressed during foetal development in both rodents and humans, but expression rapidly declines after birth with the AT_1R becoming the dominant receptor subtype in adult [23, 53]. Although AT_2R levels are significantly decreased in adulthood, specific tissues such as the uterus, adrenals, kidney,

heart, ovaries, both endothelial and vascular smooth muscles (VSMCs) and distinct areas of the brain maintain high AT_2R expression levels [2, 3, 42]. An early report documented AT_2R expression in the vasculature to be approximately 30% of total Ang II receptors in aortic VSMCs of adult rats [54]. Since then, AT_2R expression has been observed in multiple vascular sites such as uterine artery [55], renal [56], mesenteric, [57, 58], skeletal microcirculation [59] and in human [24] and mouse [60, 61] coronary arteries. In the vasculature, the AT_2R are localised in all layers of blood vessels [62]. Furthermore, under pathological conditions such as myocardial infarction, heart failure, atherosclerosis and hypertension, AT_2R expression is upregulated implying a protective or compensatory role for the AT_2R during cardiovascular disease [63-68].

Structurally, AT_2R are distinct from AT_1R, sharing only 34% in sequence homology, and activating divergent signaling pathways [69]. Similarly to AT_1R, AT_2R is also a seven-transmembrane GPCR, containing 363 amino acids with a molecular weight of 41kDa [70]. AT_2R stimulation is believed to initiate three major transduction mechanisms (Figure 1.2). These include; i) activation of protein phosphatases resulting in protein dephosphorylation, ii) regulation of nitric oxide/cyclic guanosine 3',5'-monophosphate (NO-cGMP) system and iii) stimulation of phospholipase A2 (PLA_2) and subsequent release of arachidonic acid (AA) [71] (Figure 1.2).

In terms of activation of protein tyrosine phosphatases (PTPase), stimulation of AT_2R leads to activation of three major PTPase: MAPK-1, SH2 domain-containing phosphatase 1 (SHP-1), and protein phosphatase 2A (PP2A) [1]. All three PTPase lead to dephosphorylation and subsequent inhibition of ERK1/2 [4]. Evidence for this pathway was provided from studies performed in PC12W cell (containing only AT_2R), in which Ang II rapidly activated PTPase resulting in dephosphorylation and inhibition of tyrosine residues [72, 73]. Furthermore,

AT_2R-mediated PTPase activity has also been demonstrated in N1E-115 neuroblastoma cells and R3T3 fibroblasts [74, 75], and has been shown to occur via G protein-dependent and independent pathways. Dephosphorylation and subsequent inactivation of MAP kinases ERK1/2 plays an important role in the induction of apoptosis and inhibition of growth [25].

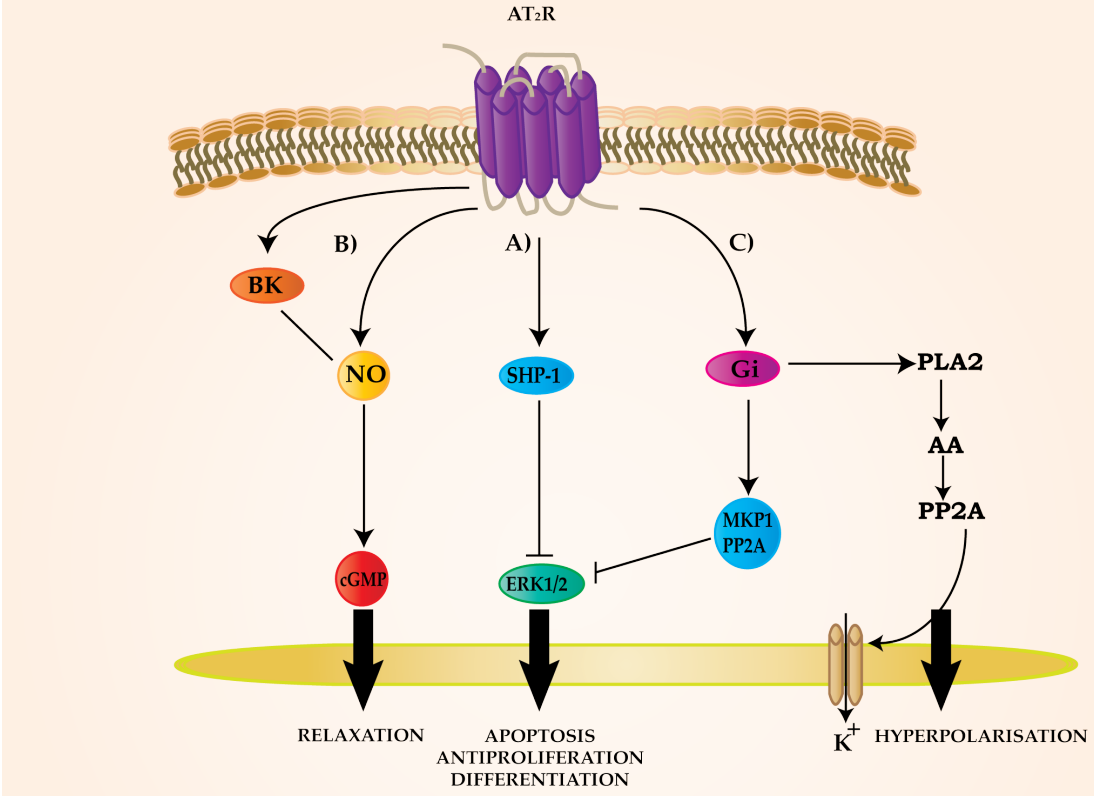

Figure 1.2. Signal transduction mechanism and physiological effects mediated by the AT_2R. (Adapted from [1])

AT_2R stimulation is well known to increase production of NO, cGMP and BK and thereby produce both vaso- and cardio-protective effects [22]. Furthermore,

many studies have demonstrated involvement of BK in the NO/cGMP signaling pathway via bradykinin B2-receptor (B_2R) [76-79]. For example, in *in vivo* settings, the Ang II-mediated increase in cGMP levels in renal interstitial fluid was reduced by AT_2R blockade, nitric oxide synthase (NOS) inhibition and B_2R blockade [77, 80, 81]. Additionally, AT_2R inhibition decreased cGMP production in transgenic mice which overexpressed AT_2R in VSMC [82], and in SHRSP rats [83]. The activation of the BK/NO/cGMP cascade by AT_2R stimulation has also been demonstrated in bovine aortic endothelial cells (BAEC) [84], isolated rat carotid arteries [85], canine coronary arteries [86], rat isolated aorta [87], rat kidney [79] and rat heart [88]. Furthermore, Siragy and Carey (1999) have demonstrated AT_2R-mediated vasodilatation to be via BK, NO and cGMP, as these mediators were diminished in AT_2R null-mice. Collectively, these studies indicate a vasodilator role of AT_2R activation to be via the cGMP/NO/BK pathway.

However, some *in vitro* studies have demonstrated NO/cGMP production to be sensitive to inhibition by both losartan and PD12331 in isolated blood vessels from animals [78, 85, 86] and humans [24], indicating that on occasions AT_1R and AT_2R may act in concert to increase NO and cGMP levels.

In vivo evidence has demonstrated AT_2R mediated vasorelaxation in various vascular beds such as mesenteric, renal, cerebral, coronary and uterine (for review, see [53]). Ang II has been observed to stimulate flow-induced dilation of perfused mesenteric arteries *in situ* [89], where AT_2R blockade caused a decrease in diameter of arterial branches exposed to pressure and flow. This effect was prevented with NOS inhibition as well as endothelial disruption [89]. Collectively, these results suggest that Ang II activates endothelial NO/cGMP pathways via AT_2R, contributing to flow induced dilation and supporting the notion that the AT_2R has a role in blood pressure regulation.

Interestingly, early investigations of AT_2R-mediated effects that used Ang II in the presence of AT_1R blockade to stimulate AT_2R, reported both vasodilator [90] and vasoconstrictor functions of AT_2R [91]. These differences may be due to the vasodilator effect of AT_2R stimulation being masked by the prevailing AT_1R induced vasoconstrictor effect of Ang II. This difficulty in observing AT_2R-mediated vasodilatation during AT_1R blockade led several groups to determine effects of the selective AT_2R agonist, CGP42112, during concomitant AT_1R blockade. Indeed, selective AT_2R stimulation using CGP42112 reduced blood pressure in SHRs [92]. A similar CGP42112-mediated effect was also observed in Sprague-Dawley (SD) rats [93]. Furthermore, this blood pressure reduction was found to be associated with vasodilator effects in the renal, mesenteric and hindquarter circulations that contributed to the blood pressure lowering effects of CGP42112 and was abolished by the AT_2R antagonist, PD123319, confirming AT_2R involvement [94]. Moreover, in perfused mesenteric resistance arteries, administration of Ang II, in the presence of AT_1R blockade, led to a significant increase in the internal diameter [95]. Importantly, not only was this vasodilator response sensitive to PD123319, but it was highly reproducible [95]. More recently, increased AT_2R expression was observed in human small peripheral resistance arteries, following chronic treatment with the AT_1R antagonist, valsartan, which resulted in enhanced AT_2R-mediated relaxation to Ang II [96].

Additionally, Ang II stimulation of AT_2R can lead to activation of PLA_2, release of AA, activation of the Na^+/HCO_3^- symporter system, which in turn regulates intracellular pH [25, 97]. This PLA_2-mediated process has been postulated as a mechanism by which AT_2R modulates intracellular acidosis following injury [97].

1.2.2.2 AT_2 RECEPTOR SELECTIVE LIGANDS

Despite potential beneficial effects of AT_2R activation, research into the exact role of AT_2R has been hampered by a lack of selective and readily available drugs. To date, CGP42112 has been the gold standard compound, which has been instrumental in elucidating function of the AT_2R subtype. CGP42112 is a modified pentapeptide analogue of Ang II with a Ki of less than 1 nmol/L for the AT_2R [28], and was found to be 1000 fold more selective for AT_2R compared to AT_1R [28]. CGP42112 has been used in an iodinated form to identify AT_2R receptor localisation [98]. However, although CGP42112 has been important tool for the investigation of the AT_2R function, there is need for alternative AT_2R-selective ligands as CGP42112 is a large peptide molecule and as such is metabolically unstable.

Additionally, PD123319 has been identified as a selective AT_2R antagonist. Indeed, PD123319 binds selectively to the AT_2R to block effects mediated by this receptor. Furthermore, PD123319 has been found to have a K_i between 10 and 100 nmol/L for the AT_2R, as recently reviewed [99], where as a close analogue of PD123319, PD123177, was found to be 3 500 times more selective for the AT_2R compared to the AT_1R [21]. PD123319 is a dihydropyridine and the use of this compound has implicated cardiovascular, renal, central and adrenal functions for this receptor [23].

Recently, Wan *et al.,* (2004), reported the first drug-like, non-peptide, selective AT_2R agonist, Compound 21. Compound 21 was derived from the non-selective AT_1R and AT_2R agonist, L-162313 [30], and was found to have a half-life of 4 hours with 20-30% bioavailability following oral administration in the rat [30]. Compound 21 has a K_i value of 0.4 nM for AT_2R and a K_i > 10 μM for AT_1R [30], thus demonstrating high selectivity for AT_2R. In the original study, in

anesthetized SHR, a bolus injection of Compound 21 lowered mean arterial pressure (MAP) at intravenous doses ranging from 0.008 to 0.05mg/kg [30]. In the same study, Compound 21 was examined at doses as high as 4mg/kg, but was reported to have significant vasodepressor effects only at lower doses (i.e. 0.008 to 0.05mg/kg) [30]; however, only the results for 0.05mg/kg dose of Compound 21, were shown by the authors. PD123319, an AT_2R antagonist, attenuated the vasodepressor effects of Compound 21, although these data were quite variable, which may have been influence by the state of anaesthesia. In a separate set of experiments, the effects of intravenously administered Compound 21, were examined on duodenal alkaline secretion in anaesthetized SD rats [30]. It was observed that Compound 21 dose dependently increased alkaline secretion in the gut of non-fasted animals, and stimulated alkaline secretion when applied directly to the mucosa by intraluminal administration [30]. Gastric secretion resulting from both topical and intravenous administration of Compound 21 was negated by PD123319, once again suggesting AT_2R involvement.

More recently, a 7-day treatment with Compound 21 in WKY rats with heart failure resulted in improved systolic and diastolic ventricular function [100]. In the same study, Compound 21 was found to exhibit anti-apoptotic and anti-inflammatory characteristics, by reducing the release of Fas-ligand, caspase 3 and a series of cytokines [100]. In fact, Compound 21 appeared to offer greater cardio-protection when compared to candesartan [100], thereby providing the first *in vivo* evidence for potential therapeutic applications of this novel AT_2R agonist.

Clearly, direct stimulation of the AT_2R, using selective AT_2R ligands could prove beneficial in hypertension but also in pathological conditions associated with inflammation such as myocardial fibrosis, atherosclerosis, myocardial infarction and myocarditis [101].

1.3 NOVEL TARGETS OF RAS: ANGIOTENSIN (1-7) – *MAS* RECEPTOR

1.3.1 ANGIOTENSIN (1-7)

As already mentioned, Ang (1-7) can be formed from either Ang I or Ang II, and the interest in this peptide has been re-ignited since the discovery of ACE2 and the realization that the Ang (1-7) peptide can be produced directly via Ang II via ACE2 [10]. Indeed, although Ang (1-7) differs to Ang II by only one amino acid, Ang (1-7)-mediated effects are markedly different to those of Ang II, and it has been suggested that Ang (1-7) may in fact play a counter regulatory role to Ang II [13, 102]. Ang (1-7) is present in various cell types such as epithelial, neuroepithelial, endothelial, and smooth muscle cells and it has been proposed that this heptapeptide plays both autocrine/paracrine role [103].

Several studies have shown Ang (1-7) to induce vasorelaxation of porcine coronary arteries [104], canine coronary arteries [105], mouse thoracic aorta [31] and mesenteric microvessels from SHR [106]. This vasorelaxation appears to be endothelium-dependent, as denuding the endothelium prevented the Ang (1-7)-mediated vascular responses [107]. Moreover, inhibition of NOS with L-nitro arginine methyl ester (L-NAME), reduced but did not completely eliminate the vasodilator response caused by Ang (1-7), suggesting that NO release is at least partially involved in Ang (1-7) signalling [108]. In addition, Ang (1-7) has been shown to interact with BK to augment its biological effects. BK initiates the release of NO, prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) from endothelial and VSMCs, causing vasorelaxation and inhibition of vascular cell growth [109]. Furthermore, Ang (1-7) has also been shown to antagonise Ang II-mediated vasoconstriction in human arteries [8] by inhibiting plasma, atrial and arterial ACE, and thus modulating the human RAS [8].

However, over the years it has become clear that Ang (1-7) is capable of activating multiple receptor subtypes. For example, in porcine coronary arteries, Ang (1-7)-mediated vasorelaxation occurs via AT_2R [104] in canine coronary arteries Ang (1-7) acts via B_2R [105], while in mouse thoracic aorta vasorelaxation is via *Mas*R [31]. Interestingly, vasorelaxation to Ang (1-7) observed in hypertensive rats was via stimulation of both B_2R and *Mas*R [106]. Furthermore, acute Ang (1-7) infusion in normotensive rats caused vascular relaxation and NO release via *Mas*R [110]. On the other hand, in an *in vivo* setting an acute infusion of Ang (1-7) in SHR led to a decrease in blood pressure that was completely inhibited by co-administration of PD123319, but was unaffected by the selective *Mas*R antagonist, [D-Alanine7]-Ang (1-7) (A779) [111].

Ang (1-7) has long been known to demonstrate anti-proliferative effects *in vitro* in both rabbit [112] and rat [113, 114] aortic VSMCs by release of PGI_2 and inhibition of ERK-1/2 [112-114]. The anti-proliferative effect of Ang (1-7) was mediated via the *Mas*R in rat aorta [113], while in the rabbit aorta this effect was via both *Mas*R and AT_2R as it was reversed by both PD123319 and A-779 [112].

Ang (1-7) is also suggested to play a role in cardiac remodeling. For example, in a model of myocardial infarction Ang (1-7) was absent in infarcted regions whilst expression was significantly greater at the borders of infarcted regions compared to non-infarcted regions [115]. In addition, Ang (1-7) has also been implicated in the prevention of cardiac fibrosis induced by Ang II infusion, with these effects mediated via PGI_2, BK and NO confirming the cardio-protective role of Ang (1-7) [116-120]. Indeed, it has been recently documented that chronic treatment with Ang (1-7) significantly attenuated heart failure in a mouse model of renal failure, by inhibiting interstitial fibrosis and cardiomyocyte hypertrophy as well as decreasing inflammatory cytokine expression and oxidative stress [121]. Furthermore, in the same study *Mas*R was upregulated in falling hearts when

compared to sham operated hearts suggesting a role for the *Mas*R [121] however, the study did not further address the possible receptors involved in the cardio-protective effect mediated by Ang (1-7).

Thus, Ang (1-7) can act via multiple receptors and activate multiple signaling pathways, and it is clear that Ang (1-7) is an important peptide of the RAS, able to counteract some of the Ang II effects. Therefore, further studies are required to assess these mechanisms in relation to Ang (1-7).

1.3.2 ANG (1-7)/*MAS* RECEPTOR

1.3.2.1 STRUCTURE, FUNCTION AND SIGNALING

*Mas*R is a seven-transmembrane GPCR containing 325 amino acid residues [31], and was first discovered in 1986 by Young and colleagues [122]. Based on its tumorigenic properties, *Mas*R was initially depicted as a protooncogene [122]. Subsequently, *Mas*R was thought to be a functional Ang receptor following transfection studies in mammalian cells [123] however, this hypothesis was rejected when Ang II binding was not observed in other cell types expressing *Mas*R [124]. Subsequently, Santos and colleagues (2003) observed that Ang (1-7) was able to bind to the *Mas*R and implicated this peptide fragment as an endogenous ligand of *Mas*R and responsible for its biological actions [31]. Santos *et al*. (2003) showed that Ang II binding was unchanged in kidneys of *Mas*R knockout (KO) mice in comparison to their wild type (WT) controls, however, Ang (1-7) binding was absent in the *Mas*R KO strain. *Mas*R mRNA was subsequently detected in the heart, testes, kidney and brain and it is interesting to note that isolated hearts of *Mas*R deficient mice presented significant changes in cardiac function suggesting a protective role of Ang (1-7) via *Mas*R [14], initiating research into both cardio-protective and counter-regulatory properties

of Ang (1-7). Additionally, Ang (1-7) was shown to inhibit cardiomyocyte growth and differentiation in rat hearts, an effect, which was attenuated by a selective *Mas*R antagonist, A-779 [125].

*Mas*R signaling can result in the phosphorylation of Akt, as was observed in rat hearts [125] and human endothelial cells leading to activation of eNOS and an increase in NO release via *Mas*R [126]. Additionally, in cultured VSMCs, a decrease in MAPK activity, such as ERK1/2, and subsequent inhibition of Ang II-induced growth, was observed as a result of *Mas*R stimulation by Ang (1-7) [127]. These findings suggest that *Mas*R-mediated modulation of MAPK and Akt phosphorylation is another mechanism for the regulation of the consequences of Ang II-mediated AT_1R activation. Therefore, Ang (1-7) and *Mas*R could indeed be of functional relevance in pathophysiological conditions and be important targets in the development of new therapeutic agents.

1.4 EFFECTS OF AGING ON THE RAS

According to the Australian Bureau of Statistics, our population is aging and it is predicted that by the year 2021, 18% of the community will be over the age of 65. With increased age, the risk of developing diseases such as stroke and cardiovascular diseases also increases (http://www.heartfoundation.org.au). Therefore, it is not surprising that the aged population is becoming one of the major targets of therapeutic intervention.

Major changes in the cardiovascular system that are closely associated with aging include modifications to cardiac structure such as cardiomyocyte loss, hypertrophy of the remaining cells and development of fibrosis which have dramatic impact on cardiac function [128]. In addition, vascular changes that can be seen as a result of aging include increased arterial wall stiffness, increased collagen accumulation [26, 129], luminal dilation, intimal and medial thickening,

and endothelial dysfunction [130], which all contribute to the vascular diseases common to elderly patients [131]. In fact luminal dilation associated with aging, is well documented in both humans [132] and animals [130, 133, 134]. The endothelium and vascular smooth muscle cells are important sources of vasodilator and vasoconstrictor mediators, and the balance between vasoconstrictor and vasodilator production is necessary for the maintenance of normal vascular structure and function. The endothelium responds to environmental stimuli releasing mediators including vasodilators such as NO and PGI_2, and vasoconstrictors such as Ang II [135]. As we age, the endothelium is distorted [136] with changes in the release or production of vasodilators such as NO, and enhanced production of PGE_2 [135] leading to an overall decrease in relaxation. Furthermore, it appears that ROS are also increased in the vasculature during aging [137-139]. In this instance, O_2^- may activate redox-sensitive kinases involved in VSMC growth [140], and inactivatie NO, resulting in the inhibition of anti-hypertrophic effects [140]. One possible explanation for the decrease in NO levels could be due to the decrease in the eNOS [141, 142]. Conversely, others have reported an increase in eNOS in the aorta of aged animals [137, 138]. However, in these studies, despite the increase in the NO production, an increase in O_2^- led to diminished NO bioavailability with increased age [137, 138]. As previously described, ROS plays an important role in Ang II signaling pathways, which contribute to vascular remodeling and vascular disease. In this context, it has been shown that the onset of human endothelial cell aging can be attenuated by inhibiting ROS production, suggesting a link between oxidative stress and vascular aging in age related vascular disease [143]. More importantly, it has been suggested that both AT_1R blockers and ACE inhibitors could potentially protect against the effect of vascular aging in both animals and humans by attenuating oxidant damage to mitochondria [144].

Interestingly, during aging circulating levels of Ang II are significantly decreased while local production of Ang II is increased in the aorta and other vessels [145]. In terms of vascular AT_2R function during aging, the Ang II-mediated vasoconstriction in human coronary microarteries was potentiated by PD123319; and this effect was more pronounced in vessels from older subjects [24]. Furthermore, an increase in AT_2R expression was observed in medial smooth muscle of mesenteric arteries from normotensive animals [146]. Similarly, there was an increase in AT_2R expression throughout both medial and endothelial layers of conduit vessels of aged normotensive animals [147].

1.5 EFFECTS OF SEX ON THE RAS

Epidemiological and clinical evidence suggests that estrogen has protective effects on the cardiovascular system, as there are significant differences in blood pressure control between males and females. Indeed, males are at greater risk for hypertension and cardiovascular disease than females before menopause [148-152]. In many hypertensive rat models including SHR, Dahl salt-sensitive (DS) rats, deoxycorticosterone-salt hypertensive rats and New Zealand genetically hypertensive rats, males exhibit much higher blood pressure than age matched females [153]. However, currently available data for the distinction between males and females in regards to RAS components, and to what degree the mechanisms observed in males applies to female counterparts is limited, most likely due to the fact that the majority of research to date has been performed in male animals. Several studies have looked at the influence of AT_2R stimulation in females compared to males. For example, in a vascular injury model it was observed that neointimal formation, inflammation and oxidative stress were decreased in female compared to male mice [154]. Furthermore, these gender differences were blunted in AT_2R KO mice suggesting that differential AT_2R

activation may be partly responsible for altered vascular remodeling [154]. Previous studies have also suggested an important role of estrogen in RAS regulation, including effects such as the suppression of plasma renin activity [155], a decrease in renal ACE [156], and downregulation of AT_1R in both VSMCs [157] and adrenal cortex [158]. Furthermore, estrogen led to an increase in renal AT_2R expression in the kidney of ovariectomised mice [159]. This increase in renal AT_2R expression resulted in altered AT_1R/AT_2R ratio, which could be beneficial for maintaining normal renal function and delaying the progression of renal disease [159]. Moreover, Silva-Antonialli *et al.* (2004) have demonstrated that estrogen led to downregulation of vascular and renal AT_1R and upregulation of AT_2R, which was accompanied by a decreased constrictor effect of Ang II in isolated aortic rings and mesenteric arteries from SHRs. In addition, female SHR and WKY rats were found to be resistant to increased blood pressure caused by short-term sodium chloride (NaCl) exposure compared to males [160].

Previous studies using the AT_1R antagonist, losartan, observed an increase in AT_2R expression in female but not in male SHR [161]. In this study, decreased microvascular reactivity of mesenteric arteries was corrected with losartan more efficiently in female SHR than in male SHR [161]. Furthermore, the decrease in blood pressure seen with administration of losartan occurred earlier in female compared to male SHR [161].

More recently, it was observed that a chronic low-dose of Ang II decreased MAP in female rats and this effect was via an AT_2R mechanism since it was abolished by PD123319 treatment [162]. Furthermore, in the same study it was shown that both renal AT_2R and ACE2 mRNA gene expression was increase in females compared to males [162]. Therefore, it is possible that the attenuated pressor response to Ang II in females compared to males is due to an increase in the vasodilatory axis of the RAS [163-165].

In terms of shorter angiotensin peptide fragments, a recent study has shown Ang (1-7) levels to be increased in female SHR compared to male SHR, under both basal conditions and following Ang II infusion [166]. Furthermore, treatment with A-779 potentiated blood pressure responses to Ang II in female rats. suggesting that Ang (1-7) inhibits the Ang II-mediated increase in blood pressure [166].

Therefore, there is an increasing amount of evidence suggesting differential regulation of RAS between males and females, and better understanding of these differences may lead to the development of new therapeutics.

1.6 AIMS OF THESIS

As outlined in the preceding discussion, the renin angiotensin system is a complex system that involves a number of components that may act in concert or in opposition resulting in a final integrated cardiovascular effect. The focus of this thesis is to examine further the counter-regulatory actions mediated by stimulation of AT_2R and *Mas*R that in turn may dampen excessive AT_1R activation. In particular, this thesis will re-assess AT_2R affinity of a number of Ang peptides, as well as novel Ang peptide analogues. In addition, the influence of aging, gender and hypertension on the cardiovascular effects of several AT_2R agonists will be determined. Therefore, the aims of this thesis are:

Chapter 2: To perform a systematic analysis of binding affinities of endogenous and synthetic ATR ligands in human embryonic kidney (HEK-293) cells stably transfected with either AT_1R or AT_2R, in order to address potential mismatches between current functional and binding data.

Chapter 3: To characterize binding and functional responses of a group of novel β-substituted Ang peptides that have been designed with the goal of identifying new AT_2R-selective ligands.

Chapter 4: To examine the effect of aging on Ang (1-7)-mediated vasodepressor effects and whether those effects are mediated via AT_2R and/or *Mas*R.

Chapter 5: To determine if the cardiovascular effects of selective AT_2R stimulation is modulated by sex.

Chapter 6: To investigate acute vasodepressor effects of Compound 21 in the setting of hypertension.

Chapter 2:

Relative affinity of angiotensin peptides and novel ligands at AT_1 and AT_2 receptors

Declaration for Thesis Chapter 2

Monash University

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed all of the experiments, analysed the results and wrote the manuscript.	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution
Emma Jones Arthur Christopoulos Marie Isabel Aguilar Walter Thomas Robert Widdop	Provided intellectual advice during experimental and manuscript preparation stages.

Candidate's Signature: .....

Date: .....

Declaration by co-authors
The undersigned hereby certify that:
(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Relative affinity of angiotensin peptides and novel ligands at AT_1 and AT_2 receptors

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A B S T R A C T

AT_1R (angiotensin type 1 receptor) and AT_2R (angiotensin type 2 receptor) are well known to be involved in the complex cardiovascular actions of AngII (angiotensin II). However, shorter peptide fragments of AngII are thought to have biological activity in their own right and elicit effects that oppose those mediated by AngII. In the present study, we have used HEK (human embryonic kidney)-293 cells stably transfected with either AT_1R or AT_2R to perform a systematic analysis of binding affinities of all the major angiotensin peptides. Additionally, we tested the novel AT_2R agonist Compound 21, as well as the *Mas*R (*Mas* receptor) agonist and antagonist AVE0991 and A779 respectively, for their ability to bind to AT_1R or AT_2R. Candesartan, CGP42214 and PD123319 were used as reference compounds. Binding studies using ^{125}I-[Sar^{1}Ile^8]AngII on the AT_1R-transfected HEK-293 cells revealed only AngII, AngIII [angiotensin III; angiotensin-(2–8)] and candesartan to have high affinity for AT_1R. In the AT_2R-transfected HEK-293 cells, competition for ^{125}I-[Sar^{1}Ile^8]AngII binding was observed for all ligands except candesartan, AVE0991 and A-779, the latter two compounds having negligible affinity at either AT_1R or AT_2R. The rank order of affinity of ligands at AT_2R was CGP42112>AngII$\geqslant$AngIII>Compound 21$\geqslant$PD123319>>AngIV [angiotensin IV; angiotensin-(3–8)]>Ang-(1–7) [angiotensin-(1–7)]. Of note, although AngIV and Ang-(1–7) exhibited only modest affinity at AT_2R compared with AngII, these two angiotensin peptides, together with AngIII, had substantial AT_2R selectivity over AT_1R. Collectively, our results suggest that shorter angiotensin peptides can act as endogenous ligands at AT_2R.

INTRODUCTION

The RAS (renin–angiotensin system) is an intricately co-ordinated hormonal cascade involved in cardiovascular control [1] with AngII (angiotensin II) as the main effector peptide regulating BP (blood pressure) regulation, and fluid and electrolyte homoeostasis. It is now widely accepted that AngII binds with high affinity to two distinct receptors: AT_1R (angiotensin type 1 receptor) and AT_2R (angiotensin type 2 receptor) [2]. AT_1R activation mediates the 'classical' effects, including vasoconstriction, cellular growth and proliferation [2]. In contrast, AT_2R activation counteracts AT_1R-mediated actions, including vasodilatation, apoptosis and anti-growth effects [3–5].

Far from being a simple linear system for the production of AngII, it is increasingly realized that other angiotensin metabolites of AngI (angiotensin I)

Key words: angiotensin II, angiotensin III, angiotensin IV, angiotensin-(1–7), angiotensin receptor, Compound 21, radioligand assay.

Abbreviations: Ang-(1–7), angiotensin-(1–7); AngII, angiotensin II; AngIII, angiotensin III [angiotensin-(2–8)]; AngIV, angiotensin IV [angiotensin-(3–8)]; AT_1R, angiotensin type 1 receptor; AT_2R, angiotensin type 2 receptor; AT_4R, angiotensin type 4 receptor; HEK, human embryonic kidney; IRAP, insulin-regulated aminopeptidase; *Mas*R, *Mas* receptor.

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and AngII can exert their own biological activity. These peptides include the C-terminal breakdown products of AngII, namely the heptapeptide AngIII [angiotensin III; angiotensin-(2–8)] and the hexapeptide AngIV [angiotensin IV; angiotensin-(3–8)], as well as the N-terminal heptapeptide Ang-(1–7) [angiotensin-(1–7)]. In most instances, these peptides evoke cardiovascular effects that are opposite to the classical effects of AngII [4,6–11]. There is also evidence that the effects of Ang-(1-7) and AngIV are via cognate receptors *Mas*R (*Mas* receptor) and AT_4R (angiotensin type 4 receptor)/IRAP (insulin-regulated aminopeptidase) respectively [12,13]. However, there are many instances where the effects of Ang-(1–7) and AngIV are reported to act via their cognate receptors and/or AT_2R [14–21]. In addition, AngIII is thought to be the endogenous AT_2R agonist in the kidney [7,22].

It has been postulated that shorter angiotensin peptides may act as endogenous AT_2R ligands [4,11]. We therefore undertook the first systematic examination of the relative AT_1R- and AT_2R-binding affinities of a number of endogenous angiotensin peptides, together with the currently available synthetic angiotensin receptor ligands. To this end, we have used HEK (human embryonic kidney)-293 cells stably transfected with either AT_1R or AT_2R and have determined the relative AT_2R selectivity of AngII, AngIII, Ang-(1–7) and AngIV. In addition, we have tested the AT_2R-selective agonists CGP42112 [23] and Compound 21 [24], and the *Mas*R ligands AVE0991 [25,26] and A-779 [12,27]. We hypothesized that some of the effects of Ang-(1–7) and AngIV that were inhibited by the AT_2R antagonist PD123319 in functional cardiovascular studies [14–16,21] may in fact be due to direct binding at AT_2R. Furthermore, by performing these analyses within the one study, our results provide a benchmark reference for the relative rank order of AT_2R/AT_1R selectivity for a number of endogenous and synthetic angiotensin ligands.

MATERIALS AND METHOD

The generation of plasmids expressing HA (haemagglutinin)-tagged versions of AT_1R and AT_2R have been described previously [28,29]. HEK-293 cells in 12-well plates were transfected with either AT_1R or AT_2R plasmids (0.6 μg/well) using Lipofectamine™ (4.8 μl/well), as described previously [30], and stably expressing clones were obtained by selection with G418 (1 mg/ml) and limiting dilution.

HEK-293 stably transfected with either AT_1R or AT_2R [expressing equivalent amounts of receptors (~1000 fmol/mg of protein)] were maintained in a humidified atmosphere in a CO_2 water-jacketed incubator (Forma Scientific) with 5 % CO_2 and 95 % air. Cells were grown in 175 cm^2 flasks at 37 °C in growth medium [DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FBS (fetal bovine serum), 4 mM L-glutamine, 16 mM Hepes and 200 μg/ml G418]. Cells were grown to approximately 80 % confluence before experiments were performed. Once 80 % confluence was reached, cells were re-plated into 48-well plates at 1×10^5 cells/well and grown for 48 h at 37 °C for a whole-cell competition-binding assay.

All of the ligands were prepared and diluted in DMEM/0.1 % BSA on the day of the experiment. The unlabelled angiotensin receptor ligands used were: AngII, AngIII, AngIV, Ang-(1–7) (each from AusPep), CGP42112 (GL Biochem), PD123319 (generously provided by Pfizer), Compound 21 (generously provided by Professor Anders Hallberg, Department of Medicinal Chemistry, Uppsala University, Uppsala, Sweden), candesartan (generously provided by AstraZenica), AVE0991 (generously provided by Adventis) and A-779 (GL Biochem) at concentrations ranging from 1 pM to 1 μM. For each experiment, each ligand concentration was tested in triplicate. On the day of the experiment, medium was aspirated taking care not to disturb cells and replaced with unlabelled ligand at specific concentration and binding buffer [DMEM, 0.1 % BSA and ^{125}I-[Sar^{1}Ile^8]AngII (at 50000 c.p.m; ProSearch International)] Plates were incubated for 45 min at 37 °C. After incubation, binding buffer was aspirated and unbound radioactivity was separated from bound radioligand by washing the wells with 250 μl of PBS. Each plate was aspirated once more and the cells were solubilized in 250 μl of 0.5 M NaOH for 10 min at room temperature (°C). The content of each well was transferred into appropriate tubes and the radioactivity was counted using a Wallac Wizard™ 1470 automatic γ-counter (PerkinElmer). Non-specific binding was defined in the presence of the unlabelled AngII (1 μM). The ability of each ligand to inhibit the specific binding of ^{125}I-[Sar^{1}Ile^8]AngII was assessed. All counts were corrected for non-specific binding and the percentage of bound AT_1R or AT_2R was calculated by normalizing the specific binding at different concentrations of the competing drugs to the specific binding determined in the presence of vehicle.

Non-linear regression of the data was performed using GraphPad Prism 5.03 and IC_{50} values, representing the concentration at which each ligand displaced 50 % binding of ^{125}I-[Sar^{1}Ile^8]AngII in either AT_1R- or AT_2R- transfected HEK-293 cells, were estimated. Log-transformed ratios of IC_{50} values for each ligand at AT_1R:AT_2R were determined as a measure of AT_2R selectivity. In cases where there was minimal displacement of ^{125}I-[Sar^{1}Ile^8]AngII by the unlabelled peptide ligand (at 1 μM), an arbitrary value of 10 μM was used as a comparative estimate of IC_{50} values (to be used in ratio calculations).

Table 1 IC_{50} values and relative AT_2R-selectivity of peptide ligands

Results are the competition binding from three separate experiments (each in triplicate). —, Minimal binding to AT_1R or AT_2R.

	AT_1R		AT_2R		AT_2R selectivity	
Peptide ligand	IC_{50} value (M)	Relative affinity to AngII (100%)	IC_{50} value (M)	Relative affinity to AngII (100%)	AT_1R/AT_2R	Fold selectivity
AngII	7.92×10^{-9}	100	5.22×10^{-10}	100	1.18	15
AngIII	2.11×10^{-8}	38	6.48×10^{-10}	81	1.51	33
AngIV	1.00×10^{-5}*	>1000	4.86×10^{-8}	1	2.31	206
Ang-(1–7)	1.00×10^{-5}*	>1000	2.46×10^{-7}	0.21	1.61	41
CGP42112	1.00×10^{-5}*	>1000	2.33×10^{-10}	224	4.63	42863
PD123319	1.00×10^{-5}*	>1000	5.60×10^{-9}	9	3.25	1786
Compound 21	1.00×10^{-5}*	>1000	2.29×10^{-9}	23	3.64	4367
Candesartan†	1.56×10^{-9}	507	1.00×10^{-5}*	>1000	−3.81†	6402†
AVE0991	1.00×10^{-5}*	>1000	1.00×10^{-5}*	>1000	—	—
A-779	1.00×10^{-5}*	>1000	1.00×10^{-5}*	>1000	—	—

*IC_{50} value of 1.00×10^{-5} M was used for the calculation

†AT_1R-selective.

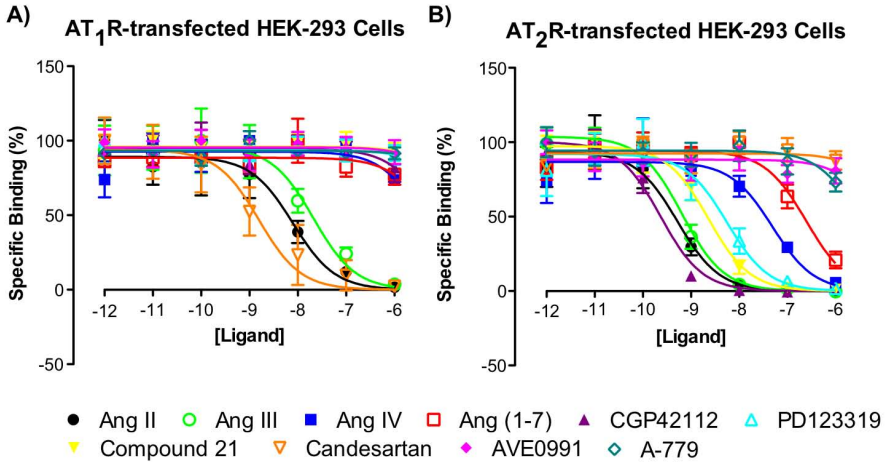

Figure 1 Radioligand competition-binding experiments performed in HEK-293 cells stably experssing (A) AT_1R or (B) AT_2R for angiotensin peptides and ligands against ^{125}I-[Sar^1,Ile^8]AngII binding

Each point represents the mean of five separate experiments, each performed in triplicate.

RESULTS

Competition for ^{125}I-[Sar^{1}Ile^8]AngII binding in HEK-293 cells transfected with AT_1R was observed for AngII, AngIII and candesartan, with the IC_{50} values in the nanomolar range. Ang-(1–7), AngIV, CGP42112, PD123319, Compound 21, AVE0991 and A-779 lacked affinity for AT_1R (IC_{50} values >1000 nM) (Figure 1A and Table 1). Relative to AngII, the rank order of affinity at AT_1R was candesartan>AngII>AngIII>>>all other ligands. By contrast, in HEK-293 cell transfected with AT_2R, competition binding for ^{125}I-[Sar^{1}Ile^8]AngII was observed with all of the ligands, except candesartan, AVE0991 and A-779. IC_{50} values were in the nanomolar range for AngII, AngIII, CGP42112, Compound 21 and PD123319. Surprisingly, competition for ^{125}I-[Sar^{1}Ile^8]AngII binding was also observed for AngIV in the nanomolar range and for Ang-(1–7) in the micromolar range. The rank order of affinity at AT_2R was CGP42112>AngII$\geqslant$AngIII>Compound 21$\geqslant$PD123319>>AngIV>Ang-(1–7) (Figure 1B and Table 1). By contrast, the *Mas*R agonist AVE0991 and antagonist A-779 lacked affinity for both AT_1R and AT_2R, at least up to 1 μM (Figures 1A and 1B).

In order to estimate the relative AT_2R selectivities of the endogenous angiotensin peptides and synthetic ligands, the ratio of IC_{50} values at AT_1R/AT_2R was determined (Figure 2). In the present study, AngII and AngIII were 15- and 33-fold selective for AT_2R over AT_1R. As expected, known AT_2R ligands exhibited

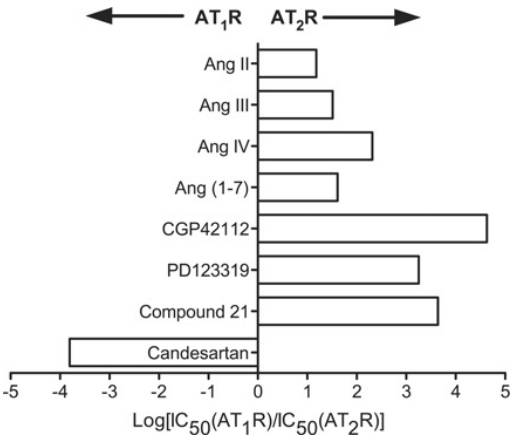

Figure 2 Ratio of log-transformed IC_{50} values of AT_1R and AT_2R for each angiotensin peptide and ligand

The results are derived from Figure 1 and Table 1, and are plotted as a measure of the relative affinity of the angiotensin peptides and ligands for AT_1R and AT_2R.

marked selectivity, ranging from 40000-fold AT_2R selectivity for CGP42112 to 4000-fold selectivity for Compound 21, whereas candesartan was at least 6000-fold selective for AT_1R (Table 1). AngIV and Ang-(1–7) exhibited a lower AT_2R affinity relative to AngII and AngIII, but these peptides still had substantial AT_2R selectivity over AT_1R [that being 200-fold for AngIV and 40-fold for Ang-(1–7); Figure 2].

DISCUSSION

To address potential mismatches in functional and binding data, we have performed a systematic binding analysis in HEK-293 cells stably transfected either AT_1R or AT_2R, which has eliminated confounders such as tissue heterogeneity and differential angiotensin receptor expression when comparing ligands, both within and between published studies. Indeed, we have examined all of the major endogenous angiotensin peptide fragments and novel synthetic ligands in the one study, and have now shown that all endogenous angiotensin peptides tested have relatively greater AT_2R selectivity over AT_1R selectivity. Strikingly, we have found that AngIII has a similar affinity as AngII at AT_2R, but at least 30-fold selectivity for AT_2R over AT_1R. Moreover, although Ang-(1–7) and AngIV exhibit ~500- and 100-fold less affinity at AT_2R compared with AngII, these peptides were 40- and 200-fold more selective for AT_2R than AT_1R. Collectively, the results of the present study have revealed the potential for all 'breakdown' products of AngII to act as endogenous ligands for AT_2R.

As expected, candesartan and PD123319 behaved as highly selective AT_1R and AT_2R ligands respectively, consistent with actions as subtype-selective antagonists [2,31,32]. Moreover, near-identical antiogensin-receptor-binding selectivities have been reported for losartan and the related non-peptide AT_2R ligand PD123177 in previous binding studies [33,34]. In the present study, CGP42112 was >40000-fold selective for AT_2R over AT_1R and had the highest affinity at AT_2R of all of the ligands tested. In their original publication, Whitebread et al. [23] reported CGP42112 to have a nanomolar affinity at AT_2R in rat and human uterus and adrenal tissue, and a micromolar affinity at AT_1R, such that its AT_2R selectivity ranged from approximately 1000-fold in rat uterus to 10600-fold in human adrenal. Thus our findings using a homogenous cell expression system has revealed exquisite AT_2R sensitivity for CGP42112.

More recently, a selective non-peptide AT_2R agonist, Compound 21 [24], has been developed, which is reported to evoke cardioprotective, anti-inflammatory [35] and vasodilator [36] effects. Initial characterization in pig uterine (AT_2R) and rat liver (AT_1R) membranes revealed K_i values of 0.4 nM and >10 μM respectively, indicating >25000-fold AT_2R selectivity [24]. We showed a similar lack of AT_1R affinity, but found that Compound 21 had an approximately 10-fold lower affinity than CGP42112 in AT_2R-transfected HEK-293 cells. Nevertheless, Compound 21 was highly AT_2R-selective (4000-fold).

In terms of angiotensin peptides, we have confirmed previous reports that AngII has high-binding affinity at both AT_1R and AT_2R [2,23,31–33,37], and that this octapeptide usually exhibits slight preferential affinity for AT_2R [23,33,34]. In the present study, AngII was approximately 15 times more selective for AT_2R compared with AT_1R.

The C-terminal heptapeptide AngIII displaced ^{125}I-[Sar^{1}Ile^8]AngII binding in AT_2R-transfected HEK-293 cells in a similar manner to AngII, whereas, in some instances, AngIII is more potent at AT_2R than AngII itself [31,32,37]. Nevertheless, given that AngIII exhibited a lower affinity for AT_1R, AngIII was >30-fold selective for AT_2R when compared with AT_1R. These findings are consistent with the binding profile of AngIII in various tissues, thus providing clear evidence that AngIII is more selective at AT_2R compared with AT_1R [33,38,39]. In this context, it is noteworthy that AngIII evoked a natriuretic effect in rats via AT_2R; an effect that was not observed using AngII [22] unless the metabolism of AngIII was inhibited [40]. These functional results indicate that AngIII may be an endogenous AT_2R agonist in the kidney, which fits with our affinity profiling of AngIII as being AT_2R-preferring. A similar finding that AngIII is the preferred AT_2R agonist in the rat coronary vascular bed has also been postulated [41].

Since the discovery of ACE2 (angiotensin converting enzyme 2) [42–44], there has been much interest in the functional effects of Ang-(1–7), which differ markedly to those of AngII [8,10]. Indeed, evidence suggests that Ang-(1–7)-mediated effects are via a specific Ang-(1–7) receptor, known as the *Mas*R [12,45]. At the same time, there are many reports that Ang-(1–7) can mediate its effects via AT_2R [8,12,18–20,46,47]. In fact, we have shown that the Ang-(1–7)-mediated vasodepressor effect was via AT_2R stimulation [17], in a similar manner to that seen with CGP42114 [48] and, more recently, Compound 21 [36]. Interestingly, in the present study, we observed substantial displacement of ^{125}I-[Sar^{1}Ile^8]AngII by Ang-(1–7) in the AT_2R-transfected HEK-293 cells, which is consistent with previous reports of this heptapeptide having low, but appreciable, affinity at AT_2R in human myometrium [31] and transfected COS-7 cells [37]. Our present study also examined AT_1R binding, and found that Ang-(1–7) exhibited minimal AT_1R binding (at 1 μM). Thus, although Ang-(1–7) had nearly 500-fold less affinity than AngII at AT_2R, it exhibited at least 40-fold greater AT_2R/AT_1R selectivity, suggesting that, in addition to *Mas*R activation, Ang-(1–7) is potentially an endogenous ligand at AT_2R. In this context, we have recently reported that chronic treatment with Ang-(1–7) evoked both vaso- and athero-protective effects in ApoE (apolipoprotein E)-deficient mice that were sensitive to blockade by both *Mas*R and AT_2R antagonists [21]. By contrast, AVE099, the first non-peptide Ang-(1–7) mimetic and *Mas*R agonist [12,25,26,49,50], and the *Mas*R antagonist A-779 [12] completely lacked affinity for either AT_1R or AT_2R in the present study. Thus the results of the present study would support an effect of Ang-(1–7) to directly stimulate AT_2R as one possible mechanism to help explain the anti-atherosclerotic effects of Ang-(1–7) [21].

Similarly to other angiotensin peptides, there is accumulating evidence suggesting the hexapeptide AngIV mediates its effects at AT_4R, which is now thought to be identical with IRAP [13], where AngIV inhibits catalytic activity. However, there are reports that the cardiovascular effects of AngIV are mediated via AT_4R/IRAP and/or AT_2R [14,51,52]. Indeed, we have shown that chronic AngIV treatment reversed endothelial dysfunction in atherosleclerosis, via both AT_2R and AT_4R [15,16]. In the present study, AngIV exhibited marked binding at AT_2R, with approximately 5-fold greater affinity than Ang-(1-7) and no appreciable AT_1R binding (at 1 μM). This analysis revealed that AngIV was at least 200-fold more selective at binding to AT_2R compared with AT_1R.

Collectively, our rank order for AT_2R binding [CGP42112>AngII$\geqslant$AngIII>Compound 21$\geqslant$ PD123319>>AngIV>Ang–(1-7)] was similar to that obtained comparing CGP42112 and angiotensin peptides in human myometrium [31] and AT_2R-transfected COS-7 cells [37], except the order was reversed for AngIV and Ang-(1–7) in the latter two studies. In any case, the results of the present and previous studies emphasize that all angiotensin peptide fragments have considerable AT_2R affinity and even those peptides that are reported to act via their cognate angiotensin receptor [AngIV and Ang-(1–7)] exhibit sub-micromolar affinity at AT_2R. The ability of angiotensin peptides to act as potential AT_2R ligands takes on greater significance if one considers that AT_2Rs are up-regulated in many cardiovascular disease settings, including cardiac failure, cardiac fibrosis, renal failure, diabetes and atherosclerosis, and even aging (reviewed in [4,11]). Although AngII overactivity contributes to many cardiovascular pathologies, there may be an increased functional importance of AT_2R, stimulated by AngII, or even shorter angiotensin peptide fragments, to limit AT_1R-mediated overactivity and cardiovascular disease. Although less is known about changes in levels of angiotensin 'breakdown products' in cardiovascular disease, studies from our own laboratory highlight the potential importance of chronic effects of Ang-(1–7) and AngIV in atherosclerosis [16,21]. In these studies, although both angiotensin peptide fragments acted via their respective cognate receptors, they also evoked vaso- and athero-protective effects that were sensitive to AT_2R blockade. Thus the results of the present binding studies implicate a direct AT_2R activation.

A limitation of the present study is that we have used individual cell lines that were selected to express equivalent amounts of receptors, which may not reflect *in vivo* binding, where both AT_1R and AT_2R may be expressed on the same cell and at different expression levels. Our receptor expression levels (~1000 fmol/mg of protein) were in the upper range of physiological levels, but these levels are likely to be achieved in various tissues, including adrenal, uterus, vascular smooth muscle and certain brain nuclei. Thus, although one must be cautious in extrapolating from artificial systems to *in vivo* conditions, our present results do allow relative affinity comparisons of ligands without confounders such as differential angiotensin receptor expression.

For the first time, we have provided an assessment of the relative AT_2R/AT_1R selectivity for all of the major endogenous angiotensin peptides and novel synthetic ligands currently available. This analysis showed the following AT_2R/AT_1R preference: CGP42112>>Compound 21>PD123319>>>AngIV> Ang-(1–7)~AngIII>AngII>>>candesartan~AVE0991 ~A-779. Thus we have confirmed the marked AT_2R selectivity of Compound 21 and have shown that the *Mas*R ligand AVE0991 does not bind to AT_2R. Finally, all of the endogenous angiotensin peptide fragments exhibited greater AT_2R preference than AngII itself, adding further weight to the concept that these metabolites of AngII actually act in concert to

oppose overactivity of the parent peptide AngII at AT_1R.

AUTHOR CONTRIBUTION

Sanja Bosnyak conducted and analysed the experiments under the supervision of Emma Jones and Arthur Christopoulos. Walter Thomas provided the transfected cell lines and provided intellectual input as did Marie-Isabel Aguilar. The experiments were conceived by Robert Widdop and Emma Jones. All authors co-wrote the manuscript.

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Chapter 3:

A single β-amino acid substitution to Angiotensin II confers AT_2R selectivity and vascular function

Declaration for Thesis Chapter 3

Monash University

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed all *in vivo* experiments and prepared figures. Analysed radioligand binding data and contributed to manuscript drafting.	50%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution
Emma Jones Mark Del Borgo Julian Kirsch Daniel Clayton Iresha Welungoda Nicholas Hausler Sharon Unabia Patrick Perlmutter Walter Thomas Marie-Isabel Aguilar Robert Widdop	Contributed to experiments or provided intellectual advice during experimental and manuscript preparation stages.

Candidate's Signature: .....
Date: .....

Declaration by co-authors
The undersigned hereby certify that:
(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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A Single β-Amino Acid Substitution to Angiotensin II Confers AT_2 Receptor Selectivity and Vascular Function

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Abstract—Novel AT_2R ligands were designed by substituting individual β-amino acid in the sequence of the native ligand angiotensin II (Ang II). Relative ATR selectivity and functional vascular assays (in vitro AT_2R-mediated vasorelaxation and in vivo vasodepressor action) were determined. In competition binding experiments using either AT_1R- or AT_2R-transfected HEK-293 cells, only β-Asp^1-Ang II and Ang II fully displaced [^{125}I]-Ang II from AT_1R. In contrast, β-substitutions at each position of Ang II exhibited AT_2R affinity, with β-Tyr^4-Ang II and β-Ile^5-Ang II exhibiting ≈1000-fold AT_2R selectivity. In mouse aortic rings, β-Tyr^4-Ang II and β-Ile^5-Ang II evoked vasorelaxation that was sensitive to blockade by the AT_2R antagonist PD123319 and the nitric oxide synthase inhibitor L-NAME. When tested with a low level of AT_1R blockade, β-Ile^5-Ang II (15 pmol/kg per minute IV for 4 hours) reduced blood pressure (BP) in conscious spontaneously hypertensive rats (β-Ile^5-Ang II plus candesartan, −24±4 mm Hg) to a greater extent than candesartan alone (−11±3 mm Hg, n=7, $P<0.05$), an effect that was abolished by concomitant PD123319 infusion. However, in an identical experimental protocol, β-Tyr^4-Ang II had no influence on BP (n=10), and it was less stable than β-Ile^5-Ang II in plasma stability assays. Thus, this study demonstrated that a single β-amino acid substitution resulted in a compound that demonstrated both in vitro vasorelaxation and in vivo depressor activity via AT_2R. This approach to the design and synthesis of novel AT_2R-selective peptidomimetics shows great potential to provide insight into AT_2R function. (***Hypertension*. 2011;57[part 2]:570-576.) ● Online Data Supplement**

Key Words: angiotensin ■ β-amino acid substitutions ■ AT_2 receptor ■ depressor ■ vasorelaxation

Angiotensin II (Ang II; Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8) acts at 2 major receptor subtypes, designated as the type 1 (AT_1R) and type 2 (AT_2R) angiotensin receptors. The binding of Ang II to AT_1R mediates vasoconstriction, cell growth, and remodeling leading to increased blood pressure (BP); cardiac, renal, and vascular hypertrophy; and fibrosis, which is the molecular basis for the clinical application of AT_1R antagonism. The binding of Ang II to AT_2R is thought to oppose the AT_1R-mediated effects of Ang II via vasodilator and antiproliferative effects.[1–5]

Many studies have used substituted analogs of Ang II as well as receptor mutagenesis to understand the pharmacophore responsible for high-affinity Ang II binding and activity at AT_1R and AT_2R. Many such structure-activity studies using Ang II analogs were performed using radioligand binding assays and functional assays, such as the measurement of vascular contraction[6,7] at a time prior to the identification of ATR subtypes. Previous studies identified that Tyr^4, His^6, and Phe^8 were important for agonism[6] in assays that recognize classical Ang II activity via AT_1R stimulation. Subsequent cloning of the angiotensin receptors revealed that the AT_1R is the primary mediator of cardiovascular actions of Ang II. To study AT_2R, the peptide compound CGP42112[8] has been used almost exclusively as a selective AT_2R agonist, and the nonpeptide compound PD123319 has been used as a selective AT_2R antagonist. Thus, research elucidating the relevance of AT_2R modulation of cardiovascular function has been severely hampered by a lack of selective compounds, either agonists or antagonists, to probe AT_2R function, particularly in chronic in vivo experiments.

The use of β-amino acids is now an emerging area in the field of peptidomimetics.[9–16] β-Amino acids are similar in structure to α-carbons except they contain an "extra" carbon atom, ie, β-amino acid substitution results in an amino acid with an identical side chain (R group) but containing an additional methylene group (CH_2) in the peptide backbone. β-Amino acid substitution can greatly affect the binding properties and stability of the β-analog peptide[9,10] through changes to backbone conformation, relayed structural effects, or nonstructural changes to the spatial positioning of the side

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Table 1. Peptide Sequences for Native Ang II and β-Substituted Ang Peptides*

Peptide Name	Sequence
Ang II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
β-Asp^1-Ang II	**β-Asp**-Arg-Val-Tyr-Ile-His-Pro-Phe
β-Arg^2-Ang II	Asp- **β-Arg**-Val-Tyr-Ile-His-Pro-Phe
β-Val^3-Ang II	Asp-Arg- **β-Val**-Tyr-Ile-His-Pro-Phe
β-Tyr^4-Ang II	Asp-Arg-Val- **β-Tyr**-Ile-His-Pro-Phe
β-Ile^5-Ang II	Asp-Arg-Val-Tyr- **β-Ile**-His-Pro-Phe
β-Pro^7-Ang II	Asp-Arg-Val-Tyr-Ile-His- **β-Pro**-Phe
β-Phe^8-Ang II	Asp-Arg-Val-Tyr-Ile-His-Pro- **β-Phe**

*Amino acid residues in bold denote the position of the β-substitution.

chains. Therefore, in the present study, we have synthesized a series of β-substituted Ang II peptide analogs, such that the corresponding β-substituted amino acid has replaced the α-amino acid at each position in the Ang II sequence (except at His^6), as shown in Table 1. We hypothesized that this series may exhibit altered AT$_2$R:AT$_1$R selectivity and peptide stability in vitro and in vivo.

Methods

Male 16- to 18-week–old spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats, weighing ≈300 to 350 g, and male ≈25-week–old FVB/N mice, weighing ≈25 to 30 g, were obtained from the Animal Resource Center. Animals were maintained on a 12-hour day/night cycle with standard laboratory rat or mice chow and water available ad libitum. Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Monash University Standing Committee on Ethics in Animal Experimentation.

Peptide Synthesis

Peptides were synthesized on a 100-μmol scale using standard Fmoc chemistry and Wang resin. The resin was washed (3×30 s) with *N*-methylpyrrolidone (NMP), and the Fmoc-protected amino acid (3.1 molar equivalents [eq.] to resin loading) was dissolved in NMP along with *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (3 eq. to resin loading), *N*-hydroxybenzotriazole (3 eq. to resin loading), and *N*,*N*-diisopropylethylamine (DIPEA) (4.5 eq. to resin loading). 4-Dimethylaminopyridine (0.1 eq. to resin loading) in NMP was added dropwise, and the reaction proceeded overnight. Following the overnight coupling, the resin was washed with NMP (5×30 s) and CH_2Cl_2 (5×30 s), and peptide synthesis was continued. One cycle of peptide elongation consisted of the following steps. The loaded resin was first washed with NMP (3×30 s), and the terminal Fmoc-protecting group was removed with 20% piperidine/dimethylformamide (2×10 minutes). The deprotected resin was then washed with NMP (5×30 s) and treated for 90 minutes with a solution containing 3.1 eq. of the appropriate amino acid, 3 eq. 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uranium hexafluorophosphate, and 4.5 eq. DIPEA. The resin was then washed 3 times with NMP (3×30 s), unreacted amino groups were acetylated on treatment with 10% v/v acetic anhydride and 1% v/v DIPEA in NMP (20 minutes), and the capped resin washed with NMP (3×30 s). These steps were repeated until the peptide sequence was complete. Once the final Fmoc-protecting group had been removed, the resin was subsequently washed with NMP (5×30 s) and CH_2Cl_2 (5×30 s), dried for 20 minutes under vacuum, and treated for 120 minutes with a cleavage solution containing 2.5% v/v water, 2% v/v triisopropylsilane, and 0.5% ethanedithiol in trifluoroacetic acid (TFA). The cleaved resin was washed twice with the cleavage solution (2×30 s), and the cleaved peptide in TFA was collected. The TFA was evaporated under a stream of N_2, and the peptide was precipitated by the addition of diethyl ether. The precipitate was filtered and reconstituted in H_2O/acetonitrile (1:1) for lyophilization. Peptides were then purified to homogeneity by preparative reverse-phase high-performance liquid chromatography (HPLC) using an Agilent HP1200 system fitted with a Vydac™C_{18} (250×22 mm) reverse-phase column. The eluents were 0.1% aqueous TFA and 0.1% TFA in acetonitile. The identities and purities of purified peptides were assessed by analytic HPLC and mass spectrometry using an Agilent 1100 MSD SL ion trap mass spectrometer.

Binding Assays

The generation of plasmids expressing hemagglutinin-tagged versions of the AT$_1$R and AT$_2$R have been described previously.[17,18] HEK-293 cells in 12-well plates were transfected with either AT$_1$R or AT$_2$R plasmids (0·6 μg/well) using lipofectAMINE (4·8 μL/well), as described previously,[19] and stably expressing clones obtained by selection with G418 (1 mg/mL) and limiting dilution. HEK cell clones expressing either AT$_1$R or AT$_2$R were plated in 12-well plates for whole-cell competition binding assays using the nonselective Ang II ligand [^{125}I]-Ang II and various concentrations of Ang II, PD123319 (an AT$_2$-selective ligand), and the various β-substituted Ang II peptides. Nonlinear regression of the data was achieved using GraphPad Prism (GraphPad Software Inc.), and IC$_{50}$ values, representing the concentration at which each ligand displaced 50% binding of ^{125}I-Ang II in either AT$_1$R- or AT$_2$R-transfected HEK-293 cells, were calculated as affinity estimates for each ligand. Log ratios of IC$_{50}$ values for each ligand at AT$_1$R:AT$_2$R were determined as a measure of ATR selectivity.

In Vitro Reactivity

Thoracic aortic rings obtained from male FVB/N mice (≈25 weeks old) were set up for AT$_2$R relaxation assays as described previously.[20] Tissues were precontracted to 40% to 50% of a maximum U46619 (≈3×10^{-7} mol/L)-evoked contraction, and concentration-response curves to CGP42112 or selected β-Ang II peptidomimetic were performed in tissues that were preincubated with the AT$_1$R antagonist losartan (100 nmol/L). Analogous studies were performed with peptides either alone or in the presence of PD123319 (1 μmol/L) or L-NAME (1 μmol/L), performed in parallel. One tissue served as a time control (precontraction only).

In Vivo BP Assays

Experiments were performed in male SHR, aged 16 to 20 weeks and weighing between 300 and 385 g. Rats were anesthetized (75 mg/kg ketamine and 10 mg/kg xylazine, both intraperitoneally, supplemented as required). Two catheters were inserted into the right jugular for intravenous drug administration. A catheter was implanted into the right carotid artery for direct BP measurement as described previously.[21–23] Rats were housed in individual cages and allowed free access to food and water while maintained on 12-hour day/night cycle. The arterial catheter was infused overnight with heparinized saline using an infusion pump. Twenty-four hours after the surgery, the arterial catheter was attached to a pressure transducer, and mean arterial pressure (MAP) and heart rate were computed from the phasic BP signal via a MacLab-8 data acquisition system (ADInstruments).

Rats were assigned to receiving a randomized within-animal treatment regime over 4 days, as previously reported to determine in vivo AT$_2$R-mediated depressor function.[20–23] For this protocol, animals received β-substituted Ang II ligand alone, AT$_1$R antagonist candesartan (0.01 mg/kg IV) alone, β-substituted Ang II ligand plus candesartan, or the previous combination plus the AT$_2$R antagonist PD123319 (50 μg/kg/min for 2 hours). We tested 2 groups of SHR. Group 1 received β-Tyr^4-Ang II (15 pmol/kg per minute for 4 hours) as the test ligand (n=11), whereas group 2 received β-Ile^5-Ang II (15 pmol/kg per minute for 4 hours) as the test ligand (n=9). It should be noted that we have repeatedly tested a saline infusion over this time, and it does not alter MAP.[20–23] In another set of experiments, WKY rats (n=6) were set up for conscious MAP

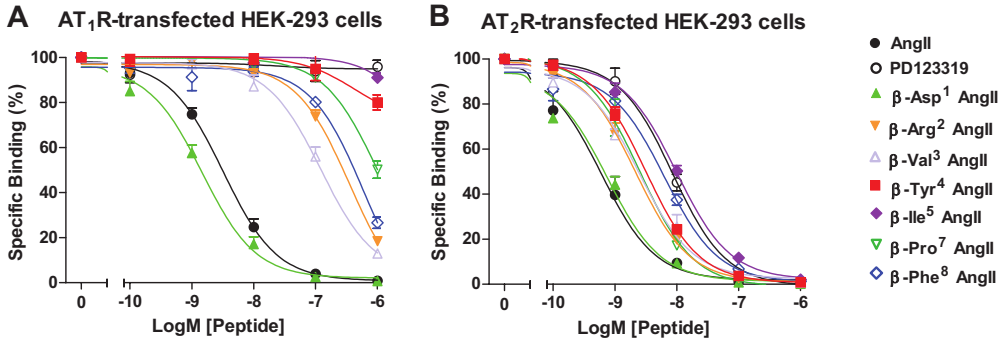

Figure 1. Competition binding from 3 separate experiments (each in triplicate) for β-substituted Ang II peptides, native Ang II, and the AT_2R antagonist PD123319 against ^{125}I-Ang II in either AT_1R-transfected (A) or AT_2R-transfected (B) HEK-293 cells. IC_{50} values are listed in Table 2.

recordings as just described; bolus intravenous doses of Ang peptides (5 or 40 ng) were injected to determine AT_1R-mediated pressor activity.

Proteolytic Stability

Selected peptide analogs were tested in a plasma stability assay to assess the susceptibility of β-substituted Ang II peptides to plasma proteases. A sample of rat plasma (diluted 1:1 with saline) was incubated with β-Tyr^4-Ang II, β-Ile^5-Ang II, or Ang II (each final concentration was 1 mg/mL), and protease activity was quenched in samples by the addition of acetonitrile, at selected time intervals. The amount of parent compound remaining at each time point was then assayed on an Agilent 1100 MSD SL ion trap mass spectrometer. The peaks observed in the resulting chromatograms were integrated, compared to a standard curve, and cross-checked by mass and retention time.

Statistical Analysis

All data are presented as mean responses±SEM. Differences in vasorelaxation or MAP between treatments were analyzed using a 2-way repeated-measure ANOVA. Nonlinear regression of binding data was also performed. All statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software Inc.). Probability values <0.05 were considered statistically significant.

Results

As expected, Ang II displayed low nanomolar affinity at both the AT_1R and AT_2R expressed in HEK-293 cells. The AT_2R antagonist PD123319 fully displaced AT_2R binding but not AT_1R binding (Figure 1). β-substitutions at each position of Ang II were well tolerated with respect to AT_2R binding since all compounds completely displaced ^{125}I-Ang II in AT_2R-transfected HEK-293 cells (β-Asp^1 Ang II equipotent with native Ang II) (Figure 1B). In contrast, apart from β-Asp^1-Ang II and Ang II, all other peptides poorly displaced AT_1R binding, with substitutions in positions 4 and 5 being relatively insensitive at 1 μmol/L (Figure 1A). IC_{50} values for all ligands at AT_2R and AT_1R are listed in Table 2, from which it was determined that β-Tyr^4-Ang II and β-Ile^5-Ang II were both ≈1000-fold selective for AT_2R over AT_1R. These selectivities were conservative estimates because the latter 2 compounds had IC_{50} values for AT_1R arbitrarily set at 10 μmol/L since there was little displacement at 1 μmol/L.

On the basis of these binding data, we have tested functionality of 3 key peptides: a nonselective ligand (β-Asp^1-Ang II) and 2 AT_2R-selective ligands that had significant AT_2R affinity but failed to displace AT_1R binding (β-Tyr^4- and β-Ile^5-Ang II) (Figure 2). Potential AT_2R-mediated vasorelaxation of mouse thoracic aortae (during AT_1R blockade) was examined. β-Asp^1-, β-Tyr^4-, and β-Ile^5-Ang II all relaxed aortae to a similar extent as the AT_2R agonist CGP42112 (Figure 2B), and this effect was blocked by the AT_2R antagonist PD123319 (data not shown). In addition, in the absence of AT_1R blockade, β-Ile^5-Ang II– induced relaxation was inhibited by PD123319 and L-NAME (Figure 3), and similar findings were noted for β-Tyr^4-Ang II (data not shown). In contrast, only β-Asp^1-Ang II (5 ng) substantially increased BP following an intravenous bolus injection in conscious rats since 8-fold higher doses of β-Tyr^4-Ang II and β-Ile^5-Ang II exerted minimal pressor activity compared with native Ang II (Figure 2A).

Next, we infused β-Tyr^4-Ang II and β-Ile^5-Ang II into separate groups of conscious SHR. In this setting, an infusion of β-Tyr^4-Ang II infusion had no effect on BP with or without AT_1R blockade (Figure 4), On the other hand, β-Ile^5-Ang II lowered BP against a background of AT_1R blockade, and this depressor effect was abolished by concomitant administration of PD123319 (Figure 5), consistent with an AT_2R-mediated depressor effect.[20–23]

In plasma stability assays, the percentage of each peptide remaining from spiked plasma samples over time was calcu-

Table 2. IC_{50} Values (in nmol/L) and Relative AT_2R Selectivity of Peptide Ligands*

Ligand	AT_1R	AT_2R	Log IC_{50} (AT_1R: AT_2R)
Ang II	3.29	0.65	0.70
PD123319	>1000	8.32	3.08†
β-Asp_1-Ang II	1.58	0.79	0.30
β-Arg_2-Ang II	275	2.24	2.09
β-Val_3-Ang II	133	2.77	1.68
β-Tyr_4-Ang II	>1000	3.19	3.50†
β-Ile_5-Ang II	>1000	10.6	2.98†
β-Pro_7-Ang II	1000	2.46	2.61
β-Phe_8-Ang II	420	6.56	1.81

*Competition binding from 3 separate experiments (each in triplicate).
†IC_{50} value of 10 μmol/L at AT_1R used for calculation.

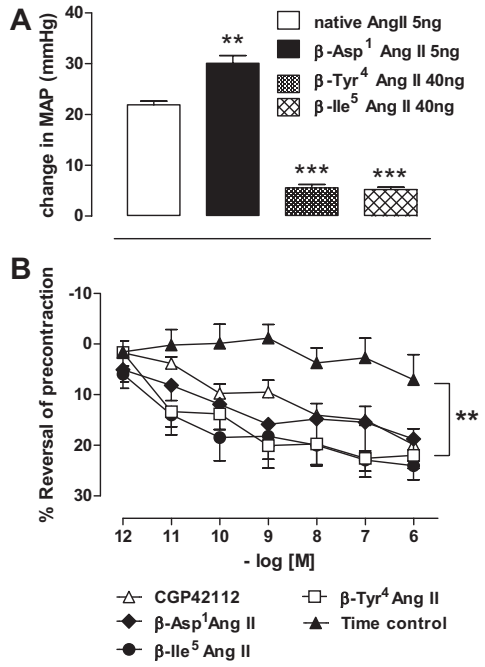

Figure 2. A, In vivo AT$_1$R-mediated pressor responses in WKY rats (n=6) in response to Ang II (5 ng), β-Asp^1-Ang II (5 ng), β-Tyr^4-Ang II (40 ng), and β-Ile^5-Ang II (40 ng). ***P<0.001, **P<0.01 vs native Ang II. B, In vitro AT$_2$R-mediated vasorelaxation in mouse aorta (in the presence of 100 nmol/L losartan) in responses to β-substituted Ang II analogs and the AT$_2$R agonist CGP42112 (n=5). **P<0.01 for all peptides vs time control.

lated. Both β-Tyr^4-Ang II and native Ang II disappeared completely at the 24-hour time point, whereas β-Ile^5-Ang II was still present at this time (Figures S1 to S3, available at http:/hyper.ahajournals.org). For each spiked plasma sample, the analysis of 1-phase exponential decay of the peptides produced apparent half-lives of 28, 65, and 295 minutes for Ang II, β-Tyr^4-Ang II, and β-Ile^5-Ang II, respectively (Figure 6).

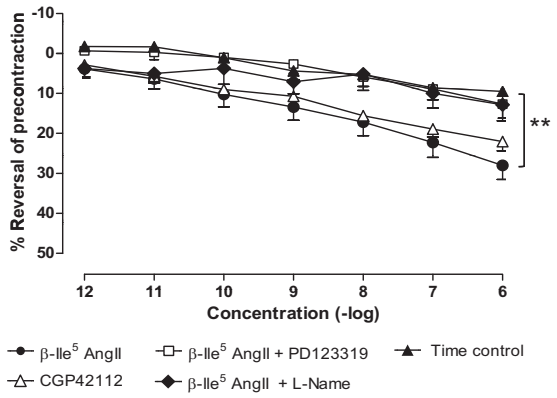

Figure 3. In vitro AT$_2$R-mediated vasorelaxation in mouse aorta (in the absence of losartan) in response to β-Ile^5-Ang II alone or in the presence of the AT$_2$R antagonist PD123319 or the NOS inhibitor L-NAME (n=5 to 7). **P<0.01 for peptide alone vs with PD123319, L-NAME, or time control.

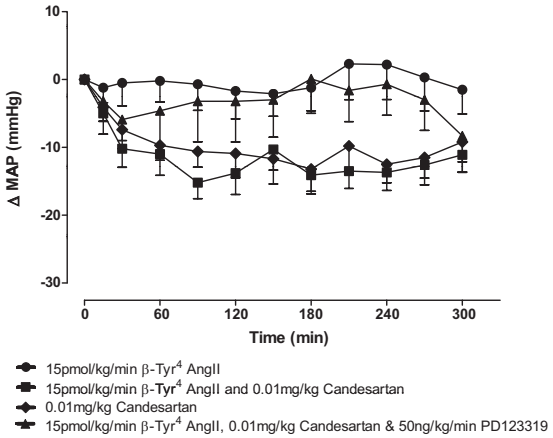

Figure 4. Effect of β-Tyr^4-Ang II (15 pmol/kg per minute for 4 hours) on MAP in spontaneously hypertensive rats (n=11) in the absence and presence of candesartan (0.01 mg/kg IV) or PD123319 (50 μg/kg/min for 2 hours).

Results from liquid chromatography mass spectrometry indicated that β-Tyr^4-Ang II was rapidly degraded, compared to β-Ile^5-Ang II. Noticeably, the formation of 2 main metabolic products, des-Asp^1-β-Tyr^4-Ang II and des-Asp^1, Phe^8 β-Tyr^4-Ang II also were degraded quickly. The main metabolites of β-Ile^5-Ang II were identified as β-Ile^5-Ang III and des-Asp^1, Phe^8 β-Ile^5-Ang II. These metabolites also showed greater proteolytic stability. This was determined by the analysis of the accumulation of Ang III (des-Asp^1-Ang II) or the β-substituted-Ang III metabolite over time (measured by LC/MS) from the respective Ang II parent compounds in spiked plasma that were compared against synthesized standards of β-substituted Ang III peptides (Figure 7).

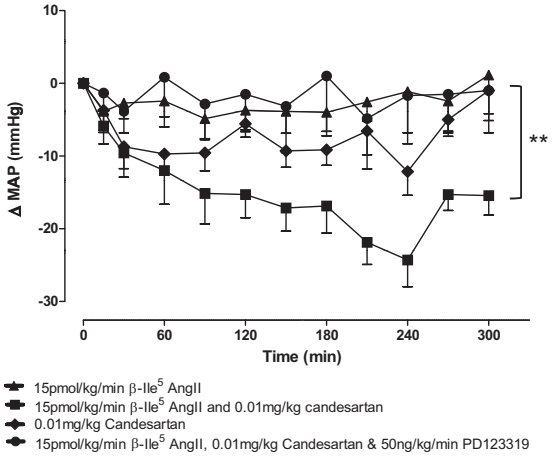

Figure 5. Effect of β-Ile^5-Ang II infusion (15 pmol/kg per minute for 4 hours) on MAP in SHR (n=9) in the absence and presence of candesartan (0.01 mg/kg IV) or PD123319 (50 μg/kg per minute for 2 hours). **P<0.01 for β-Ile^5-Ang II plus candesartan combination vs all other treatments.

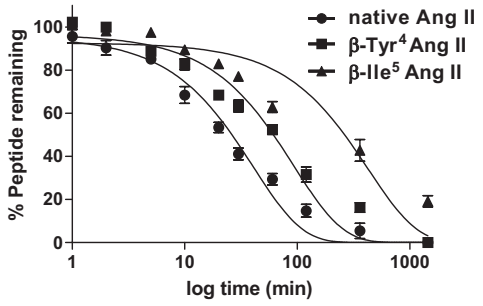

Figure 6. Plasma stability of native Ang II, compared with β-Tyr^4-Ang II or β-Ile^5-Ang II, assessed by determining the percentage peptide remaining following incubations in plasma over 24 hours.

Discussion

We have made the discovery that a single β-substitution in Ang II at the tyrosine (Y) or isoleucine (I) residue can markedly increase AT_2R selectivity and function, together with prolonging plasma stability. As such, these relatively simple peptide modifications represent a novel strategy to design AT_2R-selective peptidomimetic ligands for subsequent functional testing.

There is intense interest in the development of selective AT_2R agonists,[2] particularly since PD123319 (AT_2R antagonist) and AT_2R knockout mice only indirectly inform on AT_2R function but do not address the importance of direct AT_2R activation. Compound 21 was recently developed, modeled on the C-terminal pentapeptide structure of Ang II.[24] Compound 21 evoked a cardioprotective effect following myocardial infarction in rats,[25] and we have shown that Compound 21 acutely lowers BP (during AT_1R blockade) in conscious hypertensive animals.[20] Clearly, there is an unmet need to develop a range of AT_2R agonists to examine class effects of AT_2R agonists directly in a number of cardiovascular diseases including heart failure[25] and stroke.[26]

The majority of previous studies that have reported on novel Ang II peptides have examined binding profiles in a range of tissues with varying proportions of AT_1R and AT_2R, often with no functional correlate to assess functionality. In the present study, using homogenous ATR-transfected cell lines, we have shown that individual β-amino acid substitutions were well tolerated for AT_2R binding but not for AT_1R binding, suggesting the constraints on AT_1R binding are more strict than AT_2R. Consistent with these findings, alanine or glycine scans of Ang II caused only minor changes in AT_2R binding affinity, whereas there was sometimes marked reduction in AT_1R binding.[27,28] In the current study, on the basis of differential displacement of AT_2R and AT_1R binding, β-Tyr^4-Ang II and β-Ile^5-Ang II exhibited negligible AT_1R binding, similar to the AT_2R antagonist PD123319, thus identifying these peptides, which are modified at positions 4 and 5, as highly selective (>1000-fold) AT_2R ligands. These findings are consistent with other studies highlighting the importance of modification to the central portion of Ang II (at positions 4 and 5) for AT_2R binding. Substitution of His^6 by 4-NH_2-Phe^6 in Ang II produced a peptide with high AT_2R-to-AT_1R selectivity in binding assays.[29] Hallberg and colleagues[30] have also performed a number of modifications to Ang II, principally around the Tyr^4-Ile^5 residues, resulting in a number of highly AT_2R-selective peptidomimetics. For example, γ-turn scaffolds have been introduced around residues 4 and 5 to produce AT_2R-selective compounds.[30] In other studies, analogs with tyrosine-functionalized bicyclic dipeptides replacing the Tyr^4-Ile^5 residues presented an extended backbone conformation with heightened AT_2R-to-AT_1R selectivity.[31] The majority of the aforementioned studies relied on binding affinity estimates derived from displacement of iodinated Ang II from AT_1R in rat liver membranes and AT_2R in pig uterine membranes. However, functional assays on Ang II peptidomimetics that would indicate agonist or antagonist cardiovascular activity are lacking.

The Ang II peptidomimetics with β-Y and β-I substitutions both caused relaxation of isolated mouse aortic rings in a PD123319-sensitive manner, as also seen with CGP42112 used in parallel tissues. Thus, both binding and functional data would suggest that simple modification to the central portion of Ang II (at positions 4 and 5) yielded highly selective AT_2R agonists, signaling via nitric oxide, whereas β-Asp^1-Ang II was a nonselective agonist causing AT_2R-mediated relaxation and AT_1R-mediated in vivo pressor effects. Despite both β-Tyr^4-Ang II and β-Ile^5-Ang II causing vasorelaxation in vitro, only the latter was an effective vasodilator in vivo in SHR, during AT_1R block. This effect was confirmed to be mediated by the AT_2R as it could be reversed by concomitant PD123319 infusion. The magnitude of β-Ile-Ang II–induced hypotension was similar to that observed in response to CGP42112,[21,22] Ang (1–7),[23] and Compound 21[20] in analogous studies.

One possibility for the discrepancy between in vitro (mouse) and in vivo (rat) functional data may relate to a species difference in AT_2R abundance, although this seems unlikely since we have reported identical in vitro AT_2R-mediated vasorelaxation evoked by Compound 21 in aortae from mice and aortae and mesenteric arteries from SHR,[20] and β-Ile^5-Ang II was effective in both mouse and rat tissues in the current study. Rather, the discrepancy between the in

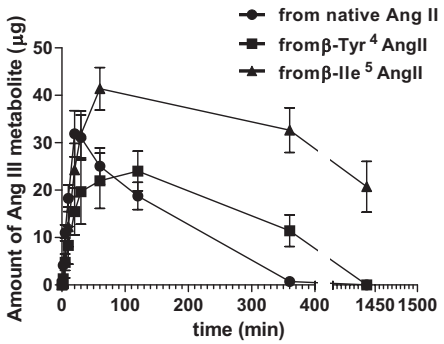

Figure 7. Accumulation of Ang III (circles), β-Tyr^4-Ang III (squares), or β-Ile^5-Ang III (triangles) over time from parent compounds Ang II, β-Ile^5-Ang II, or β-Tyr^4-Ang II, respectively.

vitro and in vivo effects of β-Tyr^4-Ang II may relate to the fact that, in plasma stability assays, β-Ile^5-Ang II had a half-life ≈10 times longer than native Ang II, while the half-life of β-Tyr^4-Ang II was only double that of Ang II. Thus, β-substitution at position 5 conferred greater protease resistance to the molecule. However, it is acknowledged that our in vitro plasma stability measurement does not account for tissue metabolism or cellular uptake, which will be addressed in future studies by determining plasma concentrations following in vivo peptide infusion. Nevertheless, it would appear that the major metabolite formed from β-Ile^5-Ang II degradation, at least in plasma, was β-Ile^5-Ang III, which accumulated to 3-times–greater levels than the corresponding metabolites, β-Tyr^4-Ang III or Ang III, from their respective Ang II parent molecules. In this context, Ang III can act as an endogenous AT$_2$R ligand to lower BP and evoke natriuresis in a number of settings.[32,33] Thus, the in vivo vasodepressor effect seen by β-Ile^5-Ang II, but not β-Tyr^4-Ang II, may be due to slowed metabolism of Ile^5-Ang II acting at AT$_2$R. However, at this stage, we cannot totally discount additional effects of other metabolites such as β-Ile^5-Ang III, or even an Ile-modified-Ang (1–7) or Ang (2–7), contributing to β-Ile^5-Ang II depressor activity.

In summary, our relatively simple chemical strategy of β-amino acid substitution to Ang II has resulted in Ang peptidomimetics with substantial AT$_2$R selectivity, which translated into functional agonist activity. As such, this type of peptidomimetic design shows great potential for the production of research tools to provide insight into AT$_2$R function. In particular, our study has revealed that β-Ile^5-Ang II acted as a novel AT$_2$R-selective agonist with in vivo vasodepressor activity.

Perspective

Research elucidating the effects of AT$_2$R modulation of cardiovascular function has been severely hampered by a lack of selective agonists or antagonists to probe AT$_2$R function, particularly in chronic in vivo experiments. In the current study, we have performed simple modifications to the peptide Ang II by incorporating an additional methylene group in the peptide backbone, resulting in a series of peptides with preferential AT$_2$R selectivity. In particular, β-substitutions at positions 4 and 5 conferred marked selectivity for agonism at AT$_2$R over AT$_1$R. This strategy could be used to investigate a number of related peptides and will allow more detailed comparison with other AT$_2$R agonists on cardiovascular function, thus providing greater information for class effects of AT$_2$R.

Sources of Funding

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Disclosures

None.

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ONLINE SUPPLEMENT

CHBPR 164301: A SINGLE BETA-AMINO ACID SUBSTITUTION TO ANGIOTENSIN II CONFERS AT_2 RECEPTOR SELECTIVITY AND VASCULAR FUNCTION

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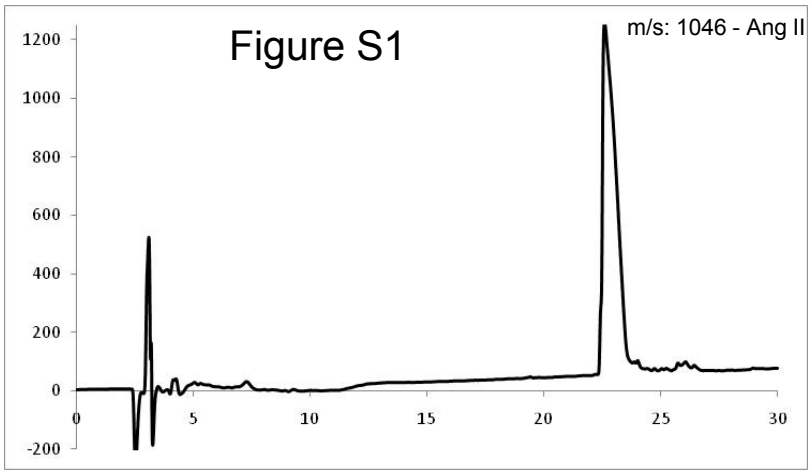

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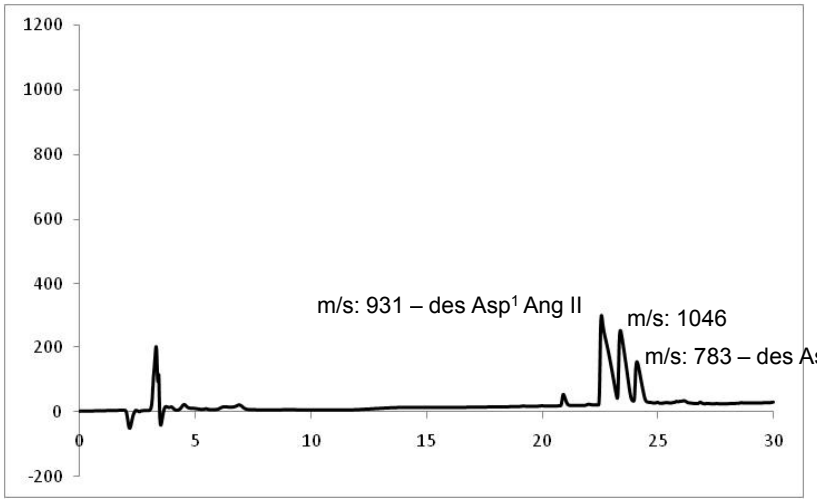

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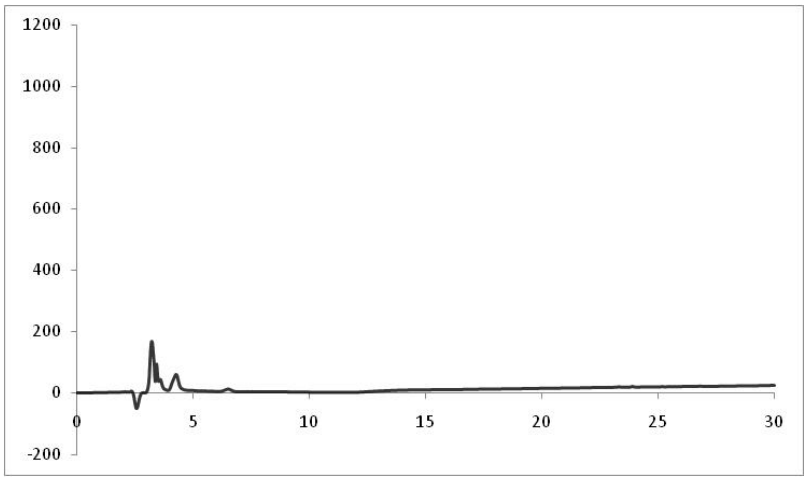
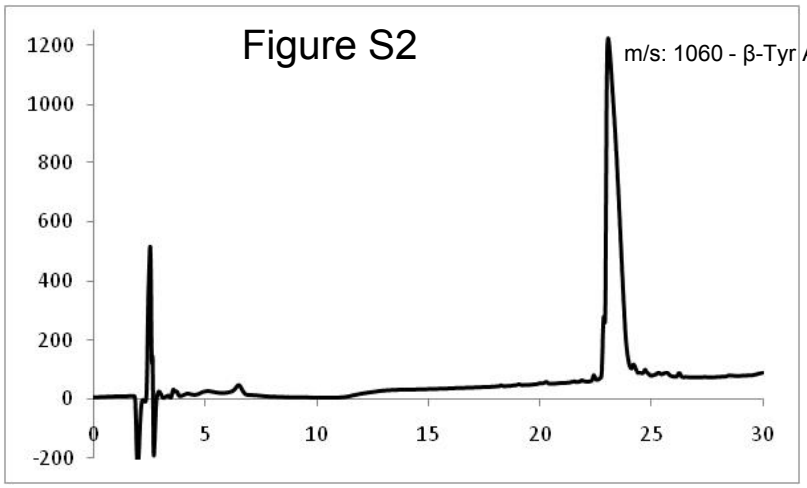


C

HPLC traces of plasma samples following incubation of a 1mg/mL solution of Angiotensin II at A) 0 mins B) 60 mins and C) 24 hours. The molecular weight of each peak is also shown and peptides identified.



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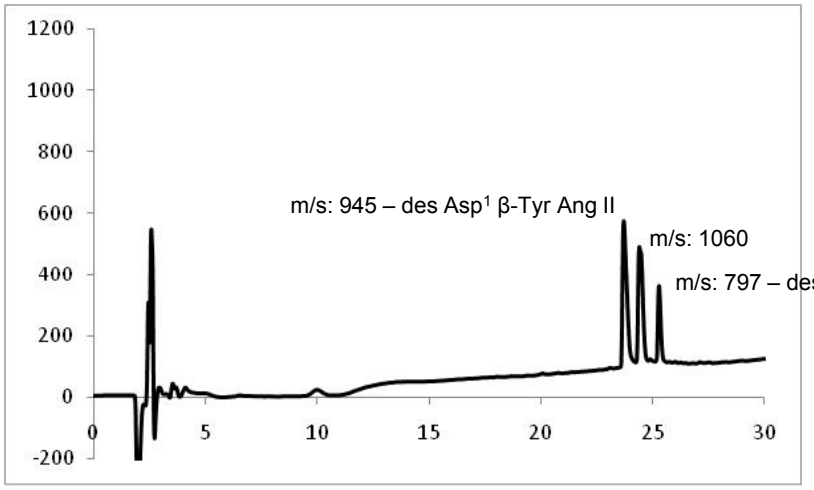

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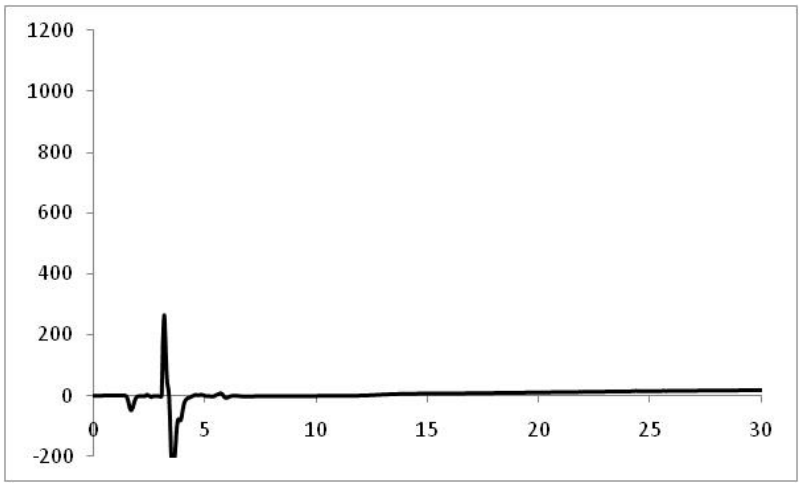
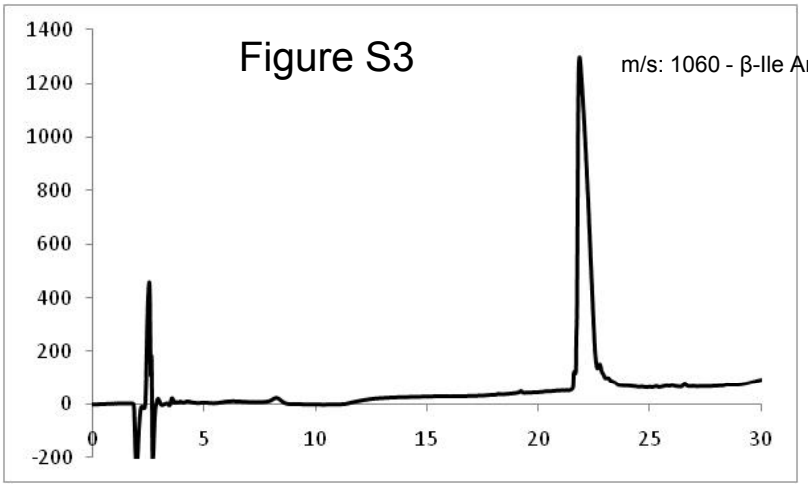


C

HPLC traces of plasma samples following incubation of a 1mg/mL solution of β-Tyr-Angiotensin II at A) 0 mins B) 60 mins and C) 24 hours. The molecular weight of each peak is also shown and peptides identified.



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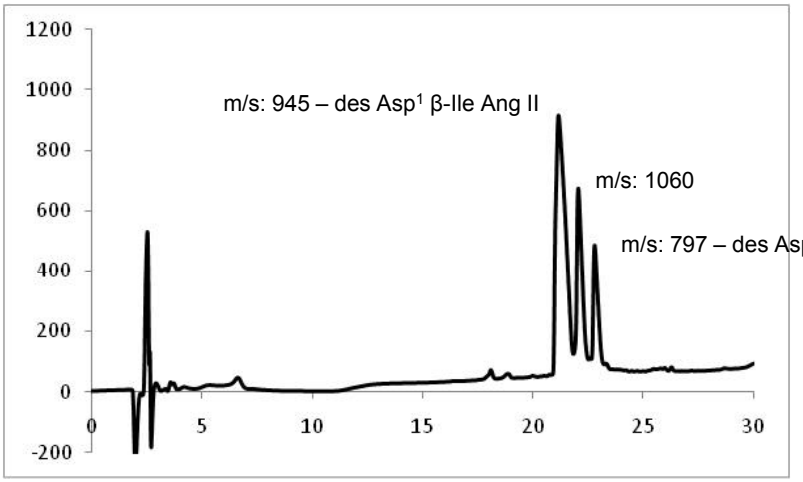

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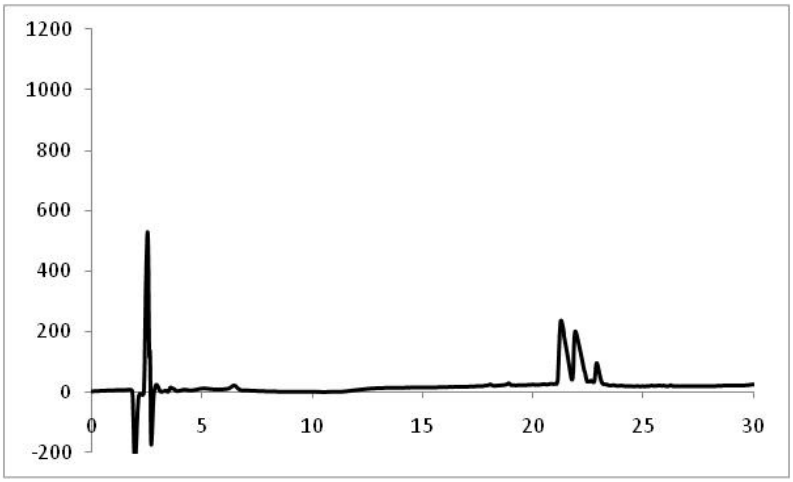

C

HPLC traces of plasma samples following incubation of a 1mg/mL solution of β-Ile-Angiotensin II at A) 0 mins B) 60 mins and C) 24 hours. The molecular weight of each peak is also shown and peptides identified.

Chapter 4:

Differential mechanism of Ang (1-7) - mediated vasodepressor effect in adult aged rats

Declaration for Thesis Chapter 4

Monash University

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed all of the experiments, analysed the results and wrote the manuscript.	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution
Emma Jones Kate Denton Robert Widdop	Provided intellectual advice during experimental and manuscript preparation stages.

Candidate's Signature: .....

Date: .....

Declaration by co-authors
The undersigned hereby certify that:
(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Name	Location	Signature	Date
Emma Jones	Monash University Department of Pharmacology		
Kate Denton	Monash University Department of Physiology		
Robert Widdop	Monash University Department of Pharmacology		

4.1 INTRODUCTION

It is well known that Angiotensin II (Ang II) mediates its physiological functions via two main receptor subtypes, the type 1- (AT_1R) and type 2- (AT_2R) angiotensin receptors where it has similar affinity for both the AT_1R and AT_2R. However, there is now increasing evidence suggesting that angiotensin peptides other that Ang II can evoke cardiovascular effects that oppose the effects mediated by the AT_1R via a number of non-AT_1R mechanisms. In fact, heptapeptide Angiotensin (1-7), (Ang (1-7)), a biologically active metabolite of angiotensin I (Ang I) and Ang II [1, 2] has been shown to possess biological activity in its own right [3]. Interest in Ang (1-7) has surged since the discovery of angiotensin converting enzyme type 2 (ACE2) and recognition that Ang (1-7) can be produced directly from Ang II via ACE2 [1, 2]. Although, Ang (1-7) differs to Ang II by only one amino acid, Ang (1-7)-mediated effects are markedly different to those of Ang II, and it has been suggested that Ang (1-7) may in fact play a counter regulatory role to Ang II [4], mediating a range of effects such as vasodilatation, inhibition of vascular smooth muscle proliferation and fluid and electrolyte homeostasis [5]. The cardiovascular effects of Ang (1-7) are often reported to be inhibited by the D-Ala^7 Ang (1-7) analogue, known as A-779 [6]. Recently, Ang (1-7) was identified as an endogenous ligand for the Ang (1-7)/*Mas*R (*Mas*R), since Ang (1-7)-mediated vasorelaxation was impaired in *Mas*$R^{-/-}$ mice [7]. However, under some circumstances, Ang (1-7) can mediate its effects via AT_2R [8-10]. In fact, we have shown that, Ang (1-7)-mediated vasodepressor effect was via an AT_2R sensitive pathway [11]. In that study, Ang (1-7) acutely lowered blood pressure in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) adult rats during concomitant AT_1R blockade, [11] in a similar manner to that seen with AT_2R agonist, CGP42114 [12, 13] and more recently with selective non-peptide AT_2R

agonist, Compound 21 [14]. Furthermore, the AT_2R antagonist, PD123319, but not the *Mas*R antagonist, A779, blocked this vasodepressor effect of Ang (1-7) [11].

While it is well recognized that the renin-angiotensin system, (RAS), has a critical role in the cardiovascular system, its role in the aging process is still under investigation**.** During aging, circulating levels of Ang II are downregulated while local production of Ang II is increased in the aorta and other vessels [15] suggesting an essential role of local RAS in the vasculature during aging. However, there is little functional evidence about angiotensin receptors and their role during aging. In this context, we have shown that AT_2R expression, was increased in both endothelial and vascular smooth muscle of aortae obtained from aged WKY rats [16].

Given that there was an increased vascular AT_2R expression in aging [16], the current study was designed to test our hypothesis that AT_2R-mediated depressor function was preserved with aging. In the present study, we have used Ang (1-7) as an endogenous ligand for the AT_2R, as we have previously reported in adult rats [11]. In preliminary experiments, we have determined that vascular expression of both AT_2R and *Mas*R/ACE2 axis was upregulated with aging. Therefore, this strategy of using Ang (1-7) will also determine whether or not there was a role for *Mas*R to evoke vasodepressor effects with aging.

4.2 METHODS

4.2.1 ANIMALS

All animal care and experimental procedures were approved by the Monash University Animal Ethics Committee and performed according to the guidelines of the National Health and Medical Research Council of Australia for animal experimentation.

Male 16- to 18-week-old WKY rats (300 to 350g) and male 20 month old WKY rats (450-500g) were obtained from the Animal Resource Centre (Perth, WA, AUS) and were used to represent adult and aged normotensive rats, respectively. Animals were maintained on a 12-hour day/night cycle with standard laboratory rat chow and water available *ad libitum*.

4.2.2 *IN VIVO P*ROCEDURES

Rats were anesthetised (ketamine and xylazine; 75mg/kg and 10mg/kg, i.p, respectively; supplemented as required). Two catheters were inserted into the right jugular for intravenous drug administration. A catheter was implemented into the right carotid artery for direct blood pressure measurement as described previously [11-14]. Rats were housed in individual cages and allowed free access to food and water while maintained on 12-hour day/night cycle. The arterial catheter was infused overnight with heparinised saline using an infusion pump.

24 hours after the surgery, the arterial catheter was attached to a pressure transducer (Gould Inc), connected to a MacLab-8 data acquisition system (ADInstruments, Sydney), interfaced to a Macintosh computer. Mean arterial pressure (MAP) and heart rate (HR) were computed from the phasic blood pressure signal.

4.2.3 EXPERIMENTAL PROTOCOL

Rats received drug combinations in a randomised fashion over a 4-day protocol, as we have performed previously [11, 12, 14]. Doses for candesartan and PD123319 were chosen on the basis of previous studies [11, 12, 14]. Six groups of rats underwent experimental protocols during which basal MAP and HR were recorded. Adult and aged WKY rats (Groups 1 and 2, respectively), were randomized to receive following treatments on different days: (1) candesartan (0.01mg/kg); (2) Ang (1-7) infusion (15pmol/kg per minute for 4 hours); (3) Ang

(1-7) infusion together with candesartan; and (4) a 4–hour infusion (0.1ml/kg per hour IV) of saline (0.9% NaCl) to confirm a lack of effect on MAP. Animals in Group 3 (adult WKY rats) and Group 4 (aged WKY rats) were randomized to receive following treatments: (1) candesartan at a 10-fold higher dose (0.1mg/kg); (2) Ang (1-7) infusion (15pmol/kg per minute for 4 hours); (3) Ang (1-7) infusion together with candesartan; and (4) Ang (1-7) infusion in the presence of candesartan and PD123319 infusion (50μg/kg per minute for 2 hours). In analogous experiments in additional adult and aged WKY rats (Groups 5 and 6), the putative Ang (1-7) antagonist, A-779 (15pmol/kg per minute), was used instead of PD123319. Doses of Ang (1-7) and A779 are based on our previous study [11].

4.2.4 LOCALIZATION OF ACE2, AT_1, AT_2 AND *MAS* RECEPTORS

Localization of ACE2, AT_1, AT_2 and *Mas* receptors using immunofluorescence was performed using thoracic aortic sections taken from naïve aged and adult rats to determine changes in expression levels between the two age groups. Male adult and aged WKY rats were killed by isoflurane inhalation followed by decapitation and the thoracic aorta was removed in order to dissect 3-5 mm long sections. Immunofluorescence was performed using 10□m thick section of thoracic aorta cut on Cryostat. Aortic sections were incubated overnight at 4°C with 1/500 dilution of polyclonal rabbit antibodies raised against AT_1R, AT_2R, *Mas*R and ACE2. Following overnight incubation, sections were incubated for 2.5 hour with a goat anti-rabbit secondary antibody conjugated with Alexa 568 flurophore. Rabbit IgG antibody was used as negative control. Sections were mounted with anti-fade medium (Vectorshield) and cover slipped. Sections were imaged using Olympus Fluoview 500 confocal microscope equipped with a ktypton/argon laser.

Fluorescence intensity was quantified using analySIS Professional software (Soft Imaging System, Singapore) with identical measurement settings.

4.2.5 STATISTICAL ANALYSIS

All data are presented as mean responses ± standard error of the mean (SEM). Differences in MAP between treatments were analysed using a 2-way ANOVA repeated measure analysis of variance. Differences in fluorescence intensity were analysed using 1-way ANOVA with Bonferroni corrections where appropriate. Statistical analysis was performed using GraphPad Prism (Version 5.0c). P values < 0.05 were deemed statistically significant.

4.2.6 MATERIALS

PD123319 and candesartan were kind gifts from Pfizer and AstraZeneca respectively. All other chemicals were purchase from commercial sources: ketamine (Troy Laboratories, Australia), xylazine (Troy Laboratories), isoflurane (Baxter, USA), Ang (1-7) (Ausep, Australia), A-779 (Auspep, Australia), rabbit polyclonal antibodies raised against AT_1R, AT_2R and ACE2 (Santa Cruz Biotechnology Inc.), rabbit polyclonal antibody raised against *Mas*R (Novus Biologicals, USA), secondary goat-anti rabbit Alexa 568 antibody (Invitrogen, USA).

4.3 RESULTS

4.3.1 *IN VIVO* EFFECT OF ANG (1-7) IN CONSCIOUS NORMOTENSIVE RATS

Basal MAP of WKY rats over the 4 experimental days for each group are listed in Table 4.01. There was no significant difference between resting MAP over the experimental period for any of the treatment groups, suggesting that none of the acute treatments had long-lasting effects, and therefore did not influence baseline MAP on subsequent days (Table 4.1).

Table 4.1. Resting MAP recorded on separate days before drug treatments, as indicated

Treatment	MAP (mmHg)
Group 1 (n=4)	
Saline	132 ± 14
Ang-(1-7) (15pmol/kg/min)	124 ± 4
Candesartan (0.01mg/kg)	131 ± 6
Ang-(1-7) & Candesartan	134 ± 4
Group 2 (n=7)	
Saline	131 ± 13
Ang-(1-7) (15pmol/kg/min)	136 ± 13
Candesartan (0.01mg/kg)	126 ± 15
Ang-(1-7) & Candesartan	136 ± 16
Group 3 (n=6)	
Ang-(1-7) (15pmol/kg/min)	138 ± 8
Candesartan (0.1mg/kg)	135 ± 8
Ang-(1-7) & Candesartan	139 ± 11
Ang-(1-7), Candesartan & PD123319 (50 μg/kg/min)	139 ± 5
Group 4 (n=7)	
Ang-(1-7) (15pmol/kg/min)	143 ± 10
Candesartan (0.1mg/kg)	142 ± 10
Ang-(1-7) & Candesartan	142 ± 9
Ang-(1-7), Candesartan & PD123319 (50 μg/kg/min)	137 ± 9
Group 5 (n=8)	
Ang-(1-7) (15pmol/kg/min)	128 ± 11
Candesartan (0.1mg/kg)	122 ± 10
Ang-(1-7) & Candesartan	133 ± 5
Ang-(1-7),Candesartan& 779(15pmol/kg/min)	132 ± 2
Group 6 (n=8)	
Ang-(1-7) (15pmol/kg/min)	129 ± 5
Candesartan (0.01mg/kg)	126 ± 10
Ang-(1-7) & Candesartan	132 ± 6
Ang-(1-7), Candesartan & A779(15pmol/kg/min)	125 ± 8

In groups 1 and 2, infusion of saline had no significant effect on MAP (Figure 4.1). Therefore, this treatment was not performed in subsequent groups in order to include additional treatment arms. In all groups, infusion of Ang (1-7) (15pmol/kg/min) or candesartan (0.01 or 0.1 mg/kg IV), had no significant effect on MAP. Co-infusion of Ang (1-7) and candesartan (0.01mg/kg IV) had no effect on MAP in adult WKY rats (Figure 4.1A) whereas in aged WKY rats, combined administration of Ang (1-7) and candesartan (0.01mg/kg IV) significantly decreased MAP ($P<0.001$) (Figure 4.1B).

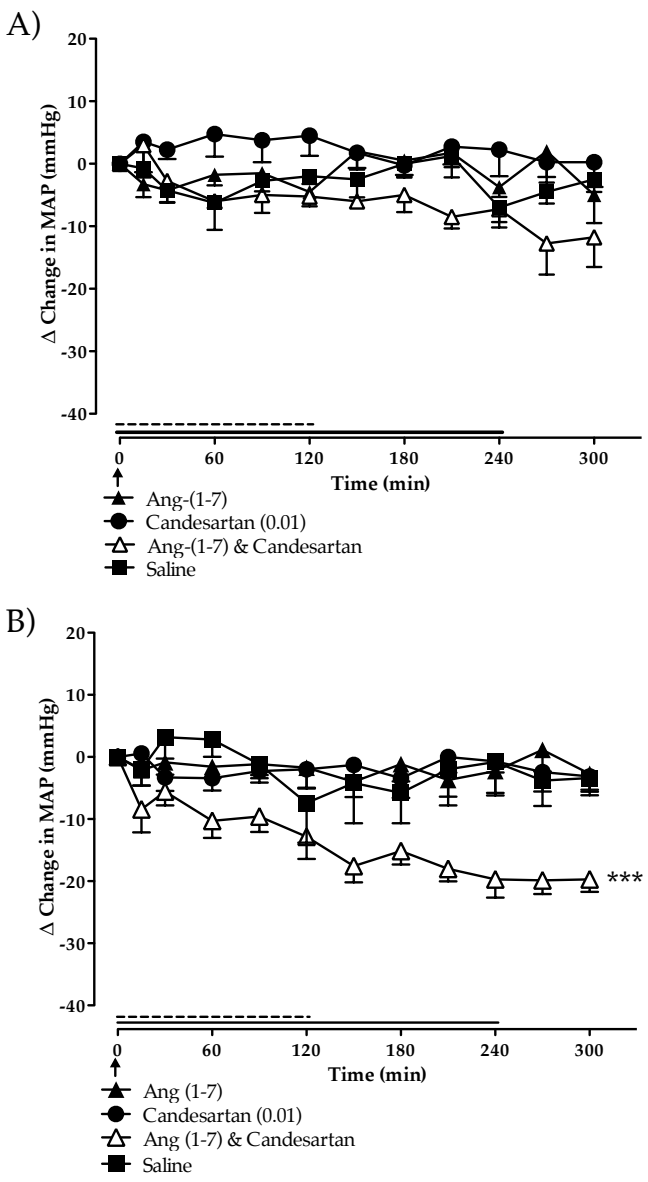

Figure 4.1: Effect of the AT_1R Ang (1-7) (15pmol/kg/min; 4-hour infusion depicted by full line), AT_1R antagonist, candesartan (0.01mg/kg bolus IV; depicted by an arrow), saline (0.1ml/kg 0.9% NaCl IV for 4 hours), and Ang (1-7) + candesartan, on MAP in (A) adult WKY rats (n=4) and (B) aged WKY rats (n=7). Values represent mean ± SEM. ***P<0.001; for treatment effect of Ang (1-7) + candesartan versus all other treatments (2-way RM ANOVA)

When Ang (1-7) was combined with a 10-fold higher dose of candesartan (0.1mg/kg IV), there were significant reductions in MAP in both adult and aged WKY male rats compared with Ang (1-7) alone or candesartan alone (P<0.01). Moreover, this depressor effect of Ang (1-7) was abolished by the addition of the AT_2R antagonist, PD123319 (50μg/kg/min), (Figures 4.2A and B).

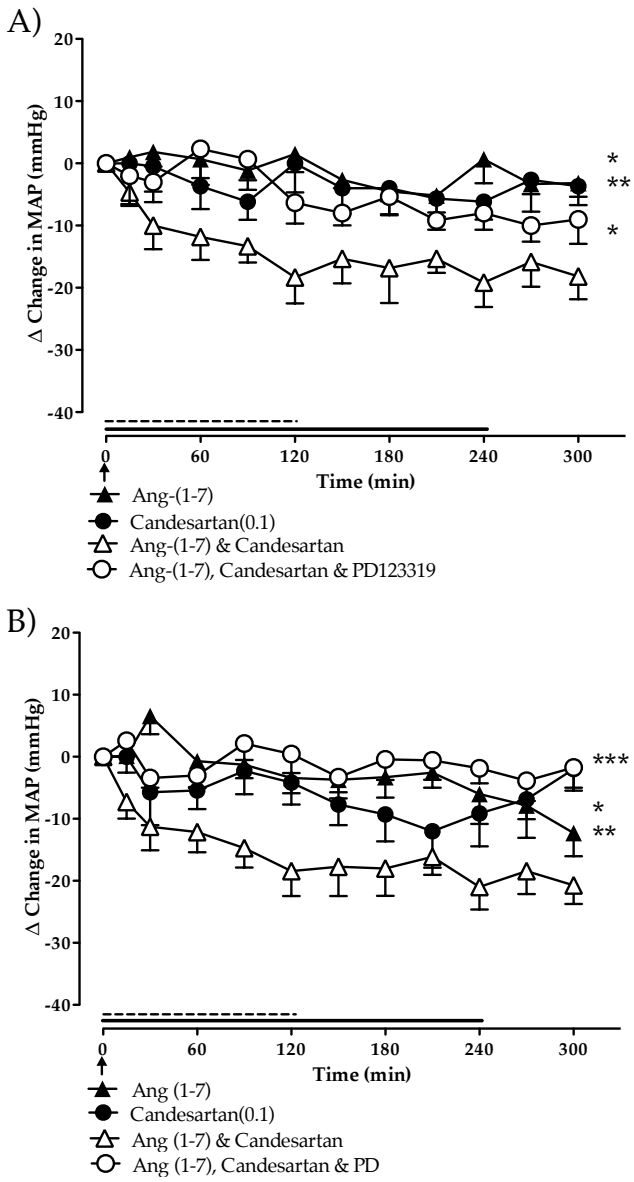

Figure 4.2: Effect of Ang (1-7) (15pmol/kg/min; 4-hour infusion depicted by full line), AT_1R antagonist, candesartan (0.1mg/kg bolus IV; depicted by an arrow), Ang (1-7) + candesartan, and Ang (1-7) + candesartan + AT_2R antagonist, PD123319 (50µg/kg/min for 2 hours; depicted by dashed line), on MAP in (A) adult WKY rats (n=6) and (B) aged WKY rats (n=7). Values represent mean ± SEM.
*P<0.05; **P<0.01; ***P<0.001; for treatment effect of Ang (1-7) + candesartan versus all other treatments as indicated (2-way RM ANOVA)

In separate groups of animals, we examined the ability of the *Mas*R antagonist A-779 to modify the Ang (1-7)-mediated depressor effect. Co-infusion of the Ang (1-7) antagonist A-779 with the Ang (1-7)/candesartan combination in adult WKY male rats did not affect Ang (1-7)-mediated depressor response (Figure 4.3A). By

contrast, the Ang (1-7)-evoked depressor response, during AT_1R blockade, in aged WKY rats was in fact abolished by the addition of A-779 (Figure 4.3B).

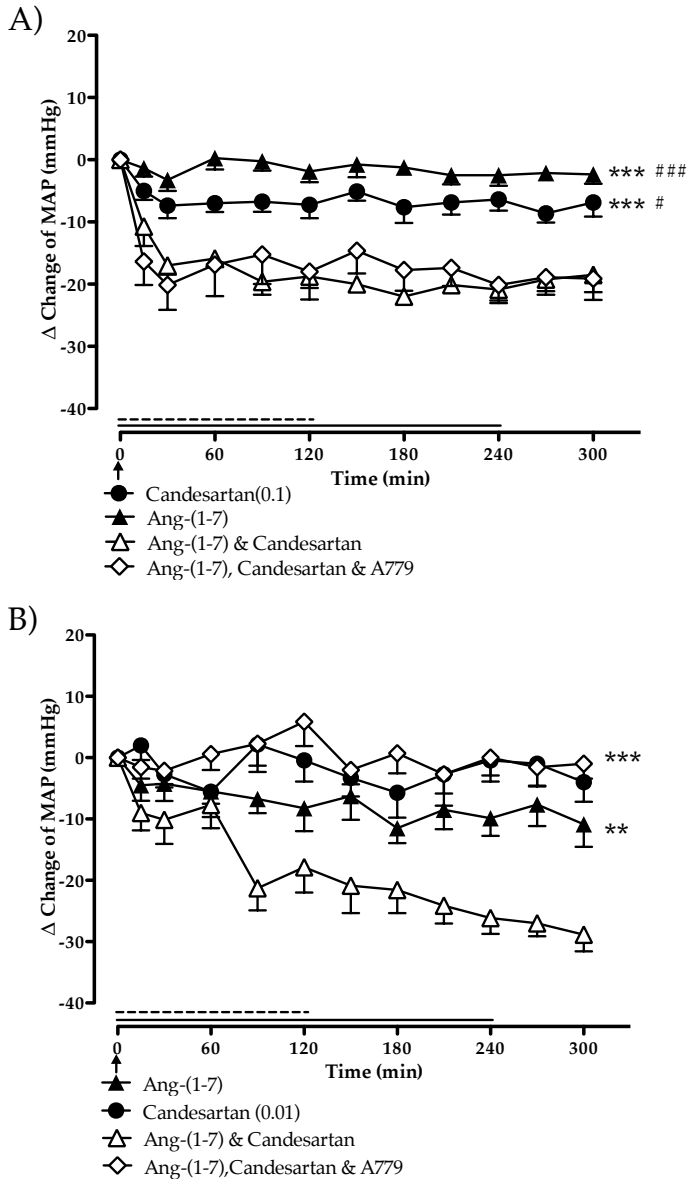

Figure 4.3: Effect of Ang (1-7) (15pmol/kg/min; 4-hour infusion depicted by full line), AT_1R antagonist, candesartan in (A) adult WKY rats (0.1mg/kg bolus IV; depicted by an arrow), and (B) aged WKY rats (0.01mg/kg bolus IV; depicted by an arrow), together with Ang (1-7) + candesartan, and Ang (1-7) + candesartan + *Mas*R antagonist, A779 (15pmol/kg/min for 2 hours; depicted by dashed line), on MAP (n=8 for both groups). Values represent mean ± SEM.
A.) ***P<0.001; for treatment effect of Ang (1-7) + candesartan versus Ang (1-7) or candesartan alone (2-way RM ANOVA)
†<0.05; †††<0.001; for treatment effect of Ang (1-7) + candesartan +A779 versus candesartan or Ang (1-7) alone (2-way RM ANOVA)
B.) **P<0.01; ***<0.001 for treatment effect of Ang (1-7) + candesartan versus Ang (1-7), candesartan or Ang (1-7) + candesartan + A-779 (2-way RM ANOVA)

4.3.2 LOCALIZATION OF ACE2, AT_1, AT_2 AND *MAS* RECEPTORS

Expression levels of ACE as well as angiotensin levels were determined using thoracic sections taken from naïve adult WKY rats (n=5) and aged WKY rats (n=4). ACE2, AT_1R, AT_2R and *Mas*R were all localised throughout the entire aortic sections (Figure 4.4A). Expression levels of the AT_1R were not changed between adult and aged WKY male rats, whereas ACE2, AT_2R and *Mas*R expression levels were all significantly upregulated in aged WKY rats compared to adult WKY rats (Figure 4.4B-E).

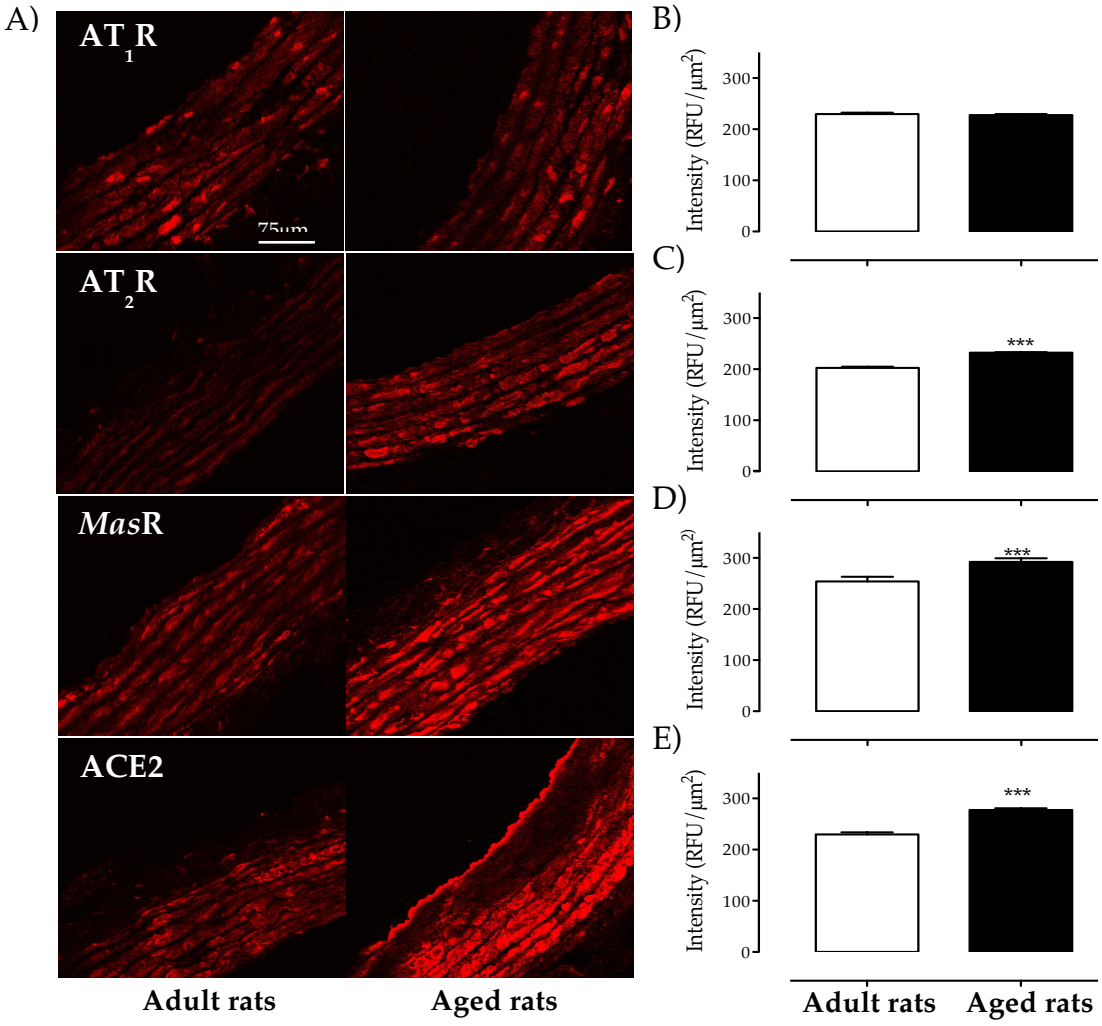

Figure 4.4: (A) Representative immunolocalisation images of AT_1R, AT_2R, *Mas*R and ACE2 in adult WKY rats and aged WKY rats. Mean data for aortic expression of the (B) AT_1R, (C) AT_2R, (D) *Mas*R and (E) ACE2 expressed as relative fluorescent units in adult (n=5) and aged (n=4) WKY rats.
***P<0.001 versus adult WKY rats

Therefore, when expressed relative to AT_1R levels, each of the vasodilator non-AT_1R components of the RAS was significantly increased in aged WKY rats compared to adult WKY rats (Figure 4.5).

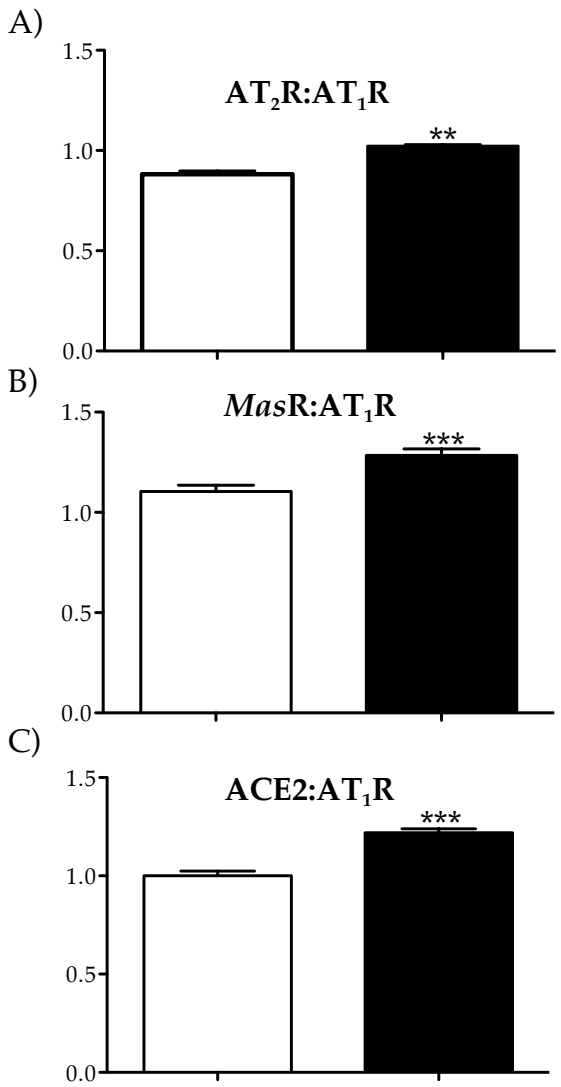

Figure 4.5: Ratios of A) AT_2R:AT_1R, B) *Mas*R:AT_1R and C)ACE2:AT_1R in adult and aged WKY rats.
**P<0.01, ***P<0.001 versus adult WKY rats

4.4 DISCUSSION

The main findings of the current study demonstrate for the first time that the depressor effect evoked by Ang (1-7) is preserved in aged normotensive animals,

and was sensitive to both AT_2R and *Mas*R blockade which contrasts with the involvement of only AT_2R in the effects of Ang (1-7) in adult rats. Moreover, these findings were consistent with increased AT_2R, *Mas*R and ACE2 expression in the thoracic aorta of aged WKY rats.

AT_2R mediated relaxation is a well established effect in isolated resistance vessels [17-21]. Previous studies have shown AT_2R-mediated vasodilatation in adult conscious rats [11-14, 22]. The AT_2R-mediated reduction in blood pressure was likely to be a result of direct vasodilatation, rather than a result of decrease in cardiac output, as CGP42112 increased mesenteric and renal conductance in SHR, which was indicative of regional vasodilatation [13]. Furthermore, it is well documented that in order to unmask any AT_2R-mediated vasodilatation, there needs to be a removal of a tonic AT_1R-mediated vasoconstriction induced by endogenous Ang II [23].

In the current study, acute Ang (1-7) infusion against a background of AT_1R blockade, resulted in a decrease in MAP in adult WKY male rats, and this Ang (1-7) response was mediated exclusively via AT_2R in adult WKY male rats since the AT_2R antagonist, PD123319, abrogated this Ang (1-7)-depressor response, which is consistent with previous findings obtained in both SHR and WKY rats [11]. Of note, the Ang (1-7) antagonist, A-779 failed to inhibit vasodepressor responses induced by Ang (1-7) during AT_1R blockade in adult rats, which confirmed our previous study that also found a 10-fold higher dose of A-779 failed to block Ang (1-7) [11]. Thus, at least in this adult model, an exclusive role for Ang (1-7) as an endogenous ligand for the AT_2R was demonstrated.

In contrast, in the aged setting, the vasodepressor effect of Ang (1-7) was mediated by both AT_2R and *Mas*R stimulation. Moreover, both candesartan doses (0.01 and 0.1 mg/kg) were effective in unmasking Ang (1-7)-mediated vasodepressor responses in aged rats. These results are consistent a 10-fold lower dose of

candesartan being used to reveal Ang (1-7)-mediated vasodepressor effects via AT_2R in SHR compared with WKY rats [11], and point towards an increased sensitivity to AT_1R blockade in aged rats, as we have noted previously [24]. Increased vascular expression of AT_2R in aging was seen in mesenteric resistance arteries [25] and in thoracic aorta [16]. Thus, Ang (1-7) infusion reduced MAP via AT_2R in aged WKY rats irrespective of the background dose of candesartan. However, there are numerous reports suggesting the *Mas*R as the functional binding site for Ang (1-7) [7]. For example, Peiro *et al.*, (2007), observed comparable impairment in Ang (1-7)-mediated vasorelaxation as a result of pharmacologic or genetic inhibition of *Mas*R using A-779 and *Mas*R-deficient mice, respectively. However, Ang (1-7) evoked vasorelaxation in pig coronary arteries that was attenuated by the AT_2R antagonist, PD123319, suggesting an AT_2R involvement [26]. Subsequent studies confirmed that Ang (1-7) can mediate its effects via AT_2R [8-10]. Ang (1-7)-stimulated NO release in bovine aortic endothelial cells was markedly attenuated by AT_2R inhibition (~90%) [27, 28] and to a lesser extent by *Mas*R inhibition (~50%) [27], suggesting activation of multiple receptors by Ang (1-7), which is also consistent with Ang (1-7)-stimulated arachidonic acid release in rabbit vascular smooth muscle cells [29].

More recently, we have demonstrated that chronic treatment with Ang(1-7) was both vaso- and athero-protective in Apolipoprotein E –deficient mice via both *Mas*R and AT_2R [30]. Similarly, in the current study, we found that Ang (1-7) evoked a vasodepressor responses in aged rats that was sensitive to both the AT_2R antagonist PD123319 and the *Mas*R antagonist A-779. This finding suggests that, unlike in adult normotensive rats, Ang (1-7) can act via AT_2R and/or *Mas*R during aging. Therefore, we also examined relative expression levels of the AT_1R, AT_2R and *Mas*R as well as ACE2 to determine if this could account for the age-related differences in the cardiovascular effects of Ang (1-7). We have now confirmed an

increased AT_2R expression in aortae from aged WKY rats [16] and in addition we have shown, for the first time, a marked increase in expression levels of both *Mas*R and ACE2 in aortic sections from aged WKY rats. These changes in ATR subtype expression fit with our *in vivo* results, and also with other evidence for increased AT_2R function in aging. For example, PD123319 can potentiate AT_1R–mediated contractions, which is an indirect measure of AT_2R relaxation [31, 32], and this 'PD123319 potentiation' was enhanced in human coronary microvessels and was positively correlated with age [33]. To our knowledge, there are no reported functional correlates for enhanced Ang (1-7) in aging. At the same time, there was no difference in the expression levels of AT_1R between adult and aged WKY rats, although a lower level of the AT_1R block was required to unmask the depressor effect of Ang (1-7) in aged rats. One possible explanation for this difference between aged and adult WKY rats is due to the presence of several potential vasodilator pathways (AT_2R, *Mas*R) resulting in preserved vasodilatation in aged WKY rats. This hypothesis is strengthened by the increased ratio of non-AT_1R components to AT_1R in aged WKY rats (Figure 4.6).

In conclusion we have found that Ang (1-7)-mediated vasodepressor activity is preserved with aging. Thus, we can postulate that an increased AT_2R/*Mas*R/ACE2 vasodilator axis relative to AT_1R in aged rats is in part responsible for the ability of Ang (1-7) to operate via multiple mechanisms in aging, as opposed to only AT_2R in adult normotensive rats.

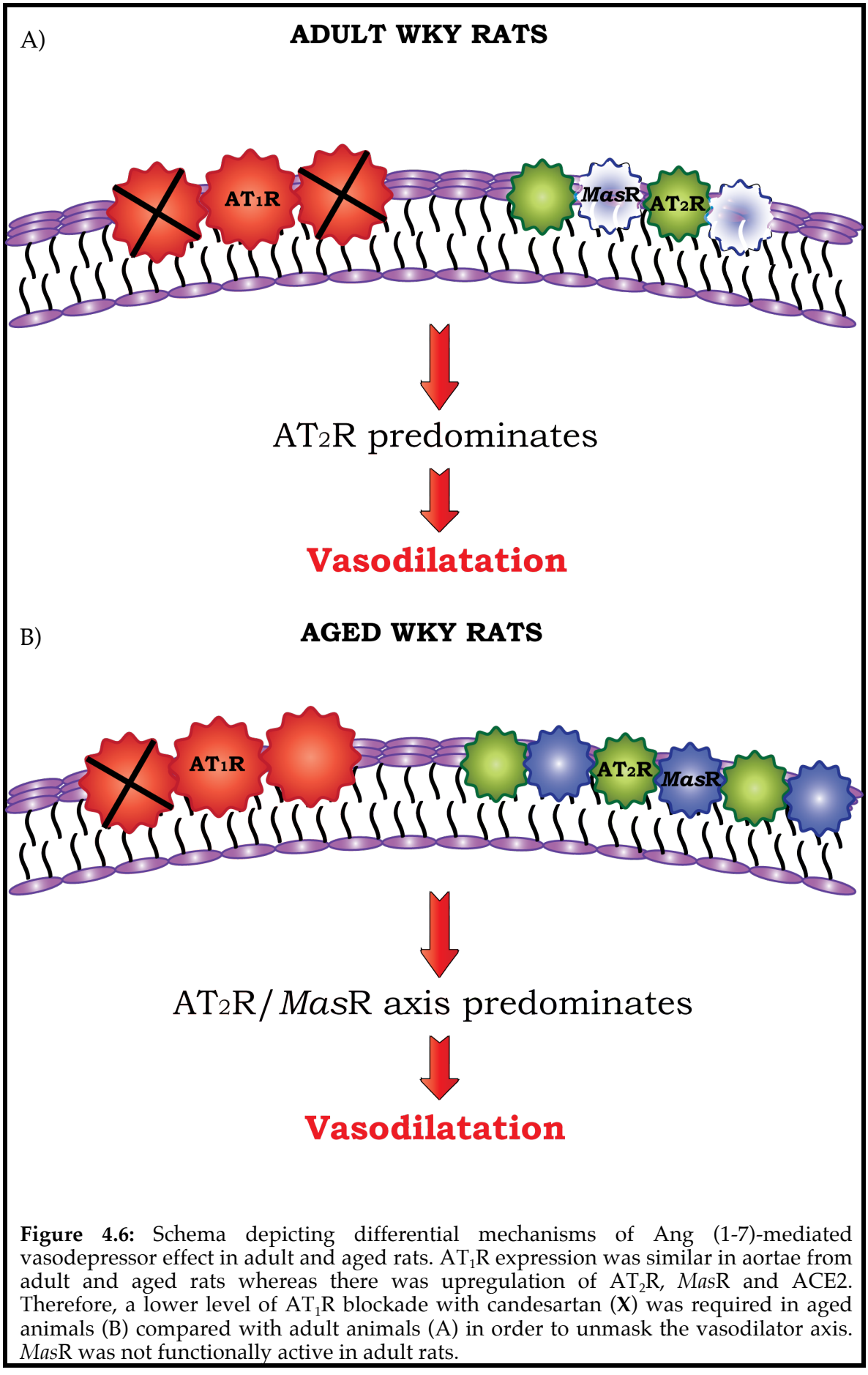

Figure 4.6: Schema depicting differential mechanisms of Ang (1-7)-mediated vasodepressor effect in adult and aged rats. AT_1R expression was similar in aortae from adult and aged rats whereas there was upregulation of AT_2R, *Mas*R and ACE2. Therefore, a lower level of AT_1R blockade with candesartan (**X**) was required in aged animals (B) compared with adult animals (A) in order to unmask the vasodilator axis. *Mas*R was not functionally active in adult rats.

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Chapter 5:

Effects of chronic Compound 21 in normotensive animals

5.1 INTRODUCTION

It is well documented, that prior to menopause females have a lower incidence and severity of cardiovascular disease (CVD), after which these risks increase significantly, with more females dying of CVD when compared to males with increasing age [1]. However, the differences seen between males and females are still puzzling and more studies are required to understand cardiovascular sex differences.

The renin-angiotensin system (RAS) has a major influence on the development and progression of hypertension in both males and females. Angiotensin II (Ang II) modulates vascular contractility, cardiovascular structure and fluid and electrolyte homeostasis through stimulation of the subtype 1 receptor (AT_1R) [2]. On the other hand, stimulation of the subtype 2 receptor (AT_2R) is believed to oppose AT_1R-mediated effects [2]. In line with the notion that the AT_2R oppose effects of AT_1R stimulation, previous studies have documented upregulation in vascular AT_2R following cuff injury in females but not males [3], downregulation of vascular and renal AT_1R and upregulation of AT_2R accompanied by a decreased effect of Ang II on isolated aortic rings and mesenteric arteries in response to estrogen [4]. Furthermore, Silva-Antonialli *et al*. (2004), have shown a greater AT_2R:AT_1R ratio in the blood vessels and kidneys of female compared to male rats which was indicative of both lower AT_1R expression in the estrogen treated animals, and increased AT_2R expression [4]. These results therefore point towards a greater AT_2R-vasodilator component in female rats.

Additionally, previous *in vivo* studies have shown attenuated mean arterial pressor responses to exogenous Ang II infusion in females compared to males [5-7]. In this context, Ang II infusion in ovariectomised rats resulted in a marked increase in the pressor response to Ang II compared to intact females [6]. This

evidence supports the notion that estrogen modulates arterial pressure via interaction with the RAS [6]. While it has been long appreciated that Ang II–mediated pressor activity is reduced in female rats, the majority of previous studies have used large doses of Ang II. In this situation, it is likely that an AT_1R-mediated effect would predominate. With this fact in mind, the effects of low dose Ang II were recently compared in male and female rats. Indeed, chronic low dose infusion of Ang II led to a reduction in mean arterial pressure (MAP) in female Sprague Dawley (SD) rats but not male rats. This effect was mediated via AT_2R as it was abolished by the AT_2R antagonist PD123319 [8].

Compound 21 is the first nonpeptide AT_2R selective agonist [9]. To date there have only been a few *in vivo* investigations with Compound 21 [9, 10]. In anaesthetised hypertensive rats, a bolous intravenous injection of Compound 21 lowered MAP over the dose range 0.008 to 0.05 mg/kg [9] although this effect was somewhat variable. Furthermore, chronic treatment with Compound 21 improved both systolic and diastolic ventricular function in normotensive rats with heart failure [10].

Based on these studies, and the fact that there are reported differences seen in the AT_2R-mediated responses between males and females, the aim of this study was to investigate the effect of chronic treatment with a selective AT_2R agonist, Compound 21, on telemeterised blood pressure in male and female normotensive mice and rats.

5.2 METHODS

5.2.1 ANIMALS

All experiments were approved by a Monash University Animal Ethics Committee in accordance with the guidelines of the National Health and Medical Research Council of Australia. Male and female Sprague Dawley (SD) rats, ~10 weeks old and weighing ~200 to 250 g, and male and female 16 week old FVB/N mice, weighing approximately 25-30 g, were obtained from the Animal Resources Centre (W.A, Australia). Rats were fed a sodium controlled diet (0.25% wt/wt NaCl; Glen Forrest Stockfeeders, W.A, Australia) and mice were fed with standard laboratory mice chow, with water *ad libitum* through out the experiment. Animals were maintained on a 12-hour day/night cycle at a temperature of 22-25°C. Animals were acclimatized to these conditions for one week prior to entering the experimental protocol.

5.2.2 MICE TELEMETRY PROBE IMPLENTATION

At ~18 weeks of age animals were anaesthetised (2-4% isoflurane in O_2) and maintained on a heated surgical surface. The front of the neck was shaved and a ventral midline skin incision was made from the lower mandible posteriorly to the sternum (~1 cm), and the submaxillary glands were gently separated using sterile cotton swabs. The left common carotid artery was exposed with care being taken to avoid disturbing vagus nerve fibers running parallel to the artery. Two silk ties (4.0 Dysilk, DYNEK, S.A, Australia) were passed under the vessel and gentle tension was applied to both ligatures to slightly retract and lift the vessel. A bent needle (21 gauge, Becton Dickson, NJ, USA) was used to create a small incision in the carotid artery just below the anterior silk suture. The catheter of the telemetry transmitter (TA11-PAC10; Data Sciences International) was inserted into the

carotid artery with the aid of a bent needle used to hold the vessel incision open. The occlusive silk tie was released, and the catheter was advanced further into the vessel. The loose ends of the occlusive silk tie were tied securely around the vessel/catheter.

Through the same ventral neck incision, a subcutaneous pouch was formed for placement of the transmitter body along the animal's right flank. Using a pair of blunt dissecting scissors, the skin was gently dissected free from underlying tissue starting at the right neck region and proceeding posteriorly to form a "pocket" along the right flank. The pocket was filled with warm saline to allow easy insertion of the transmitter body. The transmitter was inserted under the skin as close to the right hindlimb as possible, taking care to not disturb the catheter of the transmitter device. The neck incision was closed using silk (6.0 Dysilk, DYNEK, S.A, Australia). Mice were treated with the non-steroidal anti-inflammatory drug, Carprofen, (5mg/kg) via intraperitoneal injection and were kept warm and monitored closely until fully recovered from anesthesia.

5.2.3 RAT TELEMETRY PROBE IMPLANTATION

At 16 weeks of age animals were anaesthetised (2-4% isoflurane in O_2) and after shaving the abdominal area, a midline incision was made. Using blunt dissection, the abdominal aorta was exposed below the renal artery and above the femoral artery bifurcation. The abdominal aorta was delicately separated from inferior vena cava and ties (4.0 Dysilk, DYNEK, S.A, Australia) were placed around the abdominal aorta just below the renal arteries as well as above the bifurcation. The ties were retracted to occlude blood flow. A bent needle (21 gauge, Becton Dickson, NJ, USA) was used to create a hole in the abdominal aorta and the catheter of the telemetry transmitter (TA11-PAC40; Data Sciences International) was inserted approximately 2 cm into the abdominal aorta to the level of the

retracted tie. The catheter was secured using tissue glue and the device body was anchored to the internal muscle wall via sutures (2.0 Dysilk, DYNEK, S.A, Australia) through the tab in the device body. The ties occluding the abdominal aorta were released and removed to allow free flow of blood to hindlimbs. Occlusion time was less than three minutes to prevent hind limb paralysis. The skin was sutured closed and the animal was treated with Carprofen (5mg/kg) via intraperitoneal injection.

5.2.4 OSMOTIC MINIPUMP PREPARATION AND IMPLANTATION

Osmotic minipumps (Alzet, model 2ML2 and 1004, California, USA) were used to administer Ang II or Compound 21, subcutaneously. The osmotic minipumps were filled with solutions of drugs at concentrations calculated using the known pumping rate of the osmotic minipumps.

Under anaesthesia, (2-4% isoflurane in O_2), the scapular region of the rat or mouse was shaved. A skin incision was then made between the shoulder blades and using blunt dissection, a small pocket was established. The osmotic minipump was then implanted into the pocket (2ML2 for rats and 1004 for mice) and the pocket was closed using 4.0 sutures. This is a relatively short surgery of ~5-10 minutes and a full recovery occurs within 1-2 hours.

5.2.5 MICE GROUPS

FVB/N female (n=5) and male (n=6) mice received 50ng/kg/min of Compound 21 via minipump for 4 weeks. Following 2 weeks of Compound 21 treatment, the AT_1R antagonist, Candesartan Cilexetil (0.1mg/kg/day) was introduced into drinking water for the reminder of the treatment.

5.2.6 RAT GROUPS

Rats were treated with Saline (0.9% w/v NaCl); Ang II (50ng/kg/min) or Compound 21 (100 ng/kg/min), such that there waa a total of 5 treatment groups as follows:

Group 1) Female SD rats receiving saline;

Group 2) Male SD rats receiving saline;

Group 3) Female SD rats receiving Ang II infusion;

Group 4) Male SD rats receiving Ang II infusion;

Group 5) Female SD rats receiving Compound 21 infusion.

All treatments were 2 weeks in duration.

5.2.7 DATA RECORDING AND ANALYSIS

Animals were allowed 14 days recovery before baseline recording of diastolic and systolic pressure, heart rate (HR) and activity (Dataquest Labpro Version 3.0; Data Sciences International) was initiated. Data were recorded as 10 seconds averages every 10 minutes throughout the recording period of 3 days for baseline, after which animals received the allocated treatment and the data was further recorded for 14 (rats) or 28 (mice) days. The data were analysed using GraphPad Prism (version 5.0c; GraphPad Software Inc.) For both mice and rats MAP, HR and activity data is represented as 12hr averages indicating normal circadian rhythm. Additionally MAP, HR and activity data are represented as absolute change during day time, night time and as a 24hr averages during drug infusion (line graphs) as well as changes from baseline during indicated recording periods (bar graphs).

5.3 RESULTS

5.3.1 EFFECT OF COMPOUND 21 IN MICE

There was no difference in basal MAP, HR or locomotor activity between male and female FVB/N mice (Table 5.1).

Table 5.1. Baseline variables prior to treatment in male and female FVB/N mice. Mean arterial pressure (MAP), heart rate (HR) and activity. Values represented as mean ± sem

Group	MAP (mmHg)	HR (bpm)	Activity (Arbitrary units)
Group 1 (n=5)			
Female FVB/N mice	98 ± 1	615 ± 29	7.8 ± 1
Group 2 (n=6)			
Male FVB/N mice	103 ± 4	547 ± 24	5.7 ± 1

5.3.2 MICE TELEMETRY DATA

Both male and female mice exhibited similar oscillations of MAP (Figure 5.1A), HR (Figure 5.2A) and activity (Figure 5.3A) between day time sleep and night time active periods, indicating normal circadian rhythm; this was not affected by any drug treatment.

5.3.2.1 MEAN ARTERIAL PRESSURE

Compound 21 alone had no effect on MAP of female mice during day time sleep or night time active periods (Figure 5.1E and F), and consequently 24hr daily average MAP was also unaffected by Compound 21 treatment (Figure 5.1G). In contrast, Compound 21 caused a steady increase in MAP of male mice from pre-treatment baseline levels (Figure 5.1), resulting in increases of average day time (Figure 5.1E), night time (Figure 5.1F), and 24hr (Figure 5.1G) MAP values.

Addition of candesartan cilexetil to the drinking water initiated 2 weeks after treatment with Compound 21 reduced MAP in both male and female mice. As

MAP was already elevated following Compound 21 infusion in male mice, MAP remained higher than pre-treatment values even after combination treatment with candesartan cilexetil and Compound 21. In female mice MAP was slightly reduced by simultaneous Compound 21 and candesartan cilexetil (Fig 5.1G), and this observation was evident during both day and night time periods (Figure 5.1E and F).

5.3.2.2 HEART RATE

HR of female mice was significantly higher than male mice throughout the 4 week treatment period (Figure 5.2D), during both day and night time periods (Figure 5.2B and C). Compound 21 alone did not influence HR of either male or female mice, whereas there was a small decrease in HR in male mice with candesartan cilexetil and Compound 21 treatment when compared to Compound 21 alone (Figure 5.2G). This observation was evident during both day and night time periods (Figure 5.2E and 5.2F).

5.3.2.3 ACTIVITY

Similarly to HR, there was a significant difference in activity between male and female mice over the 4 week treatment period (Figure 5.3D), during day and night time periods (Figure 5.3B and C), with female mice being consistently more active than male mice. We did not observe any change in activity in either male mice or female mice following Compound 21 infusion or combination treatment with candesartan cilexetil and Compound 21 (Figure 5.3E, F and G).

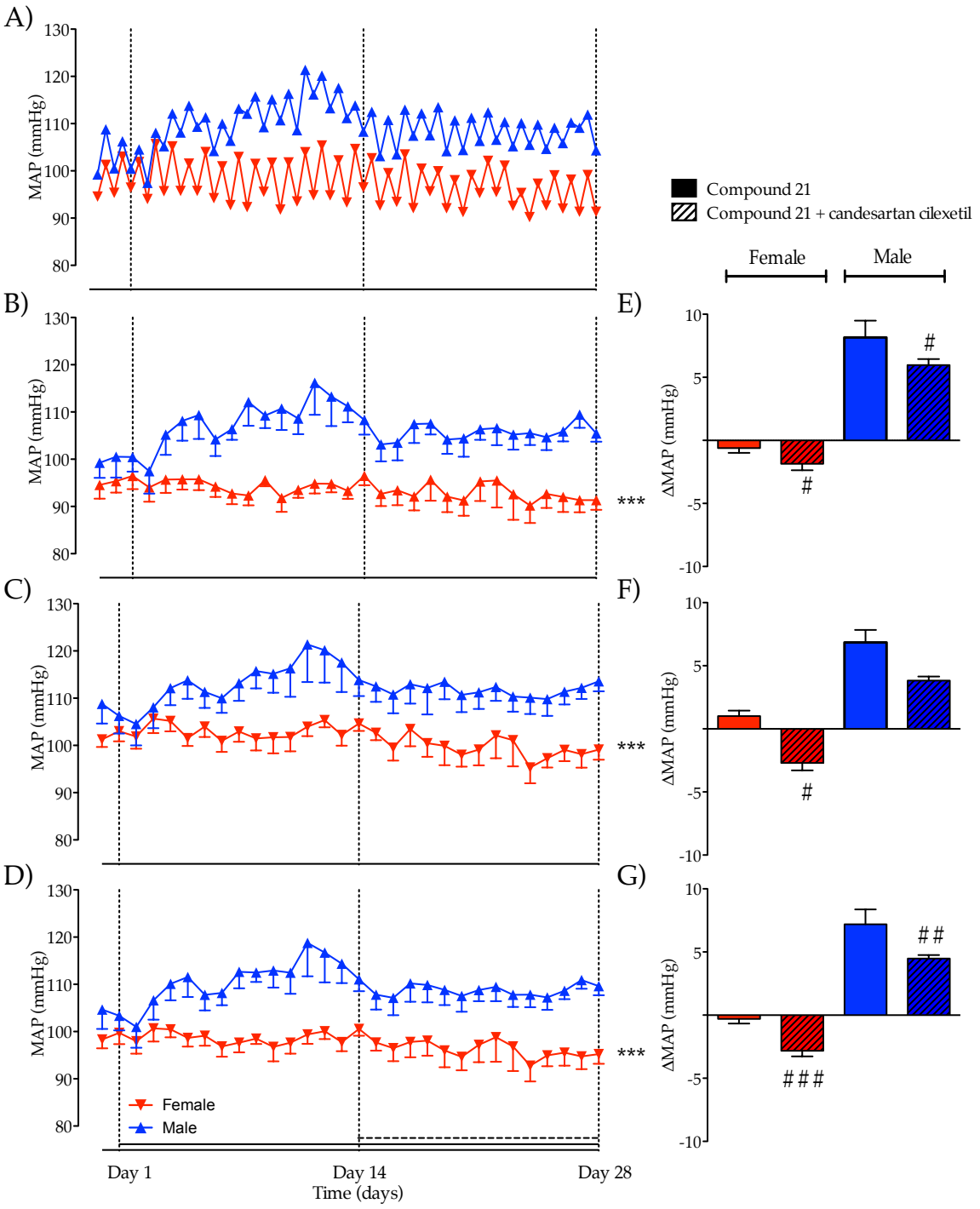

Figure 5.1: Radiotelemetry recording of 12hr (A), day time (B), night time (C) and 24hr average (D) of MAP in male (blue line) and female (red line) mice. Full line represents duration of Compound 21 infusion and dashed line represents start and finish of candesartan cilexitil treatment.
Change in MAP from baseline with Compound 21 (empty bars; weeks 1-2) and Compound 21 and candesartan cilexetil (shaded bars; weeks 3-4) recorded at day time (E), night time (F) and as 24hr average (G) are provided.
***P<0.001 versus male
#P<0.05; ##P<0.01; ###P<0.001; for treatment effect of Compound 21 and candesartan cilexetil versus Compound 21

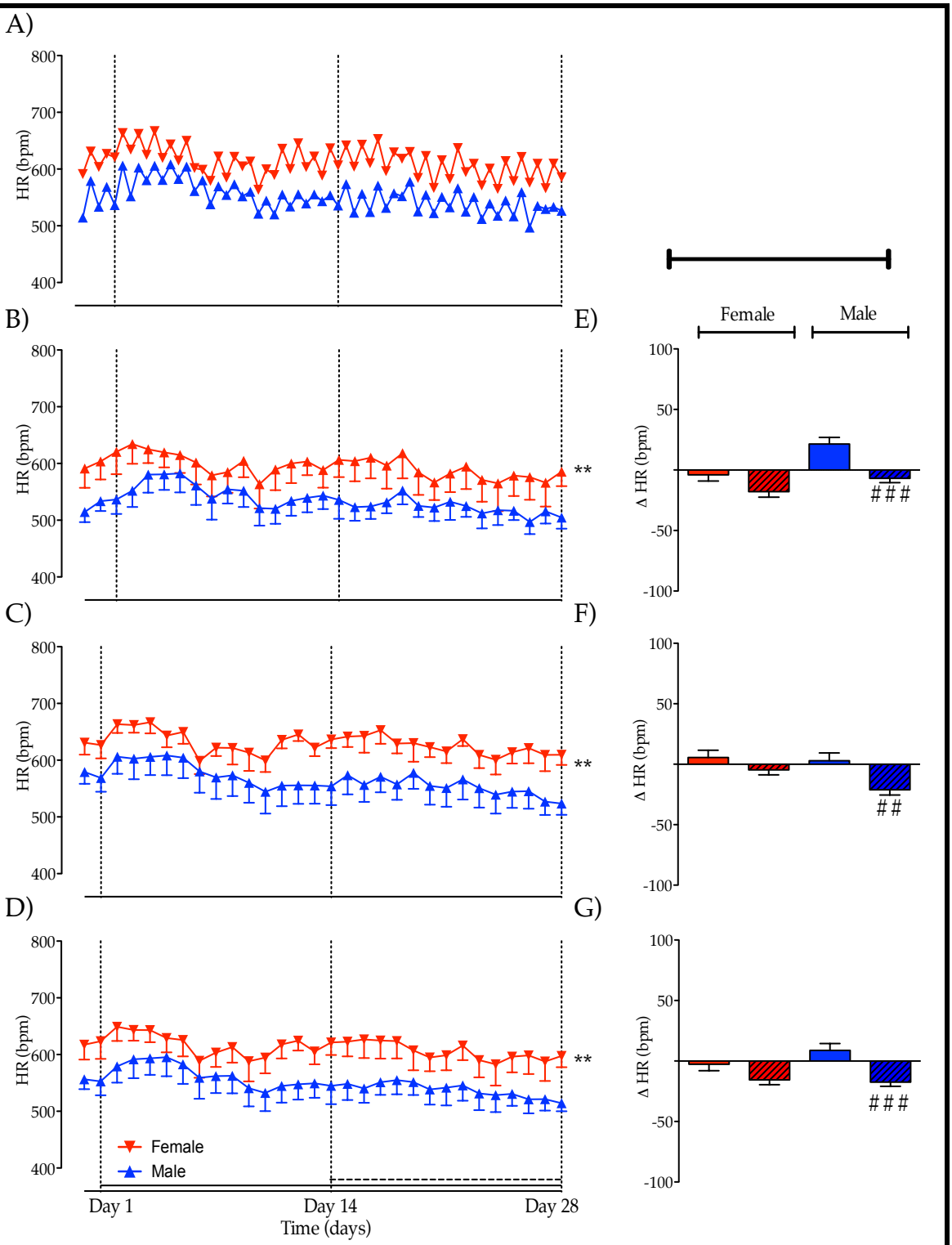

Figure 5.2: Radiotelemetry recording of 12hr (A), day time (B), night time (C) and 24hr average (D) of HR in male (blue line) and female (red line) mice. Full line represents duration of Compound 21 infusion and dashed line represents start and finish of candesartan cilexitil treatment.
Change in HR values from baseline with Compound 21 (empty bars; weeks 1-2) and Compound 21 and candesartan cilexetil (shaded bars; weeks 3-4) recorded at day time (E), night time (F) and as 24hr average (G) are provided.
**P<0.01 versus male
##P<0.01; ###P<0.001; for treatment effect of Compound 21 and candesartan cilexetil versus Compound 21

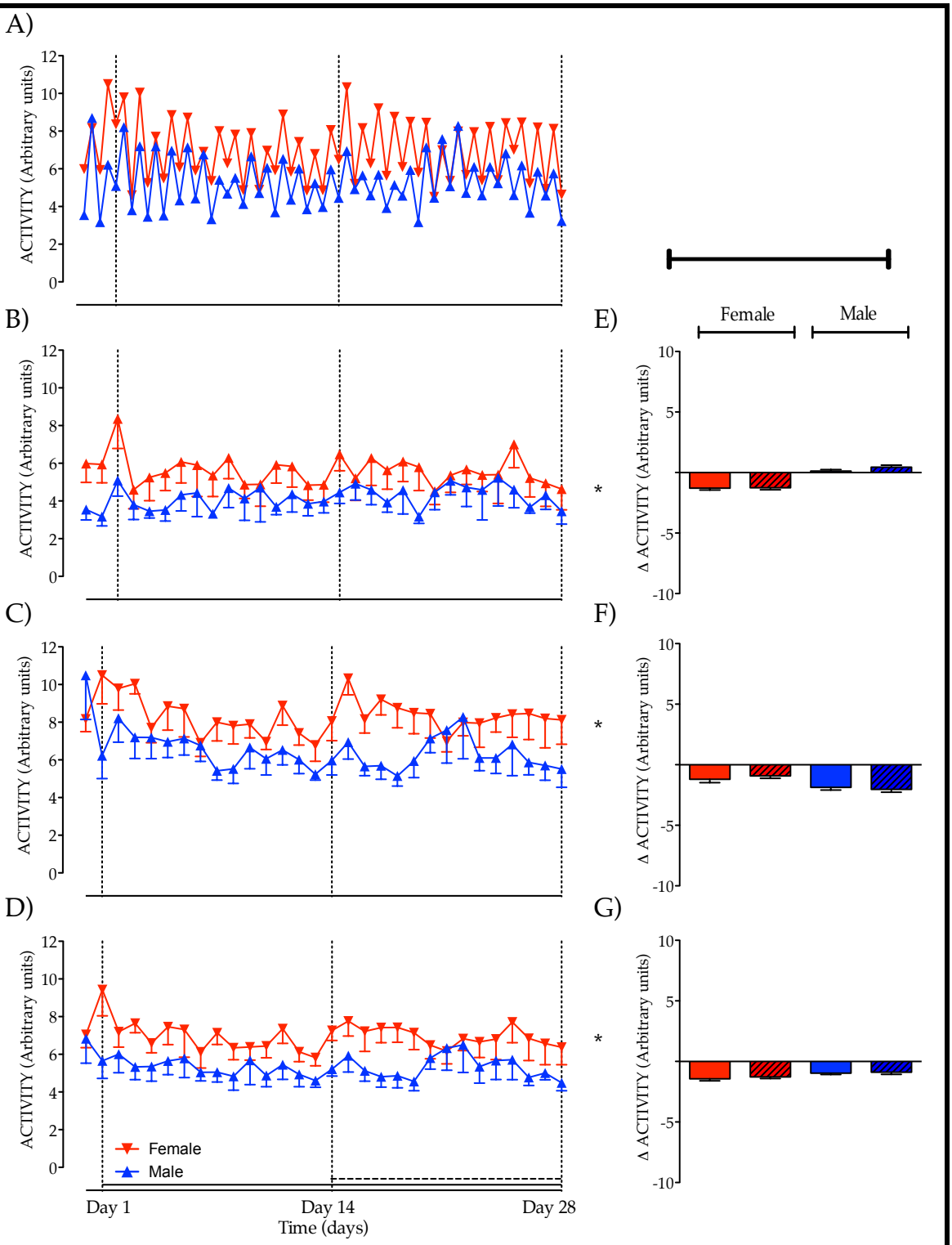

Figure 5.3: Radiotelemetry recording of 12hr (A), day time (B), night time (C) and 24hr average (D) of ACTIVITY in male (blue line) and female (red line) mice. Full line represents duration of Compound 21 infusion and dashed line represents start and finish of candesartan cilexitil treatment.
Change in activity values from baseline with Compound 21 (empty bars; weeks 1-2) and Compound 21 and candesartan cilexetil (shaded bars; weeks 3-4) recorded at day time (E), night time (F) and as 24hr average (G) are provided.
*P<0.05 versus male

5.3.3 EFFECT OF COMPOUND 21 IN RATS

There was no difference observed in basal MAP, HR or activity between male and female SD rats or between treatment groups (Table 5.2).

Table 5.2. Baseline variables prior to treatment in male and female SD rats. Mean arterial pressure (MAP), heart rate (HR) and activity. Values represented as mean ± sem

TTREATMENT	MAP (mmHg)	HR (bpm)	Activity (Arbitrary units)
Saline - ♀ SD Rats (n=6)	97 ± 2	375 ± 16	2.3 ± 0.3
Saline - ♂ SD Rats (n=3)	96 ± 1	351 ± 14	2.0 ± 0.5
Ang II (50ng/kg/min) - ♀ SD Rats (n=6)	99 ± 2	396 ± 12	2.8 ± 0.3
Ang II (50ng/kg/min) - ♂ SD Rats (n=7)	97 ± 2	370 ± 11	2.1 ± 0.3
Compound 21 (100ng/kg/min) - ♀ SD Rats (n=5)	96 ± 3	388 ± 7	2.4 ± 0.4

5.3.4 RAT TELEMETRY DATA

Both male and female rats exhibited similar fluctuations in MAP (Figure 5.4A), HR (Figure 5.5A) and activity (Figure 5.6A) during the day time sleep period compared to night time active period, indicating normal circadian rhythm.

5.3.4.1 MEAN ARTERIAL PRESSURE

Administration of Compound 21 in female rats evoked a small decrease in MAP during the day time sleep period (Figure 5.4 E) and a small increase in MAP during the night time active period (Figure 5.4 F) such that and 24hr daily average MAP was unaffected by Compound 21 treatment (Figure 5.4G).

Ang II infusion significantly decreased MAP from baseline in female rats when compared to saline treated female rats. This blood pressure-lowering effect was observed during all recording periods but was most pronounced during the night time active period (Figure 5.4F, $P< 0.001$). However, Ang II infusion had no effect on MAP of male rats when compared to saline treated male rats (Figure 5.4E, F and G). Thus the drop in MAP in female rats was also significantly different to that of Ang II –treated male rats (Figure 5.4E, F and G).

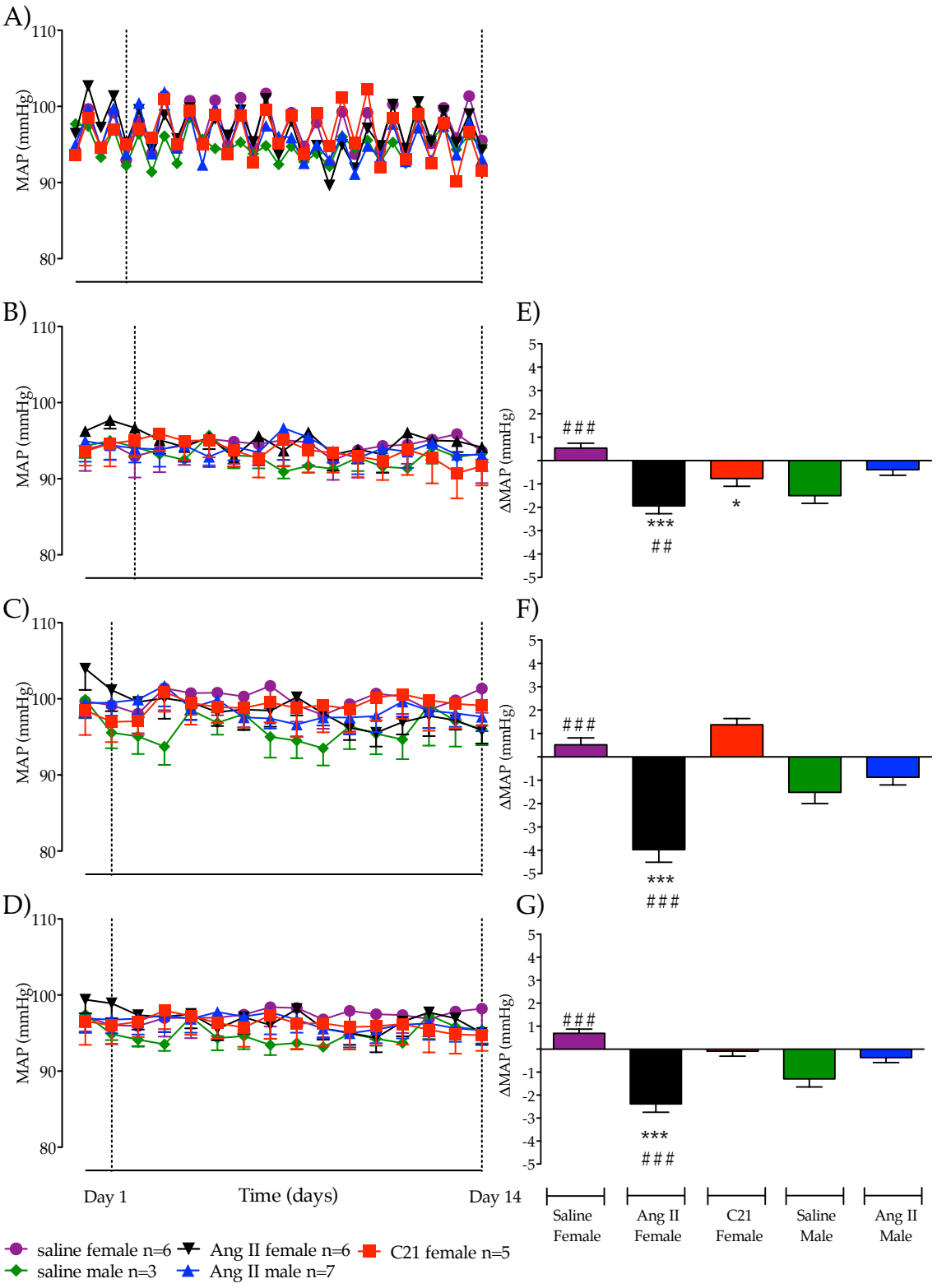

Figure 5.4: Radiotelemetry recording of 12hr (A), day time (B), night time (C) and 24hr average (D) of MAP in male and female SD rats.
Change in MAP over 2 weeks from baseline at day time (E), night time (F) and 24hr average (G) following treatment. Data are presented as mean ± sem.
*P<0.05; ***P<0.001 versus saline control
##P<0.01; ###P<0.001; female versus male counterpart

5.3.4.2 HEART RATE

Compound 21 infusion significantly increased HR (~ 10bpm) in female rats during day time sleep period, night time active period and 24hr average when compared to saline treated female rats (Figure 5.6E, F and G). There was no change in HR values in animals receiving Ang II infusion when compared to saline treated control groups (Figure 5.6E, F and G). Similarly, there was no difference in HR between female and male rats during day and night time periods (Figure 5.6E, F and C).

5.3.4.3 ACTIVITY

There was no difference in activity between male and female rats. Similarly, activity was not altered by drug treatment in any of the experimental group (Figure 5.5).

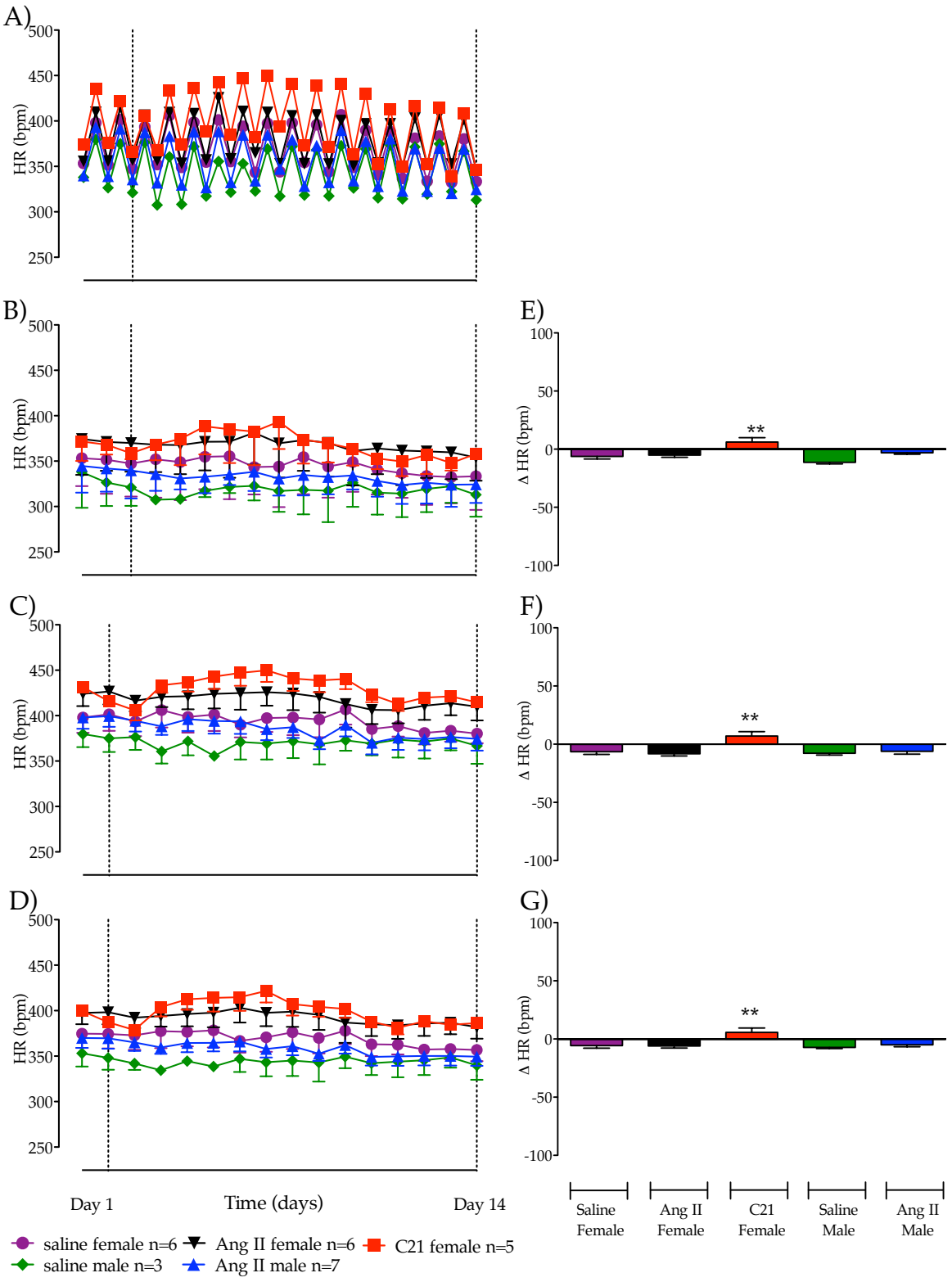

Figure 5.5: Radiotelemetry recording of 12hr (A), day time (B), night time (C) and 24hr average (D) of HR in male and female SD rats.
Change in HR values over 2 weeks from baseline at day time (E), night time (F) and 24hr average (G) following treatment. Data are presented as mean ± sem.
**P<0.01 versus saline control

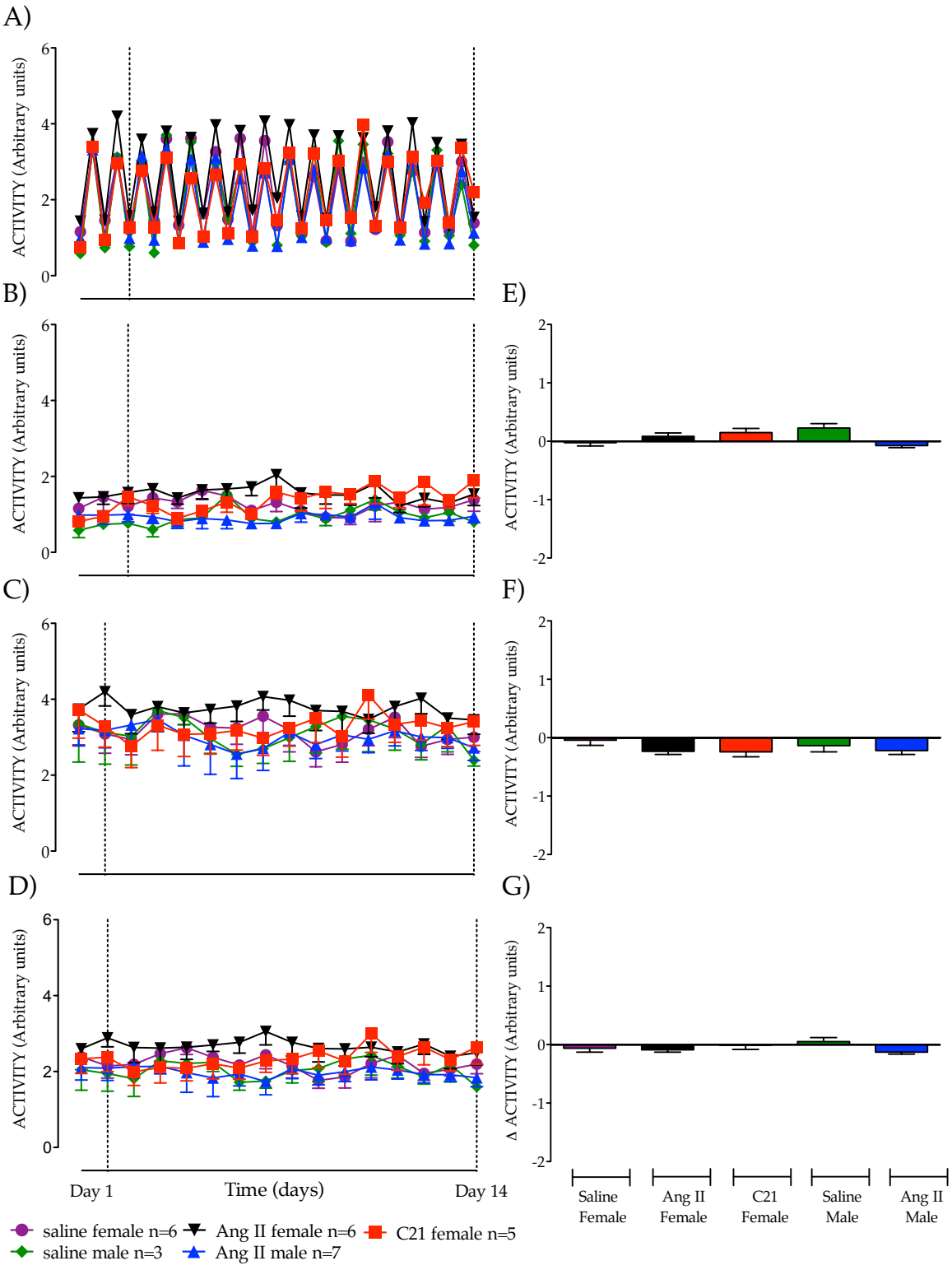

Figure 5.6: Radiotelemetry recording of 12hr (A), day time (B), night time (C) and 24hr average (D) of activity in male and female SD rats.
Change in activity values over 2 weeks from baseline at day time (E), night time (F) and 24hr average (G) following treatment. Data are presented as mean ± sem.

5.4 DISCUSSION

Differential expression of Ang II receptors in males and females has been hypothesised to be one potential mechanism contributing to sex specific differences in blood pressure [11]. Given that AT_2R expression is greater in females compared to males [4], we hypothesized that selective AT_2R stimulation would result in a greater depressor response in female animals compared to male animals. Thus the aims of the current study were to investigate the effects of chronic AT_2R stimulation on blood pressure in male and female mice and rats.

Chronic Compound 21 treatment failed to reduce MAP in either female mice or rats, as measured by radiotelemetry. These results are in direct contrast to the study by Wan *et al*, (2004), in which Compound 21 was shown to be effective in lowering MAP in SHR following intravenous bolus injection [9]. These divergent results may be due to the fact that the effect of Compound 21 in this study was only observed following a much higher bolus dose of the drug [9] than was administered in the current study. Importantly, it was not considered plausible to increase the infusion rate of Compound 21, as there was already an elevation of blood pressure in male mice at 50ng/kg/day, presumably due to loss of selectivity and off-target effects at this dose. In addition there was an increase in heart rate of female rats treated with Compound 21, although the mechanism and physiological relevance of this tachycardia is not clear.

On the other hand, in other chronic experiments which are more directly comparable to the current study, Compound 21 had minimal effect on the blood pressure of SHR, stroke prone rats or 2-kidney 1-clip rats [10, 12, 13]. Additionally, a 6-day Compound 21 infusion at 3mg/kg/day dose, in black C57Bl/6 mice, had no effect on blood pressure as recorded by radiotelemetry [14]. In these studies, although there was no effect of Compound 21 on blood pressure, there were other

beneficial effects in terms of cardiac remodelling, inflammation and apoptosis [10, 12, 13].

In the current study, concomitant treatment with Compound 21 and the AT_1R antagonist, candesartan cilexetil, lowered MAP compared to Compound 21 treatment alone in both male and female mice. This finding is consistent with previous studies which have shown that the hypotensive effects of AT_2R stimulation may be masked by the dominant pressor actions of AT_1R, thus requiring low level AT_1R blockade to enable functional demonstration of AT_2R stimulation [15-17]. However, from the current study, it is impossible to determine whether the small reduction in MAP relative to Compound 21 alone in both male and female mice, seen during combined AT_1R inhibition and Compound 21 treatment was in fact due to 'unmasking' of selective AT_2R stimulation, or was simply due to an antihypertensive effect of AT_1R blockade per se.

Although in the current study we failed to see any depressor effect of Compound 21 in mice and rats, low dose Ang II chronic infusion lowered blood pressure in female rats, as previously reported [8]. In the present study, the Ang II-mediated hypotensive effect in female rats was not of the same magnitude as that previously documented, where low chronic infusion of Ang II resulted in ~10mmHg decrease in MAP [8]. However, the discrepancies seen in the current study could be attributed to the ages of the animals, as in the current study, female SD rats received treatment at ~ 18 weeks of age, whereas in the original study animals were treated at ~12 weeks of age [8]. It is well known that the expression levels of AT_2R are high at birth and decrease dramatically with age. Therefore it is possible that different levels of expression of RAS components, particularly AT_2R expression, at different ages led to slight differences in the magnitude of blood pressure reduction between the two studies.

In previous studies, it was suggested that the Ang II-mediated depressor response seen in female rats was due to AT_2R stimulation, since the depressor effect was attenuated by the AT_2R antagonist, PD123319 [8]. Furthermore, Sampson *et* al., (2008) observed an increase in renal mRNA gene expression of both AT_2R and ACE2 in female compared to male rats, once again suggesting that an enhanced AT_2R-vasodilator axis in females may contribute to the sex-specific differences noted [8]. Thus, although we did not directly examine which receptors contributed to the Ang II-mediated vasodepressor effect in the current study, it is possible that Ang II may be directly activating AT_2R in the current study.

However, Ang II is readily metabolised *in vivo* to Angiotensin (1-7) (Ang (1-7)) via ACE2 [18], and to Angiotensin III (Ang III) via Aminopeptidase A [19]. Both Ang 1-7 and Ang III have been shownto cause vasodilatation [20, 21]. More importantly, these vasodilator effects of both Ang (1-7) and Ang III were caused via AT_2R stimulation [20, 21]. Therefore, in addition to increased expression of AT_2R and ACE2 in female rats [8] it is possible that in the current study we observed Ang II-mediated hypotension via AT_2R either directly or indirectly via conversion to Ang (1-7) and Ang III. Indeed, the lack of effect of Compound 21 may relate to this difference, as it is possible that the break down products of Ang II are activating one or more vasodilatory components of the RAS, other than, or in addition to AT_2R. For example, another possibility is that the break down product, Ang (1-7) is activating *Mas*R and/or AT_2R to induce a depressor effect. Indeed, we have previously reported that Ang (1-7)-mediated depressor effects were attenuated by either AT_2R or *Mas*R blockade (Chapter 4) in aged normotensive rats.

Interestingly in this context, a recently published study has documented that basal Ang (1-7) levels were increased in female rats compared to male counterparts, and this sex related difference was also evident following Ang II

infusion [22]. Furthermore, the same study suggested that the Ang II-mediated pressor response was modulated by Ang 1-7 levels since A-779 potentiated the hypertensive response to Ang II, and this effect of *Mas*R-inhibition was larger in female animals [22].

Thus, in the current study, we have confirmed previous findings that a chronic low dose Ang II infusion results in a decrease in MAP in female but not male normotensive animals. Although we failed to observe any effect on MAP with chronic Compound 21 infusion, previous studies have shown AT_2R activity to be influenced by the level of AT_2R in a particular tissue [23]. Therefore, it is possible that Compound 21 would be more effective in blood pressure regulation in a model of hypertension, since there is an increase in AT_2R expression in SHR [24, 25].

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Chapter 6:

Stimulation of angiotensin AT_2 receptors by the non-peptide agonist, Compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats

Declaration for Thesis Chapter 6

Monash University

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed all of the experiments with the exception of organ bath experiments, analysed the results and wrote the manuscript.	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution
Emma Jones Robert Widdop	Provided intellectual advice during experimental and manuscript preparation stages.
Iresha Welungoda	Conducted organ bath experiments.
A Hallberg M Alterman	Provided Compound 21.

Candidate's Signature: .....

Date: .....

Declaration by co-authors
The undersigned hereby certify that:
(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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RESEARCH PAPER

Stimulation of angiotensin AT_2 receptors by the non-peptide agonist, Compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats

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Background and purpose: Angiotensin type 2 receptor (AT_2 receptor) stimulation evokes vasodilator effects *in vitro* and *in vivo* that oppose the vasoconstrictor effects of angiotensin type 1 receptors (AT_1 receptors). Recently, a novel non-peptide AT_2 receptor agonist, Compound 21, was described, which exhibited high AT_2 receptor selectivity.
Experimental approach: Functional cardiovascular effects of the drug candidate Compound 21 were assessed, using mouse isolated aorta and rat mesenteric arteries *in vitro* and in conscious spontaneously hypertensive rats (SHR).
Key results: Compound 21 evoked dose-dependent vasorelaxations in aortic and mesenteric vessels, abolished by the AT_2 receptor antagonist, PD123319. *In vivo*, Compound 21 administered alone, at doses ranging from 50 to 1000 $ng{\cdot}kg^{-1}{\cdot}min^{-1}$ over 4 h did not decrease blood pressure in conscious normotensive Wistar-Kyoto rats or SHR. However, when given in combination with the AT_1 receptor antagonist, candesartan, Compound 21 (300 $ng{\cdot}kg^{-1}{\cdot}min^{-1}$) lowered blood pressure in SHR only. Further analysis in separate groups of conscious SHR revealed that, at a sixfold lower dose, Compound 21 (50 $ng{\cdot}kg^{-1}{\cdot}min^{-1}$) still evoked a significant depressor response in adult SHR (~30 mmHg) when combined with different doses of candesartan (0.01 or 0.1 $mg{\cdot}kg^{-1}$). Moreover, the Compound 21-evoked depressor effect was abolished when co-infused (50 $\mu g{\cdot}kg^{-1}{\cdot}min^{-1}$ for 2 h) with the AT_2 receptor antagonist PD123319.
Conclusion and implications: Collectively, our results indicate that acute administration of Compound 21 evoked blood pressure reductions via AT_2 receptor stimulation. Thus Compound 21 can be considered an excellent drug candidate for further study of AT_2 receptor function in cardiovascular disease.
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Keywords: Compound 21; angiotensin; angiotensin AT_2 receptor; spontaneously hypertensive rats; blood pressure; vascular function

Abbreviations: Ang II, angiotensin II; AT_1 receptor, angiotensin type 1 receptor; AT_2 receptor, angiotensin type 2 receptor; MAP, mean arterial pressure; SHR, spontaneously hypertensive rat; WKY rat, Wistar-Kyoto rat

Introduction

The octapeptide angiotensin II (Ang II) is the main biologically active mediator of the renin-angiotensin system and plays an important role in cardiovascular function by influencing vascular tone, structure, fluid and electrolyte balance via direct effects on endothelial and smooth muscle cells (Widdop *et al.*, 2003; Jones *et al.*, 2008). Two main receptor subtypes have been identified as binding sites for Ang II: angiotensin type 1 receptor (AT_1 recepter) and type 2 receptor (AT_2 receptor) (de Gasparo *et al.*, 2000); nomenclature follows Alexander *et al.*, 2008). Ang II has similar affinity for both AT_1 receptors and AT_2 receptors, whereas CGP42112 and PD123319 are the prototypical examples of an agonist and antagonist, respectively, at the AT_2 receptor subtype. On the other hand, compounds such as candesartan and losartan are selective AT_1 receptor antagonists that are used clinically for the treatment of hypertension. It is well established that most of the cardiovascular effects induced by Ang II, such as vasoconstriction, water and salt retention, are mediated via AT_1

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receptor (de Gasparo *et al*., 2000; Carey, 2005). In contrast, it has been suggested that the function of the AT_2 receptor is to counter-regulate AT_1 receptor-mediated actions, mainly based on experiments in which AT_2 receptor function was deduced from the effects of AT_2 receptor blockade, or altered responses in genetically modified animal models of AT_2 receptor over-expression or deletion. However, demonstration of AT_2 receptor-mediated effects, particularly in an *in vivo* setting, has been hampered by a lack of non-peptide AT_2 receptor selective agonists and antagonists that exhibit oral bioavailability.

In this context, Wan *et al*. (2004b) have recently described the first non-peptide, selective AT_2 receptor agonist, Compound 21 (*N*-butyloxycarbonyl-3-(4—imidazol-1-ylmethylph enyl)-5-isobutylthiophene-2-sulphonamide). Compound 21 was derived from a medicinal chemistry programme aimed at transforming the drug-like but non-selective AT_1 and AT_2 receptor agonist L-162313 (Wan *et al*., 2004a) into a selective AT_2 receptor agonist. Compound 21 exhibits a K_i value of 0.4 nM for the AT_2 receptor and a K_i > 10 μM for the AT_1 receptor. In addition, due to the non-peptide nature of the drug, Compound 21 has an estimated oral bioavailability of 20–30% and 4 h half-life in rats (Wan *et al*., 2004b). Compound 21 has been shown to induce neurite outgrowth in cell culture and to increase duodenal mucosal alkalinization in the rat via stimulation of MAPK and NO/cGMP signalling pathways (Wan *et al*., 2004b). Furthermore, Compound 21 decreased mean arterial blood pressure (MAP) in anaesthetized spontaneously hypertensive rats (SHR), although detailed and systematic evaluation of haemodynamic responses to Compound 21 were not performed in this earlier study.

AT_2 receptor-mediated relaxation is a well-established effect in isolated resistance vessels (Matrougui *et al*., 1999; Dimitropoulou *et al*., 2001; Widdop *et al*., 2002); conversely, there is less consensus regarding the influence of AT_2 receptors on blood pressure regulation *in vivo*. Studies using AT_2 receptor knockout mice support a role for AT_2 receptors in haemodynamic control, as these animals exhibit elevated basal blood pressure and enhanced sensitivity to the vasopressor effects of Ang II (Hein *et al*., 1995; Ichiki *et al*., 1995). Conversely, over-expression of AT_2 receptors in vasculature did not alter basal blood pressure, but markedly impaired Ang II-induced pressor activity (Tsutsumi *et al*., 1999). In conscious SHR, Ang II-mediated vasodilatation during AT_1 receptor blockade was not observed (Gohlke *et al*., 1998), presumably because the hypotensive effect of AT_2 receptor stimulation was masked by the concomitant, dominant AT_1 receptor-mediated pressor action during Ang II infusion. In order to avoid such confounding influences of AT_1 receptor stimulation on potential AT_2 receptor vasodilator function, we and others have also assessed the effect of selective AT_2 receptor agonists and antagonists during AT_1 receptor blockade. Using this approach, selective stimulation of AT_2 receptors by CGP42112 lowered blood pressure, provided that there was a background of AT_1 receptor blockade in conscious SHR (Wistar-Kyoto rat, WKY) (Barber *et al*., 1999), and Sprague-Dawley rats (Carey *et al*., 2001) in a PD123319-reversible manner. Furthermore, this blood pressure-lowering response to AT_2 receptor stimulation was shown to be associated with increased blood flow in renal, mesenteric and hindquarter circulations in conscious SHR suggesting widespread vasodilatation (Li and Widdop, 2004).

Therefore, in the current study we determined the effects of Compound 21 on blood pressure in conscious SHR and WKY rats, as well as in isolated vasculature. In addition, AT_2 receptor selectivity of Compound 21 was determined by simultaneous administration of the selective AT_2 receptor antagonist, PD123319, to determine whether or not these effects were AT_2 receptor-mediated.

Methods

Animals

All animal care and experimental procedures were approved by the Monash University Animal Ethics Committee and performed according to the guidelines of the National Health and Medical Research Council of Australia for animal experimentation.

Male 16- to 18-week-old SHR and WKY rats, weighing approximately 300 to 350 g and male 16-week-old FVB/N mice, weighing approximately 25–30 g were obtained from the Animal Resource Centre (Perth, WA, USA). Animals were maintained on a 12 h day/night cycle with standard laboratory rat or mice chow and water available *ad libitum*.

In vitro *reactivity*

Mice were killed by isoflurane inhalation followed by decapitation. The thoracic aorta was removed and cut transversely into ring segments for organ bath studies. Two stainless steel wires were threaded through the lumen of each aortic ring and the rings were then mounted and suspended in vertical 10 mL organ baths containing Krebs bicarbonate solution [composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.7; pH 7.4], which was maintained at 37°C and continuously bubbled with carbogen (95% oxygen and 5% CO_2). Isometric tension was continuously measured via a force transducer (Grass FT03) interfaced to a MacLab data acquisition device (ADInstruments, Sydney, Australia) displayed on a Macintosh computer. Aortic rings were set to 0.5 g resting tension and allowed to equilibrate for 90 min, during which time Krebs bicarbonate solution was changed every 15 min. After equilibration, 0.3 μM of the thromboxane A_2 receptor agonist, U46619, was used to obtain the maximum contractile response. Once a maximum response was determined, tissues were then washed with Krebs' solution and allowed to equilibrate for 30 min or until baseline had been reached.

Tissues were pre-contracted with U46619 to attain 30–40% of the maximum contractile response. In the first series of experiments, all vessels were pretreated with the AT_1 receptor antagonist, losartan (0.1 μM). Cumulative dose–response curves at log intervals to Compound 21 (1 pM to 1 μM) were performed in absence or presence of the AT_2 receptor antagonist PD123319 (0.1 μM). In a further series, AT_1 receptor blockade was omitted and cumulative dose-response curves to Compound 21 were performed in the absence and presence of either the NOS inhibitor, L-NAME (10 μM) or PD123319. A

parallel tissue served as a time control in which only U46619 was given. At the end of the experiment, 10 μM of the endothelium-independent vasodilator, sodium nitroprusside was added to the organ bath to test the integrity of the vascular smooth muscle cells.

In analogous studies, thoracic aortic rings from male SHR were set up at 2 g resting tension and a maximum response was obtained to 124 mM K+. After washing, tissues were pre-contracted with the α_1-adrenoceptor agonist phenylephrine to 30–40% of maximum K+ response and cumulative dose–response curves to either Ang II (in the presence of 0.1 μM candesartan) or Compound 21 in the presence or absence of candesartan were obtained.

Mesenteric artery

Male WKY rats, approximately 16 weeks of age, were killed by isoflurane inhalation followed by decapitation and the gut was removed in order to dissect 3 to 5 mm long sections of the third order branch from mesenteric artery. The arterial sections were cannulated at both ends and mounted in a video-monitored perfusion system (Living Systems Instrumentation, Burlington, VT, USA), as previously described (Matrougui *et al.*, 1999; Loufrani *et al.*, 2001; Widdop *et al.*, 2002). Mesenteric artery sections were bathed in 20 mL organ baths that contained Krebs solution to which the AT_1 receptor antagonist, candesartan (1 μM) was added. The solution was bubbled with carbogen (95% O_2 and 5% CO_2), with temperature maintained at 37°C and the pH at 7.4. The arterial sections were superfused at a rate of 4 mL·min^{-1} and perfused at a rate of 100 μL·min^{-1}. The intraluminal pressure was set at 75 mmHg. The diameter of the arterial sections was constantly measured and recorded with a video-monitoring system. Following an equilibration period of approximately 30 min, phenylephrine was added to achieve 20–30% of the maximum contractile response. Once the plateau was reached, a concentration response curve to Compound 21 (0.1 nM to 1 μM) was constructed. Analogous experiments were performed in which the AT_2 receptor antagonist, PD123319 (1 μM) was added 30 min before Compound 21. An additional tissue was set up that served as a time control and was only pre-contracted with phenylephrine.

In vivo *procedures*

Rats were anaesthetized (ketamine and xylazine; 75 mg·kg^{-1} and 10 mg·kg^{-1}, i.p., respectively; supplemented as required). Two catheters were inserted into the right jugular for i.v. drug administration. A catheter was inserted into the right carotid artery for direct blood pressure measurement as described previously (Barber *et al.*, 1999; Li and Widdop, 2004; Walters *et al.*, 2005). Rats were housed in individual cages and allowed free access to food and water while maintained on 12 h day/night cycle. The arterial catheter was infused overnight with heparinized saline using an infusion pump.

Twenty-four hours after the surgery, the arterial catheter was attached to a pressure transducer (Gould Inc., Eichstetten, Germany), connected to a MacLab-8 data acquisition system (ADInstruments) and interfaced to a Macintosh computer. Mean arterial pressure (MAP) and heart rate were computed from the phasic blood pressure signal.

Experimental protocol

Rats received drug combinations in a randomized fashion over a 4 or 5 day protocol, as described previously (Barber *et al.*, 1999; Walters *et al.*, 2005). Doses of candesartan and PD123319 were chosen on the basis of previous studies (Barber *et al.*, 1999; Walters *et al.*, 2005). Animals in group 1 (WKY; dose-ranging) and group 2 (SHR; dose-ranging) were randomized to receive following treatments: (i) a 4 h Compound 21 infusion (50, 100 or 300 ng·kg^{-1}·min^{-1} in WKY rats or 100, 300 and 1000 ng·kg^{-1}·min^{-1} in SHR); and (ii) a 4 h Compound 21 infusion (50 and 300 ng·kg^{-1}·min^{-1} in WKY rats or 300 and 1000 ng·kg^{-1}·min^{-1} in SHR) given simultaneously with candesartan (0.1 mg·kg^{-1} i.v.).

Based on the dose-ranging results, additional SHR (group 3) received the following treatments in randomized fashion: (i) candesartan (0.1 mg·kg^{-1} i.v.); (ii) Compound 21 infusion (50 ng·kg^{-1}·min^{-1} for 4 h); (iii) a 4 h Compound 21 infusion together with candesartan; and (iv) a 4 h Compound 21 infusion in the presence of candesartan and PD123319 infusion (50 μg·kg^{-1}·min^{-1} for 2 h). The dose of PD123319 was based on our previous experience using this compound in similar *in vivo* experiments (Barber *et al.*, 1999; Li and Widdop, 2004). In analogous experiments in separate SHR (group 4), an identical protocol was repeated to that of group 3 but a 10-fold lower dose of candesartan (0.01 mg·kg^{-1} i.v.) was used. We have previously shown that basal BP recordings are stable over these time periods. Nevertheless, in this latter group, SHR also received a 4 h infusion (0.1 mL·kg^{-1}·h^{-1} i.v.) of saline (0.9% NaCl) to confirm a lack of effect on MAP.

Statistical analysis

All data are presented as mean responses ± standard error of the mean (SEM). Differences in vasorelaxation or MAP between treatments were analysed using a two-way repeated measure, ANOVA. Statistical analysis was performed using GraphPad Prism (Version 5.0). *P*-values <0.05 were considered statistically significant.

Materials

Compound 21 was provided by A Hallberg, Department of Medicinal Chemistry, Uppsala University; PD123319 and candesartan were kind gifts from Pfizer and AstraZeneca respectively. All other chemicals were purchased from commercial sources: L-NAME (Sigma), sodium nitroprusside (Sigma, Sydney, Australia), ketamine (Troy Laboratories, Sydney, Australia), xylazine (Troy Laboratories), phenylephrine (Sigma), isoflurane (Baxter, Deerfield, IL, USA) and U46619 (Saphire Bioscience, Sydney, Australia).

Results

In vitro *relaxation evoked by Compound 21*

Compound 21 caused a dose-dependent relaxation of mouse aorta in the presence of AT_1 receptor blockade, which was

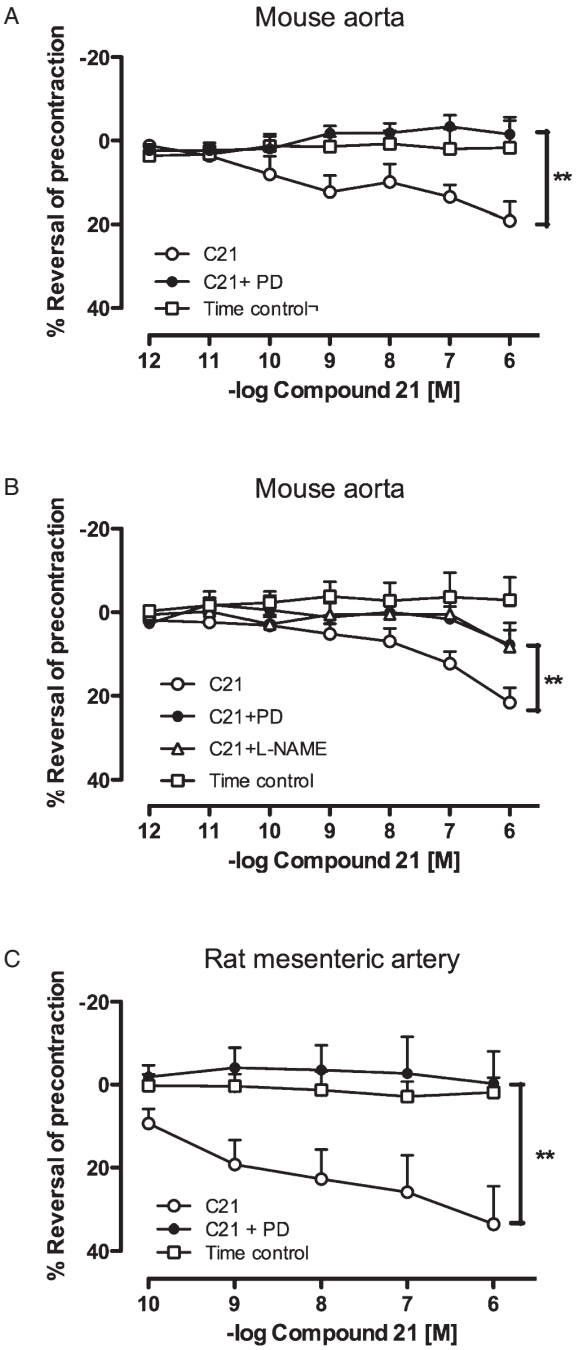

Figure 1 Dose–response curves to Compound 21 (C21) performed in (A) mouse aorta and (C) third order perfused rat mesenteric artery in the absence (n = 5 and 9 for aorta and mesenteric artery respectively) and presence (n = 5 and 3 for aorta and mesenteric artery respectively) of PD123319 (PD); all experiments in Figure 1A and C performed in the presence of angiotensin type 1 receptor (AT_1 receptor) blockade, as described in the *Methods*. (B) Effect of Compound 21 in mouse aorta in the absence of AT_1 receptor blockade and in the presence of PD123319 or L-NAME (n = 10 for each). Values represent mean ± SEM. $^{**}P < 0.01$ for treatment effect between Compound 21 and Compound 21 + PD123319 or Compound 21 + L-NAME (two-way repeated measures ANOVA).

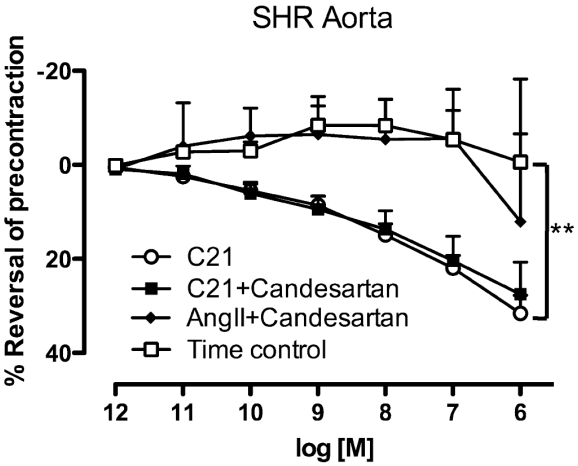

Figure 2 Dose–response curves to either angiotensin II (Ang II) or Compound 21 (C21) performed in aorta obtained from naive spontaneously hypertensive rat (SHR) in the absence (Compound 21) and presence (Ang II & Compound 21) of candesartan (0.1 μM) (n = 5 for each). Values represent mean ± SEM. $^{**}P < 0.01$ for treatment effect between Compound 21 and time control (two-way repeated measures ANOVA).

markedly inhibited by PD123319 (Figure 1A). Furthermore, Compound 21-evoked relaxation was also evident in the absence of AT_1 receptor blockade and was abolished by L-NAME (Figure 1B). In addition, Compound 21 caused vasodilatation in third order perfused rat mesenteric artery, which was also blocked by PD123319 (Figure 1C).

Experiments were also performed using aortic rings obtained from naive SHR, a preparation that is reported to be unresponsive to the AT_2 receptor-mediated vasorelaxant effects of Ang II (Cosentino *et al.*, 2005). Indeed, Ang II did not cause relaxation; on the other hand, Compound 21 evoked dose-dependent relaxation that was similar in the presence or absence of AT_1 receptor blockade (Figure 2).

In vivo effect of Compound 21 in conscious rats

Basal MAP of SHR over the four or five experimental days for each group are listed in the Table 1. There was no significant difference between resting MAP over the experimental period for any of the treatment groups, suggesting that none of the acute treatments had effects that lasted more than 24 h, and therefore did not influence baseline MAP on subsequent days.

In both WKY (Figure 3) and SHR (Figure 4), infusion of Compound 21 alone, at doses ranging from 50 to 300 $ng \cdot kg^{-1} \cdot min^{-1}$, had no significant effect on MAP. Combined administration of Compound 21 (100 and 300 $ng \cdot kg^{-1} \cdot min^{-1}$) and candesartan (0.1 $mg \cdot kg^{-1}$) also had no effect on MAP in WKY rats (Figure 3); however, the

Table 1 Resting mean arterial pressure (MAP) of spontaneously hypertensive rat recorded on separate days before drug treatments, as indicated

Treatment	*MAP (mmHg)*
Group 2 (n = 5)	
Compound 21 (100 ng·kg^{-1}·min^{-1})	174 ± 8
Compound 21 (300 ng·kg^{-1}·min^{-1})	177 ± 8
Compound 21 (1000 ng·kg^{-1}·min^{-1})	165 ± 5
Compound 21 (300 ng·kg^{-1}·min^{-1}) & candesartan (0.1 mg·kg^{-1})	164 ± 8
Compound 21 (1000 ng·kg^{-1}·min^{-1}) & candesartan (0.1 mg·kg^{-1})	163 ± 5
Group 3 (n = 7)	
Compound 21 (50 ng·kg^{-1}·min^{-1})	178 ± 6
Candesartan (0.1 mg·kg^{-1})	191 ± 6
Compound 21 & candesartan	190 ± 7
Compound 21, candesartan & PD123319 (50 µg·kg^{-1}·min^{-1})	177 ± 7
Group 4 (n = 7)	
Saline	175 ± 6
Compound 21 (50 ng·kg^{-1}·min^{-1})	179 ± 7
Candesartan (0.01 mg·kg^{-1})	184 ± 8
Compound 21 & candesartan	190 ± 7
Compound 21, candesartan & PD123319 (50 µg·kg^{-1}·min^{-1})	170 ± 10

The values shown in the Table are means ± SEM. n = 5–7 per group.

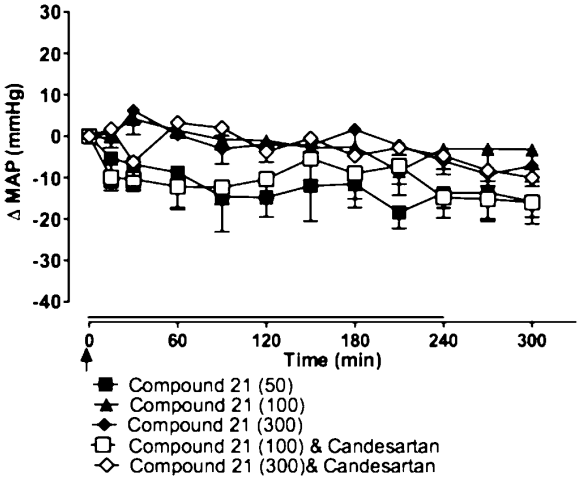

Figure 3 Effects of Compound 21 (50, 100 and 300 ng·kg^{-1}·min^{-1}), administered as a 4 h infusion (shown by horizontal line), on mean arterial pressure (MAP) in Wistar-Kyoto rats (n = 5). Compound 21 was given in the presence or absence of the angiotensin type 1 receptor antagonist, candesartan (0.1 mg·kg^{-1} i.v. bolus; shown by an arrow). Values represent mean ± SEM.

combination of Compound 21 (300 ng·kg^{-1}·min^{-1}) and candesartan, significantly decreased MAP in SHR compared with Compound 21 alone (P < 0.001) (Figure 4). Interestingly, at the highest dose tested (1000 ng·kg^{-1}·min^{-1}), Compound 21 alone caused an increase in MAP in SHR (P < 0.05), suggesting a lack of selectivity of Compound 21 at this dose. This Compound 21-mediated pressor effect was attenuated by simultaneous AT_1 receptor blockade (Figure 4).

Given the lack of response to Compound 21 in WKY rats, further examination of the effect of Compound 21 on MAP

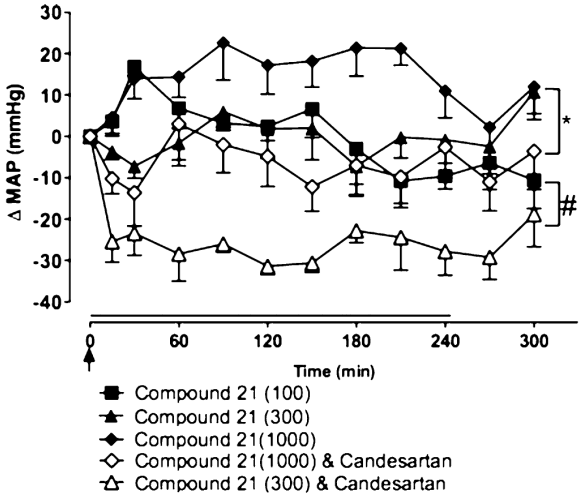

Figure 4 Effects of Compound 21 (100, 300 and 1000 ng·kg^{-1}·min^{-1}), administered as a 4 h infusion (shown by horizontal line), on mean arterial pressure (MAP) in spontaneously hypertensive rats (n = 5). Compound 21 was given in the presence or absence of the angiotensin type 1 receptor agonist, candesartan (0.1 mg·kg^{-1} i.v. bolus, shown by an arrow). Values represent mean ± SEM. #P < 0.001 for treatment effect between Compound 21 (300 ng·kg^{-1}·min^{-1}) + candesartan and Compound 21(300 ng·kg^{-1}·min^{-1}) (two-way repeated measures ANOVA); *P < 0.05 for treatment effect between Compound 21(1000 ng·kg^{-1}·min^{-1}) + candesartan and Compound 21(1000 ng·kg^{-1}·min^{-1}) (two-way repeated measures ANOVA).

was performed in separate groups of SHR, to determine the effects of lower dose of Compound 21 in combination with AT_1 receptor block. As had been previously determined, Compound 21 infusion alone (50 ng·kg^{-1}·min^{-1}) had no effect on MAP in SHR (Figure 5). However, when combined with either high-dose (0.1 mg·kg^{-1}; Figure 5) or low-dose (0.01 mg·kg^{-1}; Figure 6) candesartan, Compound 21 caused a significant reduction in MAP in SHR (P < 0.001). Importantly, when the AT_2 receptor antagonist, PD123319 (50 µg·kg^{-1}·min^{-1}), was co-infused for 2 h with Compound 21 and candesartan, this blood pressure-lowering effect was abolished, indicating AT_2 receptor selectivity of Compound 21 (P < 0.001). Furthermore, infusion of saline had no effect on blood pressure (Figure 6).

Discussion

The main finding of the current study was that the novel non-peptide AT_2 receptor agonist, Compound 21, evoked vasorelaxation *in vitro*, which translated into vasodepressor responses in conscious SHR against a background of AT_1 receptor blockade. To our knowledge, this study represents the first systematic study of the vascular effects of Compound 21, particularly in hypertension.

Compound 21 exhibits a similar binding profile at AT_2 receptors to that of Ang II and CGP42112 but with little affinity for AT_1 receptors. In mouse aortic rings, Compound 21, in the presence of AT_1 receptor antagonists, caused concentration-dependent vasorelaxation that was inhibited

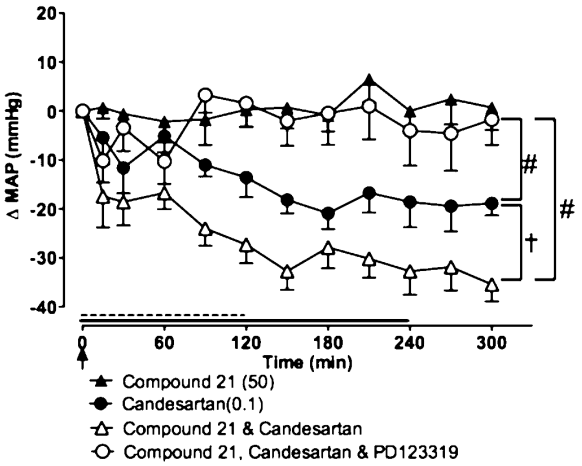

Figure 5 Effect of Compound 21 (50 ng·kg^{-1}·min^{-1}; 4 h infusion shown by full line), high-dose candesartan (0.1 mg·kg^{-1} bolus i.v.; shown by an arrow), Compound 21 + candesartan and Compound 21 + candesartan + PD123319 (50 μg·kg^{-1}·min^{-1} for 2 h; (dashed line), on mean arterial pressure (MAP) in spontaneously hypertensive rat ($n = 7$). Values represent mean ± SEM. #$P < 0.001$ for overall effect of treatment versus Compound 21 (two-way repeated measures ANOVA); †$P < 0.01$ for treatment effect between Compound 21 + candesartan and candesartan (two-way repeated measures ANOVA).

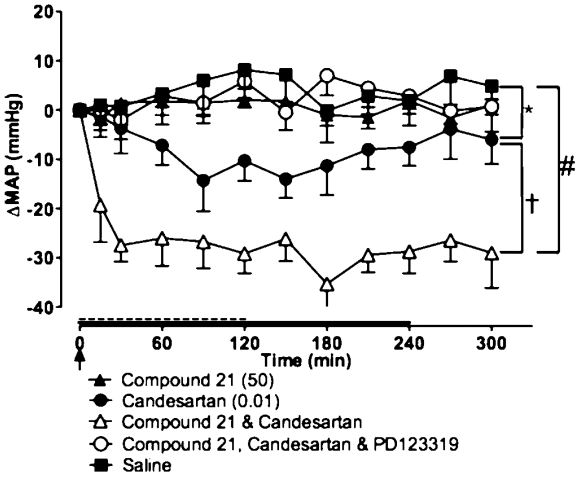

Figure 6 Effect of Compound 21 (50 ng·kg^{-1}·min^{-1}; 4 h infusion shown by full line), low-dose candesartan (0.01 mg·kg^{-1} bolus i.v.; shown by an arrow), Compound 21 + candesartan and Compound 21 + candesartan + PD123319 (50 μg·kg^{-1}·min^{-1} for 2 h; shown by dashed line), on mean arterial pressure (MAP) in spontaneously hypertensive rat ($n = 7$). Values represent mean ± SEM. *$P < 0.05$ for treatment effect between Compound 21 and candesartan; #$P < 0.001$ for overall effect of individual treatment versus Compound 21 (two-way repeated measures ANOVA); †$P < 0.01$ for treatment effect between Compound 21 + candesartan and candesartan alone (two-way repeated measures ANOVA).

by the AT_2 receptor antagonist, PD123319. This *in vitro* effect was not dependent on background AT_1 receptor blockade, as it was *in vivo*, and was also inhibited by L-NAME. Thus, Compound 21 elicited classical AT_2 receptor-mediated NO signalling, a hallmark of AT_2 receptors in vascular tissue (Widdop *et al.*, 2003; Jones *et al.*, 2008). This compound was also tested using rat isolated mesenteric arteries, a preparation well recognized as exhibiting AT_2 receptor-mediated vasorelaxation (Matrougui *et al.*, 1999; Henrion *et al.*, 2001; Widdop *et al.*, 2002). Indeed in these resistance-like vessels, Compound 21 caused vasorelaxation that was inhibited by PD123319, confirming selective AT_2 receptor-mediated actions of the compound.

In the present study, dose–response analysis was performed in conscious SHR and WKY rats in which multiple doses were tested in the same animals on different days, allowing within-animal analysis. When tested over a wide dose range, Compound 21 alone did not reduce MAP in SHR or WKY rats except during AT_1 receptor blockade in SHR, as seen previously with CGP42112 (Barber *et al.*, 1999; Li and Widdop, 2004). It is likely that these results can be explained by the fact that circulating endogenous Ang II itself exerts tonic AT_1 receptor-mediated vasoconstriction that, once removed, allows AT_2 receptor-mediated vasodilatation to be manifest. These data are consistent with previous studies in conscious SHR in which Ang II did not cause vasodilatation during AT_1 receptor blockade (Gohlke *et al.*, 1998), presumably because the hypotensive effect of AT_2 receptor stimulation was masked by the concomitant, dominant AT_1 receptor-mediated pressor action during Ang II infusion. A differential effect on vascular tone to AT_2 receptor stimulation has also been noted by others using SHR and WKY rats (Savoia *et al.*, 2005), and is consistent with a lack of effect of Compound 21 on blood pressure in anaesthetized normotensive rats (Wan *et al.*, 2004b). This lack of effect of Compound 21 on BP in normotensive animals may relate to the fact that subtle AT_2 receptor-mediated depressor responses are more easily observed from a higher basal blood pressure. Alternatively, it may represent strain-dependent differences in sensitivity to AT_2 receptor stimulation or drug-induced changes in vascular AT_2 receptor expression. In this context, aortic AT_2 receptor expression is higher in adult SHR compared with age-matched WKY, whereas mesenteric AT_2 receptor expression is increased in young SHR but decreased in adult SHR (see Widdop *et al.*, 2008).

Limited *in vivo* studies in anaesthetized SHR implied a role of this compound on vascular tone as, when given as bolus i.v. injections to anaesthetized SHR, Compound 21 lowered BP (Wan *et al.*, 2004b). The discrepancy between the two studies most likely reflects the different experimental designs between the current and previous studies (Wan *et al.*, 2004b). The highest effective dose (0.05 mg·kg^{-1}) of Compound 21 previously tested (Wan *et al.*, 2004b) was probably higher than our maximally effective depressor dose achieved during a 4 h infusion (i.e. 300 ng·kg^{-1}·min^{-1} ~ 0.072 mg·kg^{-1} total; during AT_1 receptor blockade), once half-life and pharmacokinetic considerations are taken into account, although it should be noted that a higher dose of Compound 21 (1000 ng·kg^{-1}·min^{-1}) alone also did not decrease blood pressure. Another important difference is that the previous study (Wan *et al.*, 2004b) tested barbiturate-anaesthetized SHR, which are less physiological than consciously instrumented SHR that would be more able to buffer potential blood pressure reductions by homeostatic reflex mechanisms. Moreover,

we were keen to be able to make direct comparisons with previous studies that also reported AT_2 receptor-mediated depressor effects of the selective agonist CGP42112 during AT_1 receptor blockade (Barber *et al.*, 1999; Li and Widdop, 2004).

Curiously, AT_2 receptor stimulation does not generally cause vasorelaxation in vessels isolated from SHR strains (Matrougui *et al.*, 2000; Cosentino *et al.*, 2005; Savoia *et al.*, 2005; You *et al.*, 2005). Chronic treatment with AT_1 receptor antagonists is associated with increased AT_2 receptor expression in aortae from SHR (Cosentino *et al.*, 2005; Savoia *et al.*, 2005), and human subcutaneous gluteal arteries (Savoia *et al.*, 2007), as well as in mesenteric arteries from SHR, which normally exhibited decreased AT_2 receptor expression under basal conditions (You *et al.*, 2005). Indeed, sartan-induced up-regulation of AT_2 receptors unmasked *ex vivo* AT_2 receptor-mediated vasorelaxation in otherwise unresponsive vessels (Yayama *et al.*, 2004; Cosentino *et al.*, 2005; Savoia *et al.*, 2005; Savoia *et al.*, 2007). Therefore, it is conceivable that enhanced *in vivo* sensitivity of untreated SHR, compared with WKY rats, to Compound 21 was due to higher AT_2 receptor expression, although elevated MAP *per se* could still contribute to AT_2 receptor-mediated depressor activity *in vivo*. In any case, whether or not *ex vivo* AT_2 receptor-mediated relaxation evoked by Compound 21 is more manifest following chronic treatment with an AT_1 receptor antagonist awaits further investigation. However, we did test the *in vitro* effects of Compound 21 acutely in naive SHR. As expected, Ang II did not evoke vasorelaxation whereas, strikingly, Compound 21 relaxed aortae. Thus, in aortic tissue known to be refractory to acute AT_2 receptor-mediated effects of Ang II (Cosentino *et al.*, 2005; Savoia *et al.*, 2005), Compound 21 caused vasorelaxation, in SHR, both *in vitro* and *in vivo*, at least in the presence of AT_1 receptor blockade. Thus, the current study highlights the importance of using subtype selective compounds.

At the highest dose tested (1000 $ng \cdot kg^{-1} \cdot min^{-1}$), Compound 21 alone actually increased MAP in SHR, most likely representing a lack of AT_2 receptor selectivity at this concentration. Although the sensitivity of this pressor effect of Compound 21 to blockade by PD123319 was not tested in the current study, simultaneous AT_1 receptor inhibition restored MAP responses to baseline. This finding could indicate that Compound 21 caused AT_1 receptor stimulation at higher doses, which may relate to the higher AT_1/AT_2 receptor ratio in vasculature, or that the hypotensive effect of candesartan offset Compound 21-mediated vasoconstriction via other mechanisms. The fact that a lower dose of Compound 21 (300 $ng \cdot kg^{-1} \cdot min^{-1}$), in combination with candesartan, significantly lowered MAP in these same SHR makes it likely that a more selective AT_2 receptor vasodilator effect was manifest at doses <1000 $ng \cdot kg^{-1} \cdot min^{-1}$. Similarly, pressor doses of Ang II infused in the presence of AT_1 receptor blockade do not always reduce blood pressure (Gohlke *et al.*, 1998), most likely for the same reasons of opposing vascular effects of AT_1 and AT_2 receptor stimulation (Barber *et al.*, 1999; Li and Widdop, 2004).

When infused at a sixfold lower dose, the depressor effect of Compound 21 (50 $ng \cdot kg^{-1} \cdot min^{-1}$) was similar in groups concomitantly administered either high- or low-dose candesartan, suggesting that the maximum achievable fall in MAP had been reached. By contrast, using CGP42112, we did not find additional depressor effects when combined with high-dose candesartan (Barber *et al.*, 1999), which may reflect a difference in metabolic fate of these two compounds as Compound 21 is a non-peptide compound. The additive effect of Compound 21 and candesartan may also reflect the fact that sartan-induced elevation in Ang II levels was probably not maximal. In any case, we have found that AT_2 receptors do not functionally desensitize even in the face of raised Ang II levels (Widdop *et al.*, 2002), therefore the non-peptide AT_2 receptor agonist may exert a prolonged effect. Importantly, in these same animals, we also tested PD123319, which completely abolished the depressor effect of combined Compound 21 and candesartan; consistent with the AT_2 receptor selectivity demonstrated by both the current *in vitro* data and radioligand binding assays performed with this compound (Wan *et al.*, 2004b). Interestingly, although tested in separate animal groups, it also appeared that there was no difference in the maximal Compound 21-mediated depressor effect using either 50 or 300 $ng \cdot kg^{-1} \cdot min^{-1}$. In this context, a bell-shaped dose–response relationship for the effect of Compound 21 on BP was also reported by Wan *et al.* (2004b), who found that the depressor effect of bolus Compound 21 administration in anaesthetized SHR was present at lower doses, but lost at higher doses (>0.05 $mg \cdot kg^{-1}$).

Collectively, these data implicate the AT_2 receptor as a potential target for the treatment of hypertension, although until now, there have been no drug-like candidates available to directly test this premise. In this context, the effects of Compound 21 have recently been reported in the setting of myocardial infarction. In that study, Compound 21, given for 7 days after myocardial infarction, improved systolic and diastolic function and reduced infarct size (Kaschina *et al.*, 2008), thus illustrating the potential use of this non-peptide compound in a number of cardiovascular settings. Indeed, our current findings suggest additive effects of AT_1 receptor blockade and AT_2 receptor stimulation would be beneficial for BP reduction, and fit with clinical findings of increased vascular AT_2 receptor expression after long-term sartan treatment (Savoia *et al.*, 2007), highlighting the need for future determination of the chronic effects of Compound 21 in hypertensive settings.

In conclusion, we have established that Compound 21 evoked vasorelaxation in mouse and SHR aortae or rat mesenteric arteries, and vasodepressor responses in conscious SHR, via AT_2 receptor stimulation. The BP-lowering effect of Compound 21 was additive to candesartan when the latter compound was given at a dose that itself lowered BP. Further studies are warranted on the chronic effects of Compound 21, alone and in combination with AT_1 receptor antagonists, in hypertensive-related diseases. These studies implicate the AT_2 receptor as a potential therapeutic target in the setting of hypertension and related cardiovascular diseases.

Acknowledgements

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Conflicts of interest

None.

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Chapter 7:

General Discussion

The studies performed in this thesis investigated the vascular properties of novel AT_2R-selective compounds, including the nonpeptide AT_2R agonist Compound 21 and some novel β-substituted Ang II peptides. Additionally, we also investigated the properties of the shorter Ang peptide, Ang (1-7) as an endogenous ligand at AT_2R. Furthermore, these ligands were investigated under various physiological settings including, gender, age and hypertension. For the first time we have demonstrated that acute administration of Compound 21, as well as β-Ile Ang II, evoke AT_2R-mediated vasodilatation in hypertensive animals, while Ang (1-7)-mediated vasodilatation is preserved in aged normotensive animals and involves complex interaction between both AT_2R and *Mas*R. Furthermore, we have provided a systematic examination of binding affinity of all major AT_2R and *Mas*R ligands in pure cell lines as well as relative AT_2R:AT_1R selectivity of these ligands.

The current understanding in cardiovascular physiology is that Ang II is able to evoke classical effects including vasoconstriction, hypertrophy and sodium retention via AT_1R activation [22, 53]. In contrast, the AT_2R is thought to be involved in eliciting opposing vasodilatory and anti-growth effects [22, 53]. Although AT_2R-mediated relaxation is a well established effect in isolated resistance vessels [24, 58, 89, 167], there is less direct evidence of AT_2R-mediated vasodilatation *in vivo.* This lack of data most likely relates to the inadequate number of selective AT_2R ligands available. For years, CGP42112 has been the only selective AT_2R agonist and PD123319 has been the only selective AT_2R antagonist used for research into the role of the AT_2R. However, both drugs are only available in limited quantities and the development of selective AT_2R ligands is usually associated with complex synthesis and high cost. Compound 21 is the first nonpeptide agonist, which has made the investigation of direct AT_2R stimulation possible. Other evidence suggests that smaller Ang peptide fragments such as Ang

(1-7), Ang III and Ang IV are also bioactive peptides and that these may contribute to the regulation of cardiovascular pathology.

Therefore, the overarching aim of this thesis was to investigate the relative affinities of Compound 21 and shorter Ang peptides at AT_1R and AT_2R, before determining their cardiovascular effects in various physiological settings.

7.1 AFFINITIES OF ENDOGENOUS AND EXOGENOUS LIGANDS AT AT_1R AND AT_2R

One rapidly developing area of the RAS has been the re-evaluation of Ang peptides other than Ang II. While it was recognised many years ago that the angiotensin family of peptides could evoke effects distinct from Ang II [168] there has been much recent interest in the ability of shorter Ang peptides to influence cardiovascular function via specific receptors such as the AT_2R and *Mas*R. In doing so, it is proposed that these shorter peptides may in fact oppose the actions of Ang II/AT_1R-mediated effects.

In this context, in Chapter 2 using stably transfected HEK cells with either AT_1R or AT_2R, a comprehensive binding analysis was performed to obtain binding profiles of Ang peptides as well as synthetic AT_2R and *Mas*R ligands. In those experiments, using [^{125}I]-Sar^{1}Ile^{8}Ang II competition binding, Ang II displaced both AT_1R and AT_2R binding being approximately 15 time more selective for the AT_2R, as expected [21, 23, 28, 169]. We also confirmed previous reports of high AT_1R selectivity for candesartan, and high AT_2R selectivity for CGP42112 and PD123319 [23, 28, 169, 170].

Over the years, CGP42112 has been the gold standard compound used to investigate AT_2R function and AT_2R-mediated signaling [23]. However, although CGP42112 has been an important ligand for investigative purposes, its property of being a large peptide molecule susceptible to rapid metabolism as well as being

unable to cross the blood brain barrier, has limited any clinical applications. The discovery of a selective nonpeptide AT_2R agonist, Compound 21, with oral bioavailability of 20-30% [30], rekindled the idea of direct AT_2R agonistic therapy. In Chapter 2, Compound 21 exhibited a binding profile of a selective AT_2R ligand in a similar manner to CGP42112 and PD123319. We documented that Compound 21 was a highly selective AT_2R ligand, although AT_2R affinity was ~10 fold lower that that of CGP42112, but still approximately 4000-fold more selective for AT_2R when compared to AT_1R. By contrast, the first nonpeptide Ang (1-7) mimetic and *Mas*R agonist, AVE0991, as well as the *Mas*R antagonist, A-779, both completely lacked AT_1R and AT_2R binding (at 1μM).

Interestingly, Ang III was 30-fold more selective for the AT_2R over the AT_1R, consistent with previous reports [21, 171, 172]. Other binding data suggests that Ang III has higher affinity for AT_2R than Ang II [169, 170, 173], raising the possibility that Ang III is an endogenous ligand at AT_2R playing an important role in cardiovascular function. This notion is supported by functional data whereby administration of Ang III in rats lowered blood pressure in conscious SHR during AT_1R block [174] and evoked natriuresis in the kidney [175-178], with both effects sensitive to AT_2R blockade. Moreover, AT_2R-mediated vasodilatation in coronary arteries was inferred since PD123319 potentiated vasoconstriction caused by Ang III [179].

There are numerous reports with conflicting data as to which receptor Ang (1-7) binds, including Ang (1-7) being able to evoke vasoactive effects via *Mas*R, AT_2R, and B_2R suggesting a complex interplay between these receptors [67, 102, 104, 109, 111, 180]. The experiments carried out in Chapter 2 demonstrated that Ang (1-7) exhibited modest AT_2R affinity and was approximately 40-fold more selective for AT_2R over AT_1R. This is a clear indication that Ang (1-7), in addition to activating

*Mas*R, may act as endogenous ligand for the AT_2R to exert its biological effect [111].

Strikingly, Ang IV exhibited substantial AT_2R binding (IC_{50} value in μM range) but not AT_1R binding, and was approximately 200-fold more selective for the AT_2R over AT_1R. Ang IV is an endogenous ligand at the AT_4R/IRAP but there are several functional reports suggesting that Ang IV acts via both AT_4R/IRAP and AT_2R [68, 181-184]. These binding data support the notion that part of the cardiovascular effects of Ang IV are attributed to AT_2R stimulation.

Thus, in Chapter 2 we have provided binding profiles as well as the rank order of potency for all major Ang peptides and currently available synthetic AT_2R and *Mas*R compounds. These results allowed a rank order of potency for ligands at the AT_2R to be determined which was CGP42112 > Ang II ≥ Ang III > Compound 21 ≥ PD123319 >> Ang IV > Ang (1-7). By performing these experiments we have confirmed that Compound 21 is a highly selective AT_2R ligand, and we have also provided strong evidence to support the notion that shorter Ang peptide fragments, other than Ang II, are endogenous ligands at AT_2R, which may act to oppose unfavourable effects of Ang II stimulation of AT_1R.

7.2 SINGLE BETA-AMINO ACID SUBSTITUTED ANG II PEPTIDES

In order to improve therapeutic potential of peptides, substitutions in the naturally occurring Ang II amino acid sequence with β-amino acids have been performed. Indeed, changes in biological activity and altered stability of Ang II peptides incorporating β-amino acid substitutions have previously been described [185-188], which were recently reviewed [189] but all of these studies pre-dated the sub-classification of AT_1R and AT_2R. For example, in 1979, it was documented that β-substitutions at position 4 and 5 of the Ang II amino acid sequence resulted in

peptides with a lack of hypertensive properties as opposed to the effects of native Ang II [190]. In Chapter 3, we have shown that β-amino acid substitution in position 4 (β-Tyr Ang II) and position 5 (β-Ile Ang II) of the Ang II sequence and resulted in peptides with high selectivity for the AT_2R and minimum affinity for the AT_1R (>1000 fold more selective). Furthermore, both β-Tyr Ang II and β-Ile Ang II led to vasorelaxation, in a manner similar to that observed with CGP42112, when tested in mouse isolated aortic rings. This effect was AT_2R-mediated via NO/cGMP pathway as it was blocked by both PD123319 and L-NAME. Previous studies have reported similar susceptibility of AT_2R-mediated vasorelaxation to NOS inhibition by L-NAME [89, 93]. Interestingly, despite both β-substituted peptides causing *in* vitro relaxation, only β-Ile Ang II had a vasodilator effect in conscious SHR. This effect was only obvious during a concomitant AT_1R blockade, as previously seen with CGP42112 [92] and Ang (1-7) [111]. Similar to *in vitro* results, this effect was also shown to be AT_2R-mediated, as it was completely abolished following administration of PD123319.

These effects of β-substituted Ang II peptides are remarkable for several reasons. Firstly, a β amino acid substitution introduced an additional methyl group, and this simple modification yielded highly selective AT_2R peptides with agonistic properties. Secondly, such changes that occurred in the Ang II peptide following β-substitution at each amino acid were well tolerated for AT_2R binding. Lastly, this β-substituted method produced more stable compounds than Ang II itself, which contributed to *in vivo* effects. For example, it is likely that β-Ile Ang II, unlike β-Tyr Ang II, lowered blood pressure *in vivo* partly due to its increased metabolic stability.

In this context, previous studies revealed that an Ang IV (Val-Tyr-Ile-Pro-Phe) analogue in which β-substitution at position one, β-Val, was performed, yielded a compound that was more stable and protected against metabolic breakdown. In addition, β-substitution at position six, β-Phe Ang IV, was more selective for IRAP when compared to aminopeptidase N (AP-N), and lost AT_1R affinity [191]. However, in this previous study, AT_2R affinity was not investigated. Furthermore, performing double β-substitution in Ang IV amino acid sequence at positions one and six, resulted in an analogue that was highly metabolically stable and was considerably selective for IRAP compared to both AP-N and AT_1R, making it an ideal compound for the study of Ang IV [191].

Therefore, it is likely that the method used in Chapter 3 can help in the future development of selective AT_2R compounds, but also potentially AT_1R selective compounds, with both agonistic and antagonistic properties, which would provide further insight into the complex world of RAS.

7.3 EFFECTS OF AGE ON AT_2R-MEDIATED FUNCTION

We have previously reported that Ang (1-7) has acute vasodilator properties in both normotensive and hypertensive animals and this effect was mediated via the AT_2R, since A-779 failed to inhibit these responses [111]. However, the direct functional effects of AT_2R in aging are not known. Jones *et al.,* (2004) reported that AT_2R inhibition reversed the regression of cardiac hypertrophy and fibrosis caused by chronic treatment with candesartan cilexetil in aged normotensive rats. These findings imply the presence of functional AT_2R with increased age. However, to my knowledge the *in vivo* vascular effects of AT_2R stimulation during aging are not known.

Therefore, in Chapter 4, we explored if the aging process altered the AT_2R-mediated depressor response. Initially, these experiments were planned to be undertaken using one of the β-substituted Ang II peptides, such as β-Ile Ang II, however, experiments using β-substituted Ang II peptides were not complete at the time this chapter commenced and β-Ile Ang II was in short supply. Therefore, Ang (1-7) was chosen on the basis of the previous data from our lab [111] and results from Chapter 2, showing Ang (1-7) as a possible endogenous ligand for the AT_2R. Additionally, the role of *Mas*R during aging was yet to be determined and the use of Ang (1-7) may have in fact uncovered effects mediated via *Mas*R in aging.

It was demonstrated that the vasodepressor effect evoked by Ang (1-7) was preserved in aged normotensive animals and this effect involved an interaction between both AT_2R and *Mas*R, given that both PD123319 and A-779 reversed the Ang (1-7) vasodepressor response. This result contrasted with that seen in adult normotensive animals in which the Ang (1-7)-mediated vasodepressor effect was mediated exclusively via AT_2R, in line with our previous study [111]. Given that both AT_2R and *Mas*R blockade attenuated the response mediated by Ang (1-7) to a similar extent in aged animals, it is unclear whether Ang (1-7) stimulation is concurrently stimulating both receptors or if stimulation of one receptor is required to activate the other receptor subtype. For example, it is possible that oligodimerization occurs between the *Mas*R and the AT_2R, since previous studies have reported formations of dimers between AT_1R and *Mas*R [192] as well as AT_1R and AT_2R [193]. Thus, there is precedence for heterodimerisation formation between various ATR subtypes, although the possible dimer formation between AT_2R and *Mas*R has not been investigated.

Consistent with previous reports using aortic and mesenteric vessels [146, 147] we have demonstrated increased aortic AT_2R immunoreactivity in aged normotensive animals. However, for the first time we have observed increased *Mas*R and ACE2 immunoreactivity, but no difference in the AT_1R expression between adult and aged normotensive animals. Thus, we postulate that the AT_2R/*Mas*R vasodilator axis of the RAS was dominant over the vasoconstrictor axis, which resulted in preserved vaso-depressor effect of Ang (1-7) in aged normotensive animals. In addition, the fact that an increase in ACE2 levels was observed, which readily converts Ang II to Ang (1-7), might suggest that further local production of Ang (1-7) may contribute to the regulation of blood pressure in aged animals. Therefore, it is possible that the upregulation of RAS components documented in this thesis is important for counteracting deleterious cardiovascular changes seen in the elderly.

7.4 CHRONIC EFFECT OF COMPOUND 21 ON BP IN THE NORMOTENSIVE STATE

Given the recently published data for Compound 21, we were curious to know if selective AT_2R stimulation using Compound 21 would result in sex-specific differences in blood pressure regulation in normotensive animals. The hypothesis for this study was based on recent results that a chronic low dose infusion of Ang II lowered blood pressure in female rats [162]. To this end we tested Compound 21 chronically in both normotensive female and male mice and rats. Interestingly, we did not see any effect of Compound 21 on MAP in a chronic setting in either species. In this context, Stecklings *et al*., 2010, reported that Compound 21, at a dose 20 times higher than used in the current study, also failed to decrease blood pressure in normotensive mice [194]. However, we did confirm the vasodepressor effects evoked by low dose chronic Ang II infusion in female rats compared to

male rats. Sex-specific difference in Ang II-mediated response could be due to an increase in AT_2R:AT_1R ratio in female rats [160] and an increase in renal mRNA gene expression of both AT_2R and ACE2 in female rats [162]. These changes in RAS components seen between males and females could lead to an increase in AT_2R-vasodilatory axis components of the RAS in female rats, which may contribute to the sex-specific differences seen with low dose chronic Ang II infusion.

Given the results obtained in Chapter 5, it is unclear why Compound 21 was ineffective in lowering blood pressure in both female mice and rats, particularly given that Ang II decreased blood pressure in female rats. While Ang II was not tested in female mice, it was anticipated that Compound 21 would have a similar effect, if not more pronounced, in female animals based on the fact that AT_2R were selectively stimulated. Therefore, it is possible that Compound 21 in this setting was eliciting an off target effect which in turn nullified the possible depressor effect of Compound 21. Indeed, evidence for pressor effects in response to Compound 21 were reported in male normotensive mice (Chapter 5) and SHR (Chapter 6) (See section 7.5). Furthermore, it is possible that the depressor effect seen with Ang II infusion is in fact due to a metabolic breakdown products of RAS. For example, as was previously cited Ang II can be metabolised to both Ang III and Ang (1-7), which are both shorter Ang peptides thought to oppose the actions of the Ang II-AT_1R activation [111, 175, 176, 178]. Thus, it is possible that low-dose Ang II was converted to either Ang III or Ang (1-7), which could than be responsible for the depressor response seen in the female rats possibly via multiple receptors (*Mas*R and AT_2R). Moreover, it is possible that the activation of both AT_2R and *Mas*R must occur for the depressor response to ensue.

7.5 ACUTE EFFECTS OF COMPOUND 21 ON BP IN THE HYPERTENSIVE STATE

This lack of effect of Compound 21 on blood pressure in normotensive animals may relate to the fact that subtle AT_2R-mediated depressor response are more easily observed from a higher basal blood pressure.

In Chapter 6, AT_2R stimulation by Compound 21 was observed in both *in vitro* and *in vivo* settings. In both mouse and rat isolated aorta as well as rat mesenteric artery, Compound 21 caused vasorelaxation that was reversed by the addition of PD123319 and L-NAME indicating that Compound 21 was activating AT_2R via a classic NO/cGMP pathway. Furthermore, *in vivo* administration of Compound 21 lowered blood pressure against a background of AT_1R block in a similar manner to CGP42112 [92], an effect that was attenuated by co-administration of PD123319, suggesting that Compound 21 was acting selectively via AT_2R. Interestingly, Compound 21 administered at the highest dose (1000ng/kg/min) alone, resulted in a vasopressor response in hypertensive animals. This increase in MAP is most likely indicative of a loss of AT_2R selectivity, where Compound 21 evoked an off-target effect. It is possible that this effect may not be AT_1R-mediated since Compound 21 had minimal affinity for AT_1R in the binding experiments, although this compound was not tested above 1uM (Chapter 2). Moreover, Compound 21-mediated pressor effects observed in male mice in Chapter 5 were not attenuated by co-administration of AT_1R blockade adding weight to this argument.

Collectively, the results from this thesis indicate that Compound 21 might not be a useful drug to lower blood pressure, but might be more beneficial for structural remodelling since it reduced infarct size following myocardial infarction in rats [100]. Additionally, in a recent study using 2-kidney, 1-clip (2K1C) Goldblatt rats as a model of hypertension, Compound 21 reduced inflammatory markers, TNF-α,

IL-6 and TGF-β in the clipped kidney [195]. Furthermore, increased renal production of NO/cGMP was also observed [195], suggesting activation of the classic AT_2R signalling pathway. Additionally, in spontaneously hypertensive stroke prone rats (SHRSP), chronic treatment with Compound 21 attenuated kidney inflammation and fibrosis, delayed manifestation of brain abnormalities and prolonged survival in a dose-dependent manner [196]. These effects were abolished with concomitant administration of PD123319, suggesting that the beneficial effects observed with Compound 21 were due to the activation of AT_2R [196]. Indeed, in all of these studies, Compound 21 did not alter blood pressure despite the increased production of NO/cGMP [100, 195, 196].

Clearly, further studies are required to investigate the chronic effects of Compound 21 in various cardiovascular disease models.

7.6 FUTURE DIRECTIONS

The finding that Ang (1-7) mediates its depressor effects via both the *Mas*R and AT_2R in aged rats is interesting. Recent data from our laboratory has shown PD123319 attenuated the anti-atherosclerotic effects of Ang (1-7) in a similar manner to A-779 [67]. Given that multifaceted interactions between AT_2R and *Mas*R were evident in this thesis (Chapter 4) and previous studies [67] it is imperative that all the compounds tested in this thesis be tested in HEK cells stably transfected with *Mas*R. Indeed, it is possible that some of the ligands tested, both endogenous and exogenous, exhibit affinity for both the AT_2R and *Mas*R. Therefore, these experiments would provide additional insight into the beneficial protective role of the Ang (1-7)/*Mas*R/AT_2R axis.

Additionally, given the complex relationship between these receptor subtypes and Ang peptides it is possible that the formation of heterodimers may occur. In This context, it has been previously suggested that oligodimerization of the angiotensin

receptors may occur [8, 23]. Previous reports have documented dimer formation between AT_1R and AT_2R whereby AT_2R antagonises the function of AT_1R [193, 197, 198], between AT_2R and B_2R [199] and between AT_1R and *Mas*R where it has been documented that the *Mas*R acts as a physiological AT_1R antagonist [192]. Given the fact that the effect if Ang (1-7) in aged rats were blocked by either AT_2R or *Mas*R antagonist, it is possible that formation of dimers occurs between those two receptor subtypes and further research is warranted to confirm whether this indeed does occur.

Furthermore, it would be of interest to examine chronic Ang (1-7) effects in both male and female animals considering that we documented Ang II-mediated depressor response in female normotensive rats [162]. Additionally, testing more selective compounds including *Mas*R agonist, AVE-0991 and β-substituted peptides would also provide more insight into the complexity of RAS.

Interestingly in Chapter 5, we failed to observe any effect of Compound 21 in a chronic setting while Ang II infusion resulted in depressor response in female rats [162]. It is possible that the conversion of Ang II to Ang (1-7) and subsequent activation of a *Mas*R vasodilatory axis is responsible for this effect rather than Ang II directly, particularly since others have reported increased renal ACE2 expression in a similar experimental paradigm [162]. In future studies, determination of plasma angiotensin peptide levels would provide valuable information detailing which peptides are present during Ang II infusion in males and females.

Given the acute treatment of Ang (1-7) in this thesis had differential effect dependent on age, together with increased immunofluorescence expression of AT_2R, *Mas*R and ACE2, it would be important to perform more quantitative analysis by determining the level of AT receptor mRNA expression using RT-PCR

or protein expression by Western Blot analysis. These studies would strengthen findings from this thesis

Finally, individual β-substitutions to Ang II have produces some very interesting data. An obvious extension of this work would be to perform β-substitutions using either Ang (1-7) or Ang III as the template and determine *in vitro* and *in vivo* pharmacology of these Ang analogue, with a view to further improving AT_2R selectivity and/or uncovering *Mas*R activity.

7.7 CONCLUSION

In conclusion, experimental results from this thesis have confirmed selectivity of the current available synthetic ligands including candesartan (AT_1R), CGP42112 (AT_2R), PD123319 (AT_2R) and Compound 21 (AT_2R). Furthermore, we have unveiled the possibility for all shorter Ang peptide fragments to act as endogenous AT_2R ligands. Taken collectively, these results highlight the importance of understanding the complex relationships that exit within RAS. In particular, this thesis highlights that AT_2R axis as well as other shorter Ang peptides may counter-regulate the Ang II/AT_1R axis. A greater understanding of the interactions could facilitate development of better targeted therapeutic tools for the fight against cardiovascular diseases.

Chapter 8:

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