INVOLVEMENT OF NOVEL CARDIAC PEPTIDES IN HEALTHY AND ISCHEMIC HEARTS

A thesis submitted in partial fulfilment of the requirements for the Degree of

Doctor of Philosophy in Biology

at the University of Canterbury

by H. C. G. Prosser

University of Canterbury

2009

ABSTRACT

The role and functions of Urotensin II (UII), Urotensin II-related peptide (URP) and proangiotensin-12 (PA12) are currently ambiguous, either due their relatively new identification and isolation from their host species, or due to contrasting and conflicting reports observing the physiological and pathophysiological role of these spasmogens within the mammalian cardiovascular system. Accordingly, we sought to determine the true physiological functions of these peptides in both healthy and diseased states.

The initial task was to reveal potential reasons for the contrasting responses to UII, and to define the role of UII within the isolated rat heart. UII and URP retain a highly conserved cyclic region, shown to be necessary in receptor binding and activation, with the high inter-species variance within the N-terminus reported to be of little importance. Our research revealed UII to be highly species-specific, stimulating potent, sustained vasodilation of the coronary arteries in response to the native form infused, while non-native UII peptides had either no effect, or caused significant vasoconstriction. UII-induced vasodilative effects were found to be mediated by nitric oxide and prostaglandin activity combined. Reviewing publications to date it was evident that many studies employed UII foreign to the host species, reporting potentially untrue effects, based on our findings.

Recent studies have identified UII as a potent agent in developing and promoting atherosclerosis and coronary artery disease through UII-induced mitogenic activity and promoting foam cell formation. Hence, we observed the effect of infusing the native species of UII and URP into a model of cardiac ischemia-reperfusion. Both preconditioning the heart with UII or URP, or infusing UII or URP upon reperfusion caused significant coronary vasodilation following ischemia, and significantly attenuated ischemic-induced myocardial injury. These studies indicated elevating UII and URP provided a level of cardioprotection, not only when administered into healthy hearts prior to an ischemic event, but also in hearts having already undergone ischemia and the resultant endothelial damage.

PA12 was the third peptide tested in the current thesis. Being newly identified and suggested to be a new component of the renin-angiotensin system (RAS) it was important to define the physiological role of PA12 upon the cardiovasculature, as the RAS is heavily associated with the development and progression of cardiovascular disease. Utilising the Langendorff isolated rat heart technique, PA12 was found to cause potent vasoconstriction of the coronary arteries, mediated by the angiotensin II type 1 receptor (AT_1R). Furthermore, using subjecting the perfusate samples to radioimmunoassay and RP-HPLC revealed PA12 was converted to AngII. Both PA12-induced vasoconstriction and generation of AngII were found to be dependent upon chymase activity, with inhibition of ACE1 having little effect. Myography was employed to further study the vascular response to PA12 throughout the rat arterial system from the common carotid to the femoral arteries. PA12-induced vasoconstriction displayed a potency gradient, with greatest constriction observed in vessels closest to the heart, with potency reduced and eventually lost further from the heart. PA12-induced vasoactivity was shown to be dependent upon both chymase and ACE1 activity, with ACE1 regulating PA12 activity with greater potency.

The intracellular pathways stimulated in response to PA12 were defined using western blotting, with PA12 stimulating phosphorylation of ERK1/2, JNK, p38 and $PKC\alpha/\beta_{II}$, but having no influence on $PKC\delta/\theta$. Stimulation of these pathways is consistent with the observed PA12-induced vasoconstriction, and also indicates that PA12 activation of AT_1R and the subsequent cytokines, could potentially stimulate hypertrophy, apoptosis, cell growth and differentiation, and inflammation, promoting cadiovascular remodelling and progressing atherosclerosis, hypertension and other vascular diseases if not sufficiently regulated.

Taken together, these studies indicate PA12 may have a primary role within the local, tissue-based RAS, providing an alternate substrate to angiotensin I, while ACE1 is the primary regulatory enzyme within the circulation. Our findings also display the chymase-dependent $PA12/AT_1R$ pathway as potential novel targets for pharmacological inhibition of RAS activity to ameliorate hypertension and maladaptive vascular remodelling.

STATEMENT

The work presented in the current thesis is my own and has not been presented to any other University, for any other degree. All experimental procedures were conducted myself, with the exception of creatine kinase and troponin I analysis that were performed, with thanks, by Canterbury Health Labs.

All experimentation described was carried out after approval by the Animal Ethics Committees of the University of Canterbury (Ref 2005/24R), and the University of Otago.

THESIS FORMAT

The current thesis is comprised of 11 chapters including a general introduction and general discussion, 3 literature reviews and 6 chapters of original research. Each chapter is written as it would be published as a journal article, as chapters 3, 5 and 8 are already published in internationally peer-reviewed journals, and chapter 9 has recently been submitted for publication.

PUBLICATIONS ARISING

International Journals

Prosser HC, Forster ME, Richards AM, Pemberton CJ. Cardiac chymase activity converts rat ProAngiotensin-12 to Angiotensin II: Effects of PA12 upon cardiac hemodynamics. Cardiovascular Research. 2009, Apr;82(1):40-50. (Chapter 8 of the current thesis).

Prosser HC, Forster ME, Richards AM, Pemberton CJ. Urotensin II and urotensin II-related peptide (URP) in cardiac ischemia-reperfusion injury. Peptides. 2008 May;29(5):770-7. (Chapter 5 of the current thesis).

Prosser HC, Leprince J, Vaudry H, Richards AM, Forster ME, Pemberton CJ. Cardiovascular effects of native and non-native urotensin II and urotensin II-related peptide on rat and salmon hearts. Peptides. 2006 Dec;27(12):3261-8. (Chapter 3 of the current thesis)

Conference Proceedings/Abstracts

Prosser HC. *Cardiovascular effects of native and non-native UII and URP on rat and salmon hearts*. Australian and New Zealand Comparative Physiology and Biochemistry Society, 2006, Dunedin, New Zealand.

Prosser HC. *Direct effects of proangiotensin-12 on the isolated rat heart*. Proceedings of the Health Research Society of Canterbury's AGM and Scientific Meeting. Christchurch, New Zealand, 2007.

Prosser HC. *Direct effects of proangiotensin-12 on the isolated rat heart*. European Society of Cardiology Annual Congress, Vienna, Austria, 2007.

Prosser HC. *Cardiac chymase converts proangiotensin-12 (PA12) to Angiotensin II: Effects of PA12 upon cardiac hemodynamics*. Medical Sciences Congress, Queenstown, New Zealand, 2008.

ACKNOWLEDGMENTS

First and foremost I would like to thank my supervisors for their support and guidance throughout the course of my thesis. Chris Pemberton and Malcolm Forster always made themselves available for any questions, queries… or mishaps. They provided a great working environment to conduct my research through employing the "I'm here if you need me, but otherwise go to it" approach which suited me very well. I consider myself incredibly fortunate and lucky to have had two great supervisors, both focused on producing work of the highest quality, and intent on getting my research to their own impeccable standards.

To Mark Richards, I am forever grateful for your support, both in helping to secure funding as well as general contributions to this thesis and publications. I consider myself very lucky to have been received with such openness by you and your research group and look forward to the possibility of working with you in the future.

All of the work produced in the current thesis would not have been possible was it not for the teaching (and patience) of the staff at Endolab. Thanks to Sarah Rothwell for teaching me the Langendorff isolated heart technique, and to Dianne and Noel for the ordering of supplies and teaching me how to perform radioimmunoassays. Joy Braithwaite was tremendously helpful in teaching me the angiotensin RIA, while also providing great humour, life and gossip. To Ursula Jewell and Lotte Thomson I am very grateful for your time-conserving help with developing the western blotting technique, and having someone to talk to in the dark room saved me from partial insanity. The entire staff of Endolab provided a thoroughly enjoyable working environment. To the ever-positive, ever-helpful Gavin, Liz, Jo, Sarah, Matt, Peter, Johanna, Patalee, Nairie, Sara and the two Tim's I am forever grateful for your help and support; and of course lab manager Steve Fisher - keeping work pleasurable since 1987. To Jen and the staff of the animal floor I thank you for your great rearing skills and providing me with sufficient animals when required.

I am indebted to the NZ National Heart Foundation for their financial support of this thesis, it could not have reached its current standard had it not been for their support (Postgraduate Scholarship No. 1218).

Finally, thanks are extended to my family for their continued support throughout the current thesis. Had it not been for their tactical placement of scientific books at age 3, followed up by their tremendous support throughout my education this thesis would not have been achieved. To my partner Ammie Lawry this acknowledgment is well deserved after your huge amount of support and patience in the production of this thesis. For that I am truly thankful and hope I can repay it over the many years we will have together.

I will truly miss working with such an amazing team under the guidance of superb supervisors. It has taken out all the stress I have heard so much about from those completing their thesis, and instead made it a thoroughly enjoyable 4 years of my life. I will also sincerely miss Chris introducing our institution as the "Good Times Lab" at overseas conferences.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 9

Chapter 10

Appendix A, B, C, D

LIST OF ABBREVIATIONS

5HT	Serotonin
AA	Amino Acid(s)
AbA	Abdominal Aorta
ACAT-1	Acetyl-Coenzyme A acetyltransferase 1
ACE	Angiotensin II-converting enzyme
Ach	Acetylcholine
ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone
Ang	Angiotensinogen
Ang1-7	Angiotensin peptide (
Ang	Angiotensin peptide I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)
AngII	Angiotensin peptide II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)
AngIII	Angiotensin peptide III (Arg-Val-Tyr-Ile-His-Pro-Phe)
AngIV	Angiotensin peptide IV (Val-Tyr-Ile-His-Pro-Phe)
ANP	Atrial natriuretic peptide
ARB	Angiotensin II receptor blocker
AT1R / AT1	Angiotensin II receptor type 1
AT2	Angiotensin II receptor type 2
BNP	Brain natriuretic peptide
CAD	Coronary artery disease
CAGE	Chymostatin-sensitive AngII generating enzymes
CGRP	Calcitonin gene-related peptide
CHF	Congestive heart failure
CHO	Chinese hamster ovary cells
CK	Creatine kinase
CNP	C natriuretic peptide
CNS	Central nervous system
COX	Cyclooxygenase (includes types 1 or 2)
CST	Cortistatin
CVD	Cardiovascular disease
DAG	Diacylglycerol
DP	Developed pressure (a measure of cardiac contractility)
DTA	Descending thoracic aorta
EDHF	Endothelium-derived hyperpolarizing factor
EDP	End diastolic pressure
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular-regulated kinase (MAPK 42/44)
Fem	Femoral artery
GPR14	Urotensin II receptor
HR	Heart rate (beats per minute)
HRP	Horseradish peroxidase
ICV	Intracerebroventricular
IP	Intraperitoneal
IP3	Inositol triphosphate
IR	Immunoreactive
I-R	Ischemia-reperfusion
IV	Intravenous

JNK	c-Jun N-terminal kinase
LCCA	Left common carotid artery
LDL / ox-LDL	Low density lipoprotein / oxidised low density lipoprotein
L-NAME	Nω-Nitro-L-arginine methyl. Nitric oxide synthase inhibitor
LV	Left ventricle
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
Mes	Mesenteric artery
MS/MS	Tandem mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthase
PA12	ProAngiotensin-12 (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Tyr)
PBS	Phosphate-buffered saline
PGs	Prostaglandins
PKC	Protein kinase C
PLC	Phospholipase C
PP	Perfusion pressure (a measure of coronary artery activity)
RAMPS	Receptor activity-modifying protein
RAS	Renin-angiotensin system
RCCA	Right common carotid artery
REM	Rapid eye movement
Ren	Renal artery
Rho	Ras homolog gene family
RIA	Radioimmunoassya
RMCP	Rat mast cell protease
ROS	Reactive oxygen species
RP-HPLC	Reverse phase-high pressure liquid chromatography
RVCH	Rat vascular chymase
RVD	Rheumatic valvular disease
SD rat	Sprague Dawly rat
SHR	Spontaneously hypertensive rat(s)
SP	Systolic pressure
SS	Somatostatin
SST	Somatostatin receptor
TBS(T)	Tris-buffered saline (with tween)
$TGF_{\beta 1}$	Transforming/Tumour growth factor
TnI	Troponin I
UCE	Urotensin converting enzyme
UII	Urotenin II (hUII, tUII and rUII represent the human, trout and rat forms)
URP	Urotensin II-related peptide
UT	Urotensin II receptor (GPR14)
VSMC	Vascular smooth muscle cells

1. General Introduction and aims

Peptides are short chains of amino acids joined together in a specific sequence capable of exerting effects either on neighbouring cells (paracrine), or at a distance from their point of synthesis (endocrine). Research into peptides has exploded in recent times and they have become recognised as controlling a vast amount of homeostatic regulation from hunger to blood pressure. Peptides can also be highly specific markers of health, providing practitioners with an otherwise invisible insight into the health of an individual. For example, natriuretic peptides are now widely used as a specific marker of cardiac health (Potter et al. 2009, Troughton et al. 2006), and growth hormone levels provide a measure of cellular reproduction and growth (Brooks et al. 2008). Furthermore, once the physiological role of a specific peptide has been ascertained, pharmacological agonistic or antagonistic agents can then be designed to alter the peptides activity, providing therapeutic benefits in diseased states where: 1) the peptides beneficial effects may be lacking, or 2) the peptides detrimental effects may be in excess. Therefore, the greater number of peptides identified and isolated, the further our understanding of our homeostatic processes, and the greater power we have in controlling these. It was recently estimated that the human circulation contains more than 10^6 different proteins with approximately 1173 of those proteins currently identified (Anderson L. 2005). Whilst the acceleration in technological advancements in recent times has aided isolation and identification of new peptides from many species, it is equally important that the true biological function of each new peptide is deduced in order to provide any potential therapeutic benefit. Furthermore, it must also be determined whether the effects of each peptide differ between the healthy and diseased states.

The current thesis is concerned with three recently identified peptides which have had few or no functional studies reported. Urotensin II (UII), urotensin II-related peptide (URP) and proangiotensin-12 (PA12) are all of low molecular weight (~1000-1572 kD), 8-14 amino acids in length, and exhibit wide tissue expression throughout their

host species, as well as within the circulation. What makes UII, URP and PA12 unique is their differential expression between healthy and diseased states. It has been reported that circulating UII and URP are expressed at low picomolar concentrations in healthy subjects, but become significantly elevated in those suffering cardiovascular and renal diseases (Richards et al. 2002, Matsushita et al. 2001, Russell et al. 2003, Lapp et al. 2004, Gruson et al. 2005, Ng et al. 2002). PA12 has also been reported to be significantly elevated in the heart (and significantly reduced in the kidneys) of spontaneously hypertensive rats (SHRs) when compared to normotensives (Jessup et al. 2008). These significant elevations in peptide tissue expression and circulating concentrations suggest a pathological role, either augmenting pathogenesis or providing some form of cardiovascular protection. The aim of the current thesis therefore, was to elucidate the direct actions of these three peptides upon the heart in a healthy state, followed by observing their effects in a model of cardiac injury. The approach to this was to encompass the physiological responses down to the enzymes, receptors and intracellular proteins mediating them.

While these peptides share many similarities, they are somewhat contrasting in their history and their purported physiological roles, as detailed below.

PA12 was recently identified (first reported in late 2006) and suggested to have a role in the renin-angiotensin system (RAS) based on its amino acid sequence. The RAS is a highly researched system responsible for elevating and maintaining blood pressure at homeostatic levels through activation of a cascade of specific enzymes and peptides, stimulating both mechanical and humoral responses. The discovery of a new ‘functional’ peptide within the RAS has come as a surprise as the RAS was believed to be relatively well-defined. The maiden report indicated PA12 was a potent vasoconstrictor in the rat (Nagata et al. 2006), revealing it to be a functional peptide, potentially aiding RAS-induced increases in blood pressure, and hence potentially involved with RAS-induced progression of cardiovascular disease.

Accordingly, the initial aim of the current thesis was to ascertain whether PA12 has direct effects on the isolated rat heart. Furthermore, based on its high homology with angiotensin I, can PA12 provide an alternate substrate for AngII (or other angiotensin peptide) generation? It is also unknown whether PA12 influences recovery from

cardiac ischemic injury, nor whether the current therapeutic agents administered to patients with hypertension and/or an overstimulated RAS influence PA12-induced cardiac activity. The underlying mechanisms of PA12-induced activity also require defining, isolating the cytokines mediating the physiological response to PA12. Completion of these aims will provide a solid foundation into the cardiac functions of PA12 including the mechanisms regulating its activity, and in doing so, suggesting a therapeutic course of action.

UII was initially discovered in fish 40 years ago and first isolated from teleost fish in 1980 (Pearson et al. 1980), before eventually being identified in human tissue in 1998 (Coulouarn et al. 1998). Research into the cardiovascular effects of UII were stimulated by a report in *Nature* establishing UII as one of the most potent mammalian vasoconstrictors identified (Ames et al. 1999). Moreover, reports indicate UII levels are elevated in the circulation of patients suffering congestive heart failure (CHF) (Richards et al. 2000, Russell F.D. 2003). The evolutionary and genetic history of UII are now becoming well established highlighting its highly conserved amino acid sequence, however its physiological effects and the intracellular signalling pathways mediating them are inconsistent in the literature, while its role in disease remains unclear. Initial publications reported UII as a potent vasoconstrictor in mammals (Ames et al. 1999, Douglass et al. 2000, Gray et al. 2001), whilst other reports indicated UII could also stimulate vasodilation (Gibson A. 1987, Katano et al. 2000, Prosser et al. 2006) and stimulate both positive (Russell et al. 2001) and negative (Ames et al. 1999) inotropism in mammalian hearts. Unlike PA12, UII does not appear to be part of a larger system activating or deactivating further peptides, nor has it been consistently shown to have clear synergistic activity with other peptides.

Urotensin II-Related Peptide (URP) was discovered in 2003 by Sugo et al. when probing for UII-like peptides in the rat brain. URP shares the same highly conserved 6 amino acid ring structure common to all known species of UII, but comprises a shortened N-terminus of a single amino acid. Due to its sequential homology with UII it is assumed URP elicits the same physiological effects as UII and stimulates the same intracellular pathways. However, direct comparisons of the two peptides' effects upon the heart have not been reported, nor has a UII receptor blocker been employed to observe if the receptor blocker inhibits both peptides with equal efficacy.

With regard to UII and URP the species-specificity of the peptides needs to be directly and succinctly addressed providing a base for future studies when observing the response to UII or URP. Once achieved, the pathophysiological effects of UII and URP requires attention, herein we will employ a model of cardiac ischemia-reperfusion injury. This model is designed to simulate patients experiencing elevated circulating UII levels prior to an ischemic event, or can also be used to simulate those experiencing significantly elevated UII concentration immediately following an ischemic event. The mechanisms underlying UII- and URP-induced physiological cardiovascular activity also need to be determined, reporting the effects of UII and URP from the observed physiological response to the underlying mechanisms mediating them.

2. Introduction to urotensin II (UII) and urotensin II-related peptide (URP)

Abstract

Since its initial isolation from the urophyses of Goby fish, urotensin II (UII) and its cognate receptor (GPR14) have now been identified in many species from fish to amphibians to mammals, including humans. The bioactive, mature form of UII contains a cyclic heptapeptide preserved across all known species, spanning millions of years of evolution. UII has now been researched in a wide range of settings with initial demonstration of potent vasoconstrictor activity and cardiac depression. However, further reports indicate UII to be a vasoactive peptide with highly variable effects, dependent upon species, tissue bad and cell type, suggesting a regulatory role maintaining vascular tone. Cardiovascular expression of UII and its receptor are elevated in the diseased state, indicating a potential pathological role. Current reports are conflicting, suggesting UII augments atherosclerosis and coronary artery disease through its pro-mitogenic effects, while others suggest elevated circulating levels of UII afford a level of cardioprotection, and minimise injury following a cardiac event.
Urotensin II-related peptide (URP) shares the conserved cyclic heptapeptide of UII, however was not identified until recent times. Due to its high homology with UII it is assumed URP will stimulate the same physiological effects as UII, however this is yet to be explored. Here we review the literature to date, from the initial identification and description of UII as a "somatostatin-like" peptide due to its shared tri-amino acid motif, to the recent identification of URP including discussing the physiological role of UII as a regulator of vascular tone and cardiac function, including the receptors, enzymes and intracellular mechanisms underlying the response.

2.1. Discovery of the 'somatostatin-like' peptide urotensin II

Within the caudal region of the teleost spinal cord large neurons form linear columns with their axons projecting into a highly vascularised organ called the urophysis (Bern 2008). This gland-like organ is suggested to have a key role in osmoregulation as conformational alterations were observed in response to changes in salinity of the surrounding medium (Kriebel R.M. 1980; Arnold-Reed and Belmont 1989). Analysis of extracts from the urophyses found it to contain a large array of neurohormonal substances that were broadly categorised into four groups, urotensin I-IV (Bern and Lederis 1969). Bern and Lederis (1969) suggested urotensin I and II (UI and UII respectively) influence blood pressure and smooth muscle activity, while urotensins III and IV (UIII and UIV) have potential roles in osmoregulation within the kidney and bladder (Bern and Lederis 1969). Of note, the urophysis was later found to contain very high concentrations of acetylcholine (Ach), which may have contributed to the observed smooth muscle contractions whenever extracts of the urophysis were administered directly to the tissue (Conlon et al. 1996). Over the following 3 decades since the initial identification of the urotensin family, research has confirmed the initial report of Bern and Lederis (1969) to be correct, indicating urotensin peptides are vasoactive agents capable of exerting a range of hemodynamic effects in fish, tetrapods and mammals including humans. UII was reported to be approximately 10 fold more potent than UI in elevating arterial blood pressure in the eel (Chan D.K. 1975), stimulating interest into its potent vascular effects.

Urotensin II was first isolated from the urophysis of the Goby fish *Gillichthys mirabilis* and found to be synthesised in large neurosecretory cells (*Dahlgren cells*) within the spinal cord (Pearson et al. 1980). The mature, biologically active sequence of Goby UII was identified as 12 amino acids in length, comprising a 6 amino acid ring at the C-terminus held by disulphide bonds between 2 cysteines, with an extended N-terminus (Table 2.1) (Pearson et al. 1980).

Interest was stimulated into the role of UII after it was reported that UII isolated from Goby fish constricted trout hindgut segments and major rat vessels through stimulation of smooth muscle cells, as well as altering cardiovascular activity and inducing hypertension in rats (Bern and Lederis 1969; Gibson et al. 1986; Gibson A. 1987; Itoh et al. 1987). These early studies indicated UII stimulated haemodynamic

effects in both fish and mammals, suggesting the existence of mammalian UII orthologs as well as an endogenous receptor(s). The cloning of carp UII cDNA in 1986 (Ohsako et al. 1986) enabled the first non-piscine UII isoform to be identified in the European green frog (*Rana radibunda*) (Conlon et al. 1992). UII has since been identified and purified from many species of fish including lamprey (Waugh et al. 1995), elasmobranchs and teleosts (Waugh and Conlon 1993); amphibians (Conlon et al. 1992), and many species of mammals including humans (Coulouarn et al. 1998; Ames et al. 1999; Coulouarn et al. 1999; Mori et al. 1999). The amino acid sequence of all species of UII identified to date possess an identical 6 amino acid ring at the C-terminal end (-Cys-Phe-Trp-Lys-Tyr-Cys-). Across fish species the length of UII does not vary, all being 12 amino acids long and possessing the conserved cyclic ring. However there is large sequential variation within the N-terminus (Table 2.1). The sequence and length of UII becomes more varied from tetrapods to higher order species further up the evolutionary tree, with frog UII consisting of 13, mouse and rat 14, pig 12 and human 11 amino acids respectively. There is also large inter-species variation within the N-terminus, however the heptapeptide cyclic region remains conserved. The C-terminus is also preserved across all species with the exception of the mouse and rat where 'Val' is exchanged for 'Ile' (Table 2.1).

Two variants of prepro-human UII have been identified with 124 (Coulouarn et al. 1998) and 139 (Ames et al. 1999) amino acid residues respectively. These proteins differ only at the N-terminus, while the resultant mature UII peptide remains identical. This difference in the prepro-UII sequence is suggested to be the result of alternative splicing (Ames et al. 1999). Observing the direct activity of prepro-UII and the mature UII forms, it is clear that prepro-UII requires cleavage into the mature 11 amino acid peptide, displaying considerably higher potency than its precursor (Ong et al. 2005; Tölle and van der Giet 2008). The proteolytic enzyme responsible for the cleavage of prepro-UII is currently unknown, however Russell and co-workers reported that furin and trypsin cleaved the pro-hormone into mature UII in human epicardial mesothelial cells *in vitro* (Russell et al. 2004). Administering furin antagonists abolished the presence of the mature UII peptide, suggesting that the urotensin converting enzyme (UCE) responsible for the generation of biologically active UII possesses furin-like activity (discussed in more detail below) (Russell et al. 2004).

Species	Origin	Sequence (Disulfide bridge: Cys–Cys)
UII		
SW lamprey	Brain	H-Asn-Asn-Phe-Ser-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
FW lamprey	Brain	H-Asn-Asn-Phe-Ser-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Skate	Brain	H-Asn-Asn-Phe-Ser-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Dogfish	Spinal cord	H-Asn-Asn-Phe-Ser-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Sturgeon	Spinal cord	H-Gly-Ser-Thr-Ser-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Paddlefish	Spinal cord	H-Gly-Ser-Thr-Ser-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Goby	Urophysis	H-Ala-Gly-Thr-Ala-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Sucker A	Urophysis	H-Gly-Ser-Gly-Ala-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Sucker B	Urophysis	H-Gly-Ser-Asn-Thr-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Carp α	Urophysis, spinal cord cDNA	H-Gly-Gly-Gly-Ala-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Carp β1	Urophysis	H-Gly-Gly-Asn-Thr-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Carp β2	Urophysis	H-Gly-Ser-Asn-Thr-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Carp γ	Urophysis, spinal cord cDNA	H-Gly-Gly-Gly-Ala-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Ile-OH
Flounder	Urophysis	H-Ala-Gly-Thr-Thr-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Trout	Brain	H-Gly-Gly-Asn-Ser-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Frog	Brain, spinal cord cDNA	H-Ala-Gly-Asn-Leu-Ser-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Mouse	Spinal cord cDNA	<Gln-His-Lys-Gln-His-Gly-Ala-Ala-Pro-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Ile-OH
Rat	Spinal cord cDNA	<Gln-His-Gly-Thr-Ala-Pro-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Ile-OH
Porcine A	Spinal cord	H-Gly-Pro-Thr-Ser-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Porcine B	Spinal cord	H-Gly-Pro-Pro-Ser-Glu-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Monkey	Spinal cord cDNA	H-Glu-Thr-Pro-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Human	Spinal cord cDNA	H-Glu-Thr-Pro-Asp-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
URP		
Mouse	Spinal cord cDNA	H-Ala-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Rat	Brain, spinal cord cDNA	H-Ala-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH
Human	Spinal cord cDNA	H-Ala-**Cys**-Phe-Trp-Lys-Tyr-**Cys**-Val-OH

Table 2.1. The amino acid sequence of UII and URP from different species indicating the high level of homology within the C-terminus, while the N-terminus is variable. The tissue from which the sequence was identified is indicated under the 'origin'. FW and SW represent 'fresh water' and 'salt water' respectively. From Chatenet et al. 2004

Following identification of the AA sequence of UII in fish species, similarities were apparent with another recently identified peptide, somatostatin (SS) (Bern et al. 1985). Somatostatin was first discovered in sheep hypothalamus in 1973 in two forms – SS14 and SS28, expressed throughout the CNS and peripheral tissue of many vertebrates and is suggested to suppress growth hormone release (Brazeau et al. 1973). Distribution of the SS receptors are spread in an overlapping pattern throughout the brain, CNS and peripheral tissues, enabling release of somatostatin to be tissue dependent (Lin and Peter 2001). Urotensin II was initially believed to be an isoform of the peptide SS14 due to the similarities in their peptide sequence, 27% receptor homology, and their similar distributive expression through the CNS and peripheral tissue of fish species (Bern et al. 1985; Lin and Peter 2001). Of note, SS14 contains the tri-peptide motif -Phe-Trp-Lys-, reported to be the biologically important

component of SS14 for receptor activation (Conlon et al. 1997), which overlaps the biologically important tri-peptide motif –Trp-Lys-Tyr- of UII (Flohr et al. 2002; Kinney et al. 2002; Qi et al. 2005) (Figure 2.1). Because of these similarities, and the fact that UII had no known receptor, UII was dubbed a 'somatostatin-like peptide' and believed UII may act through the SS receptors (Conlon et al. 1996). Early genetic studies suggested UII and somatostatin, although similar in their mature form, showed little similarity when comparing their precursors, and therefore most likely did not derive from a common ancestral precursor (Ohsako et al. 1986; Conlon et al. 1997; Coulouarn et al. 1998; Tostivint et al. 2006). With the recent discovery of a second urotensin II-like peptide dubbed urotensin II-related peptide (URP), described in more detail below, interest into the ancestral genetic relationship between UII/URP and somatostatin was reignited. Tostivint et al. (2006) suggests that UII and somatostatin belong to the same genetic superfamily, proposing the theory that over evolution two ancestral genes (SS and UII) were physically linked and underwent tandem duplication into UII and SS. They then were subjected to segmental duplication, splitting the two genes again, with UII closely linked to the gene encoding SS2/cortistatin (CST), and URP located in the same chromosomal region as the SS1 gene (Tostivint et al. 2006) (Figure 2.2).

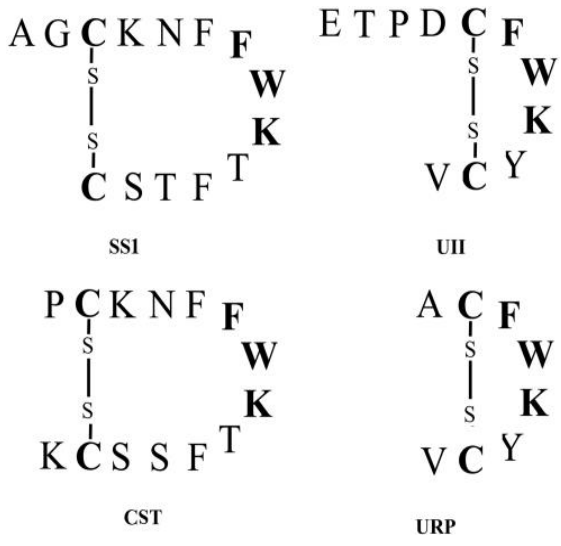


Figure 2.1 Comparing UII, URP, Somatostatin (SS) and cortistatin (CST) mature peptide sequences indicating the shared tri-amino acid motif in bold. From Tostivint et al. 2006.

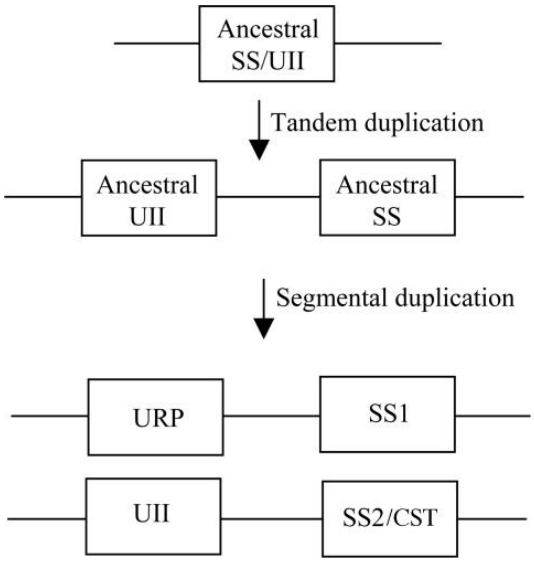


Figure 2.2 Diagram of the proposed evolutionary history of UII, URP and SS suggesting the genes resulted from an initial tandem duplication followed by segmental duplication. From Tostivint et al. 2006.

Structure-activity studies have indicated that the tri-AA motif (Trp-Lys-Tyr) sequence in the ring structure of UII, URP, SS and CST mature peptides is of high functional importance in receptor binding and activation (Flohr et al. 2002; Brkovic et al. 2003); and the UII receptor, G-protein coupled receptor-14 (GPR14) or 'UT' for the purpose of the current thesis, also shares high sequence similarity with somatostatin receptors (Marchese et al. 1995). Furthermore, the UT receptor is located in the same chromosomal region as the SST3 receptor suggesting that the receptors, like their correlate peptides, arose by tandem duplication (Tostivint et al. 2006). It has therefore been proposed that the two peptides may be capable of binding and activating the same receptor (Tostivint et al. 2006). Examining this hypothesis, somatostatin and cortistatin have been shown to cause minor stimulation of the UII receptor (UT) (Liu et al. 1999; Rossowski et al. 2002) indicating partial agonistic activity. Recently, with the development of better UT antagonists it was revealed that both UII and URP are able to activate the somatostatin receptors SST2 and SST5 in transfected CHO-K1 cells (Malagon et al. 2008); and Rossowski et al. (2002) reported that SST antagonists inhibited human UII-induced calcium transients in rat thoracic aortic rings (Rossowski et al. 2002). In sum, the high level of homology between the two peptides and their correlate receptors appears at this stage to enable minor cross activation of one another. Whether this cross activation provides a specific function, such as providing a feedback mechanism, synergism or an alternate route if one system was to fail; or whether it is simply co-incidental and the evolutionary branching from the original gene has not diversified sufficiently remains to be defined.

2.2 Discovery and distribution of the UII receptor (UT)

The UII receptor was not identified until 1995 when a G-protein coupled receptor was identified and cloned from rat brain extracts (Marchese et al. 1995). This G-protein coupled receptor was labelled GPR14 and at the time of discovery showed no specific binding to any common ligands, thus was dubbed an 'orphan' receptor (Marchese et al. 1995). It was not until 4 years later that a study identified the orphan GPR14 receptor as the endogenous receptor for UII in rats and humans (Ames et al. 1999) and several studies have now established that UII specifically binds and activates GPR14 in several species (Ames et al. 1999; Liu et al. 1999; Mori et al. 1999; Nothacker et al.

1999). The development of a GPR14 knockout model ($UT^{-/-}$) in mice confirmed it as the as endogenous receptor for UII, as administration of human UII to arteries from $UT^{-/-}$ mice *in vitro* showed a significant loss of potency compared with wild type mice (Behm et al. 2003).

The distribution of GPR14, otherwise known as UT, displays greatest within the CNS but is also expressed throughout the peripheral tissue including the heart, (localised in cardiomyocytes, endothelium and vascular smooth muscle cells (Gong et al. 2004)), kidney, liver, pancreas, thyroid, adrenal gland, vascular endothelial cells, and pituitary (Matsushita et al. 2001; Sugo et al. 2003; Richards and Charles 2004; Silvestre et al. 2004; Charles et al. 2005; Albertin et al. 2006; Jégou et al. 2006). UII and UT mRNA are expressed in comparatively high amounts within the heart (Liu et al. 1999; Douglas et al. 2002), peripheral vasculature (Maguire et al. 2000) and kidneys (Shenouda et al. 2002), with UT mRNA expression greatest in skeletal muscle (Douglas et al. 2004). This suggests UII is a potential regulatory peptide controlling cardiac homeostasis, peripheral vascular activity, kidney function, and thus blood pressure. Because UII is also present in the circulation at low levels, the distribution and density of the UT receptor in peripheral tissues may provide a regulatory mechanism in itself (Itoh et al. 1988). UT displays greatest expression within the brain and CNS, specifically within the endothelial cells (Spinazzi et al. 2006) and axonal terminals while UT mRNA expression is greatest in skeletal muscle (Douglas et al. 2004). This suggests UT may act as both a presynaptic receptor with UII or URP being the ligand (Clark et al. 2001), as well as modulating vascular tone and having other physiological effects. The direct influence of UII in the brain has been observed in both fish and mammals where intracerebroventricular (ICV) administration of UII significantly altered blood pressure, heart rate, and ventilation in the trout, indicating UII may have a specific role within the brain (Watson et al. 2003; Lancien et al. 2004; Watson and May 2004). Interestingly, an early study suggests UII function in the earliest vertebrates was as a neurotransmitter or modulator in the CNS rather than a hormone within the caudal neurosecretory system, as early fish species lacked a caudal neurosecretory system and the structure of UII is better conserved in phylogenetically ancient fish when compared with teleosts (Waugh et al. 1995). This would suggest that over time with the development of the caudal neurosecretory system UII (and potentially URP) became capable of exerting effects throughout the

animal dependent upon its cognate receptor distribution and explains why UII and URP exhibit strong expression within the CNS and are able to exert effects in the CNS as well as the peripheral tissue. Several studies have indicated stimulation of UII receptors within the brain and CNS produce many physiological and behavioural effects (Le Mével et al. 1996; de Lecea and Bourgin 2008; do Rego et al. 2008; Ono et al. 2008; Watson et al. 2008) indicating the neurotransmitter function of UII to be preserved in higher order species. Interestingly, UII administered via ICV has been repeatedly shown to produce different effects compared with IV application. This effect is maintained from fish to mammals. Le Mevel (1996) and colleagues reported ICV administration of UII to conscious trout caused an elevation in blood pressure with a slight increase in HR while intravenously administered UII increased blood pressure but reduced HR (Le Mével et al. 1996). In sheep ICV administration produced tachycardia, elevated cardiac output, reduced peripheral resistance and stimulated greater adrenocorticotropic hormone (ACTH) release while IV administration only caused tachycardia with no change in cardiac output or ACTH levels (Watson et al. 2003; Hood et al. 2005). Furthermore, ICV injection of UII increased arterial pressure and heart rate in conscious rats while IV injection elicited a depressor response (Lin et al. 2003). The historical evidence that UII was primarily a neurotransmitter in ancient fish species (Waugh et al. 1995) combined with the fact that UII and UT are expressed at their greatest amounts within the CNS, illustrates that the neurotransmitter function of UII/UT is conserved and elicits potent activity in the brain and CNS of mammals as well as producing haemodynamic effects directly on the vasculature. These studies indicate the potential for UII to produce disparate effects dependent upon the location of the receptor, providing a level of receptor control in regulating cardiovascular haemodynamics. UT is localised to the sarcolemma of rat skeletal muscle cells, where it is suggested that UII stimulates hindquarter vasodilation mediated by co-localised nitric oxide synthase (NOS) in the skeletal muscle (Maguire et al. 2008).

Inter-species distribution of UT and UII are reported to be relatively consistent, with the notable exception that rat vascular endothelial expression of UT-immunoreactivity was significantly less than compared with the same cells in humans (Liu et al. 1999; Maguire et al. 2000; Maguire et al. 2008). UT sequential homology is 92% between rat and mouse, 95% between human and monkey and 74% between rodent and

monkey (Proulx et al. 2008). Differences in receptor homology between species may influence ligand binding and may help explain the different haemodynamic responses when different species-forms of UII are administered (Douglas et al. 2000; Prosser et al. 2006). Structure-activity studies report little difference in UT receptor binding and activation between UII isoforms, revealing the WKY motif within the conserved 6 amino acid ring as vital for receptor binding and activity with alterations in the N- and C-terminus having little effect (Coy et al. 2002; Flohr et al. 2002; Brkovic et al. 2003; Labarrère et al. 2003; Guerrini et al. 2005). Extracellular loops 2 and 3 have been recognised as important in binding UII and URP, with loop 1 having no influence. As UII is known to produce a broad range of effects, not surprisingly binding of UII to UT activates intracellular pathways capable of eliciting vasoconstriction, vasodilation, cell proliferation and growth. UII is reported to act through the $G_{q/11}$/PLC/IP_3/PKC/RhoA/Rho pathway causing an influx of intracellular Ca^{+2} eliciting constriction (Russell and Molenaar 2004; Yoshimoto et al. 2004; Brailoiu et al. 2008), whilst also capable of causing vasodilation through stimulating eNOS (Katano et al. 2000; Gray et al. 2001; Prosser et al. 2006), as well as promoting VSMC proliferation mediated by extracellular signal-regulated kinase (ERK1/2) (Watanabe et al. 2001a; Watanabe et al. 2001b; Tamura et al. 2003). Furthermore, UII is known to elevate cyclooxygenase activity, initiating prostaglandin production, capable of exerting dilative, constrictive and hypertrophic effects dependent upon the isoforms produced (Gardiner et al. 2004; Horie et al. 2005; Gardiner et al. 2006; Deeb et al. 2008).

Recent reports suggest the possibility of a second UII receptor as Gendron et al. (2005) and Prosser et al. (2008) reported unusual responses when administering UT antagonists to rat hearts suggesting the presence of further receptor activity (Gendron et al. 2005; Prosser et al. 2008). Behm et al. 2004 also reported contrasting effects in response to the UT antagonist SB-710411 where it significantly attenuated UII-induced inositol phosphate formation at the rat UT receptor, but functioned as an agonist at the monkey UT receptor (Behm et al. 2004). Furthermore, the UT antagonists urantide and palosuran functioned as a partial agonists in the rat cardiovasculature, while antagonising UII-induced activity (Song et al. 2006; Prosser et al. 2008). These studies illustrate the complex nature of UII/UT binding and activity, and suggest development of a UT antagonist may have to be species-specific.

2.3 Source and stimulus of UII

Trans-organ sampling indicated UII is secreted from the heart, liver and kidney in sheep (Heller et al. 2002; Charles et al. 2005). Expression of UII mRNA has been identified in human coronary artery and endothelial cells (Totsune et al. 2004), human VSMCs, endocardial endothelial cells (Douglas et al. 2002), and cardiac fibroblasts (Tzanidis et al. 2001). It is expressed at high levels in inflammatory cells including lymphocytes, monocytes, macrophages and foam cells (Bousette et al. 2004), as well as the CNS in the brain stem and spinal motorneurons (Coulouarn et al. 1998; Dubessy et al. 2008). Stimulation of UII production and secretion is yet to be concretely defined, however UII levels have been repeatedly shown to be elevated in many cardiac diseases and with increased pressure load (Cheung et al. 2004). The conversion of prepro-UII to its bioactive, mature form has had little research focus and it is yet to be fully established which urotensin-II-converting enzyme(s) (UCE) contribute to proteolytic cleavage, including whether cleavage is localised within the cell or at the cell surface. Russell et al. (2004) investigated UCE activity in cultured human epicardial mesothelial cells and in blood and reported an intracellular enzyme capable of converting pro-hUII to hUII with minimal conversion at the cell surface (Russell et al. 2004). This intracellular UCE exhibited furin-like characteristics while it was suggested that a serine protease such as trypsin also contributed in converting pro-hUII to hUII in the blood assuming the prohormone was secreted into the circulation (Russell et al. 2004). Assuming the elevations in UII concentration reported are in fact augmenting cardiovascular disease, it would of great benefit to define the proteolytic enzyme(s) involved in generating the mature UII peptide. This would enable the development of antagonists to inhibit UCE activity as a means of attenuating any potential pathological effects stimulated by UII, as current UT receptor blockers are reported to have variable efficacy in blocking UII activity, some of which behaving as partial agonists (Song et al. 2006; Behm et al. 2008).

2.4 Effect of UII on hemodynamics

Initial studies identified UII as a potent spasmogen, constricting isolated trout rectum and intestine rings *in vitro* (Bern and Lederis 1969). Further studies confirmed UII as a vasoactive agent altering arterial tone, constricting isolated arteries and stimulating hypertension and altering heart rate (HR) in live trout (Conlon et al. 1996). In elasmobranchs UII altered cardiovascular function, but had little influence on isolated rings of dogfish intestine or rectum, indicating a more defined role for UII in elasmobranchs as a regulator of cardiovascular function (Conlon et al. 1992; Hazon et al. 1993; Conlon et al. 1996). With reports indicating fish UII could alter isolated rat vessel tone (Gibson et al. 1986; Gibson 1987; Itoh et al. 1987; Gibson et al. 1988) it was hypothesised that UII and its cognate receptor UT were present in rats, and potentially all mammalian species including humans. UII is now synonymous with being named the most potent vasoconstrictor identified to date with a potency many times that of endothlelin-1, serotonin or norepinephrine (Russell et al. 2001; Watanabe et al. 2001a; Zhu et al. 2004). UII-induced vasoconstriction has now been demonstrated in isolated vessels of many different species including the rat, mouse, dog, pig, cynomolgus monkey and human (Bottrill et al. 2000; Douglas et al. 2000; Maguire et al. 2000). The vasoconstrictive effect of UII is attributed to an influx of intracellular calcium mediated by binding to the UT receptor, activating PLC and IP_3 (Opgaard et al. 2000; Brailoiu et al. 2008). However, the vascular response to UII is inconsistent, as Gibson et al. (1987), Katano et al. (2000) and Li et al. (2004) all reported UII induced vasodilation in rat coronary arteries (Gibson 1987; Katano et al. 2000; Li et al. 2004). UII-induced vasodilation can be attenuated by reducing the presence of endothelial nitric oxide (eNOS), and inhibiting cyclooxygenase (COX) activity (Katano et al. 2000; MacLean et al. 2000; Gray et al. 2001; Prosser et al. 2006), indicating UII-induced vasodilation is the result of elevating nitric oxide (NO) and activating prostaglandins, specifically PGE_2, prostacyclin as well as endothelium-derived hyperpolarizing factor (EDHF) (MacLean et al. 2000; Ishihata et al. 2005a; b; Lacza and Busija 2006). NO is a known vasodilative agent, well established in mammals (Gamboa et al. 2007) and reported to provide regulatory control of coronary flow in teleosts, including salmon, modulating appropriate blood supply to the ventricle under basal and stressed conditions (Agnisola 2005). Furthermore, it has

been displayed that UII-induced vasoconstriction is elevated when in the presence of L-NAME and indomethacin (MacLean et al. 2000; Gray et al. 2001; Deuchar et al. 2006). The dilative effects of UII have also been reported in *in vivo* studies in rats and cynomolgus monkeys where UII elicited hypotension and a depressor response, constricting some arteries while severely reducing peripheral resistance ((Gibson et al. 1986; Gibson 1987; Ames et al. 1999; Gardiner et al. 2004; Zhu et al. 2004). This hypotensive response in cynomolgus monkeys was accompanied by a severe reduction in cardiac output resulting in death (Ames et al. 1999; Zhu et al. 2004), suggesting UII also directly influences cardiac contractility and other physiological effects (Table 2.2).

Indeed like the vascular response, the effect of UII on contractility is varied with studies suggesting UII acts as a positive (Russell et al. 2001; Watson et al. 2003; Gong et al. 2004; Gardiner et al. 2006) or negative inotrope (Hassan et al. 2003; Zhu et al. 2004; Fontes-Sousa et al. 2008). The method of application of UII to the subject animal appears to heavily influence the contractile response to UII, as IV injection produces opposing effects compared with ICV infusion. This is best illustrated by two studies in sheep where UII administered IV elevated HR and produced negative inotropic activity, while ICV application elevated HR and caused positive inotropic effects (Watson et al. 2003; Hood et al. 2005). The reason why UII elicits opposing responses in the same subject animal is suggested to be due to the different pathways activated. ICV infusion of UII reportedly stimulates cardiac sympathetic nerve activity, epinephrine release, and is dependent upon beta-adrenoreceptor stimulation, whereas UII administered IV does not stimulate beta-adrenoreceptors and is dependent upon activating its own intracellular pathways (Watson et al. 2003; Hood et al. 2005) (Table 2.2). The intracellular pathway mediating the UII-induced positive inotropic response is reported to be PKC- and myosin light chain 2-dependent (Russell and Molenaar 2004).

UII	Systemic Administration	ICV Administration
Heart	Constricts / dilates the coronary arteries Negative Inotrope ↑ or ↓ Heart Rate	Positive Inotrope ↑ Heart Rate
Peripheral	Vasoconstrictor - ↑ MAP Vasodilator - ↓ MAP	↑ Blood pressure
CNS	Behavioural effects altering ventilation, REM	↑ Sympathetic activity ↑ epinephrine release
Kidney	Epithelial cell proliferation Decreased renal blood flow Altered Na^+ excretion	

Table 2.2. Physiological and behavioural responses to UII in healthy subjects revealing a wide range of effects, suggested to be dependent upon the method of drug administration.

2.5 The role of UII in the kidney and its influence in renal disease

The kidneys play a vital role in the regulation of cardiovascular homeostasis as they control cardiac preload through regulating plasma volume, and are a major influence on peripheral resistance through release and clearance of vasoactive agents. The physiological effects of UII were initially witnessed in their regulation of blood pressure in the fish when the osmolarity of the media was altered indicating UII may regulate salt and water balance (Bern et al. 1985; Bond et al. 2002). UII, UII mRNA and UT have since been identified in the kidney of mammals, with expression there higher than other tissues (Nothacker et al. 1999; Shenouda et al. 2002). UII is capable of stimulating intracellular extracellular signal-regulated kinase 1/2 (ERK1/2) in pig renal tubular epithelial cells (Matsushita et al. 2003) indicating a potential for cell proliferation. The renal role of UII is yet to be fully determined, however UII has been suggested to alter Na^+ excretion and decrease renal blood flow (Douglas et al. 2004). However, it has been established that UII levels are elevated in the plasma and urine

of patients with renal dysfunction and disease (Matsushita et al. 2001; Totsune et al. 2001), and the kidney has been identified as a source of UII in the circulation (Charles et al. 2005). Interestingly, the majority of studies observing circulating UII levels in renal disease indicate UII provides cardiorenal protection and is inversely correlated with cardiovascular events (Mallamaci et al. 2006; Ravani et al. 2008; Zoccali et al. 2008), when compared with pure cardiovascular diseases which heavily portray UII as pathogenic (Bousette et al. 2006a; Bousette et al. 2006b; Watanabe et al. 2006).

2.6 UII is species-, tissue- and cell type-dependent

2.6.1 Dependence on species-specific form of UII

The general assumption has been that the N-terminus of UII has little or no influence on receptor activation, and thus little effect on the intracellular signalling pathways activated. Reasons underlying this are: a) all species of UII retain the highly conserved ring structure (Douglas and Ohlstein 2000; Onan et al. 2004), b) several reports showed fish UII was capable of producing a hypotensive response in rats and rabbits (Gibson et al. 1986; Itoh et al. 1988; Conlon et al. 1996) whilst fish UII administered at low doses caused vasodilation in rat vascular smooth muscle and vasoconstriction at high doses (Gibson 1987). Furthermore, non-native UII was capable of elevating intracellular calcium concentrations in cultured cells containing transfected human UT receptors (Ames et al. 1999; Liu et al. 1999; Mori et al. 1999; Nothacker et al. 1999). Structure-activity studies have reported that binding of UII to its cognate receptor UT is highly dependent upon the conserved Trp-Lys-Tyr amino acid motif within the conserved ring, with modification of the N-terminus having little or no influence on binding affinity or potency (Flohr et al. 2002; Kinney et al. 2002; Greco et al. 2003; Labarrère et al. 2003; Qi et al. 2005). However, initial studies reported UII may have species-specific actions; for example frog UII caused significant vasoconstriction in the frog, a response that could not be repeated when rat UII was given to rats (Yano et al. 1994; Conlon et al. 1996). Thus, analysis of publications to date suggest that the species-dependent N-terminus of UII may substantially influence receptor binding, activation and the resultant physiological response(s) (Table 2.3). This hypothesis was supported by a study observing the effect of UII in different species and different tissue types, indicating human UII could elicit

vasoconstriction or have no effect in the same vessel of different species (Douglas et al. 2000). Figure 2.3 displays the percentage of receptor sequential homology between species indicating the homology between fish and mammals can vary up to 44% (Lu et al. 2008). Whether the difference in response to different species-forms of UII administered is due to the sequential differences in the UII ligand or the slight differences in receptor homology between species is yet to be determined.

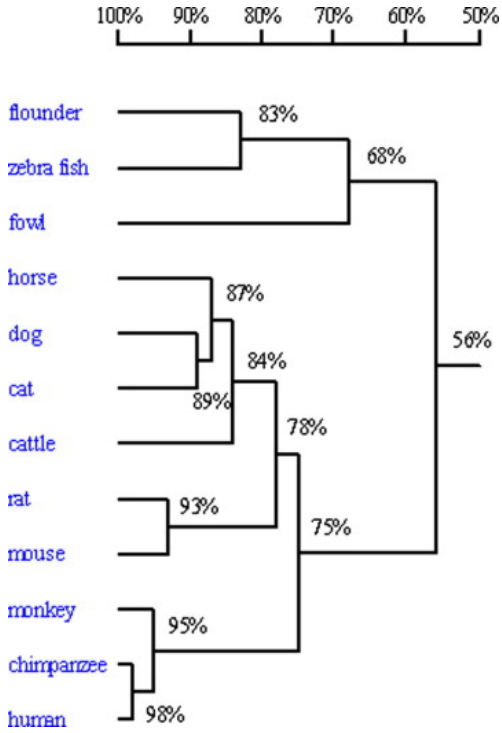


Figure 2.3 Evolutionary tree comparing the % UT receptor sequence homology between species. From Lu et al. 2008.

Table 2.3 displays the different responses to UII in many species. Some vessels such as the thoracic aorta respond to UII solely with vasoconstriction, independent of the species of UII administered; however, other vessels such as the coronary, mesenteric, pulmonary and abdominal resistance arteries exhibit variable responses producing either vasodilation, vasoconstriction or no effect dependent upon the species of UII administered (Table 2.3). Since research began recording the cardiovascular effects of UII, studies have generally administered the non-native form of UII to the subject animal, and in doing so have potentially reported false physiological responses that would not occur under the natural setting. Only recently has it become increasingly

apparent that UII is highly species-specific, a factor potentially responsible for the large variation in results currently reported (Douglas et al. 2000; Prosser et al. 2006).

2.6.2 Dependence on Cell and Tissue Type

The physiological effects of UII also appear to be dependent upon the cell type expressing UT. UT is expressed on both VSMCs and endothelial cells affording the ligand direct binding and activation of either cell type (Maguire et al. 2008). Activation of receptors located on VSMCs display consistent vasoconstriction, while activation of receptors located on the endothelium stimulated vasodilation (Gendron et al. 2005). The role of the endothelium in mediating the response to UII remains to be concretely defined, however currently it appears UII activity becomes altered upon removal of the endothelium. Some studies reported UII activity to be independent of endothelial cells with human UII and fish UII, constricting rat thoracic aorta independently of endothelium removal (Itoh et al. 1987; Bottrill et al. 2000), a finding repeated in rabbit thoracic aorta (Opgaard et al. 2000). Studies indicating UII-induced vasoconstriction is endothelium-dependent, found removal of the endothelium augmented UII-induced vasoconstriction in rat thoracic aorta (Wang et al. 2007), and rat & human main pulmonary arteries (MacLean et al. 2000).

Not surprisingly, UII-induced vasodilation is shown to be strongly endothelium-dependent, for example hUII-induced vasorelaxation of rat coronary arteries could be converted to vasoconstriction when the endothelium was removed (Bottrill et al. 2000). This UII-induced vasoconstriction or loss of UII-induced vasodilation in endothelial denuded/damaged vessels has also been displayed in human coronary (Russell et al. 2001), pulmonary and abdominal resistance arteries (Stirrat et al. 2001). Gendron et al's (2005) suggestion that UII causes differential effects dependent upon the cellular level (i.e. smooth muscle cells respond differently to UII compared to endothelial cells) is now appearing highly likely (Gendron et al. 2005).

Interestingly, the studies suggesting UII activity to be endothelium-independent were observed only in the thoracic aorta. The thoracic aorta appears to differ somewhat from other vessels in response to UII in that it shows no dilative response and is

seemingly independent of the species form of UII administered. This indicates UII may also be tissue-dependent, whereby producing different effects on different tissue beds. This suggests that the differential responses to UII observed in different vessels throughout the body may lie within the receptor as opposed to the ligand, and/or the intracellular signalling pathways activated. As previously indicated: a) UII may trigger 1 receptor with several alternate intracellular pathways whereby the dominant (alpha) pathway produces the observed effect and the alpha pathway may change in order to maintain homeostasis; or b) UII may activate greater than one receptor, (Gendron et al. 2005).

Further possible explanations for the inconsistencies reported between research groups include the concentration of calcium used within the buffer solution when observing the vessel or organ *ex vivo*. The concentration of calcium used is highly important when measuring the response to UII as UII-induced vasoconstriction is calcium-dependent (Brailoiu et al. 2008), and therefore it is vital that the concentration of calcium within the buffer is at a homeostatic level for that animal.

In sum, analysis of reports observing the physiological response to UII, indicates UII-induced activity is dependent upon the species form of UII administered, the cell type exposed, the tissue type and the method of administration (Table 2.3).

2.7 Urotensin II-related peptide (URP)

Urotensin II-related peptide (URP) was isolated from rat brain in 2003 by Sugo and his team when searching for products displaying immunoreactivity to anti-UII antibody (Sugo et al. 2003). Structure analysis determined the sequence of URP to be 8 amino acids in length, comprising Ala-Cys-Phe-Trp-Lys-Tyr-Cys-Val with Cys-Cys bound by disulphide bonds producing the cyclic ring common to all known species of UII. Human, rat and mouse prepro-URP were found to comprise 119, 118 and 113 amino acids respectively, displaying sequence homology of 54.2% for human and rat, 47.5% for human and mouse, and 73.6% for rat and mouse (Sugo et al. 2003). Like UII, the mature URP peptide is located at the C-terminus and the proposed site of proteolytic cleavage is at the Lys-Arg residues immediately preceding the mature

URP peptide (Mori and Fujino 2004). Little similarity is shown in the sequence homology between prepro-URP and prepro–UII, suggesting they are not derived from a common ancestor and indeed the two precursor peptides are located on separate chromosomes (UII on 1p36 and URP on 3q29) (Sugo et al. 2003; Mori and Fujino 2004). The genetic history of UII and URP was explored in more detail recently by Tostinivt et al. (2006). As mentioned above, Tostivint and colleagues suggest the URP gene was originally part of a UII/somatostatin/cortistatin genetic 'superfamily' which then underwent evolutionary pressures resulting in the URP gene to become located in the same chromosomal region as the SS1 gene, however separated from UII (Tostivint et al. 2006).

In rodents, prepro-URP gene expression was greatly elevated in the testis, spleen and thymus, but displayed equal expression in the spinal cord when compared with prepro-UII (Figure 2.4). The distribution of prepro-URP gene expression differs between species, for example, human prepro-URP is expressed in its highest amount within the ovary whilst it is non-existent in the rat ovary; in contrast, the rat spinal cord expresses more prepro-URP (Sugo et al. 2003). Of note, the greatest expression of prepro-URP in the human was located within the ovary and testis; while it was the testis and spleen in the rat (Figure 2.4) (Sugo et al. 2003). The reason for the high expression of URP within the reproductive organs of both human sexes and the testis but not ovary of rats is yet to be explored. It is not uncommon for the testis and/or ovaries to express high levels of circulating peptides with little known reproductive function, for example CNP is primarily known for its role in bone growth and natriuretic effects throughout the body (Potter et al. 2009), but its expression is greatest within the testis, and has since been shown to regulate the blood-testis barrier in rats (Xia et al. 2007).

Immunohistochemistry studies observing URP in the CNS revealed URP-immunoreactive axon terminals were located within specific regions of the mouse brain; these regions are known to control gonadotropic cells of the anterior pituitary (Egginger and Calas 2005). Furthermore, neurons expressing URP are located within the mouse hypothalamus and thoracic spinal cord, and found to contain both URP-mRNA and URP peptide indicating URP can be synthesized and released from these cells (Egginger and Calas 2005; Pelletier et al. 2005). This supports the theory that

URP is a potential neuroendocrine peptide in mammals, a theory also suggested for UII as the cardiovascular effects following ICV administration of UII have been shown to significantly alter cardiac haemodynamics (Lin et al. 2003; Watson et al. 2003; Hood et al. 2005). It is yet to be tested whether URP shows variable responses dependent upon IV or ICV administration as observed with UII. The wide peripheral distribution of URP indicates that it potentially has a role outside of the CNS, however without an assay currently capable of differentiating URP from UII it is difficult to define the role of each peptide in the CNS and peripheral tissue.

UT was confirmed as the endogenous receptor for URP, the same receptor believed to be endogenous for UII based on reports observing URP-induced physiological changes and binding-activity studies (Sugo et al. 2003; Chatenet et al. 2004; Sugo and Mori 2009). Administration of URP to Chinese hamster ovary (CHO) cells expressing human or rat UT caused an increase in intracellular calcium mobilization with high binding affinity for both receptors (Sugo et al. 2003). The binding affinity of URP for human UT-transfected cells has been shown by two reports to be slightly higher than human UII, indicating UT is likely to be the functional receptor for URP (Sugo et al. 2003; Chatenet et al. 2004).

In vivo, URP caused sustained hypotension when administered intravenously into the anaesthetised rat, the same effect observed with rat UII (Sugo et al. 2003), while i*n vitro* URP constricted rat aortic rings, exhibiting slightly reduced potency, but slightly higher binding affinity for UT when compared with human UII (Chatenet et al. 2004).

Taken together, these initial reports indicate URP binds and activates GPR14, stimulating the same physiological effects as UII with reduced potency (Chatenet et al. 2004). Furthermore, like UII, structure-activity studies indicate the Phe-Trp-Lys motif (particularly Tyr) within the cyclic domain of URP is pivotal for receptor binding and activation, whilst altering in the N- or C-terminus had little influence on receptor binding (Chatenet et al. 2004).

2.8 Summary of the UII/URP system

From rather humble beginnings as a simple neurosecretory peptide from the urophysis of fish, UII has now been identified as a potent endocrine agent present in all species tested from fish to mammals, including humans. UII is manufactured throughout the body including the CNS, heart, liver & kidneys and has a wide receptor distribution affording UII a wide range of effects throughout the body, from influencing ventilation and rapid eye movement (de Lecea and Bourgin 2008), to vascular constriction and dilation, to having a potential role in synaptic nerve activity within the CNS (Nothacker and Clark 2005) (Table 2.2). Urotensin II has become synonymous with being the most potent vasoconstrictor identified to date. However with increasing numbers of studies observing the effects of UII both *in vivo* and *in vitro*, it is apparent UII can also stimulate sustained vasodilation as well as VSMC proliferation and augment the development of atherosclerosis. The true biological function of UII has become increasingly difficult to define due to its dependence on cell type, tissue bed, species, method of administration, and development of receptor antagonists now reported to display species-specificity. Thus, there is a clear need for basic species-specific studies, identifying the effects of UII and URP directly upon the heart and cardiac vasculature. Furthermore, whether UII and/or URP provide beneficial or detrimental effects in the context of cardiac injury and cardiovascular pathology must be addressed.

Salmon and rats were chosen as experimental subjects to provide a foundation for observing the comparative physiological effects in response to direct infusion of UII. This method provided a clear view of the endogenous effects of UII from teleost fish to mammals, observing whether the response to UII was conserved with its highly conserved structure over evolutionary time, as well as determining the species-specificity of UII, as an early study indicated that fish UII could elicit a response in rat vessels (Itoh et al. 1987). Coronary arteries were chosen as the experimental vessel as the effect of UII on this vessel could be accurately measured in both the rat and salmon, enabling direct comparative analysis.

The doses of rUII and URP administered were based upon preliminary dose-response studies (Figure 3.1A). These doses were based upon calculated dilutions of the

original peptide with subsequent RIA analysis revealing the viable concentration of UII reaching the heart to be approximately one third of that calculated (Chapter 3). Subsequent studies report the effects of administering 1 nM rUII and 10 nM URP based upon calculated dilutions alone, however this may be a gross overestimation based on the previous analysis. This must be taken into account when addressing the physiological versus pathophysiological doses administered in the following experiments.

Previous studies determining the concentrations of UII from plasma of healthy subjects and patients suffering cardiac disease have reported concentrations ranging from 0.6 pmol/L to 29 nmol/L, respectively (Totsune et al. 2001; Dschietzig et al. 2002; Heller et al. 2002; Richards et al. 2002; Gruson et al. 2005; Zoccali et al. 2006; Dai et al. 2007). Our own study found the circulating concentration of UII in healthy rats to be 3 pmol/L (Prosser et al 2006). The chosen dose of 1 nM rUII and 10 nM URP were based upon our own dose-response studies and are consistent with previous reports both *in vivo*, *ex vivo* and *in vitro* (Douglass et al. 2000; Itoh et al; 1987; Ishihata et al. 2005 and 2006; Gray et al. 2001; Johns et al. 2004; Tzanidis et al. 20003; Russell and Molenaar 2004). The EC_{50} of UII across monkey and dog coronary arteries and rat aorta is reported to be just below 1 nM (Douglass et al. 2000; Itoh et al. 1987). Experiments to date have elicited responses following administration of low picomolar doses to fish, while other studies require higher doses to observe any effect, commonly 1-100 nM in explanted organs or vessels (Douglass et al. 2000; Itoh et al; 1987; Ishihata et al. 2005 and 2006; Gray et al. 2001; Johns et al. 2004; Tzanidis et al. 20003; Russell and Molenaar 2004), however higher doses (up to 1 μM) have also been employed (Gray et al. 2001; Douglas et al. 2000). The use of 1 nM UII in the following studies could, therefore be considered pathophysiological, providing a good measure of UII-induced cardiac effects both in healthy and ischemic hearts. As UII is chronically elevated in diseased patients as opposed to acute release, the following studies administered UII and URP over 30 min to better simulate the natural setting of elevated UII during cardiac disease, and provide a more accurate analysis into the role of elevated UII/URP in the healthy and ischemic heart. The concentration of URP administered (10 nM) is consistent with a previous study (Sugo et al. 2003), with preliminary dose-response studies indicating URP to have reduced vasoactive potency when compared with rUII (Figures 3.1 and 3.2).

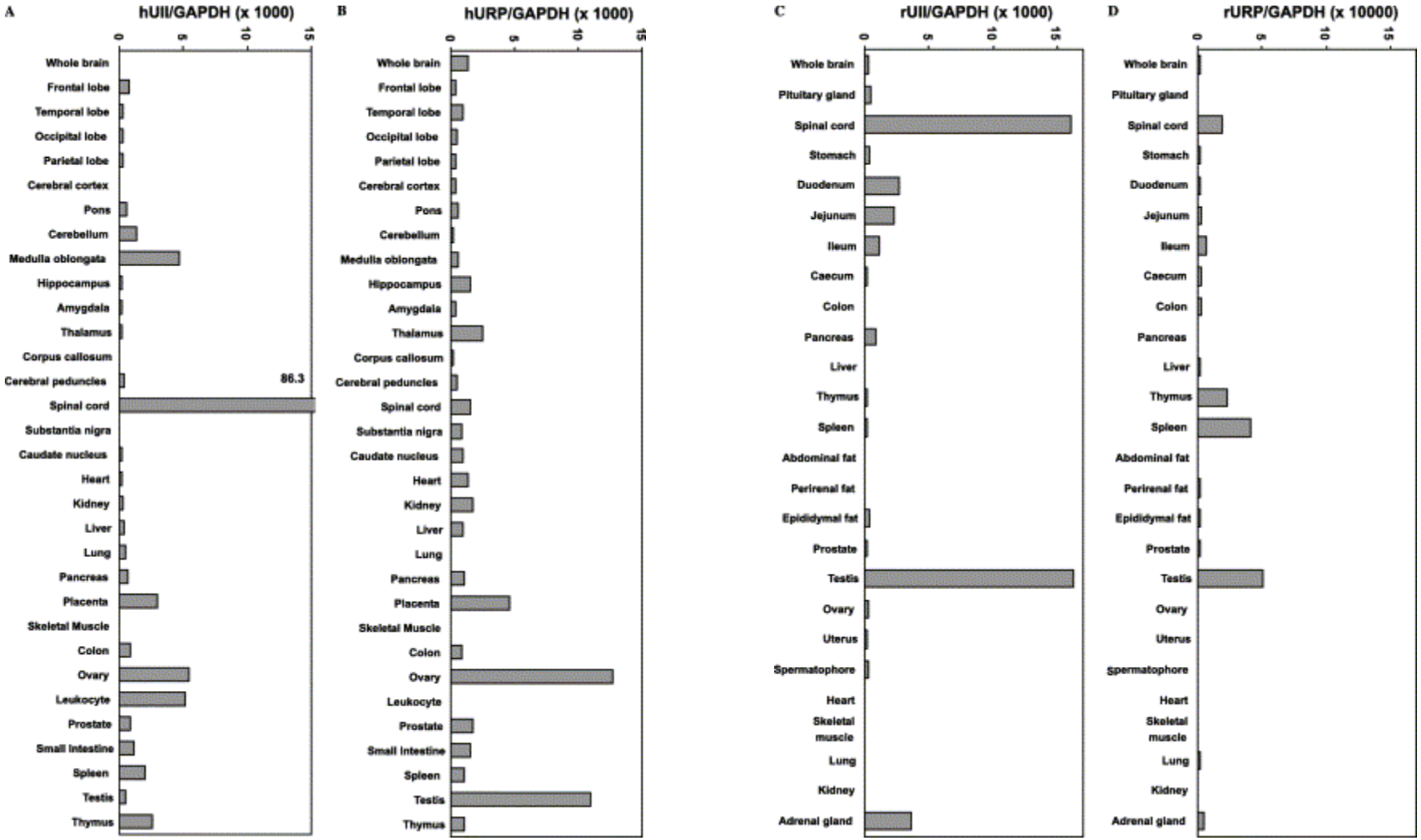


Figure 2.4 Distributive expression of genes encoding the preproproteins: human UII (A) and URP (B), rat UII (C) and URP (D) displaying interspecial differences, and comparing the gene expression encoding prepro-UII and prepro-URP throughout the rat and human. Gene expression was determined using TaqMan PCR with the value obtained for each tissue normalised to that of GAPDH (the value for the human spinal cord in (A) is out of range). From Sugo et al. 2003.

Table 2.3 Vascular response to UII throuout many species

Vessel	Animal	UII	Response	Reference(s)
Thoracic Aorta	Rat	HUII	Constricted	(Ames et al. 1999; Bottrill et al. 2000; Douglas et al. 2000; Maguire et al. 2000; Hillier et al. 2001; Rossowski et al. 2002)
		URP	Constricted	(Brkovic et al. 2003; Sugo et al. 2003; Chatenet et al. 2004)
		Goby	Constricted	(Itoh et al. 1987)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Rabbit	HUII	Constricted	(Opgaard et al. 2000)
	Dog	HUII	N/C	(Douglas et al. 2000)
	Pig	HUII	Constricted	(Douglas et al. 2000)
	Marmoset	HUII	Constricted	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Constricted	(Douglas et al. 2000)
Coronary arteries	Rat	RUII	Dilation	(Li et al. 2004)
		HUII	N/C	(Douglas et al. 2000)
		HUII	Constricted	(Bottrill et al. 2000; Gray et al. 2001)
		HUII	Dilation*	(Bottrill et al. 2000)
		HUII	Dilation	(Katano et al. 2000)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	Constricted	(Douglas et al. 2000)
	Pig	HUII	N/C	(Douglas et al. 2000)
	Marmoset	HUII	N/C	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Constricted	(Ames et al. 1999; Douglas et al. 2000; Zhu et al. 2004)
	Human	HUII	Constricted	(Maguire et al. 2000; Russell et al. 2001; Maguire et al. 2004)
Renal artery	Rat	HUII	N/C	(Douglas et al. 2000)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	Variable constriction	(Douglas et al. 2000)
	Pig	HUII	Variable constriction	(Douglas et al. 2000)
	Marmoset	HUII	N/C	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Constricted	(Douglas et al. 2000)
Femoral artery	Rat	HUII	N/C	(Douglas et al. 2000)
		Goby	N/C	(Itoh et al. 1987)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	Variable constriction	(Douglas et al. 2000)
	Pig	HUII	Constricted	(Douglas et al. 2000)
	Marmoset	HUII	Variable constriction	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Constricted	(Douglas et al. 2000)
Mesenteric artery	Rat	HUII	N/C	(Bottrill et al. 2000; Douglas et al. 2000)
		RUII	Dilation	(Gardiner et al. 2001)

Table 2.3 Vascular response to UII throuout many species				
Vessel	**Animal**	**UII**	**Response**	**Reference(s)**
		Goby	variable constriction	(Itoh et al. 1987)
		HUII	Dilation	(Gardiner et al. 2001)
		HUII	Dilation**	(Bottrill et al. 2000)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	Variable constriction	(Douglas et al. 2000)
	Pig	HUII	Constricted	(Douglas et al. 2000)
	Marmoset	HUII	Constricted	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Constricte	(Douglas et al. 2000)
Abdominal aorta	Rat	HUII	Constricted	(Douglas et al. 2000)
		Goby	Variable constriction	(Itoh et al. 1987)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	N/C	(Douglas et al. 2000)
	Pig	HUII	Constricted	(Douglas et al. 2000)
	Marmoset	HUII	Constricted	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Constricted	(Douglas et al. 2000)
Pulmonary artery	Rat	HUII	N/C	(Douglas et al. 2000)
		HUII	Constricted	(MacLean et al. 2000)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	N/C	(Douglas et al. 2000)
	Pig	HUII	Constricted	(Douglas et al. 2000)
	Marmoset	HUII	N/C	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Constricted	(Douglas et al. 2000; Zhu et al. 2004)
	Human	HUII	Variable constriction	(MacLean et al. 2000)
		HUII	Dilation	(Stirrat et al. 2001)
Mammary artery	Rat	HUII	N/C	(Douglas et al. 2000)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	Variable constriction	(Douglas et al. 2000)
	Pig	HUII	Variable constriction	(Douglas et al. 2000)
	Marmoset	HUII	N/C	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Constricted	(Douglas et al. 2000)
	Human	HUII	N/C	(Hillier et al. 2001)
Carotid artery	Rat	HUII	Constricted	(Douglas et al. 2000)
		Goby	Constricted	(Itoh et al. 1987)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	Variable constriction	(Douglas et al. 2000)
	Pig	HUII	Variable constriction	(Douglas et al. 2000)
	Marmoset	HUII	Constricted	(Douglas et al. 2000)
	Cynomolgu	HUII	Constricted	(Douglas et al. 2000)

Table 2.3 Vascular response to UII throuout many species				
Vessel	**Animal**	**UII**	**Response**	**Reference(s)**
	s Monkey			
		HUII	Constricted (in vivo)	(Zhu et al. 2004)
Pulmonary Vein	Rat	HUII	N/C	(Douglas et al. 2000)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	Constricted	(Douglas et al. 2000)
	Pig	HUII	N/C	(Douglas et al. 2000)
	Marmoset	HUII	N/C	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Constricted	(Douglas et al. 2000)
Jugular vein	Rat	HUII	Variable constriction	(Douglas et al. 2000)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	Variable constriction	(Douglas et al. 2000)
	Pig	HUII	N/C	(Douglas et al. 2000)
	Marmoset	HUII	N/C	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Variable constriction	(Douglas et al. 2000)
Saphenous vein	Rat	HUII	N/C	(Douglas et al. 2000)
	Mouse	HUII	N/C	(Douglas et al. 2000)
	Dog	HUII	Variable constriction	(Douglas et al. 2000)
	Pig	HUII	Variable constriction	(Douglas et al. 2000)
	Marmoset	HUII	N/C	(Douglas et al. 2000)
	Cynomolgus Monkey	HUII	Variable constriction	(Douglas et al. 2000)
	Human	HUII	N/C	(Hillier et al. 2001)
Abdominal resistence artery	Human	HUII	Dilation	(Stirrat et al. 2001)
Small subcutaneous resistence artery	Human	HUII	N/C	(Hillier et al. 2001)
Forearm blood flow	Human	HUII	Constricted	(Böhm and Pernow 2002)

Table 2.3 The vascular response to UII in vessels throughout the arterial and venous system. The response to UII is shown to be highly species-specific in some vessels, while showing little species-dependence in others. *Response only when preconstricted with 5HT. **Response only when preconstricted with methoxamine. N/C represents no change.

3. Cardiovascular effects of native vs non-native urotensin II and urotensin II-related peptide on rat and salmon hearts

(*The following chapter is a copy of the published article Prosser et al. Peptides, 2006 Dec;27(12):3261-8)*

Abstract

Urotensin II (UII) was first discovered in the urophyses of goby fish and later identified in mammals, while urotensin II-related peptide (URP) was recently isolated from rat brain. We studied the effects of UII on isolated heart preparations of Chinook salmon and Sprague Dawley rats. Native rat UII caused potent and sustained, dose-dependent dilation of the coronary arteries in the rat, whereas non-native UII (human and trout UII) showed attenuated vasodilation. Rat URP dilated rat coronary arteries, with 10-fold less potency compared with rUII. In salmon, native trout UII caused sustained dilation of the coronary arteries, while human UII and URP caused significant constriction. L-NAME and indomethacin significantly attenuated the URP and rat UII-induced vasodilation in the rat heart. We conclude that UII is a coronary vasodilator, an action that is species form specific. We also provide the first evidence for cardiac actions of URP, possibly via mechanisms common with UII

3.1 Introduction

Urotensin II (UII) was first identified as a neurosecretory hormone thought to be present only in fish (Pearson et al. 1980). UII is now known to be present in most animal species and has been isolated from the brain of frogs, mice, rats, pigs and humans (Coulouarn et al. 1998; Coulouarn et al. 1999; Mori et al. 1999). Measurement of plasma concentrations of UII has been purported to hold clinical significance, being elevated in patients with congestive heart disease (Richards et al. 2002; Russell et al. 2003) cardiac dysfunction (Heringlake et al. 2004), renal dysfunction (Totsune et al. 2001), cirrhosis (Heller et al. 2002), and diabetes (Langham et al. 2004; Totsune et al. 2004). Recently, urotensin II-related peptide

(URP) was isolated and cloned from rat brains by Sugo et al. (Sugo et al. 2003). URP has been shown to bind to the same receptor as UII (Mori and Fujino 2004) and have approximately the same binding affinity, but less potency than UII in contracting rat aortic strips in vitro (Chatenet et al. 2004). In vivo, a single study has suggested that URP (like UII) is hypotensive (Sugo et al. 2003), however no studies have yet investigated potential direct cardiac effects of URP. A prominent feature of UII is its structural and sequence preservation across species (Chatenet et al. 2004) which appears to flow onto its biological actions as studies have shown that fish UII is capable of exerting in vitro contractile effects on the rat vasculature (Itoh et al. 1987). Because of the apparent structural conservation of the disulphide bonded bioactive 6 amino acid ring that binds to the UII receptor (Coy et al. 2002), it has been assumed that this plays the major role in defining the biological actions of UII/URP. However, it is becoming more apparent that UII has opposing actions in vessels isolated from different vascular beds (Douglas et al. 2000). Furthermore, the amino terminal sequence of UII, upstream from the 6 amino acid ring structure differs in length and composition across species (Lihrmann et al. 2006). Accordingly, we sought to determine if direct administration of native (species-specific form) versus non-native UII has any influence on observable bioactivity. Furthermore, we provide the first evidence of direct cardiac actions of URP and report circulating plasma levels of UII & URP. Finally, previous studies highlighted NO and prostanoids as possible mediators of the response to UII in the heart (Katano et al. 2000; Abdelrahman and Pang 2002; Gardiner et al. 2004; Lin et al. 2004; Ishihata et al. 2005a; b). Accordingly, we investigated whether URP can activate these same mechanisms.

3.2 Materials and methods

3.2.1. Materials

Male Sprague-Dawley (SD) rats weighing ~300 g (60-75 days old) were housed under controlled temperature (21ºC), humidity (~40%) and natural day length, with free access to standard rat food and water.

Rat UII (rUII), human UII (hUII), URP, Nω-Nitro-L-arginine methyl (L-NAME) and indomethacin were obtained from Pharmac (Belmont, CA) and Sigma (St. Louis, MI). Trout UII (tUII) was synthesized by the solid phase methodology on a Fmoc-Val-

PEG-PS, using a Pioneer PerSeptive Biosystems peptide synthesizer (Applera-France, Courtaboeuf, France) and the standard Fmoc procedure as previously described (Labarrere et al. 2003, Lancien et al. 2004). The synthetic peptide was purified by reversed-phase HPLC (purity was >99.5%) and characterized by MALDI-TOF mass spectrometry on a Voyager DE PRO spectrometer (Applera-France). All peptides and L-NAME were diluted in distilled water, aliquoted and stored at –20ºC prior to use. Indomethacin was diluted in 100% ethanol, aliquoted and stored at –80ºC prior to use. (The percentage of ethanol infused into the heart containing indomethacin was 0.0025-0.0063%. Control infusions of 0.01% ethanol had no influence on cardiac function when compared with infusion of vehicle alone).

3.2.2. Isolated rat heart perfusion

In vitro perfusion of rat hearts was performed as previously described (Pemberton et al. 2005). Briefly, rats were anesthetized by i.p. injection of 50 mg/kg sodium pentobarbital. The heart was then rapidly excised, rinsed in ice-cold buffer, mounted in the Langendorff apparatus and perfused at 12 ml/min (constant retrograde flow) with perfusion buffer comprising (mM); 123 NaCl, 22.0 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 1.1 MgSO4.7H2O, 1.5 CaCl2.2H2O, and 11.0 glucose (final pH 7.40). Buffer was maintained at 37°C, and gassed with 95%O2/5%CO2. The left atrium was removed allowing an ethanol-filled balloon attached to a pressure transducer to be inserted into the left ventricle, enabling measurement of hemodynamic contractile parameters. A side-arm cannula was inserted into the rubber aortic cannula to measure vascular reactivity. Hearts were paced at 350 bpm (Digitimer DS2A – Mk. II). Hearts were allowed to settle for a further 30 minutes before administration of drugs or control vehicle. Data was recorded and analyzed using a Powerlab Chart 5 system (AD Instruments). Rats were chosen at random for each dose of UII/URP or control. The control vehicle or drugs were infused over 30 minutes at 0.5 ml/min using a syringe pump feeding into the perfusion line. See Appendix B.1 for supplementary information and Langendorff methodology.

3.2.3. Antagonist studies

Hearts were prepared as described above. Antagonist agents were diluted in buffer enabling infusion of 0.5 ml/min for 30 minutes. The effect of the antagonists on rUII / URP was observed by co-infusion of L-NAME (an inhibitor of nitric oxide synthase)

or indomethacin (a non-selective inhibitor of cyclooxygenase) mixed with either 300 pmol rUII or 10 nM URP and infused into the heart as described above. Experiments were run in pairs: one heart infused with both the antagonist and peptide; the other simultaneously infused with the peptide only from the same stock solution and same buffer reservoir providing a parallel control for all antagonist experiments. The heart given the antagonist was chosen at random.

3.2.4. Myography of salmon coronary arteries

Salmon (mean weight 1918 ± 533g) were obtained from Isaacs Salmon Farm (Christchurch, NZ). Salmon were removed from raceways and held in 20,000 L flow through tanks with no less than 3 fish per tank at any time. Water was provided from an underground bore directly below the salmon farm with temperature maintained at 10°C in the tanks. Salmon were fed every 3 days with standard salmon chow. Coronary arteries running along the dorsal or ventral side of the bulbous arteriosus were isolated and refrigerated in salmon Ringer comprising (mM): NaCl 120, KCl 4, MgSO4 1.2, CaCl2 1.53, Glucose 5.5, HEPES 3, NaHEPES 7 (values in mM, final pH 7.8) overnight allowing them to relax and metabolize agents released during the capturing process. Vessels were cut to 2 mm lengths and mounted on wires in the jaws of a Mulvany myograph (model 410A, JP Trading, Århus, Denmark); see Appendix B.2 for supplimentry methods. The vessels were then pretensioned in 12ºC Ringer bath and allowed 2 hours to settle and reach their resting tension. One 'control' vessel was untouched throughout the experiment to measure any spontaneous change in tension. Changes in tension were recorded using Chart 4 (AD Instruments). Aliquots of UII were administered directly into the bath. The effects of 1 nM and 100 nM concentrations were measured, 40 minutes apart. A full concentration-response curve was not attempted as tachyphylaxis was discovered in preliminary experiments. At the end of the experiment 0.12 M KCl was administered as to obtain each vessel's maximum potential contraction and ensure viability.

3.2.5. RIA measurement of rat plasma URP and rUII

An RIA kit specific for rUII was supplied by Phoenix Pharmaceuticals Inc. in order to measure the concentration of rUII circulating in plasma from 13 healthy rats and in the perfusing solution. Blood was collected into tubes containing EDTA, placed on ice and centrifuged at 5000 rpm for 10 min at 4 °C before being stored at -80 °C until

required. The RIA kit is 100% cross-reactive with URP, thus the result reveals the potential combined concentration of both rUII and URP in rat plasma. In order to overcome this RP-HPLC was employed (run at 0-60% CH_3CN over 60 min) and RIA repeated on the subsequent aliquots collected at 1 min intervals.

3.2.6. Statistical analysis

All data are presented as mean ± SEM. Analysis of time-point changes in perfusion pressure was performed using a two-way ANOVA with repeated measurements. Comparisons of peptide responses were analyzed for significance using one-way ANOVA with Bonferroni's multiple comparison test, post-hoc. The significance of all data concerning salmon coronary vessels was analyzed using a one way repeated measures ANOVA with Newman-Keuls multiple comparison test, post-hoc. In all statistical tests a value of $P<0.05$ was considered significant.

3.3. Results

3.3.1. Effect of rat UII upon isolated, perfused rat hearts

30 minute infusion of rat UII effected a dose-dependent reduction in perfusion pressure (PP) at 3, 30 and 300 pmol/L of 10% ($P=0.08$), 17% ($P<0.001$) and 33% ($P<0.001$), respectively, compared with control (Figure 3.1A). The influence of rUII on PP was immediate and sustained over the 30 min infusion, with all concentrations stimulating maximum vasodilation by 20-30 min.

3.3.2. Effect of non-native UII in isolated perfused rat hearts

Both human and trout UII reduced PP in the perfused rat heart, but the changes were not significantly different from those observed in control perfusions at 30 minutes ($P>0.05$) (Figure 3.1B). Neither native nor non-native UII had significant effects on cardiac contractility in rat hearts (Appendix C, Figure C.1).

3.3.3. Effect of URP on PP in rat hearts

URP significantly reduced PP in the rat heart at both 1 nM ($P<0.001$) and 10 nM ($P<0.001$) (Figure 3.2), but with a reduced potency compared with rUII. 1 nM URP caused a maximal reduction in PP of 23% at 20 min and 10 nM 30% at 10 min during infusion, when compared with control. The response of rat hearts to URP compared with rUII, was less potent, requiring 10-fold more URP to elicit a comparable

reduction in PP. Like UII, URP had no direct contractile effects on isolated, perfused rat heart preparations (Appendix C, Figure C.1).

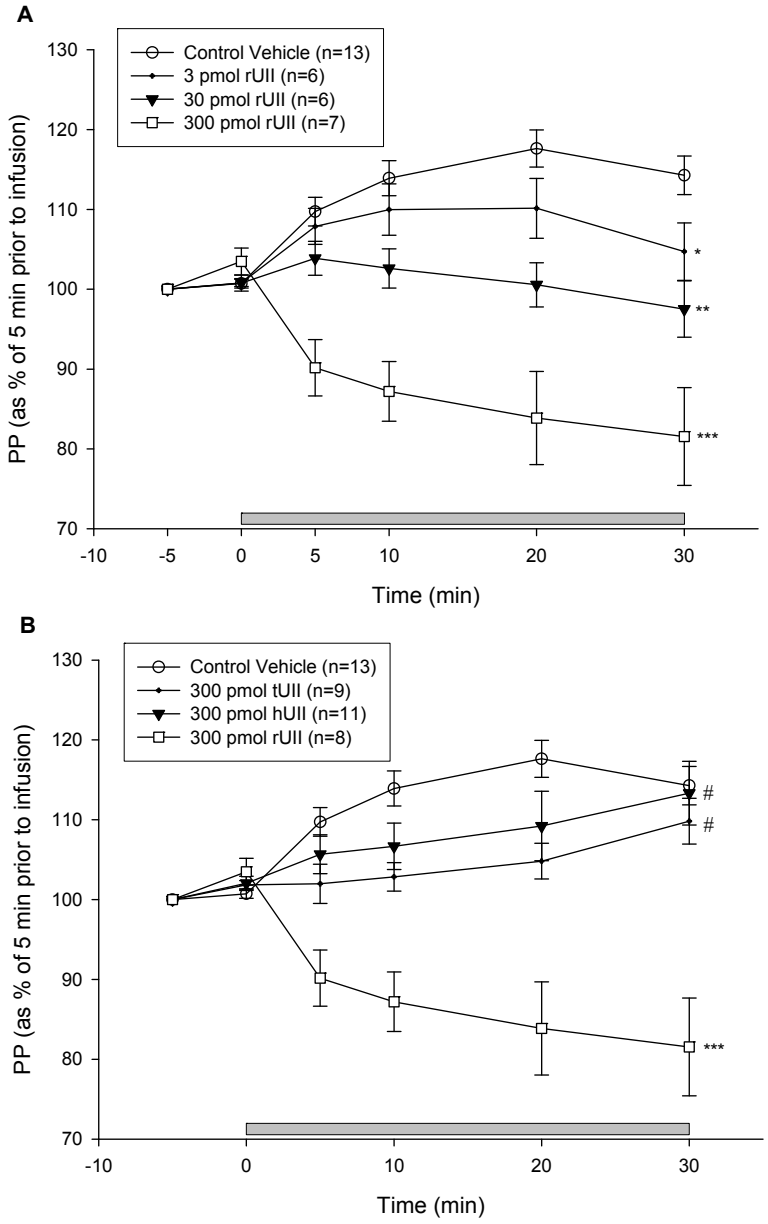


Figure 3.1 (A) Dose-dependent response of perfusion pressure to rUII in the rat heart. 3, 30 and 300 pmol/L rUII all significantly reduced PP compared with control infusion using a one-way ANOVA (*P=0.08, **P<0.001 and ***P<0.001, respectively) indicating potent, sustained dilation of the coronary arteries. (B) Observing 300 pmol/L native (rUII) and non-native species of UII (tUII and hUII) on perfusion pressure in the isolated, perfused rat heart. RUII significantly reduced PP compared with vehicle (***P<0.001 analysed by one-way ANOVA) and significantly differs from both non-native hUII and tUII (two-way ANOVA found both #P<0.002). The effects of both non-native hUII and tUII do not significantly differ from control at 30 min post infusion (P>0.05). Gray bar indicates infusion period.

3.3.4. Effect of antagonists on rUII and URP in the isolated rat heart

To determine whether NO was involved in the observed vasodilation in response to rUII and URP, L-NAME was co-infused with each factor. 0.59 μM L-NAME alone raised PP above control vehicle levels, consistent with blockade of nitric oxide synthase (Figure 3.3A) and had no effect on contractile parameters. L-NAME significantly inhibited the vasodilatory effect of 300 pmol rUII throughout the infusion period ($P<0.05$), maintaining PP at a level not significantly different from control vehicle ($P>0.05$) (Figure 3.3A). L-NAME was less effective at inhibiting 10 nM URP-induced dilation (Figure 3.3B). L-NAME co-infused with URP significantly increased PP above URP alone at 5 and 10 minutes post-infusion ($P<0.05$), but remained significantly different from control vehicle until after 10 minutes of infusion ($P<0.05$). By 20 minutes of co-infusion, PP became insignificant from both control vehicle and URP alone ($P>0.05$). Infusion of 3 μM indomethacin significantly reduced PP ($P<0.05$). 3 μM indomethacin significantly inhibited rUII-induced reductions in PP ($P<0.05$, Figure 3.4A). Likewise, 3 μM indomethacin also significantly inhibited the URP-induced reduction in PP up to 10 minutes post-infusion ($P<0.05$, Figure 3.4B). Indomethacin had no significant influence on altering DP when co-infused with either rUII or URP.

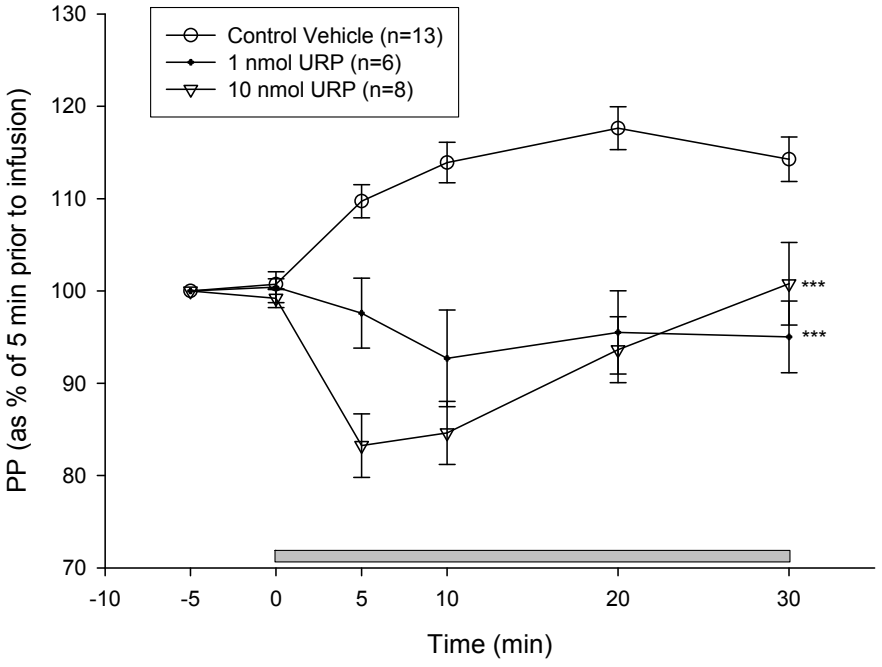


Figure 3.2 Dose-dependent response to URP observing perfusion pressure (1 and 10 nM) in the isolated, perfused rat heart. Both 1 nM and 10 nM URP significantly reduced PP compared to control. ***$P<0.001$ following two-way ANOVA; gray bar indicates infusion period.

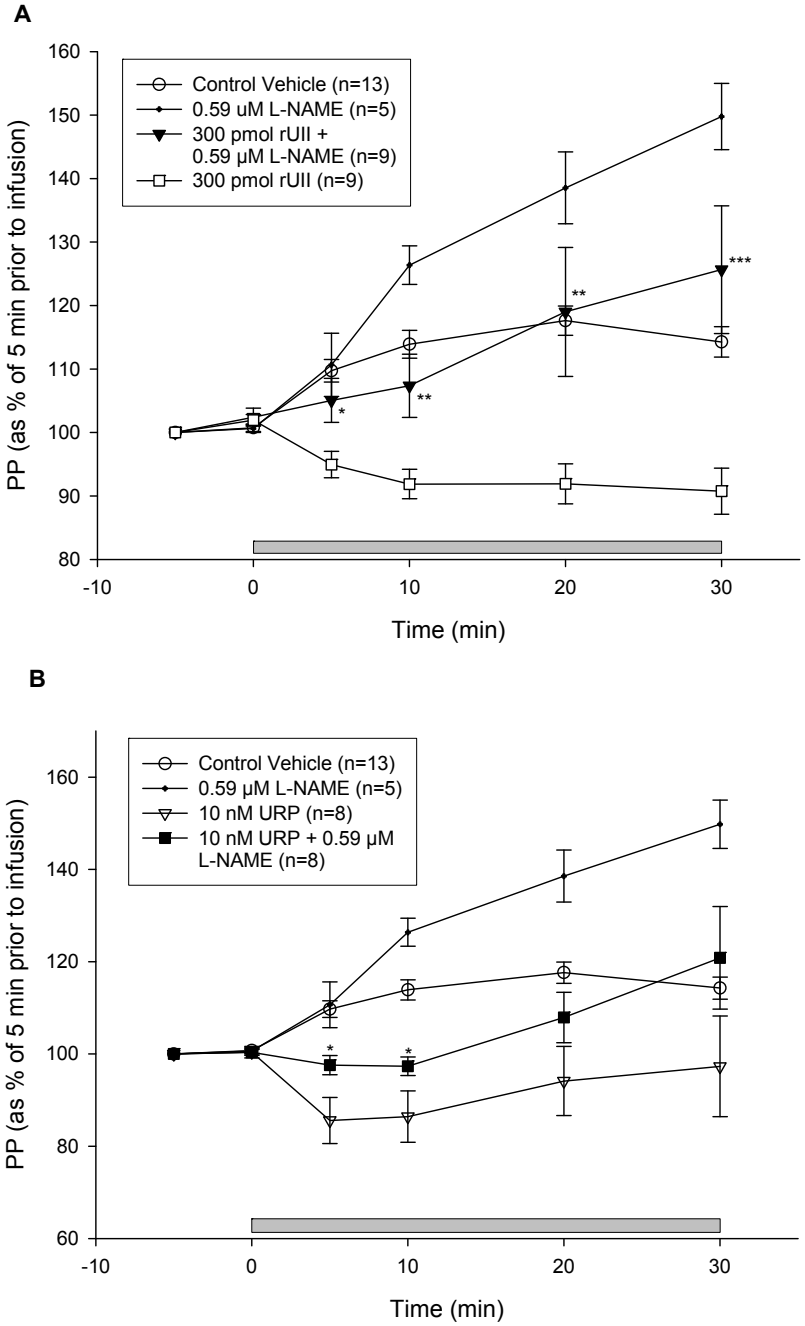


Figure 3.3. (A) The effect of 0.59 µM L-NAME on 300 pmol/L rUII-induced vasodilation in isolated, perfused rat hearts. 0.59 µM L-NAME alone significantly raised PP, consistent with blockade of NOS activity. L-NAME significantly attenuated the dilatory response to rUII throughout the infusion period compared with 300 pmol/L rUII alone (individual time-point comparison using student's t-test: *P=0.014, **P=0.004, ***P=0.001). (B) The effect of 0.59 µM L-NAME on 10 nM URP-induced vasodilation in isolated, perfused rat hearts. URP alone significantly reduced PP when compared with control infusion (P<0.001). L-NAME significantly attenuated the drop in PP caused by URP at 5 and 10 minutes post infusion using Student's t-test analysis (*P<0.05). The gray bar indicates infusion period.

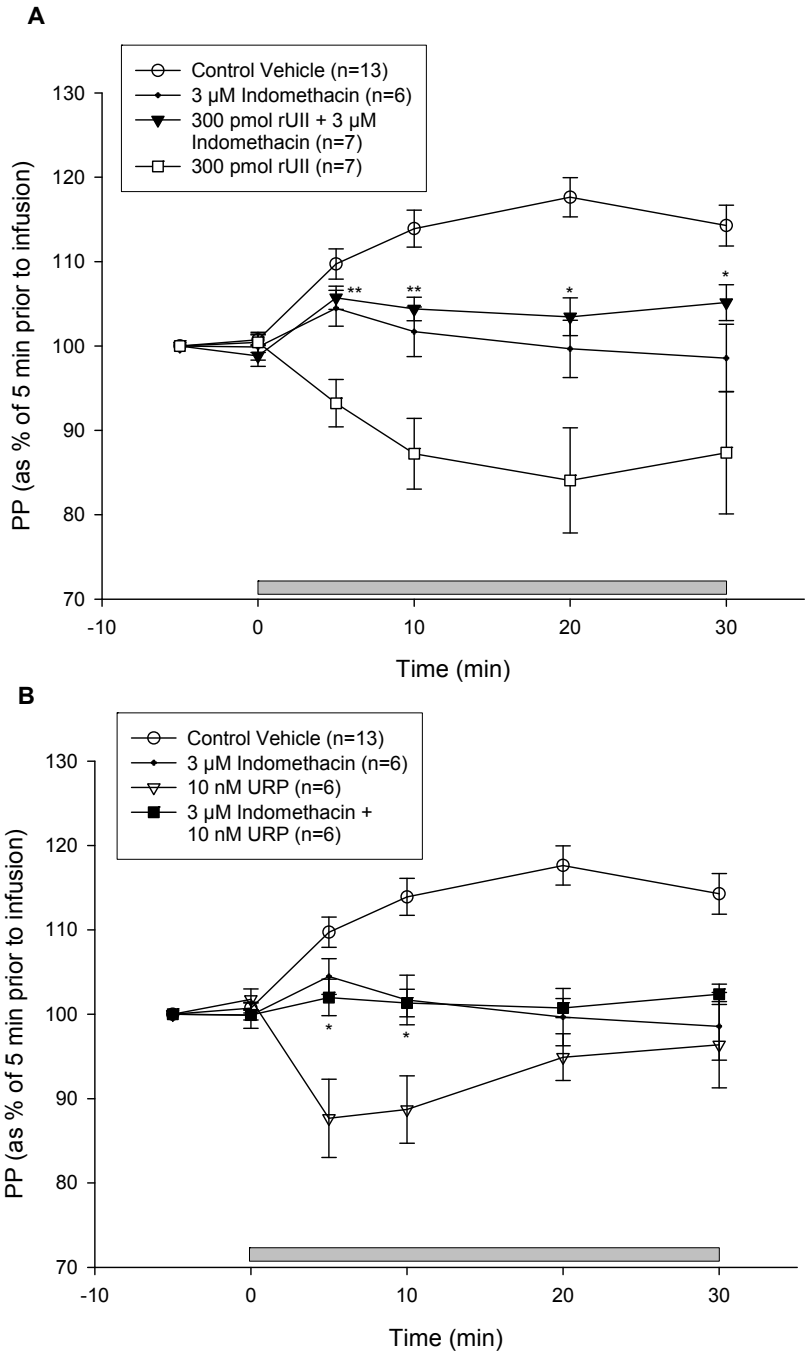


Figure 3.4. (A) The effect of indomethacin on 300 pmol/L rUII-induced vasodilation of the coronary arteries in isolated, perfused rat hearts. Indomethacin significantly prevented all rUII-induced reduction in PP at each time point (*P<0.05, **P<0.005). When combined with indomethacin, rUII could not reduce PP below that of 3 μM indomethacin infusion alone. (B) The effect of indomethacin on 10 nM URP-induced vasodilation of the coronary arteries in isolated, perfused rat hearts. Indomethacin significantly inhibited the URP-induced reduction in PP at 5 and 10 min post-infusion (*P<0.05 comparing individual time points). Gray bar indicates infusion period.

3.3.5. Plasma concentration of UII/URP in healthy rats

Mean circulating concentrations of rUII/URP in plasma from 13 SD rats were 2.9 ± 0.3 pmol/L.

3.3.6. Effect of native and non-native UII on salmon coronary arteries

Salmon coronary vessels responded to tUII in a biphasic fashion. Native tUII dilated salmon coronary arteries, although this did not achieve significance below 100 nM (Figure 3.5). In contrast, non-native rUII caused a significant constriction of salmon coronary arteries at both 1 nM ($P=0.032$) and 100 nM ($P=0.033$), with constriction being immediate upon addition of rUII at both doses. This contraction peaked at 2 minutes then reduced gradually to 31.53% of its maximum contraction by 40 minutes post administration.

3.3.7. Effect of URP on salmon coronary arteries

URP constricted salmon coronary arteries significantly at 100 nM ($P<0.01$) (Figure 3.5), with maximum constriction of the vessel occurring within 1 min of drug administration. Constriction was not sustained, falling rapidly to just 20.95% of initial maximum contraction by 10 min; and by 40 min the tension was back to resting levels.

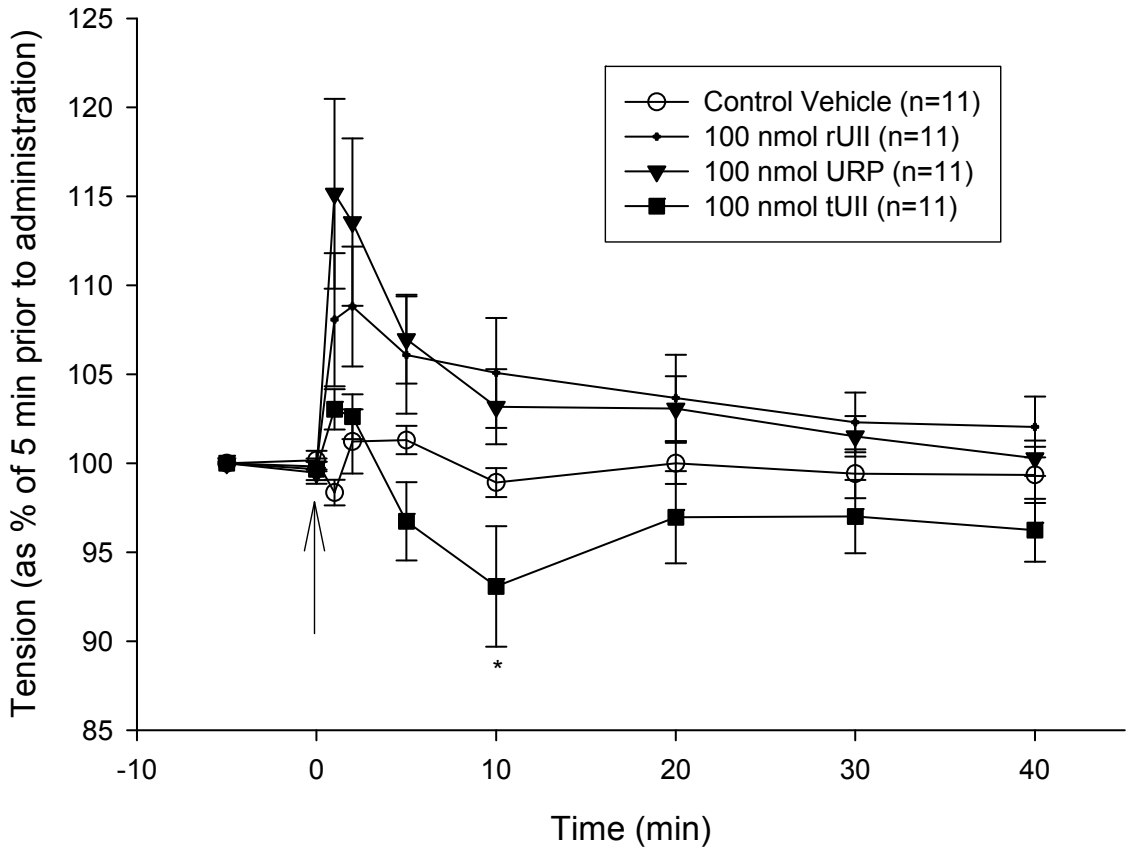


Figure 3.5. The effect of URP and native and non-native species of UII on Chinook salmon coronary arteries. Non-native rUII and URP significantly constricted coronary vessels ($P<0.01$ using a one-way ANOVA), while native tUII significantly dilated the vessels at 10 min (*$P<0.05$ when comparing individual time points using student's t-test). Arrow indicates addition of the peptide or vehicle at 0 min.

3.4 Discussion

In coronary artery preparations, UII has been shown to be a vasoconstrictor (Bottrill et al. 2000; Gray et al. 2001), vasodilator (Katano et al. 2000; Li et al. 2004), or have no effect (Douglas et al. 2000). Interestingly, in nearly all of these studies, hUII was used instead of the appropriate native rat UII. We build on prior knowledge to show:

1) UII has consistent vasodilatory activity in the coronary vasculature when the species appropriate form is used in both rat and fish species.
2) This dilation is severely attenuated or reversed (i.e. constriction) when nonnative forms are used.
3) URP has dilatory actions in the rat but at approximately 10-fold lower potency than UII.
4) Nitric oxide and prostanoids have a significant role in both rUII and URP-induced vasodilation of rat coronary arteries
5) UII/URP circulates in the plasma of healthy SD rats at ~3 pmol/L

As our results show, the use of non-native UII markedly attenuated or reversed the vasodilator effect of native UII on coronary vasculature. Our results found rUII to be a potent, dose-dependent vasodilator of the coronary arteries in the perfused rat heart, dilating the coronary arteries up to 33% after 30 min, indicating native UII causes not only a rapid response, but can maintain dilation of the coronary vasculature. Consistent with previous reports (Bottrill et al. 2000; Douglas et al. 2000; Gray et al. 2001) non-native UII had little influence on PP in rat hearts when compared to control, with both tUII and hUII not significantly different from control at 20-30 minutes post infusion.

We found both native and non-native UII to have no significant influence on left ventricular contractility. Published in vivo studies across rats and sheep have shown UII to be both a positive (Watson et al. 2003; Hood et al. 2005) and negative inotrope (Ames et al. 1999; Hassan et al. 2003; Zhu et al. 2004), however the response appears to be dependent upon the method of infusion with intracerebroventricular infuse on increasing (Watson et al. 2003; Hood et al. 2005), and intravenous infusion either having no effect or reducing left ventricular contractility (Ames et al. 1999; Heller et al. 2002; Watson et al. 2003; Zhu et al. 2004). Ours is the first report to document the direct effects of URP in the mammalian heart and fish coronary arteries. Because the

URP sequence comprises the “active region” of UII, it has been hypothesized that, compared with UII, URP might have approximately equal or greater binding affinity with GPR14, provoking an equally rapid response, and being equally potent (Sugo et al. 2003). In our hands, URP rapidly reduced PP in the rat heart in a concentration-dependent manner. However, URP was noticeably less potent than rUII in dilating the coronary arteries, and it was unable to maintain the dilation over the 30 minute infusion period. In agreement with our findings, Chatenet et al. report that URP has a slightly higher binding affinity for GPR14, but is less potent than hUII on rat thoracic aorta in vitro, but it must be noted that the species of URP used in salmon and rat tests was rat URP (Chatenet et al. 2004). Thus far, URP has been identified in rats, mice and humans and all retain the same amino acid (AA) sequence. It remains unknown whether URP exists in fish retaining the same AA sequence, a different sequence or whether URP is specific only to mammals.

In salmon, native tUII, elicited a biphasic response on coronary arteries with initial minor contraction, followed by sustained dilation. In contrast, the non-native rUII caused pronounced constriction of the coronary arteries. Although the 6 amino acid ‘active’ ring structure is conserved throughout all known UII species (Lihrmann et al. 2006), there is great variation in the N-terminal sequence and length. For example, hUII is only 67% identical to rUII (Coulouarn et al. 1999). Structure-activity relationship studies have suggested that shortening the N-terminus of hUII has no significant effect on binding affinity to rat or human UII receptors (Brkovic et al. 2003), or in constricting rat aortic strips compared with hUII itself (Coy et al. 2002; Kinney et al. 2002; Brkovic et al. 2003). However, removal or substitution of hUII’s C-terminus caused a substantial loss in potency and binding affinity to the rat and human UII receptors (Coy et al. 2002; Brkovic et al. 2003). The C-terminus of rUII differs from both hUII and tUII, and this may be partially responsible for our observed differences between rat and salmon coronary artery responses.

The biphasic response provoked by tUII in salmon coronary arteries could suggest that more than one receptor may be activated by UII in this species. This is not uncommon in biological systems; for example catecholamines activate alpha and beta receptors which have opposing effects on one another (Forster 1981; Agnisola et al. 1996). Alternatively, tUII may bind to the only known UII receptor, GPR14 (Ames et

al. 1999; Liu et al. 1999; Mori et al. 1999; Behm et al. 2003), but activate more than one intracellular pathway, causing initial contraction, prior to the secondary pathway overriding the first resulting in a biphasic response. Gendron et al. showed UII constricted rat thoracic aorta in vitro, but reduced mean arterial pressure (MAP) in vivo. They suggest UII may use the "same or similar metabolic pathways, but be localized in different cells with opposite functions" (Gendron et al. 2005), as GPR14 is present in both aortic smooth muscle and the endothelium of tissues (Onan et al. 2004). Thus, UII has the potential to constrict smooth muscle directly by increasing $[Ca^{2+}]_i$ activating PKC, calmodulin and phospholipase C (Ames et al. 1999; Rossowski et al. 2002; Russell and Molenaar 2004), and/or dilate vessels via stimulating NOS and prostanoid expression in the endothelium and adventitia (Behm et al. 2003; Lin et al. 2004; Horie et al. 2005). These opposing responses produced by UII may explain the biphasic effect observed with tUII in salmon coronary arteries. Our results suggest both rUII and URP stimulate the release of NO in rat coronary arteries as L-NAME significantly attenuated the vasodilatory actions of rUII and URP in the isolated rat heart. These findings agree with previous reports observing the relationship between NO and UII (Katano et al. 2000; Li et al. 2004; Ishihata et al. 2005a; Ishihata et al. 2006).

In rat hearts, L-NAME significantly attenuated URP-induced vasodilation at 5 and 10 minutes post-infusion, with PP climbing towards control levels after 5-10 minutes post-infusion. Interestingly, the inhibitory potency of L-NAME upon URP was less than for rUII suggesting rUII is more dependent upon NO than URP to dilate coronary arteries. However, L-NAME could not totally inhibit vasodilation induced by rUII / URP indicating other intracellular pathways may be utilized by both factors. Prostanoids (specifically PGE2, PGD2, PGI2) have been previously shown to dilate coronary arteries of isolated, perfused rat hearts (Bouchard et al. 1994) and may be a mechanism utilized by UII in the rat heart (Ishihata et al. 2005b). Our results agree with this hypothesis as indomethacin significantly inhibited rUII-induced dilatory activity in our preparations. Intriguingly indomethacin alone had vasodilatory actions in rat heart preparations. Prostanoids encompass a large family of molecules, some of which previously shown to vasodilate and others vasoconstrict coronary arteries (Cornish et al. 1983). The dilation may suggest that the balance of constrictive PGs versus dilatory PGs occurring in the coronaries at the time of infusion was slightly in

favour of constriction, and thus removal of all PG activity by indomethacin resulted in a slight dilation. Co-infusion of indomethacin with rUII or URP did not result in synergistic decreases in perfusion pressures. Why indomethacin-induced dilation should inhibit UII / URP dilation is unclear, but it may relate to the constant biological control, balancing activated and suppressed peptides and molecules to maintain homeostasis. This hypothesis agrees with our results observing the antagonism of rUII with indomethacin suggesting UII activates vasodilatory PGs and may inhibit vasoconstrictive PGs.

This is the first study to reveal the effects of inhibiting prostanoids on URP bioactivity in the isolated, perfused rat heart. Indomethacin significantly inhibited URP-induced vasodilation but lost significance past 10 minutes post-infusion. This was the same pattern of response observed with L-NAME above, due to URP naturally losing potency and rising towards control levels. Co-infusion of URP and indomethacin caused PP to remain at levels equal to infusion of indomethacin alone, revealing: a) URP influences prostanoid activity in the isolated, perfused rat heart; and b) prostanoids possess greater potency than NO in dilating the coronary arteries when stimulated by URP. Circulating levels of rUII/URP in the rat were ~3 pmol/L. We attempted to discriminate the molecular forms of UII/URP in the rat circulation through coupling RP-HPLC with a specific commercially available URP RIA but we could not achieve consistent results. Discrimination of URP/UII forms in the circulation thus remains a logical target for future study.

In conclusion, UII has consistent vasodilatory activity in the coronary vasculature when the species appropriate form is used in both rat and fish, but this dilation is severely attenuated or reversed (i.e. constriction) when non-native forms are used. URP has dilatory actions in the rat but at approximately 10-fold lower potency than UII. Nitric oxide and prostanoids have a significant role in both rUII and URP-induced vasodilation of rat coronary arteries. These findings reveal the level of species-specificity of UII and the possible importance the C- or N-terminals for correct receptor activation.

4. Urotensin II and Disease

Abstract

At the commencement of this thesis the American Heart Association (AHA) reported cardiovascular disease (CVD) to be prevalent in 80 million Americans and responsible for the death of 864,500 Americans in 2005, recognising it as the greatest cause of death in America and the Western world (American Heart Association 2009). Understanding the pathogenesis of CVD is vital in order to design means of reducing its pathological effects and reduce mortality. This includes identifying the peptides, enzymes, receptors and intracellular agents underlying the progression of CVD, including both cardioprotective and pathogenic. UII has been suggested to have a significant role in the pathology of CVD in mammals as circulating UII is elevated in patients suffering congestive heart failure (CHF), and other cardiovascular diseases. UII is also reported to be correlated with elevated left ventricular end-diastolic dysfunction, and severity of disease, suggesting circulating UII concentration may provide a potential marker of disease and predictor of future complications and patient survival. This review will look specifically at the role of UII in two major types of cardiovascular disease, congestive heart failure (CHF) and atherosclerosis, examining UII-induced pathogenic and protective properties, as well as its potential as a marker of cardiac disease.

4.1. Congestive Heart Failure (CHF)

Many studies have now reported the UII/UT system (including UT mRNA and prepro-UII mRNA) to be upregulated in cardiovascular disease and CHF (Maguire et al. 2000; Douglas et al. 2002; Zhang et al. 2002). UII/UT expression within the myocardium itself is shown to be altered with disease; for example Zhang et al. (2002) reported rats that with CHF displayed elevated cardiac UT expression, which was greatest in the right ventricle, suggesting UT expression is related to pulmonary hypertension (Zhang et al. 2002). Douglas et al. (2002) reported prepro-UII mRNA and UII expression to be greater in the left ventricle of patients with ischemic heart disease, while patients with dilated cardiomyopathy showed similarly elevated expression across both ventricles and atria (Douglas et al. 2002). Furthermore, UT density was reported to be elevated in the myocardium of CHF hearts, specifically localised to the cardiomyocytes, VSMCs and endothelial cells (Douglas et al. 2002), while another study reported no significant difference in UT density in coronary artery medial smooth muscle cells between CHF patients and healthy volunteers (Maguire et al. 2000; Maguire et al. 2004). Experimental animal models inducing CHF in rats recorded elevations in plasma UII levels compared to their controls (Bousette et al. 2004; Bousette and Giaid 2006), while spontaneously hypertensive rats (SHRs) also exhibited elevated UII levels (Song et al. 2006; Watanabe et al. 2006). These studies reveal UII is produced throughout the heart and indicate UII production is elevated in cardiovascular disease suggesting a potential pathological role in CHF. However, it is yet to be confirmed whether UII and its mRNA are elevated in response to the disease in order to provide a level of protection, or whether the elevation augments the progression of the disease. This is the key finding currently eluding researchers and in doing so hindering the development of using UII as a potential therapeutic tool.

Studies have provided evidence for UII as both a cardioprotective and pathogenic agent. For example, patients expressing low UII levels exhibited poorer outcomes with increased likelihood of a cardiovascular event occurring (Khan et al. 2006; Mallamaci et al. 2006; Ravani et al. 2008); while maintaining an elevated UII concentration in a rat model of volume overload preserved myocardial contractility (Harris et al. 2007) indicating UII possesses protective properties. In contrast, reports

have also indicated blockade of the UII receptor aided recovery from ischemia, significantly lowered collagen I:III ratio and attenuated cardiac fibrosis in live rats (Bousette et al. 2006a; Bousette et al. 2006b). Further *in vitro* studies have shown UII to promote collagen gene expression and synthesis in cardiac fibroblasts (Tzanidis et al. 2001; Tzanidis et al. 2003; Bousette and Giaid 2006) and stimulate VSMC proliferation, causing vessel wall thickening and contributing to CHF (Sauzeau et al. 2001; Watanabe et al. 2001a; Tamura et al. 2003).

Observing the effect of UII on the pulmonary artery in mammalian species, UII has been shown to stimulate vasoconstriction, or have no effect (Douglas et al. 2000; Zhu et al. 2004), while in human arteries hUII is reported to cause variable vasoconstriction (MacLean et al. 2000) and vasodilation (when preconstricted with endothelin) (Stirrat et al. 2001), suggesting UII may regulate pulmonary arterial tone. Dschietzig et al. reported that increasing pressure over isolated pulmonary artery endothelial cells within a flow chamber caused a reduction in prepro-UII mRNA and UII peptide expression (Dschietzig et al. 2001). Their results suggest that if viewing UII as a potent vasoconstrictor, the observed reduction in UII in response to increased pressure suggests UII does not contribute in the pathophysiology of pulmonary hypertension. However, if UII naturally dilates the pulmonary artery in the healthy setting, the reduction in response to elevated pressure may contribute to the pathophysiology of pulmonary hypertension by reducing UII-induced vasodilative effects (Dschietzig et al. 2001). This again illustrates the complexity of the UII system and calls for analysis on a vessel-by vessel, species-by-species basis.

4.2 Atherosclerosis and Coronary Artery Disease (CAD)

The endothelium lining the lumen of all vessels is of vital importance in the regulation of blood pressure and vascular health as it produces and secretes vasoactive agents regulating vascular tone. Hypertension, smoking, hyperlipidemia, hypercholesterolemia, diabetes, all increase the likelihood of endothelial dysfunction (ED), which in turn, can result in the development of coronary artery disease (CAD) and atherosclerosis (Katz 1997; Meigs et al. 2002; Hadi et al. 2005; Haluska et al. 2008). UII levels are reported to be elevated in hypertensive subjects (Cheung et al.

2004; Suguro et al. 2007) and ICV application of UII to normotensive and SHRs significantly elevated blood pressure (Lin et al. 2003). UII-induced rises in blood pressure were more pronounced in SHR animals, suggesting existing hypertension may increase sensitivity of the vasculature to UII/UT activity.

Hypertension-induced vascular stress and stretch results in ED, the symptoms of which include loss of endothelial integrity resulting in increased endothelial permeability, reduced ability to produce vasodilative agents, monocyte migration, platelet activation and increased vascular reactivity, which combined, lead to CAD and atherosclerosis (Schiffrin 2008a; b). The development and progression of atherosclerosis and hypertension can be divided into two hypertension-induced pathways: one leading to the development of atherosclerotic plaques, while the other simultaneously stimulates maladaptive vascular remodelling (Watanabe et al. 2006) (Figure 4.1).

UII and UT expression are elevated in patients with hypertension and positively correlated with atherosclerotic plaque score and severity of CAD (Heringlake et al. 2004; Suguro et al. 2007), suggesting that UII may have a significant pathological role on the development of CAD and atherosclerosis. No difference in UT density was observed in human medial VSMCs when comparing normal and atherosclerotic coronary arteries (Katugampola et al. 2002), however UT expression is elevated in the atherosclerotic plaque and regulated by inflammatory mediators, produced in abundance within and around the sight of atherosclerotic plaque (Papadopoulos et al. 2008). UII is now reported to significantly influence both plaque formation and vascular remodelling (Figure 4.1), discussed below.

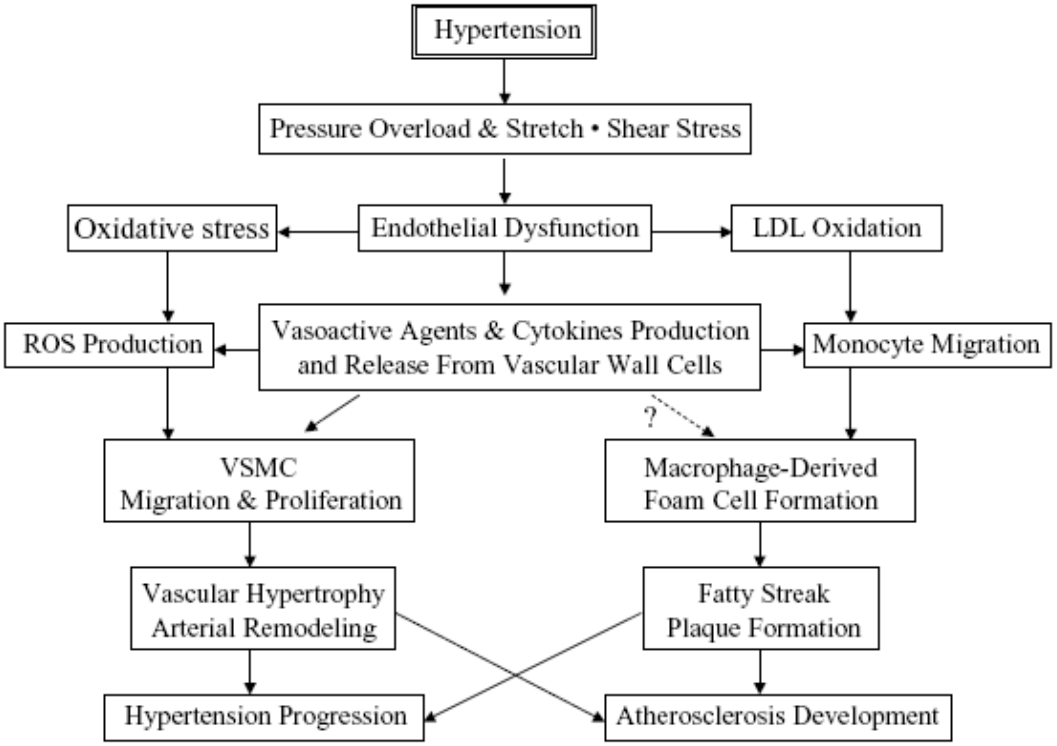


Figure 4.1. Flow diagram illustrating the pathways stimulated in response to hypertension leading to the development and progression of atherosclerosis and hypertension. ROS - reactive oxygen species; LDL - low-density lipoprotein; VSMC - vascular smooth muscle cell. From Watanabe et al. 2006.

It is now well established that UII stimulates NO production, causing vasodilation, and potentially a level of vascular protection to alleviate pressure, stretch and shear stress (Gray et al. 2001; Zhang et al. 2003; Lin et al. 2004; Ishihata et al. 2006; Lacza and Busija 2006). However, in dysfunctional endothelial cells the production of nitric oxide (NO) is known to become uncoupled, and ironically, eNOS produces reactive oxygen species (ROS) instead of NO (Otani 2009). NO is also scavenged by excess ROS, and thus in the diseased state NO availability is quenched, limiting endothelium-dependent vasodilation (Lavi et al. 2008). This has been observed in the human left anterior descending coronary artery where patients suffering ED expressed significantly reduced NO bioavailability due to increased oxidative stress, whilst NO production remained the same as vessels from healthy patients (Lavi et al. 2008). The

elevation in oxidative stress observed is primarily due to activation of vascular nicotinamide adenine dinucleotide (NADPH) oxidase, forming ROS. To our knowledge, only one study has observed the direct effects of UII on NADPH activity. Djordjevic et al. administered UII to human pulmonary artery smooth muscle cells *in vitro* and concluded that UII elevated ROS activity via increased NADPH oxidase. ROS then initiated cellular proliferation and pulmonary arterial remodelling through the activation of cytokines, as well as reducing NO bioavailability, suggesting UII augments pulmonary hypertension (Djordjevic et al. 2005). NADPH oxidase can also convert LDL to oxidised LDL in the arterial wall. Oxidised LDL has been found to interact with other agents including angiotensin II and serotonin, and recently it has been suggested that UII can act synergistically with mildly oxidised LDL to further increase VSMC proliferation, reducing the diameter of the lumen, elevating hypertension (Watanabe et al. 2001a). Hypertension-induced ED also stimulates the release of growth factors from endothelial cells and macrophages in the intimal layer that induce cellular proliferation and migration of VSMCs to the intima (Hadi et al. 2005). Atherosclerotic plaques occur when the dysfunctional endothelium enables lipid and leukocyte deposition in the arterial wall, producing a fatty streak which over time accumulates foam cells, lipids, myointimal cells and extracellular matrix proteins to form a plaque (Watanabe et al. 2006). UII has been suggested to progress atherogenesis by upregulating ACAT-1 levels, an enzyme responsible for converting intracellular free cholesterol to cholesterol ester capable of then being stored in lipid droplets accelerating foam cell formation, eventually leading to the formation of fatty streaks (Watanabe et al. 2005; Papadopoulos et al. 2008; Shiraishi et al. 2008). UII has also been reported to increase endothelial cell permeability (Gendron et al. 2005), promote VSMC proliferation and inhibit apoptosis through ERK1/2 and RhoA/Rho kinase activity (Matsushita et al. 2001; Watanabe et al. 2001a; Watanabe et al. 2001b; Zou et al. 2001; Tamura et al. 2003; Tzanidis et al. 2003; Shi et al. 2006; Chen et al. 2008). Further proatherogenic effects of UII currently reported include UII-induced increases in collagen synthesis and expression (Wang et al. 2004; Zhang et al. 2008), promotion of cardiac fibrosis (Zhang et al. 2007), and elevating acetylcholine levels (Russell and Molenaar 2004) augmenting vasoconstriction. The distributive expression of UII also suggests a close relationship with inflammation and atherosclerotic lesions as UII and UT are both localised within human carotid plaque (Bousette et al. 2004). UII is expressed in foam cells and myointimal and medial

VSMCs of atherosclerotic human coronary arteries (Hassan et al. 2005), and is produced by macrophages and lymphocytes (Bousette et al. 2004; Maguire et al. 2004) indicating localised production of UII within the atherosclerotic lesion. Furthermore, UII colocalises with macrophages in human atherosclerotic coronary artery lesions (Maguire et al. 2004) and expression of UII/UT is regulated by inflammatory mediators including interlukin-6 and 1β and interferon-γ produced by T lymphocytes in the atherosclerotic plaque (Papadopoulos et al. 2008). Shiraishi et al. (2008) recently confirmed that infusion of UII into live rats for 4-14 weeks elevates the presence of aortic atheromas and lesion size, while UT and UII expression were both elevated in ApoE knock-out mice, specifically within the endothelium, smooth muscle cells and atheroma. Blockade of the UT receptor effectively attenuated the UII-induced effects, reducing aterosclerotic lesions in the ApoE ko animal model, and effectively inhibiting the development of atherosclerosis (Shiraishi et al. 2008).

In summary, these reports indicate UII to be a potentially potent proatherogenic, hypertensive agent stimulating vascular hypertrophy, and promoting the development of atherosclerotic plaque by accelerating foam cell formation, and increasing collagen expression. This suggests that inhibiting UII activity through blockade of UT may attenuate the progression of atherosclerosis and CAD. Rakowski and co-authors provide further support for this by demonstrating that blockade of the UT receptor reduced myointimal thickening and increased lumen size in a rat model of carotid artery stenosis (Rakowski et al. 2005). Currently, just one report has suggested a potential cardioprotective function for UII in atherosclerosis. Russell et al. (2004) proposed that UII-induced elevations in collagen and fibrinogen may afford a means of protection by maintaining the integrity of the fibrous atherosclerotic plaque cap, preventing the release of the lipid lesion into the vessel (Russell 2004). This theory is yet to be tested, however it has been reported that $TGF_{\beta 1}$ stabilizes atherosclerotic plaque (Cipollone et al. 2004b) while PGE_2 is associated with atherosclerotic plaque destabilization (Cipollone et al. 2004a); both agents reported to be produced in response to UII.

4.3 Protective and pathogenic pathways stimulated by UII

Identifying the intracellular agents and pathways stimulated in response to UII may provide beneficial explanations to the complex, dichotomous activity of UII observed in studies using healthy and diseased subjects (Figure 4.3). The cardioprotective properties of UII are evident in that UII stimulates nitric oxide production, EDHF, $TGF_{\beta 1}$, and prostanoid activity (namely PGE_2 and prostacyclin), all known to provide cardioprotection through eliciting vasodilation and regulating cellular proliferation (Lamontagne et al. 1992; Yano et al. 1995; Gray et al. 2001; Horie et al. 2005; Gardiner et al. 2006; Jia et al. 2008). Bottrill et al. (2000) reported that while UII constricted rat coronary arteries, it also caused dilation when the same arteries were preconstricted suggesting UII may provide a means of homeostatic regulation, maintaining blood pressure within normal levels (Bottrill et al. 2000). This has also recently been reported in cultured human aortic endothelial cells where UII induced an increase in $[Ca^{2+}]_i$ when the vessel was undergoing light shear stress, but UII had no effect on the vessel when static (Brailoiu et al. 2008). This pattern of response suggests UII has a protective, regulatory role in the cardiovasculature of healthy vessels (Figures 4.2 & 4.3). However, as discussed above, many reports implicate UII as a potent pathogenic factor, augmenting cardiovascular disease, including exacerbating atherosclerosis via the activation of ROS and accelerating foam cell formation (Watanabe et al. 2005; Watanabe et al. 2006), and stimulating extracellular-regulated kinase (ERK)- and c-jun N-terminal kinase (JNK)-mediated maladaptive VSMC remodelling (Figures 4.2 & 4.3). While the vasoconstrictive effects of UII promoting hypertension are reported to be mediated via Ca^{2+}-dependent stimulation of protein kinase C (PKC) and calmodulin within the VSMC (Figure 4.2). The contrasting effects of UII are proposed to be dependent upon endothelial dysfunction.

Endothelial dysfunction (ED) is a known characteristic of CHF (Katz 1997) and results in severe reduction of local NO expression and endothelium-derived hyperpolarizing factor (Andersson et al. 2003; Triggle et al. 2003). Due to this reduction in available vasodilative agents, UII may have reduced potency in dilating the vessel. This exact phenomenon was observed in a human *in vivo* study by Lim et al. (2004), where UII administered to healthy controls caused increased blood flow through the skin microvasculature, but application of UII to the same vessels of CHF

patients elicited vasoconstriction (Lim et al. 2004). Studies observing the effects of UII on endothelium-denuded vessels are, in theory, observing the effect of UII on diseased vessels. Endothelial-dependence studies have all reported UII to stimulate equal or increased vasoconstriction and a loss of vasodilation upon removal of the endothelium (MacLean et al. 2000). The reason for the elevated vasoconstriction is that the UT receptors are located on both endothelial and smooth muscle cells and thus if the endothelium becomes damaged or degraded, UII can bind directly to the smooth muscle cells stimulating an influx of calcium via the PLC/IP_3/PKC pathway, resulting in sustained vasoconstriction and possible cellular hypertrophy (Figure 4.2). Furthermore, dysfunctional endothelial cells still capable of binding UII can produce detrimental disruption of intracellular pathways that were once beneficial. For example, UII-induced stimulation of the NO pathway within a dysfunctional endothelial cell can lead to the production of reactive oxygen species (ROS) due to eNOS uncoupling, leading to eNOS-mediated superoxide anion production resulting in damage to surrounding cells and hypertrophy (Bauersachs and Fraccarollo 2008).

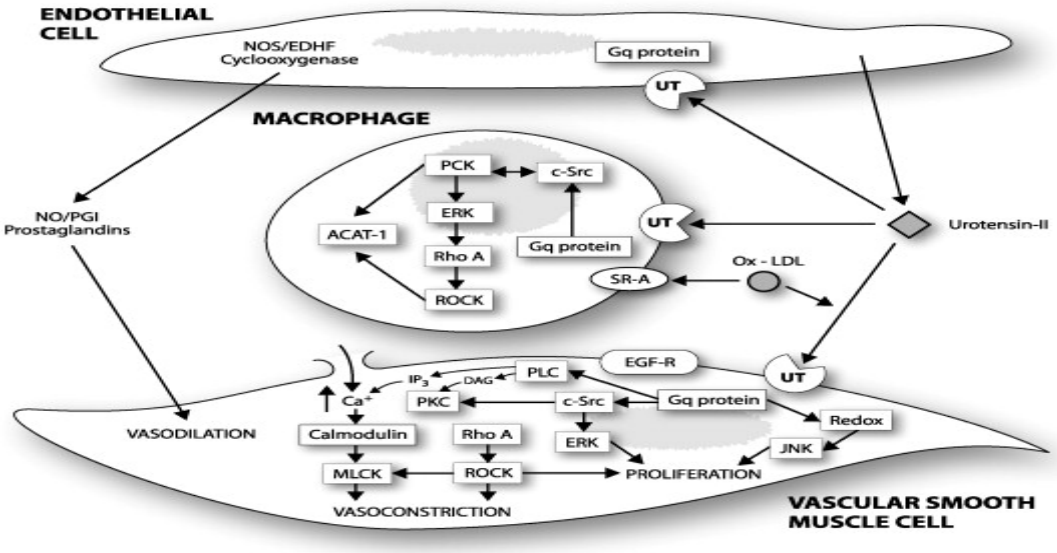


Figure 4.2 Schematic illustration of the intracellular pathways stimulated by UII in both endothelial and vascular smooth muscle cells. UII can stimulate NO- / PGI-induced vasodilation or PKC- / calmodulin-induced vasoconstriction dependent upon the cell type stimulated. UII can also promote VSMC proliferation and foam cell formation through stimulating ACAT-1 activity in macrophages, together developing and progressing atherosclerosis. From Papadopoulos et al. 2008.

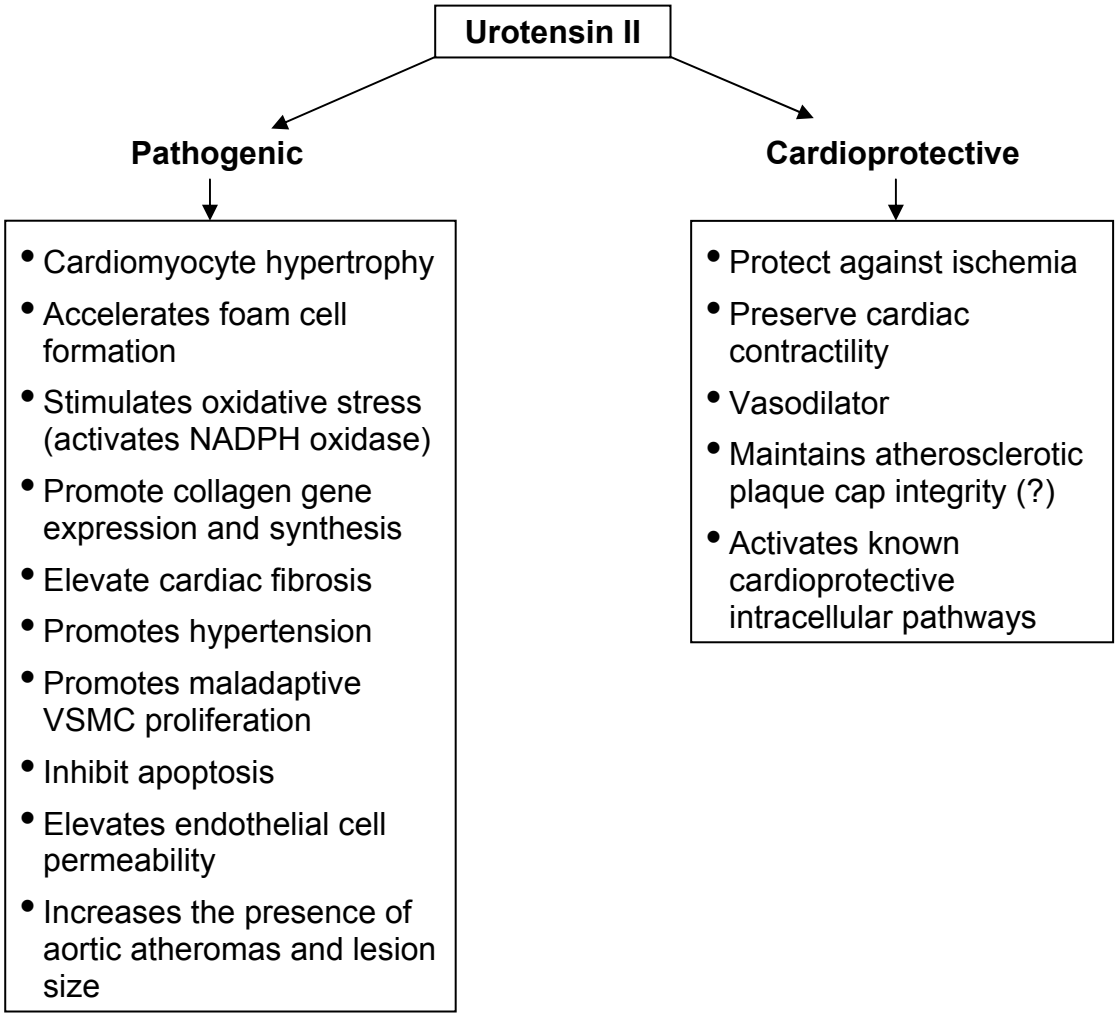


Figure 4.3 Summarizing the urotensin II-induced cardioprotective and pathogenic effects on the cardiovasculature reported to date.

In renal and cardiac disease the elevation in UII is significantly correlated with an elevation in $TGF_{\beta 1}$ expression, and UII has since been shown to directly stimulate $TGF_{\beta 1}$ expression including its mRNA (Mallamaci et al. 2006; Dai et al. 2007). In the healthy state $TGF_{\beta 1}$ has been reported as vasculoprotective (Mallamaci et al. 2006) regulating cellular proliferation through stimulating apoptosis and growth factors within the cell. However, in the diseased state $TGF_{\beta 1}$ functions can reverse becoming

a profibrotic cytokine, stimulating cardiac and renal hypertrophy and fibrosis (Border and Noble 1994; Li et al. 1998; Hein et al. 2003). In renal disease the elevation in UII levels was reported to provide $TGF_{\beta 1}$-mediated vasculoprotection and be antiatherogenic (Mallamaci et al. 2006), while diabetes-induced upregulation of UII and UT aided $TGF_{\beta 1}$-mediated renal fibrosis and dysfunction in the kidney (Tian et al. 2008). In healthy rat myocardial fibroblasts UII elevated $TGF_{\beta 1}$-mediated collagen mRNA expression, indicating UII aids pro-fibrotic cardiac remodelling, as antagonism of the UT receptor and $TGF_{\beta 1}$ both inhibited collagen synthesis (Dai et al. 2007). These studies indicate UII-induced $TGF_{\beta 1}$ activity can stimulate both protective and detrimental effects in both healthy and diseased cells revealing the difficulty in interpreting the UII-induced $TGF_{\beta 1}$-mediated role in cardiovascular disease.

UII also stimulates activation of cyclooxygenase-1 and -2 (COX-1 and -2) enzymes producing prostaglandins capable of both preventing and promoting vascular disease (Deeb et al. 2008). COX-1 is suggested to cause pathological effects through stimulating vasoconstriction, inflammation, and cellular proliferation, while COX-2 is reported to be vasoprotective eliciting PG-mediated vasodilation, and inhibiting platelet activation and adhesion (Deeb et al. 2008). As with $TGF_{\beta 1}$, the variable effects produced by UII-induced PG activity may also depend upon the functional state of the cell. To date all studies observing COX activity in response to infusion of UII have been performed in healthy vessels or animals, providing little evidence that UII-induced PG production can differ in the diseased state. COX-2 expression is reported to be elevated in atherosclerotic lesions in animals and humans providing vasoprotection (Deeb et al. 2006), however the increased expression also results in elevated proinflammatory PGs contributing to plaque rupture (Cipollone et al. 2004a). Based on this it is plausible that UII-induced upregualtion of COX expression could potentially progress atherosclerosis, although this is yet to be tested.

In summary, three of the main pathways activated by UII (NO, PGs and $TGF_{\beta 1}$) are known to produce opposing effects dependent upon the functional state of the cell. NO is a known dilative agent, however stimulation of eNOS can produce ROS in dysfunctional endothelial cells, progressing atherosclerosis and CAD. Activation of COX enzymes stimulate PGs capable of producing vasodilative, vasoconstrictive and

proliferative effects depending upon which isoforms is stimulated. Lastly, $TGF_{\beta 1}$ is described as a vasculoprotective agent regulating cellular growth in healthy cells; however, UII-induced stimulation of $TGF_{\beta 1}$ resulted in collagen synthesis in both healthy and diseased myocardial fibroblasts proving it is not a simple vasculoprotective agents but can also induced pathogenic effects, adding to the complex nature of UII activity.

4.4 UII as a novel biomarker of cardiac disease

Correlation studies between plasma levels of circulating UII and other markers of CHF suggest a possible association between BNP and UII, as UII was found to be positively correlated with BNP in CHF patients (Lapp et al. 2004; Gruson et al. 2005). In contrast, plasma UII levels negatively correlate with BNP in chronic renal failure (Zoccali et al. 2006). Whether the positive and negative correlations between UII and BNP are dependent on the disease state is yet to be determined and warrants further research. Currently, UII is not used as a measure of cardiovascular disease. One report suggests UII may be a better marker of CHF than NT-BNP as UII is independent of age, sex or NYHA class (Ng et al. 2002). The potential for UII to be used as a measure of CHF, CAD, or other cardiovascular diseases requires further research, and it has been recently reported that UII may be a marker of rheumatic valve disease (RVD), where plasma levels of UII correlated significantly with valvular regurgitation and pulmonary artery pressure, indicating that plasma UII may be predictive of pulmonary arterial hypertension (Ozer et al. 2009). Plasma UII levels have also been shown to correlate significantly with the level of atherosclerotic plaque score (Suguro et al. 2007), with UII and UT mRNA shown to be elevated in atherosclerotic legions of human aorta (Bousette et al. 2004), indicating UII could potentially be used as a marker for these conditions.

4.5 Summary

Taken together, it is becoming clear UII has a key role in CHF, atherosclerosis and CAD, heavily influencing the vasculature and myocardium. UII and UT expression are consistently reported to be elevated in cardiovascular and renal disease, however UII is yet to be established as a consistent biomarker of CVD. It is also yet to be concretely determined whether the elevation in UII and UT expression in response to cardiovascular disease provides cardioprotective effects, or whether UII augments CVD. Aggregating the reports to date it is evident that the number of reports suggesting UII augments CVD with blockade of the UT receptor providing antiatherogenic, antihypertensive effects outweigh the number of reports suggesting upregulation of UII/UT provides cardioprotective properties in the diseased state. The intracellular pathways stimulated by UII provide some explanation into the differential results reported as the vasculoprotective, vasodilative and antihypertrophic stimulation of NO synthesis, COX activity and TGF_1 can also produce vasoconstrictive, atherogenic, hypertrophic effects, in the pathological, maladaptive state. This indicates that development of a UT blocker may provide therapeutic benefits to 'at risk' patients with CVD or CHF.

As discussed in Chapter 2, UII is highly dependent upon species, tissue and cell-type (Chapter 2.6, page 18), while the development of specific UT antagonists are reportedly capable of having both agonistic and antagonistic effects, again seemingly dependent upon the species type (Chapter 2.2, page 10). Due to these complexities it is important to be aware of studies reporting pathogenic or protective effects of elevated UII concentration or blockade of UT where the non-native species form of UII has been administered, or a UT blocker with reported agonistic effects has been used. Until research is repeated using these guidelines the pathological effects of UII may remain ambiguous. Thus, the following chapters aim to provide significant insight into the cardioprotective or pathogenic effects of UII/URP in the setting of acute, global ischemia in the isolated, perfused rat heart. We determine the effects of both agonistic (UII/URP) activity and antagonism of UT upon recovery from ischemia.

5. Urotensin II and urotensin II-related peptide (URP) in cardiac ischemia-reperfusion injury (A)

(The following chapter is a copy of the published article Prosser et al. Peptides, 2008 May;29(5):770-7)

Abstract

Circulating urotensin II (UII) concentrations and the tissue expression of its cognate receptor (UT) are elevated in patients with cardiovascular disease (CVD). The functional significance of elevated plasma UII levels in CVD is unclear. Urotensin-related peptide (URP) is a paralog of UII in that it contains the 6 amino acid ring structure found in UII. Although both peptides are implicated as bioactive factors capable of modulating cardiovascular status, the role of both UII and URP in ischemic injury is unknown. Accordingly, we provide here the first report describing the direct cardiac effects of UII and URP in ischemia-reperfusion injury. Isolated perfused rat hearts were subjected to no-flow global ischemia for 45 min after 30 min preconditioning with either 1 nM rUII or 10 nM URP. Both rUII and URP induced significant vasodilation of coronary arteries before (both $P<0.05$) and after ischemia (both $P<0.05$). Rat UII alone lowered contractility prior to ischemia ($P=0.053$). Specific assay of perfusate revealed rUII and URP both significantly inhibited reperfusion myocardial creatine kinase (CK) release ($P=0.012$ and 0.036 respectively) and atrial natriuretic peptide (ANP) secretion ($P=0.025$). Antagonism of the UT receptor with 1 μM palosuran caused a significant increase in perfusion pressure (PP) prior to and post ischemia. Furthermore, palosuran significantly inhibited reductions in both PP and myocardial damage marker release induced by both rUII and URP. In conclusion, our data suggests rUII and URP reduce cardiac ischemia-reperfusion injury by increasing flow through the coronary circulation, reducing contractility and therefore myocardial energy demand, and inhibiting reperfusion myocardial damage. Thus, UII and URP present as novel peptides with potential cardioprotective actions.

5.1. Introduction

Urotensin II (UII) is a cardiovascular peptide with an array of biological effects that are species and tissue specific (Douglas et al. 2000; Prosser et al. 2006). Thus, UII has been shown to constrict and dilate vascular tissue (Bottrill et al. 2000; Katano et al. 2000; Opgaard et al. 2000), increase and decrease blood pressure and contractility *in vivo* (Ames et al. 1999; Hassan et al. 2003; Watson et al. 2003; Zhu et al. 2004), stimulate vascular smooth muscle cell proliferation (Watanabe et al. 2001a; Watanabe et al. 2001b), and promote cardiomyocyte hypertrophy (Tzanidis et al. 2003; Zoccali et al. 2006). Blood levels of UII and the tissue expression of its cognate G protein-coupled receptor (UT) are both elevated in patients with congestive heart failure (CHF) and other cardiovascular diseases (Douglas et al. 2002; Richards et al. 2002; Russell et al. 2003; Heringlake et al. 2004; Lapp et al. 2004).

Recently a precursor cDNA peptide encoding the same conserved 6 amino acid ring as UII was isolated from rat, mouse and human tissues. This UII paralog has been named urotensin-related peptide (URP) and is a second endogenous ligand for the UT receptor (Sugo et al. 2003; Chatenet et al. 2004), with studies administering URP both *in vitro* and *in vivo* reporting biological actions similar to UII (Sugo et al. 2003; Chatenet et al. 2004). The distribution of prepro-URP in human and rat tissue is widespread, including brain, and the cardiovascular system (Sugo et al. 2003). However, its biological effects in these systems are unclear.

Despite a number of reports documenting cardiovascular actions of UII (Ames et al. 1999; Douglas et al. 2004; Richards and Charles 2004; Zhu et al. 2004) its role in cardiac ischemia-reperfusion injury is unclear. Experimental animal models of heart failure suggest UII may have a pathological role, as antagonism of UT results in reduced mortality and improved recovery of heart function in rats (Watanabe et al. 2001a; Bousette et al. 2006a; Bousette et al. 2006b). In contrast, patients with significantly elevated plasma UII levels have improved outcomes post myocardial infarction, suggesting UII may play a cardioprotective role (Khan et al. 2006; Zoccali et al. 2006). Furthermore, any role for URP in cardiac ischemia-reperfusion injury is unreported. Accordingly, we provide here the first documentation of the species-

specific actions of rUII and URP in cardiac ischemia-reperfusion injury and their comparative effects upon contractile and cardioendocrine function.

5.2 Methods

5.2.1 Materials

All animals used in this study were male Sprague-Dawley rats (250-400 g, 60-75 days old) obtained from the Christchurch School of Medicine, Christchurch, New Zealand. Rats had free access to standard rat chow and water and were housed under controlled temperature (21ºC), humidity (~40%) and natural day length.

Rat urotensin II (rUII) and URP were obtained from Phoenix Pharmaceuticals (Belmont, USA). UII receptor antagonist palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt) was a generous gift from Dr M Iglarz, Actelion Pharmaceuticals Ltd (Switzerland) (Clozel et al. 2004). All peptides and palosuran were diluted in distilled water, aliquoted and stored at -20ºC prior to use.

5.2.2 Langendorff isolated heart preparation

Isolated heart perfusion was carried out as previously described (Pemberton et al. 2005; Prosser et al. 2006). Briefly, rats were anesthetized with sodium-pentobarbitol (50mg/kg, i.p.). The heart was exposed and cannulated above the aortic valve with oxygenated (95% O_2/5% CO_2) buffer comprising (mM): 123 NaCl, 22.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.1 $MgSO_4.7H_2O$, 1.5 $CaCl_2.H_2O$ and 11.0 glucose (final pH 7.40). Buffer was maintained at 37ºC. The heart was rapidly excised and attached to the Langendorff apparatus perfused retrograde at 12 ml/min. The left atrium was removed allowing an ethanol-filled balloon (attached to a pressure transducer) to be inserted into the left ventricle, enabling the measurement of left ventricular developed pressure (DP) and rate of change in pressure (±dP/dt). A side-arm cannula attached to a pressure transducer was inserted into the rubber aortic cannula measuring perfusion pressure (PP), a direct measure of coronary artery resistance. All data were recorded using a Powerlab Chart 5 System (ADInstruments). All hearts were paced at 330 bpm using a Digitimer DS2A-Mk. II stimulator placed on the right atrium. In all studies

hearts were allowed to equilibrate for 30 minutes before any experimental procedure was started (see Appendix B.1 for supplementary information and methodology). All experiments in the current study were approved by the University of Otago Animal Ethics Committee.

5.2.3 Ischemia-reperfusion protocols

Hearts were allowed to stabilize before being infused with either 1 nM rUII, 10 nM URP, 1μM palosuran or vehicle continuously for 30 min (0.5 ml/min). The doses of rUII and URP were employed based on our previous work (Prosser et al. 2006). The concentration of 1 μM palosuran was based on the manufacturers' report (Clozel et al. 2004). Infusion of the drug/vehicle was then stopped and hearts underwent no-flow global ischemia for 45 min with pacing halted. Hearts were then reperfused with pacing for 105 min following ischemia (Figure 5.1).

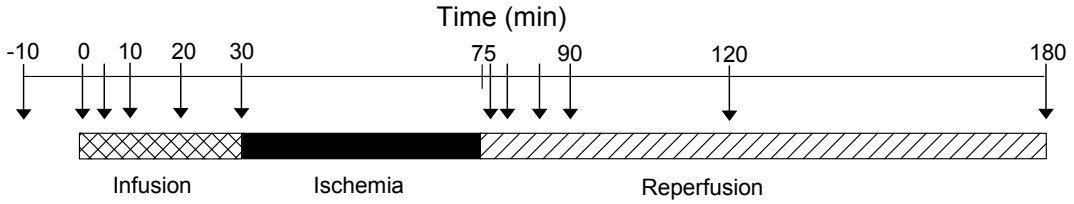


Figure 5.1. Experimental protocol of ischemia-reperfusion. Rat hearts underwent preconditioning infusion (30 min), followed by no-flow global ischemia (45 min), and a reperfusion period (105 min). Arrows indicate hemodynamic and hormonal sampling timepoints.

5.2.4 Analysis of Atrial Natriuretic Peptide (ANP) secretion and myocardial Creatine Kinase (CK) release

Perfusate samples were collected at specific time points after passing through the heart (Figure 5.1). ANP release was measured by extracting perfusate samples using SepPak columns and measured by radioimmunoassay (RIA) as previously described (Pemberton et al. 2000, refer Appendix D.2). Perfusate creatine kinase (CK) concentrations were determined on an Abbot Aeroset (Canterbury Health Labs, Christchurch, New Zealand).

5.2.5 Statistical Analysis

All data are presented as mean + S.E.M. Analysis of changes in cardiac hormones and hemodynamics measured were performed on SPSS using a two-way ANOVA with repeated measures, with Bonferroni's multiple comparison test, post hoc. Cumulative data comparisons were made using a paired student's t-test. In all statistical tests a value of $P<0.05$ was considered significant.

5.3. Results

5.3.1 RUII and URP reduce PP prior to and following ischemia

Both rUII and URP significantly reduced PP during preconditioning by a maximum of 8 % at 10 min ($P<0.05$, Figures 5.2A and 5.2B, respectively) compared with vehicle. During the first 15 min of reperfusion this vasodilation was retained in both rUII and URP preconditioned hearts with maximum reductions of 16 and 18% respectively compared with vehicle ($P<0.05$, Figures 5.2A and 5.2B), before returning to vehicle levels.

5.3.2 Effect of UT receptor antagonism upon PP

Infusion of 1 μM palosuran alone caused a significant increase in PP (mean=9.5 ±1.7 %, $P<0.05$) during the 30 min preconditioning phase (Figures 5.2A and 5.2B). Co-infusion of palosuran with 1nM rUII significantly attenuated rUII-induced reductions in PP during preconditioning ($P<0.05$, Figure 5.2A). Palosuran maintained this attenuation of rUII during early reperfusion, entirely blocking the rUII-induced reduction in PP ($P<0.05$).

Palosuran also significantly inhibited URP-induced reductions in PP during preconditioning ($P<0.05$, Figure 5.2B). Post-ischemia, palosuran had no influence on URP actions upon PP.

5.3.3 The effect of rUII, URP and palosuran on left ventricular (LV) contractility following ischemia

Compared with control, preconditioning with rUII tended to reduce DP (P=0.053, Figure 5.3A), both + and –dP/dt and left ventricle (LV) systolic pressure. This trend remained during reperfusion.

Preconditioning with 1 μM palosuran alone induced a reduction in DP by 5 ±1.8 % (P<0.05, Figure 5.3A) and also reduced both + and –dP/dt (P=0.004 and 0.017 respectively, Figures 5.3C and 5.3D) and LV systolic pressure, (P=0.002, Figure 5.3E). Palosuran had no effect on LV end diastolic pressure throughout the study. During reperfusion, palosuran tended to increase DP above control (P=0.074), and it attenuated the rUII-induced reduction in DP (P=0.058, Figure 5.3A). The same response was repeated observing + and –dP/dt, where palosuran attenuated the rUII-induced reduction (P=0.088 and 0.117 respectively, Figure 5.3C and 5.3D), however showed no attenuation of LV systolic pressure (P=0.17, Figure 5.3E).

Preconditioning the heart with 10 nM URP had no significant effect on DP or ±dP/dt before or after ischemia when compared with control (Figure 5.3B). Co-infusion of palosuran with URP had no significant effect during preconditioning, however raised DP above control (P>0.05) during reperfusion.

5.3.4 Myocardial CK and ANP release following ischemia

Both rUII (Figure 5.4A) and URP (Figure 5.4B) significantly reduced perfusate CK levels during reperfusion, when compared with control vehicle (P=0.012, and 0.036 respectively). Preconditioning the heart with 1 μM palosuran alone also significantly reduced CK release below that of control vehicle (P=0.013, Figure 5.4A). Co-infusion of 1 μM palosuran with 1 nM rUII (Figure 5.4A) or 10 nM URP (Figure 5.4B) did not significantly alter CK release compared with each respective peptide alone.

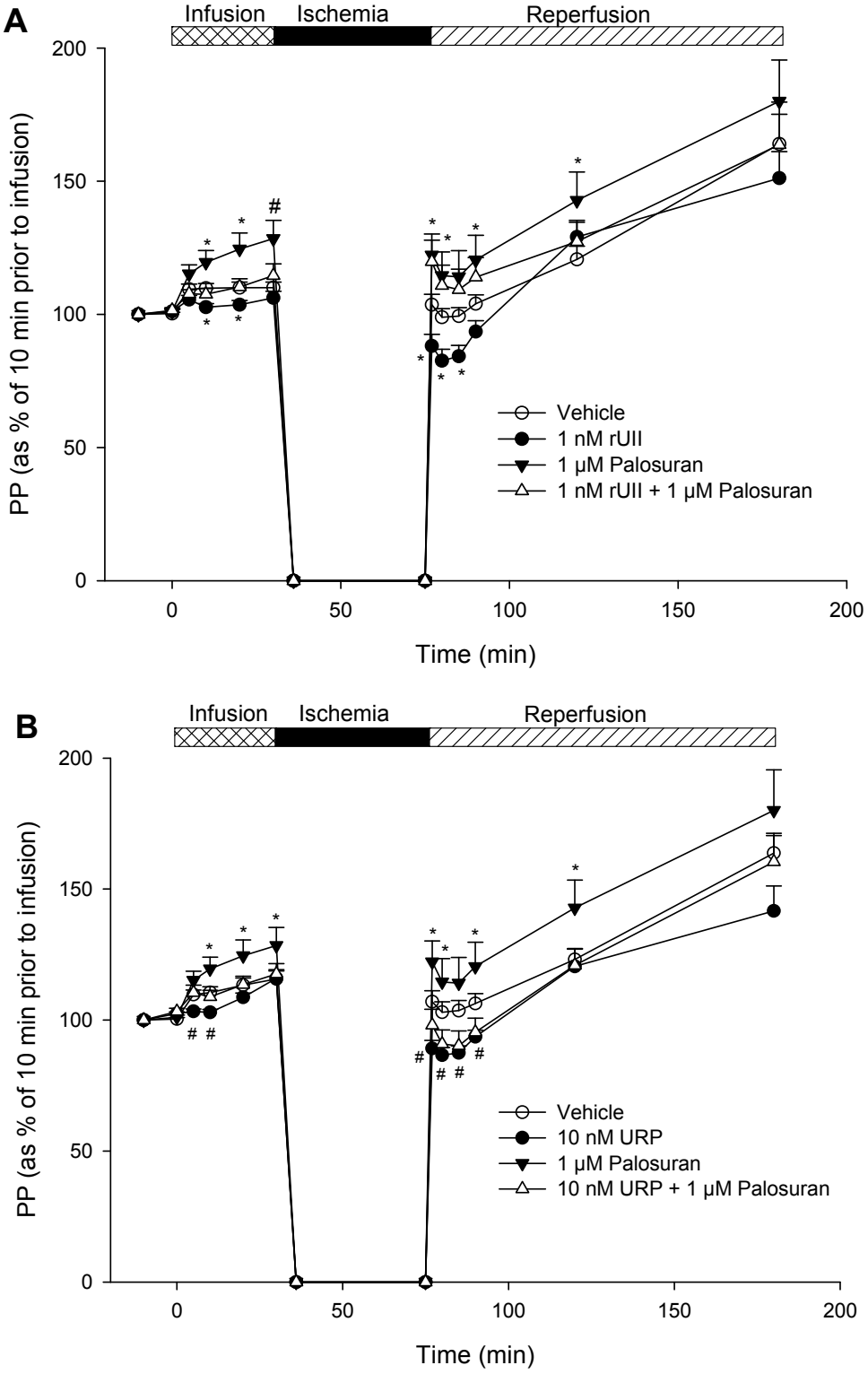


Figure 5.2. Changes in coronary perfusion pressure (PP) in response to (A) 1 nM rUII, and (B) 10 nM URP with and without the UT receptor antagonist palosuran during ischemia-reperfusion. All data represented as mean +SEM % relative to time = -10 min prior to infusion of drug or vehicle. ANOVA revealed significant time points of difference between vehicle and rUII/URP, or vehicle and 1 µM palosuran (*P<0.05, # P<0.01).

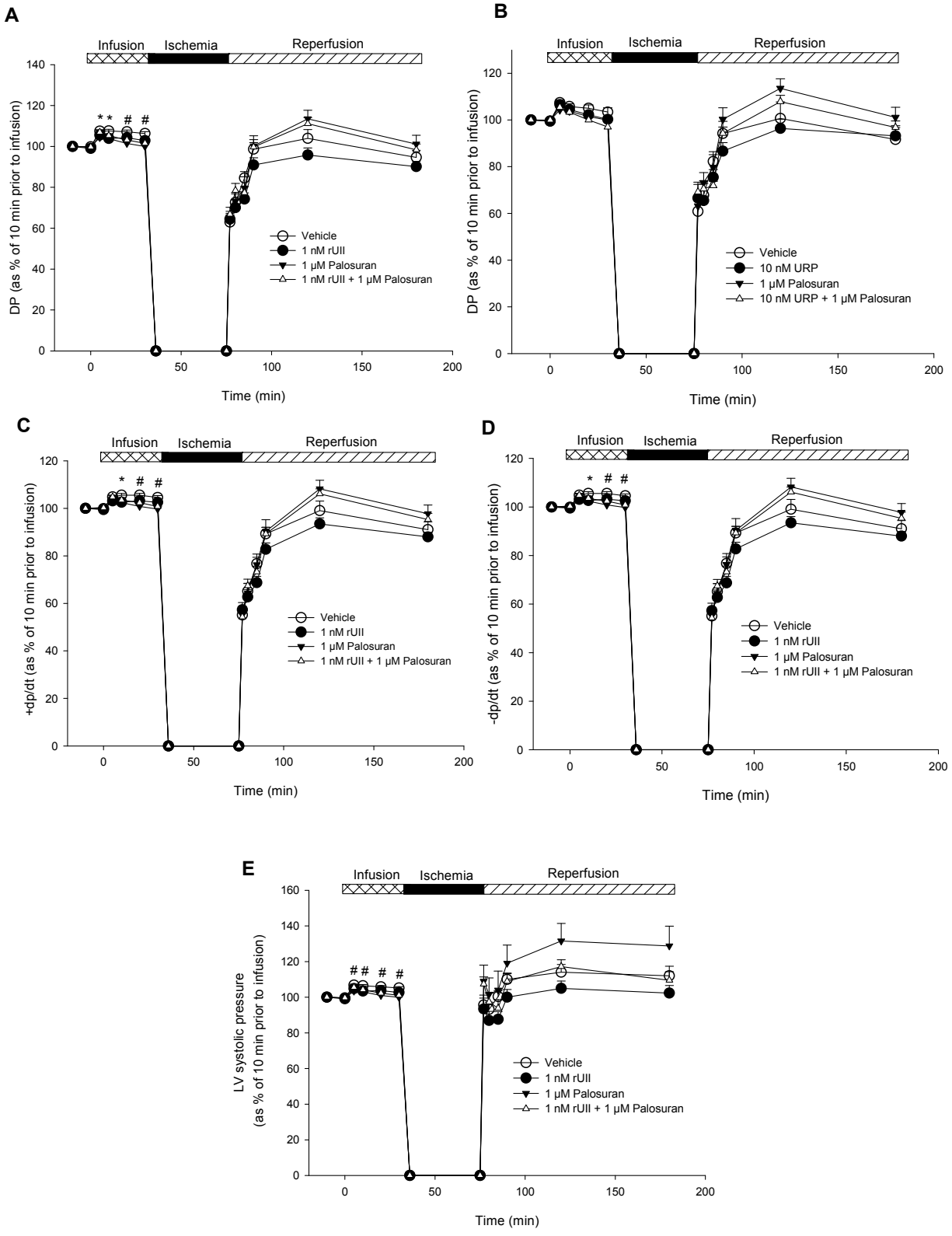


Figure 5.3. Left ventricular developed pressure (DP) in hearts preconditioned with 1 nM rUII (A) and 10 nM URP (B), with and without 1 µM palosuran. Further analysis revealed significant changes in left ventricular ±dP/dt (C & D) and left ventricular maximum systolic pressure (E), (end diastolic pressure not shown as did not differ from control). All data represented as mean +SEM % relative to 10 min prior to infusion of drug or vehicle. One-way ANOVA revealed significant points of difference between vehicle and rUII, or vehicle and 1 µM palosuran (*P<0.05, # P<0.01).

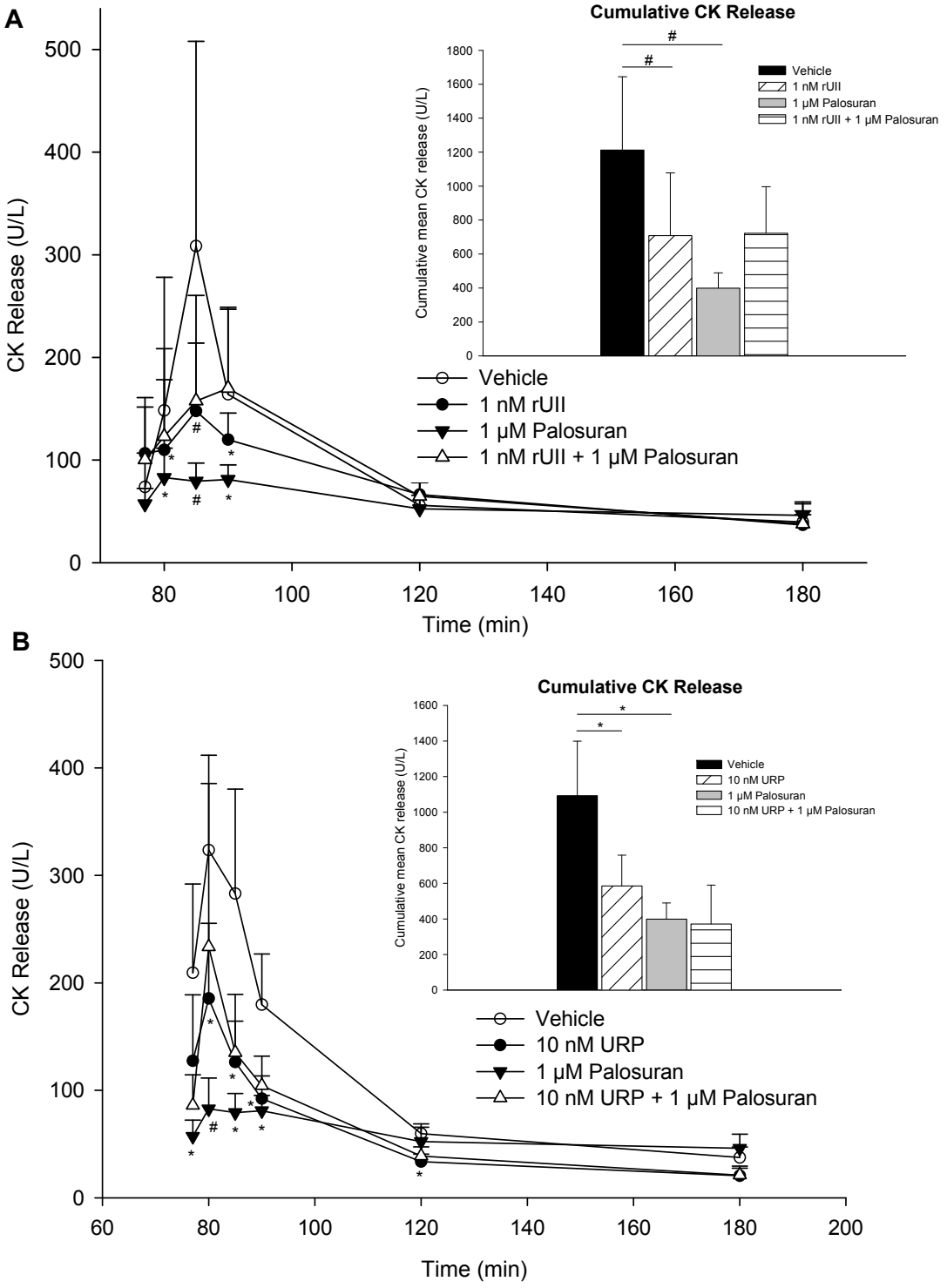


Figure 5.4. Myocardial creatine kinase (CK) release during reperfusion in isolated hearts preconditioned with (A) 1 nM rUII or (B) 10 nM URP, with or without the presence of 1 µM palosuran. Perfusate samples were collected after passing through the heart at specific time points throughout the reperfusion period (line graph) and cumulative CK release was calculated (inset, bar graph). One-way ANOVA revealed significant points of difference between vehicle and rUII, or vehicle and 1 µM palosuran (*P<0.05, # P<0.01).

RUII preconditioning had no significant effect upon basal ANP release. Preconditioning hearts with 1 nM rUII significantly attenuated the sharp increase in ANP secretion observed in vehicle treated hearts upon reperfusion ($P=0.025$, Figure 5.5A). Hearts preconditioned with palosuran alone also showed a reduction in ANP secretion during reperfusion (Figure 5.5A). In contrast, co-infusion of rUII with palosuran resulted in a marked increase in ANP release well above control levels following ischemia ($P<0.05$, Figure 5.5A).

Infusion of 10 nM URP had no significant effect on ANP release (Figure 5.5B). As observed with rUII, co-infusion of 1 μM palosuran with 10 nM URP resulted in greatly increased ANP secretion above that of control vehicle, ($P<0.05$, Figure 5.5B).

5.4 Discussion

We provide here the first documentation that: 1) rUII and URP dilate cardiac coronary arteries both prior to and post-ischemia. 2) RUII weakly reduces left ventricular (LV) contractility post-ischemia but URP does not. 3) Both rUII and URP significantly reduce cardiac CK release upon reperfusion, whereas post-ischemia ANP release is attenuated in the isolated heart when preconditioned with rUII only. 4) Palosuran has a constrictor action in the coronary vasculature, consistent with its blockade of a vasodilatory ligand such as UII. 5) Palosuran exhibits differing actions on the vascular activity of UII and URP in the heart. 6) Hearts preconditioned with palosuran, rUII or URP alone had reduced CK and ANP release post-ischemia-reperfusion, whereas co-infusion of palosuran with rUII or URP elevated CK and ANP release post-ischemia-reperfusion. Taken together the findings suggest that when infused alone, palosuran is a partial agonist of the UT receptor.

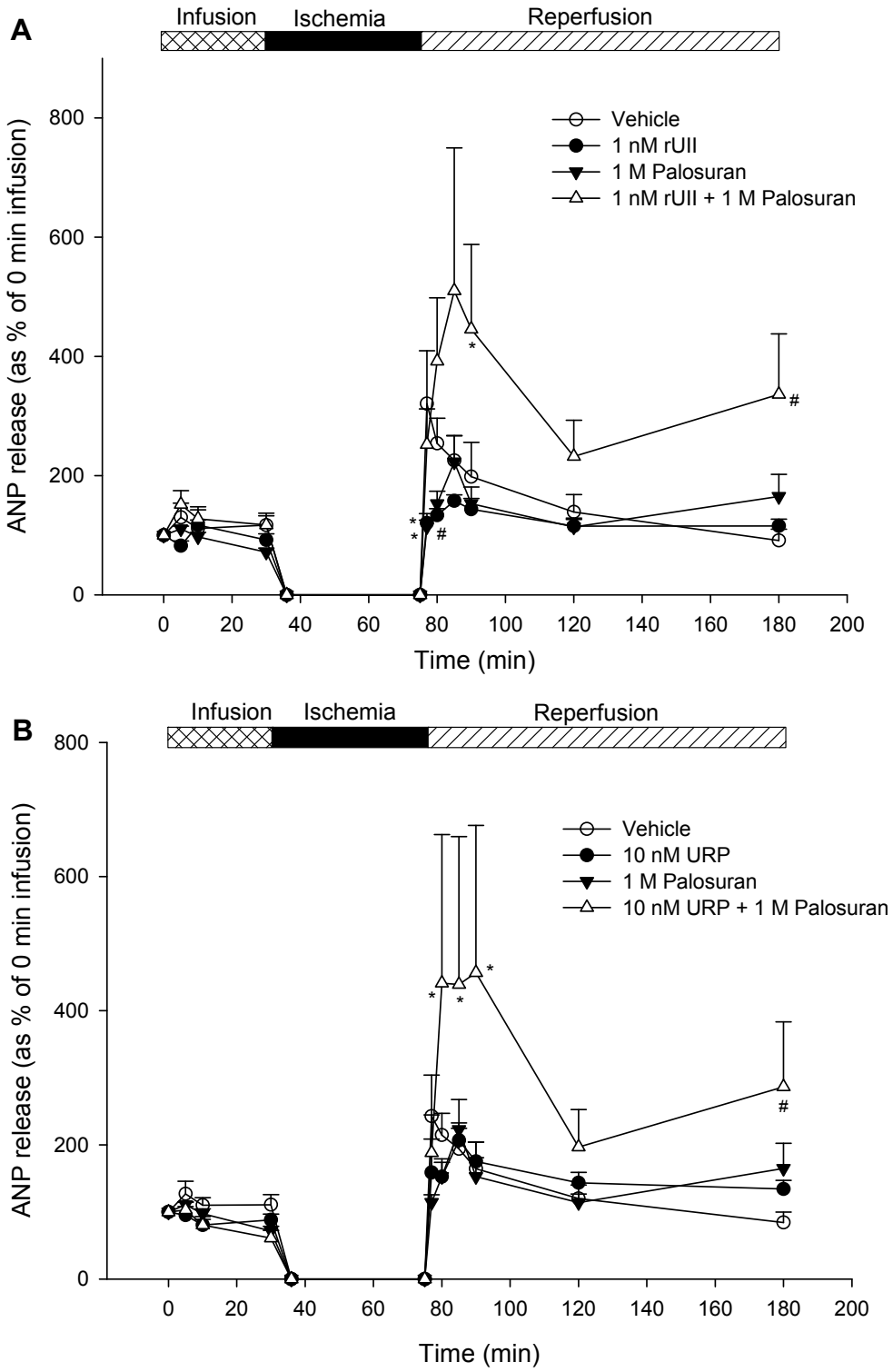


Figure 5.5. Atrial Natriuretic Peptide (ANP) secretion in the isolated rat heart undergone ischemia-reperfusion preconditioned with (A) 1 nM rUII or (B) 10 nM URP with or without the presence of 1 μM palosuran. Data are shown as mean +SEM % relative to 0 min infusion. One-way ANOVA analysis revealed significant points of difference between vehicle and 1 nM rUII or 10 nM URP, vehicle and 1 μM palosuran, or vehicle and palosuran + rUII/URP (*P<0.05, # P=0.01).

Circulating UII concentrations have been reported to range between 0.6 pmol/L to 29 nM/L (Totsune et al. 2001; Dschietzig et al. 2002; Heller et al. 2002; Richards et al. 2002; Gruson et al. 2005; Zoccali et al. 2006; Dai et al. 2007) and are elevated above normal levels in patients with coronary artery disease (Lapp et al. 2004; Hassan et al. 2005), atherosclerotic lesions of human aorta (Bousette et al. 2004), ischemic heart failure (Heringlake et al. 2004), hypertension (Suguro et al. 2007) and congestive heart failure (Richards et al. 2002). Thus, the concentration of rUII infused in the current study is within the physiological range. No study has yet measured the circulating concentration of URP in humans. However, the pEC_{50} of hUII and URP in isolated cells have been shown to be equivalent in binding affinity (pEC_{50} 8.55 and 8.68 respectively) while URP showed reduced potency in constricting rat aortic rings (pEC_{50} of 8.11 for hUII and 7.75 for URP) (Chatenet et al. 2004).

To our knowledge, this is the first comparative study observing the species specific effects of rUII and URP in a model of global ischemia-reperfusion. A single report studying the effect of human UII in the ischemic rat heart (Zhou et al. 2003) reported UII-induced reductions in coronary flow and contractile function, alongside increased lactate dehydrogenase release during reperfusion. In contrast, we utilized the species specific form of UII (rat), and consistent with our previous work (Prosser et al. 2006) documented vasodilatory and potential cardio-protective effects upon recovery from ischemia. The assumed effect of UII has been vasoconstriction with reports showing UII constricts multiple artery types *in vitro* (Douglas et al. 2000) a trend that continues in *in vivo* animal models of heart failure (Ames et al. 1999; Bousette et al. 2006a; Bousette et al. 2006b). We have previously shown UII and URP to be potent vasodilators of coronary arteries when the species-specific form of UII is used (Prosser et al. 2006). In the present study, rUII and URP significantly reduced PP prior to ischemia, and we have now extended those findings to show that preconditioning the heart with rUII and URP for 30 minutes prior to ischemia results in continued vasodilation during the early stages of subsequent reperfusion.

UII has been reported as both a negative and positive inotrope (Ames et al. 1999; Hassan et al. 2003; Gong et al. 2004; Russell and Molenaar 2004), or to have no effect (Li et al. 2004). In our hands, infusion of rUII caused a minor reduction in DP during the pre-conditioning phase and further maintained DP below control levels

during the initial 5-30 min of reperfusion. RUII weakly reduced +dP/dt and –dP/dt and left ventricular maximum systolic pressure, with no effect on LV end diastolic pressure. The observed reductions in ±dP/dt are consistent with other *in vivo* reports observing the effect of UII on contractility in the monkey (Ames et al. 1999) and rat (Hassan et al. 2003). In contrast, URP had no influence on DP or ±dP/dt, nor did these parameters significantly differ from control during the reperfusion phase following ischemia. This points to differential contractile activities of UII and URP upon the heart. Interestingly, in our hands rUII activity appears to mimic beta-blocker effects including dilation of the coronary arteries and negative inotropism. Reports observing the dependence of UII on β-adrenoreceptors for bioactivity have been performed, but results vary depending upon the method of administration (intravenous or intracerebroventricular) (Watson et al. 2003; Hood et al. 2005). In keeping with our previous work (Prosser et al. 2006), we suggest N- or C-terminus differences in sequence are responsible for the observed contrasting effects of rUII and URP on LV contractility. This minor, although significant reduction in LV contractility in response to rUII was not observed in our previous study (Prosser et al 2006, Chapter 3) where rUII and URP were found to have no significant effect on LV contractility. However, analysis of the earlier study reveals a trend for rUII and URP to reduce LV contractility, although not significantly differing from vehicle levels (Appendix C, Figure C.1). The reason for this discrepancy is suggested to be due to the inconsistent effects produced by UII (as mentioned above), and the reduced variability of the latter study resulting in statictically significant results, despite the changes being very minor.

We measured perfusate CK release from the heart as an index of myocardial damage. We found rUII and URP both significantly reduced CK release, with rUII possibly being more potent. This suggests rUII and URP possess similar cardio-protective qualities, reducing myocardial damage following ischemia.

Differences between the two peptides were revealed when the UT receptor was blocked with palosuran. Hearts infused with palosuran alone had significantly increased PP prior to ischemia, consistent with blockade of a receptor that induces vasodilation. The elevation in PP suggests palosuran blocks endogenous UII/URP

produced by the heart. No reports have yet investigated direct cardiac production of UII/URP in the rat, however many reports have shown UII/URP is produced by the sheep (Charles et al. 2005) and human heart, with UII mRNA and cDNA expressed in many cell types of the human heart (Sugo et al. 2003; Liu et al. 1999; Douglas et al. 2002; Totsune et al. 2004; Tzanidid et al. 2001). Furthermore, co-infusion of palosuran with either rUII or URP abolished reductions in PP induced by rUII and URP during the 30 min infusion period, and significantly inhibited rUII-induced reductions in PP during the initial reperfusion period. In contrast, palosuran had no influence on reductions in PP induced by URP. The reason(s) underlying the observed comparative and disparate hemodynamic effects in response to rUII and URP in the rat heart undergone ischemia-reperfusion may lie in the sequential differences between the two peptides. Both peptides retain the highly conserved 6 amino acid ring structure common to all known species of UII. However, rUII differs from URP having an extra 6 amino acids at the N-terminus, and the C-terminus of URP is Valine, while it is Isoleucine for rUII (Chatenet et al. 2004). The longer N-terminus of rUII may afford it greater time bound to the receptor and in doing so, enable activation of a greater number of intracellular pathways for an extended period of time. The antagonistic potency of palosuran against URP has not previously been tested, in fact, no UT receptor antagonist has yet been directly tested on URP activity, this report being the first study to do so. The effects caused by blockade of the UT-receptor suggests a more complex system with potentially separate pathways being switched on or off in response to inhibition or activation of the UT receptor, and the possibility of more than one receptor capable of binding UII/URP or palosuran.

Hearts preconditioned with rUII, URP or palosuran alone had reduced CK and ANP release, suggesting reduced myocardial damage and stress and enhanced recovery from ischemia. However, the ability of palosuran to also induce cardio-protection was surprising, given its ability to inhibit UII and URP induced vasodilation. The finding that palosuran alone acts weakly in a similar direction to UII/URP for some actions strongly suggests palosuran itself may be a partial agonist of the UT receptor. A similar finding has been reported for the UT receptor antagonist, SB-710411, where it potently antagonized UII-induced inositol phosphate formation at the rat UT receptor, however functioned as an agonist at the monkey UT receptor (Behm et al. 2004). This pattern of activity has been reported for other peptides such as the renin-angiotensin

system where the angiotensin-II receptor antagonist Saralasin caused partial agonistic activity, in a system now found to contain greater than one receptor (Case et al. 1976).

Furthermore, when palosuran was co-infused with rUII or URP, CK and ANP secretion were elevated, suggesting blockade of the UT receptor combined with the elevated presence of rUII/URP results in greater myocardial damage and increased cardiac stress. These findings support the hypothesis that over-expression of UII in diseased cardiac tissue may cause further deleterious effects and inhibit recovery as seen in *in vivo* rat models of ischemia (Bousette et al. 2006a; Bousette et al. 2006b). However, our results serve to highlight the complicated nature of UII and URP signaling in the heart, and suggest that multiple receptors, of distinct types, may be present in the cardiac vascular beds. UT receptor sequence homology differs between species producing differential effects in response to the same species of UII administered (Douglas et al. 2000), while UII can produce vasoconstriction or vasodilation when activating receptors located in smooth muscle cells or endothelial cells respectively (Gendron et al. 2005). Many peptides involved in cardiac homeostasis, such as angiotensin II, endothelin and the family of natriuretic peptides ANP, BNP and CNP have multiple receptors. We therefore suggest UII/URP may activate greater than one receptor triggering multiple signaling pathways (analogous to RAMPS identified for adrenomedullin and CGRP peptides (Conner et al. 2004)) present in cardiac tissue.

Taken together, our data demonstrates that rUII dilates the coronary arteries, induces mild negative inotropic effects and reduces post-ischemia CK and ANP release. In contrast, URP displayed dilation of the coronary arteries and reduced CK release with reduced potency compared to rUII, but had no other significant effects comparable with rUII. These effects were reduced by palosuran. This suggests these peptides have a cardioprotective role, minimizing damage to the myocardium by reducing energy requirements following ischemia.

6. Urotensin II and urotensin II-related peptide (URP) in cardiac ischemia-reperfusion injury

Abstract

Urotensin II (UII) expression is elevated in cardiovascular disease, both within tissue and the circulation. However, the pathological role of UII in mammalian cardiovascular disease is currently undefined. Studies have reported disparate responses to UII, suggesting inhibition of the UII receptor aids recovery from ischemia and attenuates maladaptive myocardial remodelling, while other studies have reported UII can stimulate cardioprotective effects, with elevated circulating UII levels correlated with an overall better outcome and reduced cardiac risk. The current report builds on a previous publication observing the effect of preconditioning hearts with UII or its paralog, urotensin II-Related peptide (URP), on recovery from ischemic injury. However, reports have identified that UII-induced vasodilative activity may be dependent upon the endothelial state. Herein, we explore the effect of administering rat UII and URP upon reperfusion in a Langendorff, isolated rat heart model of ischemia-reperfusion. This provided insight into whether the response to UII and URP is altered following endothelial and myocardial ischemic damage. UII and URP caused significant, sustained dilation of the coronary arteries during reperfusion (both $P<0.001$), and both peptides attenuated myocardial injury, indicated by a significant reduction in creatine kinase (CK) release (both $P<0.005$). UII and URP displayed no significant influence on left ventricular contractile parameters, and did not significantly alter atrial natriuretic peptide (ANP) secretion (both $P>0.05$). In sum, infusion of the native form of UII or URP upon reperfusion stimulated sustained vasodilation, and significantly attenuated ischemic injury.

6.1. Introduction

Prosser et al. recently reported that hearts infused with rat UII (rUII) or urotensin II-related peptide (URP) for 30 min prior to global ischemia displayed significantly less myocardial damage and had greater coronary flow when compared to hearts infused with vehicle (Prosser et al. 2008). Their study effectively mimicked patients with chronically elevated plasma UII concentrations prior to experiencing myocardial ischemia, however a known characteristic of ischemia and cardiovascular disease is endothelial dysfunction (ED). Vascular hypoxia can cause ED, inducing the production of reactive oxygen species (ROS) and stimulating inflammatory agents causing significant damage to the endothelium, negatively altering its intracellular pathways (Schiffrin 2008a). ED is reported to uncouple the production of nitric oxide (NO), converting the process to produce ROS, and in so doing severely reducing the vasodilative potential, as well as promoting ROS-induced cellular damage (Lavi et al. 2008; Schiffrin 2008a). UII has been repeatedly shown to stimulate NO (Katano et al. 2000; Gray et al. 2001; Li et al. 2004; Prosser et al. 2006), dilating coronary arteries in the rat, while experimental removal of the endothelium or infusion of L-NAME caused a loss or reduction in UII-induced vasodilation respectively (Bottrill et al. 2000; Russell et al. 2001; Stirrat et al. 2001).

Here, we provide further research into the effect of rat UII (rUII) and URP on recovery from cardiac ischemia. The Langendorff isolated rat heart model was employed to observe the influence of rUII and URP on recovery from ischemia, when administered following ischemia. In theory, infusion of rUII or URP at the time of reperfusion provides a model to test whether the previously reported vasodilative and cardioprotective effects of rUII/URP in healthy hearts are conserved when administered immediately following ischemia, or whether their protective physiological effects are lost with ischemic injury sustained.

6.2. Methods

6.2.1 Materials

All animals used in this study were male Sprague-Dawley rats (250-400 g, 60-75 days old) obtained from the Christchurch School of Medicine, Christchurch, New Zealand. Rats had free access to standard rat chow and water and were housed under controlled temperature (21ºC), humidity (~40%) and natural day length.

Rat urotensin II (rUII) and URP were obtained from Phoenix Pharmaceuticals (Belmont, USA). Both peptides were diluted in distilled water, aliquoted and stored at -20ºC prior to use.

6.2.2 Langendorff isolated heart preparation

Refer *Chapter 5.2.2* (page 59) of the current thesis.

6.2.3 Ischemia-reperfusion protocol

Once attached to the Langendorff apparatus hearts were allowed to stabilize for 30 min before being paced at 330 bpm for a further 30 min. Following this period perfusion was stopped and hearts underwent 45 min of no-flow global ischemia with pacing halted. At reperfusion, hearts were infused continuously for 30 min (0.5 ml/min) with either 1 nM rUII (n=5), 10 nM URP (n=5) or vehicle (n=10) with pacing recommenced. After the 30 min infusion hearts underwent a further 75 min perfused with buffer alone (Figure 6.1).

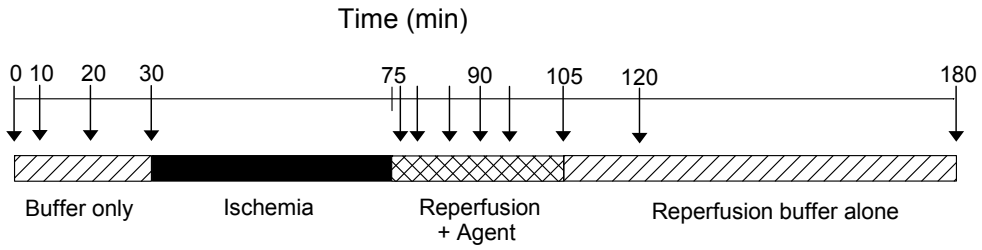


Figure 6.1. Ischemia-reperfusion protocol performed on isolated rat hearts. Arrows indicate times of data/samples taken.

6.2.4 Analysis and Measurement of Atrial Natriuretic Peptide (ANP) secretion and myocardial Creatine Kinase (CK) release

Refer *Chapter 5.2.4* (page 60) of the current thesis

6.2.5 Statistical analysis

Refer *Chapter 5.2.5* (page 61) of the current thesis

6.3 Results

6.3.1 Effects of rUII and URP on PP and LV contractility following ischemia

Infusion of both 1 nM rUII or 10 nM URP at reperfusion caused a potent and sustained reduction in PP of 24.43 ± 2.03 and 16.46 ± 1.46 % below vehicle levels throughout the reperfusion period respectively (both $P<0.01$, Figure 6.2).

Left ventricular contractile parameters (DP, ±dP/dt, systolic and end diastolic pressures) were not significantly influenced by rUII or URP when infused upon reperfusion compared with control (Figures 6.3A-E). However, rUII and URP maintained systolic pressure slightly above vehicle levels throughout reperfusion (Figure 6.3D).

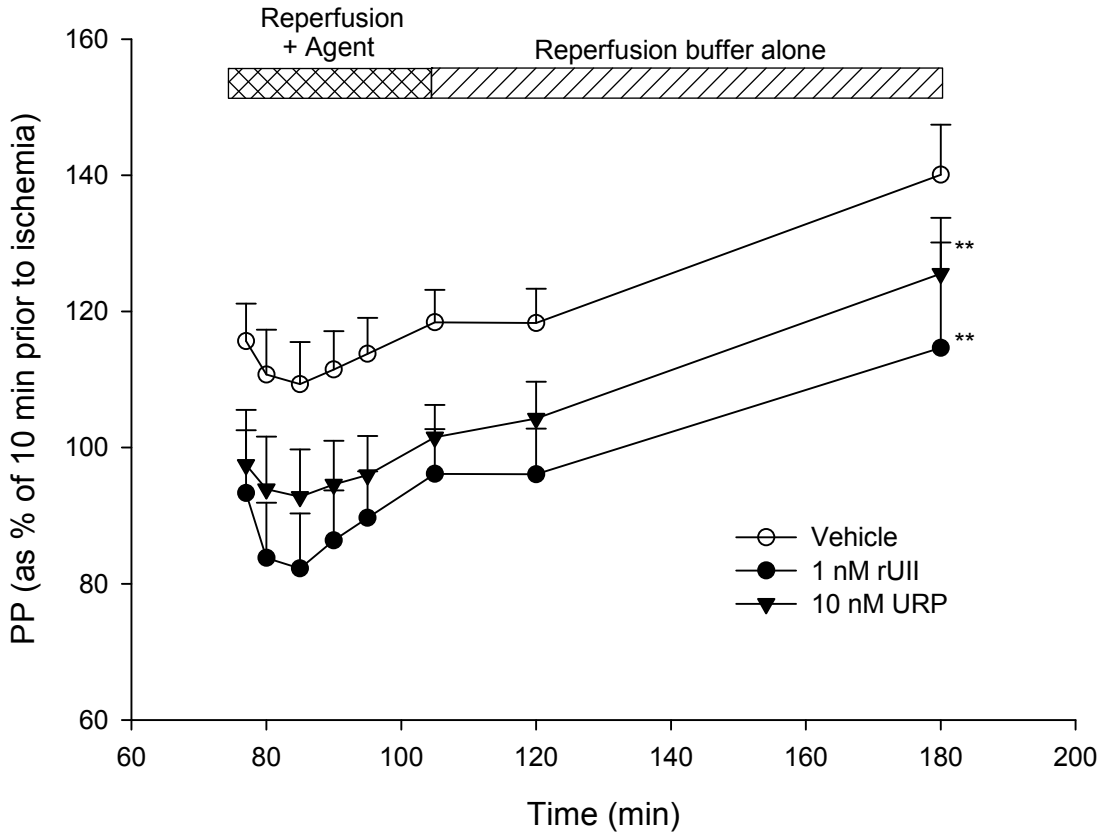
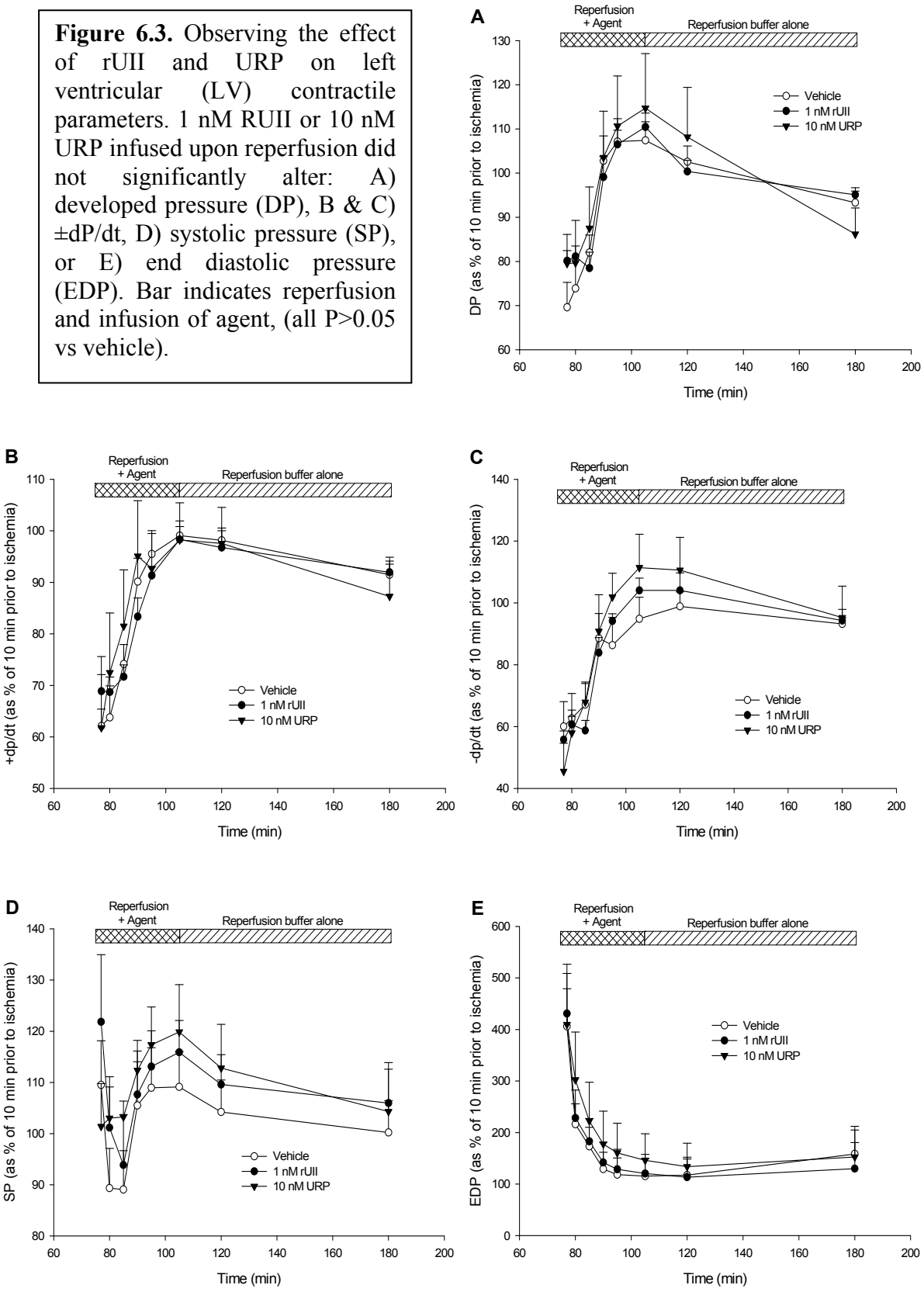


Figure 6.2. Infusion of 1 nM rUII or 10 nM URP significantly reduced perfusion pressure (PP) throughout the reperfusion period maintaining levels significantly below that of vehicle. Bar indicates reperfusion period and the infusion of agent; two-way ANOVA indicated significant differences between control and treatment groups **P<0.005.

Figure 6.3. Observing the effect of rUII and URP on left ventricular (LV) contractile parameters. 1 nM RUII or 10 nM URP infused upon reperfusion did not significantly alter: A) developed pressure (DP), B & C) ±dP/dt, D) systolic pressure (SP), or E) end diastolic pressure (EDP). Bar indicates reperfusion and infusion of agent, (all P>0.05 vs vehicle).



6.3.2 Myocardial CK and ANP release during post-conditioning reperfusion

Both rUII and URP reduced perfusate CK levels significantly below that of vehicle during reperfusion (both $P<0.05$, Figure 6.4) indicating a significant reduction in myocardial damage.

Post-ischaemia, ANP concentrations increased in the perfusate of all three treatments, by a comparable amount. There is a suggestion that rUII might have stimulated a prolonged secretion of ANP in the following period of perfusion with buffer alone, but the variability in ANP titres, coupled with the relatively small sample size precludes any firm conclusions (Figure 6.5).

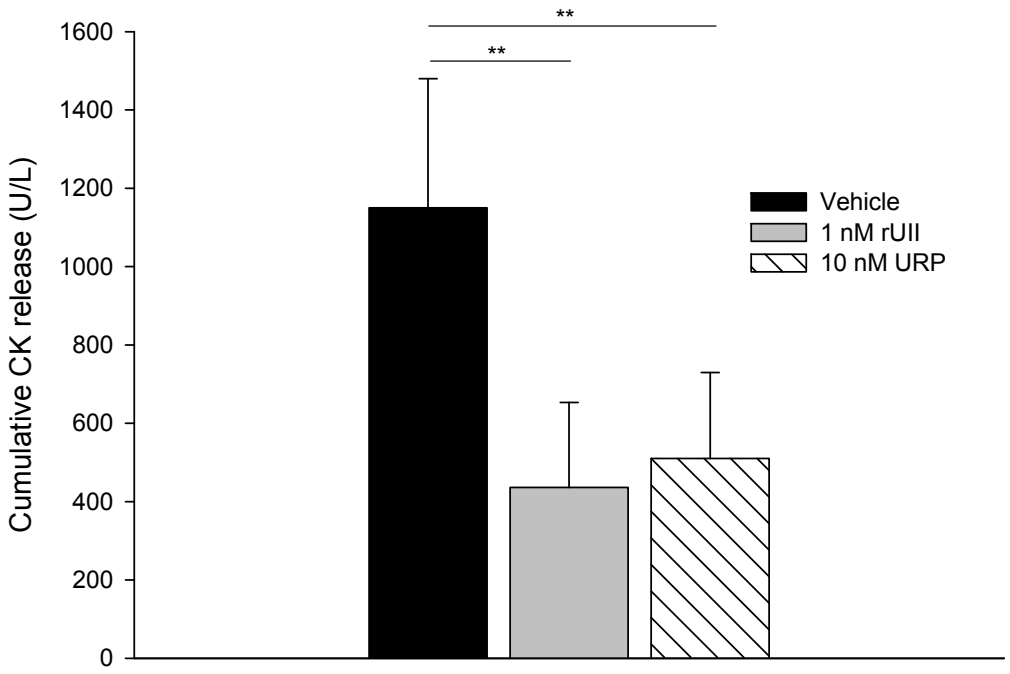


Figure 6.4. Creatine kinase (CK) release was measured from the perfusate at specific time points throughout reperfusion. Analysing cumulative CK release displays hearts infused with 1 nM rUII or 10 nM URP released significantly less CK compared to vehicle, indicating reduced myocardial damage. Bar indicates infusion/reperfusion period, **$P<0.005$ using student's t-test.

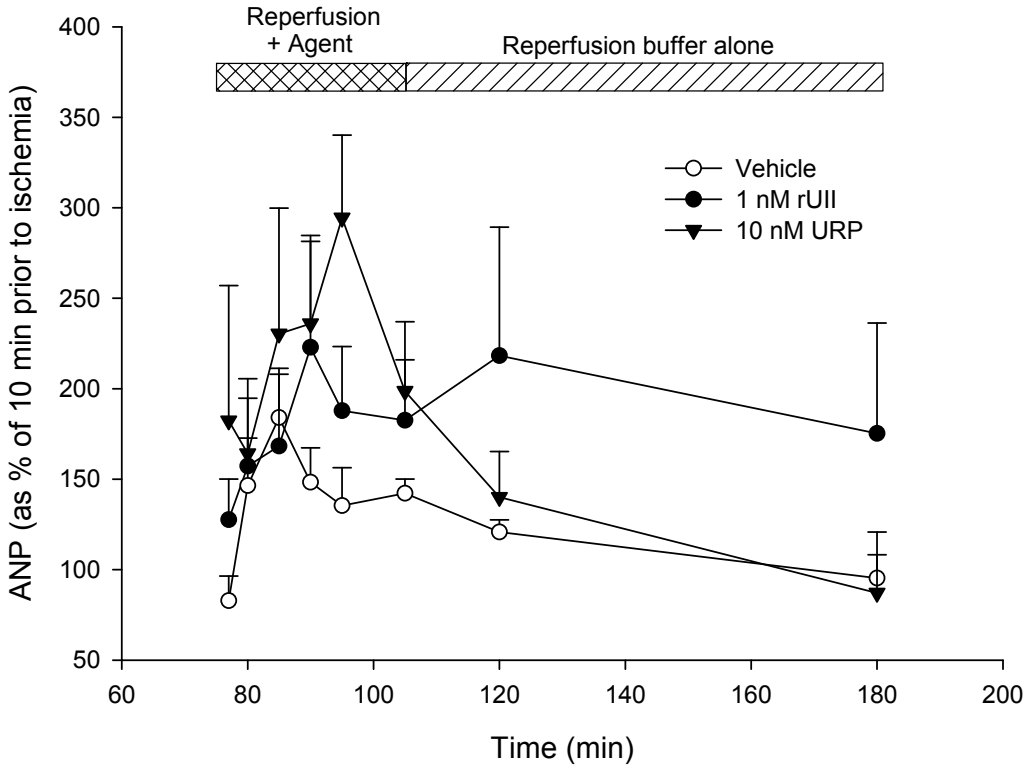


Figure 6.5. Atrial Natriuretic peptide (ANP) secretion during reperfusion in hearts treated with vehicle, 1 nM rUII or 10 nM URP. Data is displayed as % of ANP levels 10 minutes prior to ischemia. Neither rUII nor URP significantly differed from vehicle levels ($P>0.05$).

6.4 Discussion

UII levels are known to be elevated in patients with cardiac or renal diseases (Matsushita et al. 2001; Ng et al. 2002; Richards et al. 2002; Russell et al. 2003; Lapp et al. 2004; Gruson et al. 2005), however it is unclear whether the elevation in UII expression progresses or alleviates disease. Many studies have now reported on the role of UII in cardiac disease, with some indicating UII augments pathogenesis (Watanabe et al. 2001a; Bousette et al. 2006a; Bousette et al. 2006b), while other reports suggest UII possesses cardioprotective properties (Mallamaci et al. 2006; Harris et al. 2007; Prosser et al. 2008); thus the true role of UII and URP in cardiovascular disease remains to be determined.

In our previous paper we reported that infusion of rUII and URP immediately prior to global ischemia significantly reduced cardiac injury during reperfusion through reducing PP, inhibiting CK release and rUII reduced LV ±dP/dt and systolic pressure (Prosser et al. 2008). It was concluded that hearts preconditioned with rUII and URP displayed cardioprotective properties, aiding recovery from ischemia through increased coronary flow allowing greater oxygen supply to the myocardium, reducing myocardial damage, and rUII-induced negative inotropy, together alleviating cardiac energy requirements and aiding recovery (Prosser et al. 2008). This finding was supported by several reports indicating patients with elevated UII levels correlated with a better outcome following a cardiac event compared to those exhibiting lower UII expression (Khan et al. 2006; Mallamaci et al. 2006; Ravani et al. 2008). The cardioprotective, vasodilative actions of UII/URP have been shown to be mediated by NO and prostaglandins released from endothelial cells (Katano et al. 2000; MacLean et al. 2000; Gray et al. 2001; Gardiner et al. 2004; Ishihata et al. 2005a; b). Both NO and cyclooxygenase, the enzyme responsible for prostaglandin production, have been reported to reduce mitochondrial oxygen consumption and provide cardioprotection during early reperfusion in the isolated rat heart (Bolli et al. 2002; Dawn and Bolli 2002). However, it is well documented that during cardiovascular disease where vascular shear stress or ischemia occurs, such as in hypertension, CHF, CAD, atherosclerosis and myocardial infarction the endothelium becomes damaged and dysfunctional. ED causes increased endothelial permeability, due to loss of cellular membrane integrity, as well as significant detrimental alterations in endothelial intracellular functioning (Schiffrin 2008a). This is most evident when observing endothelial production of nitric oxide (NO). In diseased endothelial cells, the intracellular pathway to produce NO becomes uncoupled and results in the production of reactive oxygen species (ROS) (Otani 2009). Ironically, ROS activity directly opposes that of NO, quenching NO availability and thus inhibiting vessel dilative potential, as well as stimulating vasoconstriction, cellular proliferation and further cellular damage, which if unregulated, can progress to the development of atherosclerosis (Bauersachs and Fraccarollo 2008; Lavi et al. 2008). Because UII-induced vasodilation is known to be mediated by NO and stimulation of COX activity, it could be hypothesized that the response to rUII or URP may beome altered in the diseased state. This phenomenom has been observed in human skin *in vivo*

where UII caused dilation of the microvasculature in healthy subjects, but elicited vasoconstriction when infused into CHF patients (Lim et al. 2004).

We found infusion of rUII and URP following ischemia caused potent dilation of the coronary arteries, maintaining PP significantly below vehicle levels throughout reperfusion, including 75 minutes after the infusion of rUII or URP had been halted. Both rUII and URP had no significant effect on DP or ±dP/dT but significantly reduced CK release during reperfusion. These results indicate rUII and URP attenuated myocardial injury and increased coronary arterial flow when infused following ischemia. These results are consistent with our previous report revealing the vasodilative, cardioprotective effects of rUII and URP are conserved when infused either prior to, or following ischemia (Prosser et al. 2008).

The main discrepancy observed between pre- and post-ischemic infusion of rUII/URP in the isolated rat heart was that pre-ischemic infusion caused a reduction in ANP levels (Prosser et al. 2008), while post-ischemic infusion had no effect. This contrast may indicate one example where the response to rUII/URP was influenced by healthy versus damaged cells. Infusing rUII upon reperfusion caused a slight, although non-signficant, elevation in ANP secretion, potentially revealing an association between UII and ANP at the myocardium. UII has been previously shown to stimulate ANP and BNP genes in cardiac myocytes (Zou et al. 2001). This may afford additional cardioprotection as ANP can stimulate vasodilation and possesses antihypertrophic, antifibrotic characteristics (Nishikimi et al. 2006).

Furthermore, DP, ±dP/dT, systolic and end diastolic pressures were not found to significantly differ from vehicle levels, although rUII and URP visibly elevated systolic pressure above vehicle levels throughout reperfusion. This may indicate rUII/URP stimulated a minor increase in LV contractility, potentially explaining the slight elevation in ANP secretion. Infusing rUII into a healthy heart preparation prior to ischemia caused a significant negative inotropic effect, and this was continued following ischemia, although not significantly below vehicle levels (Prosser et al. 2008). Infusion of rUII post-ischemia had no significant influence on DP, ±dP/dT, systolic or ED pressures, but saw a minor, non-significant increase in systolic pressure. This suggests that UII-induced cardiac contractility may be dependent upon

the functional state of the cell.

1 nM rUII was visibly more potent compared with 10 nM URP in lowering PP, despite the 10 fold difference in concentration. Both peptides retain the highly conserved 6 amino acid ring structure, however rUII possesses an extended N-terminus and different C-terminus compared to URP. This sequential difference may be responsible for the difference in potency observed, perhaps affording rUII greater receptor binding time providing extended activation, and/or resisting proteolysis. Receptor-activity and binding studies found URP to have slightly higher binding affinity for the native receptor GPR14 but exhibiting lower potency when compared with hUII (Chatenet et al. 2004), supporting the current findings.

In summary, the sustained reduction in PP in response to rUII or URP following ischemia was comparable to that of infusing either peptide prior to ischemia (Prosser et al. 2008). However, rUII-induced negative inotropy was lost when infused post-ischemia. This indicates that administering UII/URP immediately following the cardiac event was effective in dilating the coronary vasculature and reducing cardiac damage, alleviating injury and aiding cardiac recovery, whilst not influencing left ventricular contractility. Subjecting the heart to no-flow global ischemia for 45 min with zero oxygen supply to the vasculature was assumed to cause substantial vascular and myocardial damage as illustrated by the significant rise in CK release and PP levels exhibited during reperfusion in control hearts. Our results illustrate that rUII and URP protect the cardiovasculature in spite of the vascular damage sustained through stimulating vasodilation and attenuating myocardial damage. Therefore, we advocate UII and URP as potential therapeutic agents providing cardioprotection through both preconditioning the heart prior to a cardiac event, as well as attenuating injury sustained. Although rUII and URP were observed to aid recovery from ischemia, the preventative qualities of UII or URP are yet to be reported.

7. The Renin-Angiotensin-System (RAS)

Abstract

The arterial and venous system is under constant regulatory control, influenced by the production and secretion of constrictive and dilative agents balancing vessel tone, which, in turn, maintains flow and influences blood pressure. Angiotensin II is the main potent, effector product of the renin-angiotensin system (RAS), responsible for elevating blood pressure when it falls below homeostatic levels through activating humoral and mechanical mechanisms. The generation of AngII is seemingly linear, with the primary enzyme renin produced and released from the kidneys in response to a drop pressure cleaving circulating angiotensinogen. To form angiotensin I (AngI). Ang I is then cleaved to angiotensin II (AngII) by the carboxypeptidases angiotensin-converting enzyme 1 (ACE1) and chymase (and other less potent enzymes). Overstimulation of the RAS is well known to develop and promote cardiovascular disease, producing hypertension and progressing atherosclerosis, with many human trials reporting the significant benefits of inhibiting RAS activity and attenuating AngII-induced effects, specifically with regard to cardiovascular disease. Recently, a peptide sharing high sequence homology with angiotensin I (AngI) was identified. Proangiotensin-12 (PA12) was identified and isolated from rat small intestine, and was suggested to be a new component of the RAS. Preliminary studies suggest PA12 may directly stimulate vascular constriction and have other physiological effects within the heart. Little is currently established concerning the physiological effects of PA12, its mechanisms of function, or the receptors and enzymes potentially mediating its activity. The current review provides a general introduction to the RAS, observing the peptides and enzymes responsible for regulating its activity, as well as introducing PA12 as a novel RAS peptide, and reviewing the pathological effects of the RAS.

7.1 Introduction to the RAS

Maintenance of blood pressure at the homeostatic level to provide sufficient perfusion to all tissue throughout the body, whilst not exerting unnecessary work on the heart, is regulated by both mechanical and humoral mechanisms. One key system controlling these mechanisms is the renin-angiotensin system (RAS), which elevates blood pressure via: stimulating arteriolar vasoconstriction, increasing sympathetic activity, secreting aldosterone from the adrenal gland, increasing tubular reabsorption of Na^+ and Cl^- while excreting K^+ to increase water retention, stimulating the release of antidiuretic hormone (ADH) from the pituitary, and reducing glomular filtration rate (Robertson and Nicholls 1993). The RAS is also involved in the central nervous system where it can stimulate thirst (Serova et al. 2004). The RAS is initially stimulated at the kidneys where juxtaglomerular cells produce renin in response to a drop in pressure. Renin is the initial enzyme responsible for triggering the hormonal cascade converting hepatically secreted angiotensinogen to the effector peptide, AngII (Figure 7.1). Angiotensinogen (Ang) circulates as a 452 amino acid precursor molecule, which is cleaved by renin to form angiotensin I (AngI), comprising 10 amino acids of Ang's N-terminal end. Secondary enzymes are then responsible for converting AngI into biologically active AngII including angiotensin converting enzyme 1 (ACE1), chymase, and other less common carboxypeptidases. AngII circulates throughout the body causing potent pressor effects listed above. Once homeostatic blood pressure at the kidney is restored, the juxtaglomerular cells halt the secretion of renin preventing further stimulation of the RAS, providing a negative feedback system.

Further enzyme activity can produce shorter AngII-derived peptides including Ang1-7, AngIII (Ang 2-8) and AngIV (3-8). AngIII and AngIV elicit a similar, although weakened response compared with AngII, whereas Ang1-7 has been suggested to stimulate vasodilation and inhibit cardiac remodelling, opposing the effects of AngII and potentially providing some regulatory control of the RAS (Dias-Peixoto et al. 2008; Mercure et al. 2008). More recently, a peptide has been identified in the rat with the same amino acid sequence as AngI plus an additional -Leu^{11}-Tyr^{12} at its C-terminus, comprising a 12 amino acid peptide dubbed proangiotensin-12 (PA12) (Figure 7.2) (Nagata et al. 2006). Currently, PA12 has only been isolated from the rat

and it is yet to be determined whether PA12 is also present in other mammals, including humans, or what role it has within the RAS.

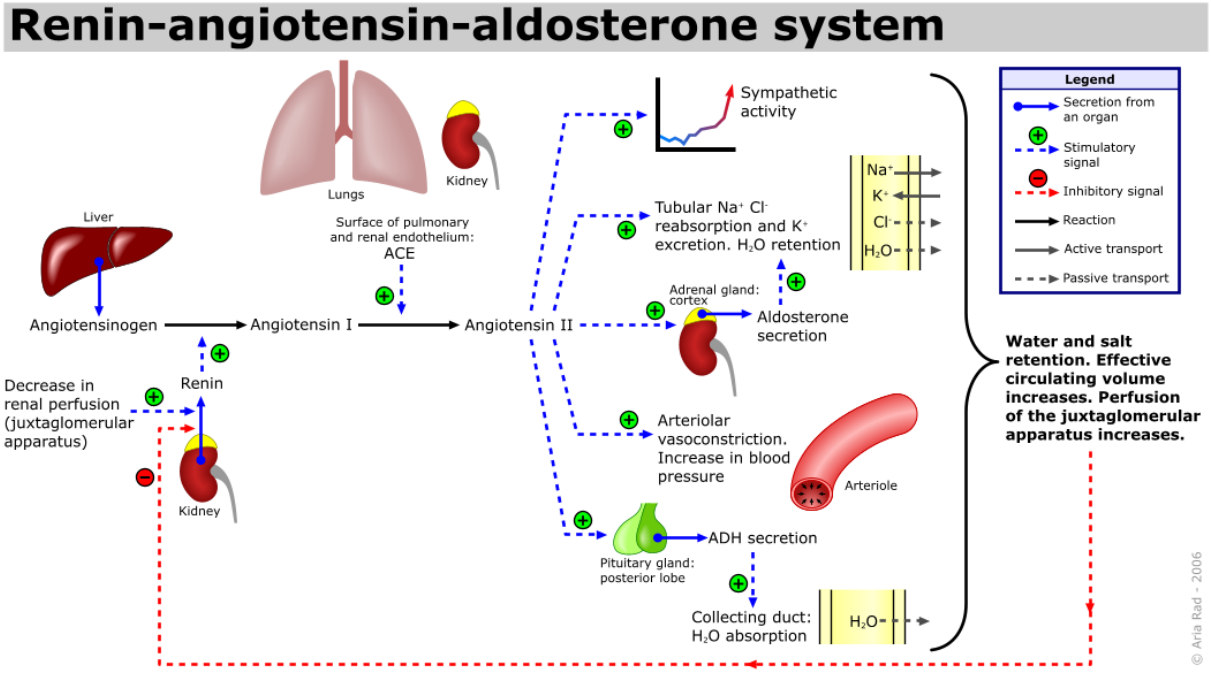


Figure 7.1. Schematic diagram of the Renin-Angiotensin System (RAS) displaying the generation of the effector peptide, Angiotensin II, and its wide range of hypertensive effects throughout the body. Figure from Rad A. 2006

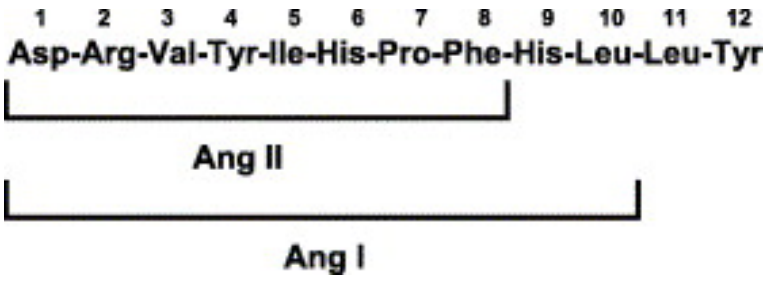


Figure 7.2. Amino acid sequence of proAngiotensin-12 (PA12) compared with Angiotensin II (Ang II) and Angiotensin I (Ang I).

7.2. Angiotensin II-generating enzymes

7.2.1 Angiotensin-converting enzyme I (ACE1)

ACE1 is a carboxypeptidase with 2 catalytic domains enabling the cleavage of 2 amino acids from the C-terminus of AngI to produce AngII. ACE1 not only cleaves off the C-terminus of AngI to produce AngII, but it also inactivates bradykinin via cleavage of similarly placed residues contained within bradykinin's C-terminus. The physiological result is that ACE1 effectively augments the pressor effect via activation of the vasoconstrictor AngII, combined with simultaneous inactivation of the vasodilator bradykinin (Robertson and Nicholls 1993).

ACE1 is currently believed to be the primary enzyme in converting AngI to AngII and is bound to the plasma membrane of endothelial cells present throughout many tissue beds, including arteries and veins. The kidneys and lungs express the greatest levels of ACE1 and ACE1 mRNA (Uchide et al. 2006), indicating the greatest production at these sights, however ACE1 is also known to be present in the brain (Whiting et al. 1991), reproductive organs (Strittmatter et al. 1985; Vivet et al. 1987) and cardiovasculature (Danser and Schalekamp 1996; Borland et al. 2005).

The function of ACE1 makes it an ideal target for treating hypertension and chronically elevated high blood pressure due to overactivation of the RAS. The use of ACE1 inhibitors for treating hypertension is touched on below in section 7.3 (page 92).

7.2.2 Angiotensin-converting enzyme II (ACE2)

Recently, a second angiotensin-converting enzyme has been identified in the RAS, angiotensin-converting enzyme 2 (ACE2). ACE2 is an 805 amino acid protein exhibiting 60% homology with ACE1 with its gene located on the sex chromosome X as opposed to the ACE1 gene, located on chromosome 17 (Hubert et al. 1991; Palmer et al. 2008). Like ACE1, ACE2 is an endothelium-bound carboxypeptidase, but differs in that it only possesses one cleavage site, and is thus capable of cleaving only 1 amino acid from its target peptide (Oudit et al. 2003). Furthermore, compared to the wide tissue distribution of ACE1, ACE2 is expressed primarily within the endothelium and VSMCs of arteries, arterioles and venules of the heart and kidney

(Donoghue et al. 2000), consistent with its suggested proteolytic role in cleaving vasoactive, pressure-regulating peptides.

ACE2 has been shown to cleave AngI into inactive Ang1-9 (Dales et al. 2002), which can then be cleaved by ACE1 into Ang1-7 (Donoghue et al. 2000). ACE2 can also directly convert AngII into Ang1-7 (Vickers et al. 2002). The generation of Ang1-7 suggests ACE2 may mediate vasodilation, directly opposing the effects of ACE1 providing a check on AngII activity. Interestingly, ACE2 mRNA levels are shown to be significantly reduced in hypertensive rats compared with normotensive animals (Crackower et al. 2002), and ACE2 expression is not elevated in the failing human myocardium (Donoghue et al. 2000), together suggesting ACE2 production may be independent of blood pressure. In ACE2 knock-out mice, circulating AngII levels were double that of normal mice and the animals exhibited reductions in cardiac contractility, and reduced ±dP/dt compared with wild types (Crackower et al. 2002). Further studies have reported ACE2 stimulates cardioprotective effects by producing Ang1-7 and attenuating AngII-induced endothelial ROS production, attenuating the development of atherosclerosis, aiding endothelial-dependent vasorelaxation, and protecting the heart from ischemia-induced pathophysiology (Der Sarkissian et al. 2008; Katovich et al. 2008; Lovren et al. 2008). However, one study reported overexpression of ACE2 reduced systolic blood pressure in SHRs, but also lead to severe cardiac fibrosis in the same animals (Masson et al. 2009).

In summary, ACE2 presents an important enzyme within the RAS providing a counterbalance to ACE1, negatively modulating AngII activity, providing cardioprotection during ischemia, and maintaining endothelial homeostasis. The removal of the ACE2 gene severely diminishes heart function. Therefore, manipulation of ACE2 enzymatic activity to promote its beneficial effects provides another potential avenue of therapy for those requiring the downregulation of RAS-generated AngII.

7.2.3 Chymase

Boucher et al. were the first to suggest enzymes other than ACE may be capable of forming AngII from its precursor (Boucher et al. 1974). Since their report other proteases (angiotensinases) have been identified that can cleave angiotensinogen into

AngI and AngII (Akasu et al. 1998) including chymase and other les abundant carboxypeptidases. Many studies observing AngII formation both *in vivo* and *in vitro* have reported ACE1 inhibitors failed to completely block AngII production in the rat, hamster, dog, baboon and human (Gondo et al. 1989; Hoit et al. 1995; Shiota et al. 1997; Guo et al. 2001; Borland et al. 2005), while in human left ventricle tissue one report suggested ACE1 is responsible for only 10-20% of AngII formation (Urata et al. 1990a; Urata et al. 1990b). Whilst carboxypeptidases have been shown to form AngII from AngI (Robertson and Nicholls 1993), proteases contained in cytoplasmic granules of mast cells have also been shown to have significant proteolytic effects on angiotensin peptides. These proteases belong to either the chymase or tryptase family and are suggested to be responsible for converting remaining AngI to AngII or biologically inactive peptides. Okunishi et al. (1984) reported that chymostatin (a chymase inhibitor) combined with an ACE inhibitor completely abolished AngII formation in the spontaneously hypertensive rat and thus the tissue must contain chymostatin-sensitive AngII generating enzymes dubbed CAGE (Okunishi et al. 1984). Later, CAGE was identified as a vascular chymase (Takai et al. 1997b) and was distinguished from other proteases due to its complete inhibition by chymostatin and its high specificity for AngI (Urata et al. 1990b; Akasu et al. 1998). Using a specific chymase antibody, human chymase was shown to be present in the interstitium, aorta and coronary arteries of the heart, and synthesized and stored within the granules of mast cells, mesenchymal cells and endothelial cells (Urata et al. 1993; Guo et al. 2001).

Interestingly, ACE1 expression in the human heart is greatest in the atrium, while chymase expression is greatest within the ventricles, suggesting AngII-generating pathways may vary between chambers (Urata et al. 1993), potentially providing a further level of regulatory control within the heart itself. Human chymase expression has also been located in tissues outside of the heart including the lung, liver, kidney and skin (Urata et al. 1994). Chymase has been shown to be a potent enzyme with high efficacy in converting AngI to AngII, as well as catalysing the degradation of AngII in mammalian hearts. However, chymase is highly species-specific displaying differential effects between species (Kunori et al. 2005). In studies to date, human, hamster, dog and baboon chymases show high catalytic efficacy of cleaving AngI at Phe^{8} to produce AngII with little or no further cleavage of the AngII peptide in both

isolated vessels or within the human heart (Wintroub et al. 1984; Urata et al. 1990b; Okunishi et al. 1993; Hoit et al. 1995; Takai et al. 1996; Balcells et al. 1997; Sanker et al. 1997; Takai et al. 1998; Caughey et al. 2000). In contrast, rat chymase is shown to primarily degrade AngI into Ang1-4 and Ang5-10, both biologically inactive fragments, through cleavage at the Tyr^4-Ile^5 bond (Wintroub et al. 1984; Sanker et al. 1997; Takai et al. 2001). Although, rat chymase does display the ability to convert AngI to AngII but at 20-fold less efficacy compared to the cleavage at Tyr^4 (Okunishi et al. 1984; Wintroub et al. 1984; Sanker et al. 1997). Based on their structure and specificity in converting AngI, the chymase isoforms from different mammalian species have been classified into two groups – α and β (Chandrasekharan et al. 1996). Human, monkey, hamster and dog chymases all convert AngI to AngII with little or no further degradation and are classified as α-chymases, while β-chymases comprise rat mast cell protease I and II (RMCP I and II), gerbil and mouse chymase, all of which cleave AngII at Tyr^4 with little or no AngII production (Chandrasekharan et al. 1996). The reasons for the difference in activity is suggested to be due to subtle differences in binding sites at the C-terminus of AngI between species where the amino acid sequence differs (Yamamoto et al. 1998).

With regards to the rat, it was originally believed that two isoforms of rat mast cell chymases exist, RMCP I and RMCP II, both shown to be biologically active serine proteases (Karlson et al. 2002; Kishi et al. 2006). However, Guo et al. explored the presence of chymase in rat vascular smooth muscle cells (VSMC) and identified and cloned a new third rat chymase dubbed rat vascular chymase (RVCH) (Guo et al. 2001). The same group showed that the cDNA of RVCH exhibits 74% and 79% sequence homology with RMCP I and II respectively, and 56% compared to human heart chymase (Guo et al. 2001). Structurally, RVCH is most similar to RMCP (I and II) which would suggest it is a β-chymase, however its effect on AngI differs markedly. Purified RVCH only cleaves AngI at Phe^8 to produce AngII with no cleavage at Tyr^4 and thus exhibits α-chymase activity (Guo et al. 2001). RVCH also shows increased expression in isolated aortic smooth muscle cells from spontaneously hypertensive rats, compared with normotensive rats, suggesting a potential pathological role for RVCH as is suggested with chymases from other mammals (Urata 2000; Guo et al. 2001). It must be noted that all experiments observing the role of RVCH were performed *in vitro* on cells isolated from the rat aorta and pulmonary

artery and the actions of RVCH have not yet been tested *in vivo* where, like other mammalian chymases, it may have reduced or altered effects. However, the underlying difference between the RMCPs and RVCH is that the latter clearly forms AngII with no further peptide degradation, indicating the rat also contains chymase-induced AngII-generation.

Caution must be given when studying the potency of chymase in *in vitro* settings due to the localisation of chymase within mast cells. Experiments documenting chymase activity in human blood vessels and tissue homogenates have shown it to be responsible for up to 90% of AngII production from AngI (Urata et al. 1990b; Borland et al. 1998; Maassen Van Den Brink et al. 1999). However, *in vivo* and *ex vivo* studies report chymase to have reduced efficacy, with ACE1 primarily responsible for generating AngII (Shiota et al. 1993; de Lannoy et al. 2001; Kirimura et al. 2005). The differences between these studies may be due to their cellular states, in which *in vitro* studies using homogenised tissue releases maximal amounts of chymase stored within the mast cells, and provides an optimal pH for chymase activity, stimulating maximal AngII formation. *In vivo*, chymase is retained within the mast cells with its release dependent upon mast cell degranulation, usually the result of stress or disease stimulating oxidative agents and cytokine activity such as protein kinase C (Singh et al. 1999; Peng and Beaven 2005; Palaniyandi et al. 2008). Because of this, chymase-dependent AngII formation may be more localised to specific tissues undergoing stress or damage, as opposed to ACE1-induced AngII production occurring throughout the circulation on the vessel walls. This further harmonises with the differential localisation of chymase and ACE1, primarily confined to the interstitial tissue and circulation respectively (Miyazaki and Takai 2006).

In some local events of cardiovascular damage, such as coronary bypass grafts of the saphenous vein (Nishimoto et al. 2001), percutaneous coronary intervention (Miyazaki et al. 1999) and angioplasty (MERCATOR 1992), chymase-induced AngII generation is elevated above that of ACE1-induced AngII generation. Increased mas cell and chymase expression, including chymase mRNA has been reported in atherosclerosis (Kaartinen et al. 1994; Takai et al. 1997a), coronary bypass grafts (Nishimoto et al. 2001), neointimal hyperplasia (Jin et al. 2005), abdominal aortic aneurysm (Nishimoto et al. 2002; Tsunemi et al. 2002), myocardial infarction (Jin et

al. 2001), and cardiac fibrosis (Takai et al. 2003). In all of these studies inhibition of chymase reduced further damage and aided recovery, strongly suggesting chymase to possess a pathogenic function. RVCH has also been shown to stimulate hypertension in rats (Guo et al. 2001) and hypertensive arteriopathy in chymase-overexpressing mice (Ju et al. 2001). Inhibition of chymase activity prevented cardiac fibrosis and dysfunction in rats following myocardial infarction and lowered collagen mRNA levels (Kanemitsu et al. 2005), a finding repeated in dogs with tachycardia-induce heart failure (Matsumoto et al. 2003) indicating the therapeutic benefits of suppressing chymase activity in cardiac disease. Thus, chymase inhibition represents a novel target for therapeutic attenuation alongside or combined with ACE1 and/or ARB inhibitors. Mast cell degranulation can also be inhibited through antagonising εPKC, reducing the release of chymase and other pro-inflammatory, pro-hypertopic, profibrotic and vasoactive agents, significantly attenuating pathological cardiac remodelling and aiding myocardial function in a rat model of hypertension-induced heart failure (Palaniyandi et al. 2008). AngII is known to activate intracellular PKC (Mehta and Griendling 2007), suggesting a potential feed-forward mechanism stimulating further mast cell degranulation and the release of these proinflammatory, pro-hypertrophic, and profibrotic mediators, including chymase stimulating further AngII production, exacerbating pathological cardiac remodelling (Palaniyandi et al. 2008). However, the fact that some forms of chymase also cleave AngI into inactive fragments suggests a regulatory mechanism in reducing AngII generation.

7.2.4 Summary of angiotensin II-generating enzymes

Taken together, it appears ACE1 is primarily responsible for AngII-formation within the circulation and vessel walls, whilst chymase potently forms AngII in localised tissue dependent on local conditions. The identification of a new rat chymase, RVCH, provides a novel endogenous AngII-forming enzyme in this species, independent of ACE activity, extending our knowledge of the RAS and providing evidence that the RAS is not a simple linear system as originally thought. Several reports have now shown chymase expression to be elevated in many cardiac diseases and injury, with inhibition of chymase activity significantly reducing AngII production and AngII-mediated pathogenic effects, attenuating hypertension and profibrotic activity including collagen synthesis. Inhibition of chymase may therefore be an additional supplement for the treatment of hypertension and alleviation of heart failure.

7.3 The RAS and Cardiovascular Disease

It is now well established that the RAS plays an integral role in mediating and augmenting cardiovascular disease and dysfunction. Not only does the RAS cause global arteriolar constriction but it also has profound direct effects on cardiac tissue progressing cardiovascular disease, developing hypertension and promoting atherosclerosis and CAD. Overstimulation of the RAS results in chronically elevated levels of AngII, causing hypertension, which promotes endothelial dysfunction, oxidative cellular stress, progressing atherosclerosis and CAD. AngII also directly stimulates cardiac hypertrophy and fibrosis through altering collagen levels and influencing fibroblasts within the myocardial interstitium (Brilla et al. 1993; Cai and Harrison 2000; Higashi et al. 2002; Nickenig and Harrison 2002; Sowers 2002). These effects are mediated through the angiotensin II Type 1 receptor (AT_1R), displayed in Figure 7.3.

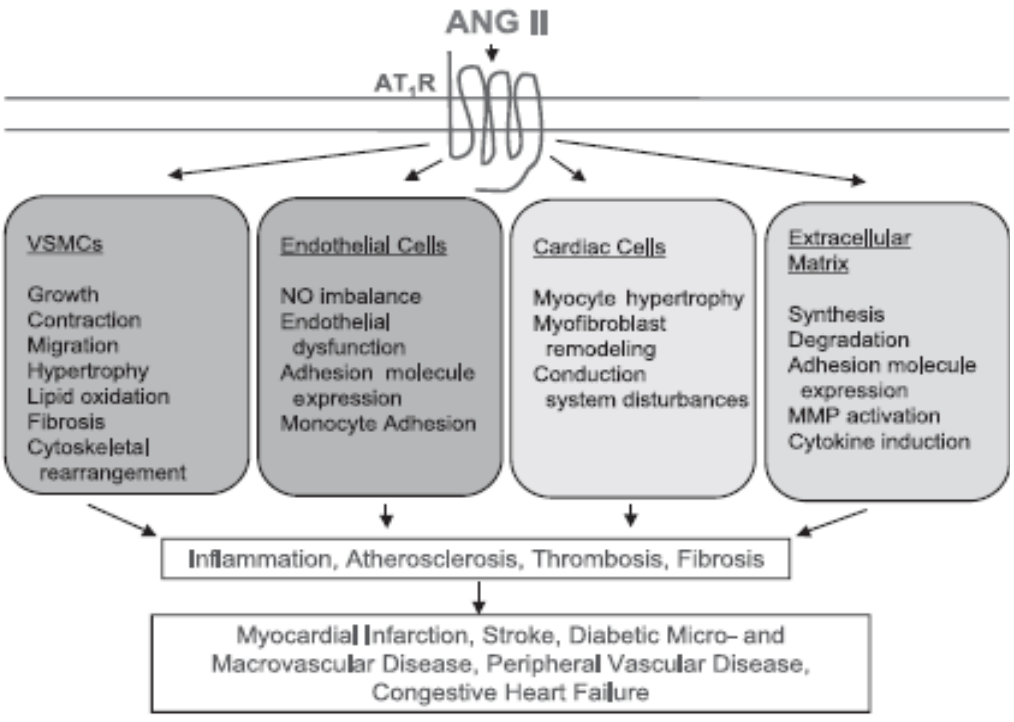


Figure 7.3 The pathogenic effects of AngII in the cardiovascular system. AngII stimulates many pathogenic effects in several different cell types, all converging to induce and progress vascular disease and ultimately lead to severe cardiovascular diseases. Figure from Mehta and Griendling 2007

AngII-induced stimulation of ROS, mediated by vascular NADPH oxidase (Griendling et al. 1994), activates pro-inflammatory cytokines including $NF_{\kappa}B$, promoting the development of atherosclerosis (Ferrario 2006). The RAS has also been reported to cross-talk with oxidised low-density lipoprotein (ox-LDL) in coronary artery disease resulting in synergistic elevations in oxidative stress, foam cell formation and the inflammatory cascade (Chen and Mehta 2006), all augmenting atherosclerosis.

AngII phosphorylates many intracellular proteins (Figure 7.4), activating pathogenic pathways stimulating vasoconstriction, cellular growth and differentiation, hypertrophy, inflammation and apoptosis (Wollert and Drexler 1999; Lassègue et al. 2001; Ruiz-Ortega et al. 2001). In healthy subjects these pathways are tightly regulated, however if AngII levels are chronically elevated, these pathways can become overstimulated, resulting in hypertension and maladaptive vascular remodelling due to unregulated cellular growth, differentiation, ROS production and hypertrophy.

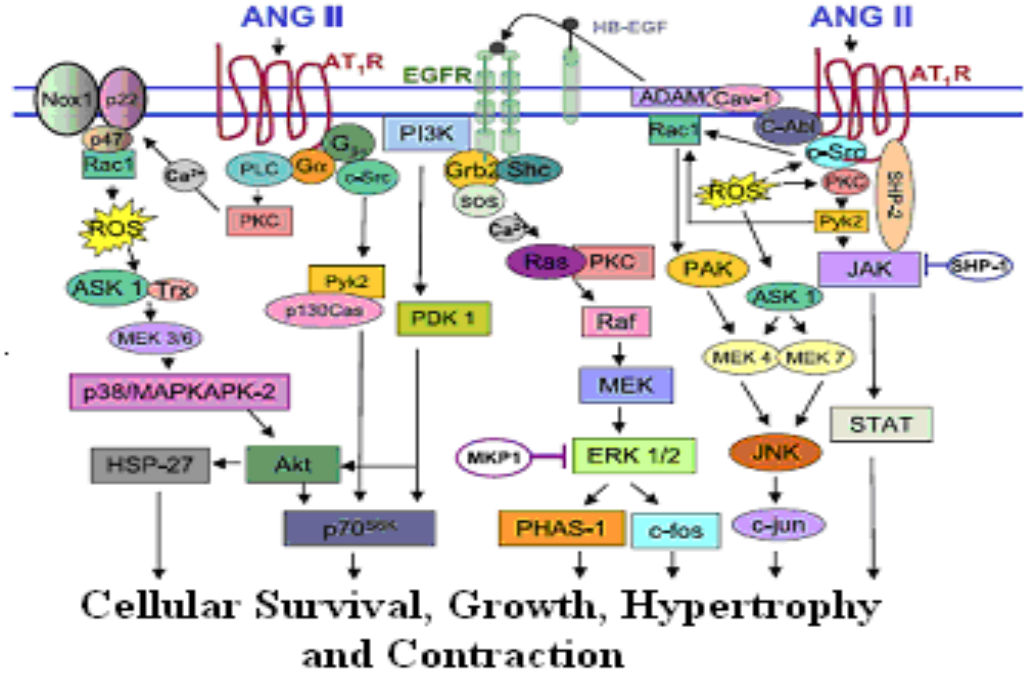


Figure 7.4 AngII binding to AT_1R triggers the activation of many intracellular pathways. The downstream effector agents of these pathways stimulate cellular survival, growth, hypertrophy and contraction. Figure modified from Mehta and Griendling 2007.

The use of ACE1 inhibitors and AT_1R blockers confirms that inhibition of AngII and blockade of its receptor provides therapeutic benefits including lowering blood pressure, attenuating the development of atherosclerosis, alleviating hypertension and minimising cellular hypertrophy and cardiac remodelling (Giles 1992; Lee et al. 1993; Nicholls et al. 1994; Hollenberg 2000). Many human trials have now been performed using ACE inhibitors and AngII receptor blockers, all indicating ACE inhibition attenuates hypertension and lowers the risk of stroke, atherosclerosis and myocardial infarction (Yusuf et al. 2000; Yusuf et al. 2003; Ferrario et al. 2004; Ferrario 2006; Al Khalaf et al. 2009). However, treatment of hypertension using ACE1 inhibitors does not always lower blood pressure to desired levels and has not been shown to prevent restenosis following coronary angioplasty in a human trial (1992). ACE inhibition also produces some undesirable side effects including a dry cough due to its influence in the lungs (Al Khalaf et al. 2009). Furthermore, patients using ACE1 inhibitors over a prolonged period may require higher doses in order to sustain blood pressure within the desired level (Personal correspondence with Prof. Mark Richards), with higher doses exacerbating undesirable side effects, indicating the need for an alternate line of anti-hypertensive treatment. The development of AT_1R blockers (ARBs) have helped partially resolve this issue as ARBs do not produce coughing or detrimental effects within the lungs. However, they are not as favourable as ACE1 inhibitors as they do not minimise stroke or MI risk to the same degree (Al Khalaf et al. 2009). Renin inhibitors have also been recently developed, effectively halting the initial step in the RAS cascade and proving effective in treating mild to moderate hypertension either alone or in conjunction with another antihypertensive agent such as an ARB (Musini et al. 2009; Sanoski 2009).

A ‘perfect’ therapy is yet to be established in sufficiently inhibiting the RAS whilst avoiding side effects. Organ damage has been shown to occur in response to high doses of ACE inhibitors suggesting that antihypertensive agents may need to target vasoconstrictive agents outside of the RAS, such as chymase.

7.4 PA12 and the local, tissue-based RAS

It was initially believed that the RAS was a purely endocrine system comprising of renin from the kidney, angiotensinogen from the liver, and ACE from the pulmonary system to produce the final effector peptide AngII within the circulation. It is now evident that a local RAS also exists in many tissues including the vascular wall, heart, kidney and brain capable of producing AngII independent of the circulation (Bader 2002; Bader and Ganten 2008). Cardiac tissue possesses all the necessary agents required for a functional RAS as angiotensinogen and ACE1 their mRNA have been detected, along with minimal expression of renin which is currently debated (Bader 2002; Varagic and Frohlich 2002). Renin mRNA has been detected in minute amounts in the atria, ventricles and cardiomyocytes, while mast cells are also shown to store renin (Mackins et al. 2006). Overall, it appears the heart is capable of local AngII production independent of circulatory-derived agents. PA12 represents a potentially important component of any local AngII-generating system as cardiac immunoreactive staining for PA12 suggests it is localised to different cellular areas compared with Ang (Jessup et al. 2008). PA12 is primarily localised to ventricular myocytes but also displayed some staining of the medial layer and surrounding adventitia of some coronary arteries, and predominantly concentrated in the cytoplasm of cardiac myocytes (Jessup et al. 2008), whereas Ang was barely detectable in the ventricle and displayed staining patterns different to PA12 where it was most prominent. Furthermore, Jessup et al. report PA12 expression within the left ventricle of rats was higher than that of AngI and AngII further supporting PA12 as a potential precursor peptide. It is yet to be determined whether renin is responsible for producing PA12 or another enzyme more readily expressed within the tissue.

7.5 RAS and UII crosstalk

Few studies have reported UII to work in combination with other peptides or regulatory systems. In 2001 Watanabe and colleagues reported UII acts with serotonin (5-HT) and mildly oxidised LDL to synergistically promote VSMC proliferation (Watanabe et al. 2001a; Watanabe et al. 2001b), while a recent study employed a mathematical formula combined with hemodynamic data and estimated that UII works synergistically with angiotensin II in constricting rat aortic rings (Lamarre and Tallarida 2008)). The synergistic activity of UII with serotonin and angiotensin II are both suggested to be mediated by PKC activity (Watanabe et al. 2001a; Watanabe et al. 2001b; Wang et al. 2007). These studies highlight the potential for UII to work synergistically with other known vasoconstrictive agents to produce greater vasoconstriction, and potentially progress CVD. No study has yet explored the potential of UII to work synergistically with vasorelaxant agents in vessels known to relax in response to UII such as the coronary arteries (Katano et al. 2000; Li et al. 2004; Prosser et al. 2006), mesenteric and other peripheral vessels (Gardiner et al. 2001; Stirrat et al. 2001; Gardiner et al. 2004; Hood et al. 2005).

Although no synergistic effect has yet been directly observed between UII and PA12 or the RAS, they have both been reported to stimulate similar effects and indeed similar pathways in augmenting atherosclerosis and cardiovascular disease. Both UII and AngII have been shown to interact with, and elevate oxidised low-density lipoproteins, enhancing the development of foam cells and atherosclerosis (Watanabe et al. 2005; Chen and Mehta 2006; Watanabe et al. 2006). UII activity and AngII-induced activation of the AT_1R both stimulate cellular growth and hypertrophy through ERK1/2, elevate ROS production through NAPH oxidase activation, and cause fibrosis of the intistitial tissue through elevating collagen synthesis and fibrosis. The apparent difference between the two peptides is that UII also possesses cardioprotective properties, while RAS stimulation of the AT_1R does not.

8. Cardiac chymase converts rat Proangiotensin-12 (PA12) to angiotensin II: effects of PA12 upon cardiac hemodynamics

(The following chapter is a copy of the published article Prosser et al. Journal of Cardiovascular Research, 2009 Apr;82(1):40-50)

Abstract

Aims: The aim of this study was to observe the direct physiological and biochemical cardiac effects in response to a newly identified putative component of the renin–angiotensin system, proangiotensin-12 (PA12); and investigate whether PA12 can serve as a substrate for angiotensin II (AngII) generation. *Methods and results:* The direct cardiac actions of PA12 and its role as a substrate for chymase-dependent AngII generation were investigated in Sprague–Dawley rats using an isolated heart model of cardiac ischaemia–reperfusion injury. PA12 potently constricted coronary arteries with no significant effect on left-ventricular contractility. PA12 impaired recovery from global ischaemia, maintaining coronary constriction and markedly increasing release of creatine kinase and troponin I (TnI), indicating greater myocardial injury. Analysis of perfusate collected after transcardiac passage revealed a marked increase in AngII production from hearts infused with PA12. Cardiac AngII production was not blocked by angiotensin-converting enzyme inhibitors, whereas inhibition of chymase with chymostatin significantly reduced AngII production and attenuated PA12-induced vasoconstriction and myocardial damage following ischaemia. Furthermore, angiotensin II type 1 receptor (AT_1R) blockade abolished PA12 activity. *In vitro*, PA12 was efficiently and precisely converted to AngII as assessed on reverse phase-high performance liquid chromatography coupled to tandem mass spectrometry. This conversion was blocked by chymostatin. *Conclusion:* PA12 may act as a circulating substrate for cardiac chymase-mediated AngII production, in contrast to ACE-mediated AngII production from AngI.

8.1 Introduction

Knowledge of the renin-angiotensin system (RAS) began with the initial discovery of renin in 1898, an enzyme shown to significantly alter blood pressure (Inagami 1998). Since then exploration into the RAS has found it to be one of the most important homeostatic systems in pressure-volume homeostasis. Renin released from the kidney in response to reductions in renal perfusion, acts on the angiotensin-precursor peptide angiotensinogen, cleaving it to release the biologically inactive decapeptide angiotensin1-10 (AngI). Angiotensin-converting-enzyme 1 (ACE1) cleaves a further 2 amino acids from the C-terminus of AngI to produce angiotensin1-8 (AngII), a potent biologically active peptide triggering a broad range of effects throughout the body including aldosterone secretion, salt and water retention, and potent arteriolar vasoconstriction (Robertson and Nicholls 1993). Biologically active AngII-derived peptides have been isolated, including AngIII (Ang2-8) and AngIV (Ang3-8), suggesting angiotensinogen is a precursor to many functional peptides (Robertson and Nicholls 1993). AngII (and its active derivative peptides) bind to two known G-protein coupled receptors, AngII receptor Type 1 and 2 (AT_1 and AT_2). AT_1 is expressed abundantly by most cell types throughout the body and is the primary receptor mediating the pressor response to AngII (Higuchi et al. 2007). AT_2 is far less abundant in the adult and has been suggested to stimulate vasodilation, counteracting the pressor and other effects of AT_1 (Higuchi et al. 2007; Yayama and Okamoto 2008).

Proangiotensin-12 (PA12) is a newly discovered peptide believed to be an immediate proteolytic fragment of angiotensinogen. PA12 was isolated from the rat small intestine where it was most abundant, but it is also present in the circulation and throughout many tissues and organs of the rat (Nagata et al. 2006). The sequence of PA12 is identical to that of angiotensin I (AngI) with an extended C-terminus, thus comprising Ang1-10-Leu^{11}-Tyr^{12}. To date, a single report has documented that PA12 can constrict rat aorta *in vitro* and elevate blood pressure in anaesthetized normotensive Wistar rats *in vivo* (Nagata et al. 2006). Whether PA12 is present in human (or other mammalian) blood or tissue is unknown. It has been suggested the effects of PA12 are dependent upon both ACE1 and AT_1R activities, and that PA12 levels are elevated in hypertensive rat hearts (Jessup et al. 2008). However, whether

PA12 has direct cardiac actions is not known, nor whether PA12 can serve as a substrate for generation of AngII or other AngII-related peptides.

Accordingly, we provide the first documentation of direct hemodynamic and endocrine effects of PA12 on the isolated, perfused rat heart, and report its effects in a model of cardiac ischemia-reperfusion (I-R) injury. We also provide the first evidence that PA12 is converted to AngII *in vitro* and *ex vivo* by enzymatic chymase activity, and provide a comparison with AngI under the same protocols.

8.2 Materials and Methods

8.2.1 Materials

Male Sprague-Dawley (SD) rats weighing 300-400 g (60-75 days old) were obtained from the Christchurch Animal Research Facility, University of Otago, New Zealand. Rats were housed under controlled temperature (21 ºC), humidity (~40%) and natural day length with free access to standard rat chow and water.

Synthetic rat PA12 was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA), while angiotensin I and the angiotensin-converting-enzyme 1 antagonists Captopril and Ramipril, as well as the chymase inhibitor Chymostatin were all obtained from Sigma-Aldrich (St. Louis, MI, USA). The angiotensin II receptor type 1 blocker, CV-11974 (Candesartan, 2-ethoxy-1-[[2′-(1*H*-tetorazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid)*h* was a generous gift from Takeda Chemical Industries Ltd (Osaka, Japan). PA12 and captopril were diluted in distilled water, aliquoted and stored at -20 ºC prior to use. Ramipril and chymostatin were dissolved in DMSO, while CV-11974 was dissolved in 1 M Na_2CO_3 solution, aliquoted and stored at -20 ºC prior to use.

8.2.2 Langendorff isolated rat heart perfusion

Isolated rat heart perfusion was performed as previously described (Pemberton et al. 2005; Prosser et al. 2006). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The heart was rapidly excised and mounted on the Langendorff apparatus, cannulated above the aortic valve and perfused at 12 ml/min (constant

retrograde flow) with perfusion buffer comprising (mmol/L): 123 NaCl, 22.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.1 $MgSO_4 \cdot 7H_2O$, 1.5 $CaCl_2 \cdot 2H_2O$ and 11.0 glucose (final pH 7.40). Buffer was maintained at 37 ºC and oxygenated with 95% O_2/5% CO_2. The left atrium was removed allowing a 40% ethanol-filled balloon (attached to a pressure transducer) to be inserted through the mitral valve into the left ventricle (LV) enabling measurement of left ventricular hemodynamic contractile parameters. A side-arm cannula attached to a second pressure transducer was inserted into the aortic cannula above the heart to measure perfusion pressure (PP), an indirect measure of coronary arterial tone. Hearts were allowed to settle for 30 min before being paced at 320 bpm using an electrode attached to a Digimeter DS2A-Mk. II stimulator placed on the right atrium. Hearts were allowed a further 30 min to resettle before any experimental protocol was started. All data were recorded using a Powerlab Chart 5 System (ADInstruments); see Appendix B.1 for supplementary information and methodology. Drugs were diluted using perfusion buffer to enable infusion of the drug or vehicle over 30 min at 0.5 ml/min using a syringe pump feeding directly into the perfusion line. This investigation was approved by the University of Otago Animal Ethics Committee and conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

8.2.3 Ischemia-reperfusion protocols

All antagonist agents (100 µmol/L captopril, 100 nmol/L CV-11974, 100 nmol/L chymostatin, and 100 µmol/L and Ramipril) were co-infused with either 10 nmol/L PA12 or 10 nmol/L angiotensin I (AngI). Experiments were run in pairs: one heart infused with both the antagonist and PA12 or AngI, the other simultaneously infused only with PA12 or AngI from the same stock solution and same buffer reservoir providing a parallel control for each antagonist experiment. The heart given the antagonist was chosen at random.

The doses of 10 nmol/L PA12, 10 nmol/L AngI, 100 nmol/L CV-11974, 100 µmol/L captopril and 100 nmol/L chymostatin administered were based upon preliminary dose-response studies or manufacturers recommendations (Appendix C, Figure C.2). After 30 min infusion of agents, perfusion was stopped and hearts underwent no-flow

global ischemia for 45 min with pacing halted. Hearts were then reperfused with accompanying pacing for 105 min following ischemia (Figure 8.1).

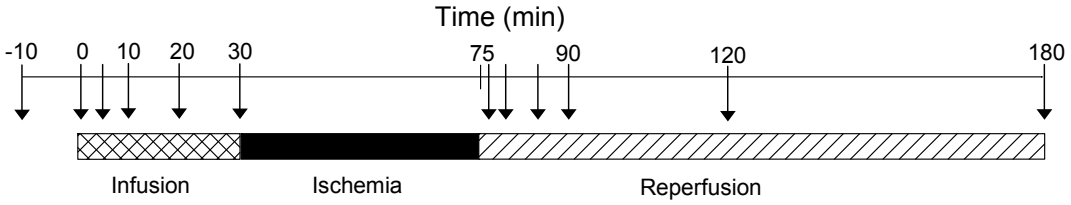


Figure 8.1 Ischemia-reperfusion experimental protocol. Following a settling period, rat hearts underwent preconditioning infusion (30 min), no-flow global ischemia (45 min) and reperfusion with buffer only (105 min). Arrows indicate

8.2.4 Analysis of cardiac AngII, Atrial Natriuretic Peptide (ANP), Myocardial Creatine Kinase (CK), and Troponin I (TnI) release

Perfusate samples were collected at specific time points after passing through the heart for hormonal and biochemical analysis (Figure 8.1). Samples of infusion solution from the syringe were also taken prior to passing through the heart. Angiotensin II (AngII) production was determined by extracting perfusate samples (collected into 500 IU Aprotinin/0.1% Triton X-100) through Bond Elut columns and measured using our previously described, specific radioimmunoassay (RIA) (Nicholls and Espiner 1976, refer Appendix D.1). This assay shows no detectable cross-reactivity with PA12 or angiotensin I.

ANP secretion was measured by RIA after extraction through SepPak columns as previously described (Yandle et al. 1986, refer Appendix D.2). Perfusate creatine kinase (CK) and troponin I (TnI) concentrations were measured using the Abbot Aeroset platform (Canterbury Health Labs, Christchurch, New Zealand).

8.2.5 Ex vivo cardiac conversion of PA12 analysis

To assess the *ex vivo* cardiac conversion of PA12, we employed reverse phase-high pressure liquid chromatography (RP-HPLC) coupled with specific RIA. RP-HPLC was carried out at 40ºC, with a gradient of 0-60% CH_3CN / 0.1% TFA over 60 min, using a 22 cm Brownlee C_{18} RP-HPLC column. The gradient elution profile was calibrated with Ang(1-7), AngII, AngI and PA12 standard peptides. To assess

enzymatic cardiac conversion of PA12 after a single cardiac passage, 1ml samples of perfusate (during infusion of 10 nmol/L PA12) were collected into Aprotinin/Triton post-infusion and subjected to RP-HPLC as above, with fractions collected at 1 min intervals. Fractions were dried under air at 37ºC, reconstituted in AngII RIA buffer and subjected to AngII RIA, as described (Nicholls and Espiner 1976).

8.2.6 In vitro conversion of PA12 by chymase – Tandem Mass Spectrometry analysis

To confirm that chymase could generate authentic AngII from PA12, we employed *in vitro* incubation of PA12 with recombinant chymase. PA12 (12.72 µmol/L) was incubated either: a) alone; b) in combination with recombinant human chymase (3.33 pmol/L); or c) in combination with both 3.3 pmol/L chymase and 33.4 µmol/L chymostatin. Incubations proceeded for 30 min at 37ºC in 50 µL PBS. Reactions were quenched by addition of 2 µL glacial acetic acid (final concentration of 0.6 mol/L), and the reaction products of each submitted to RP-HPLC using the gradient described above. Fractions were collected at 1 min intervals and portions submitted to AngII RIA analysis. The remainder of the RP-HPLC fractions were dried under air and those with AngII immunoreactivity and UV detection were submitted to Tandem Mass Spectrometry (MS/MS).

8.2.7 Tandem Mass Spectrometry:

Peptides were resuspended in 30% [v/v] acetonitrile and 0.1% [v/v] trifluoroacetic (TFA) acid in water. 1 µL of peptide solution was premixed with 2 µL of matrix (10 mg/ml αcyano-4-hydroxycinnamic acid dissolved in 65% [v/v] aqueous acetonitrile containing 0.1% TFA and 10 mmol/L ammonium dihydrogen phosphate). 0.8 µL of sample/matrix mixture were spotted onto a MALDI sample plate (Opti-TOF 384 well plate, Applied Biosystems, MA) and air dried.

Samples were analyzed on a 4800 MALDI tandem Time-of-Flight Analyzer (Applied Biosystems, MA). All MS spectra were acquired in positive-ion mode with 800-1000 laser pulses per sample spot. The 6 strongest precursor ions of each sample spot were used for MS/MS collision-induced fragmentation (CID) analysis. CID spectra were acquired with 2000-4000 laser pulses per sample spot using the 2 kV mode and air as the collision gas at a pressure of $1x10^{-7}$ torr.

For protein identification MS/MS data was searched against the SWISS-PROT amino acid sequence database using the Mascot search engine (matrix science). The search was set up for full tryptic peptides with a maximum of 4 missed cleavage sites. Carboxyamidomethyl cysteine, oxidized methionine, and pyroglutamate (E, Q) were included as variable modifications. The precursor mass tolerance was 75 ppm and the maximum fragment mass error 0.3 Da.

8.2.8 Statistical analysis

All data are presented as mean +S.E.M. Analysis of changes in cardiac hormones and hemodynamics were performed on SPSS using a two-way ANOVA with repeated measure, with Bonferroni's multiple comparison test, post hoc. Individual and cumulative data comparisons were made using a student's T-test. In all statistical tests a value of $P<0.05$ was considered significant.

8.3 Results

8.3.1 PA12 Increases Coronary Perfusion Pressure (PP) pre and post cardiac ischemia

PA12 dose-dependently increased PP indicating vasoconstriction of the coronary arteries. This effect was maximal at 10 nmol/L and sustained throughout the 30 min infusion period (Figure 8.2A). 1 and 10 nmol/L PA12 elevated PP by an average of 11 ± 0.7 and 32.3 ± 4.4 % ($P<0.05$, n=6 and $P<0.0001$, n=8) respectively, compared with vehicle infusion. PA12 had no significant effect on left ventricular developed pressure (DP), nor ±dP/dt (ΔDP from 0 to 30 min was 8 ± 2.27 % and 9 ± 2.15 % for vehicle and PA12 respectively).

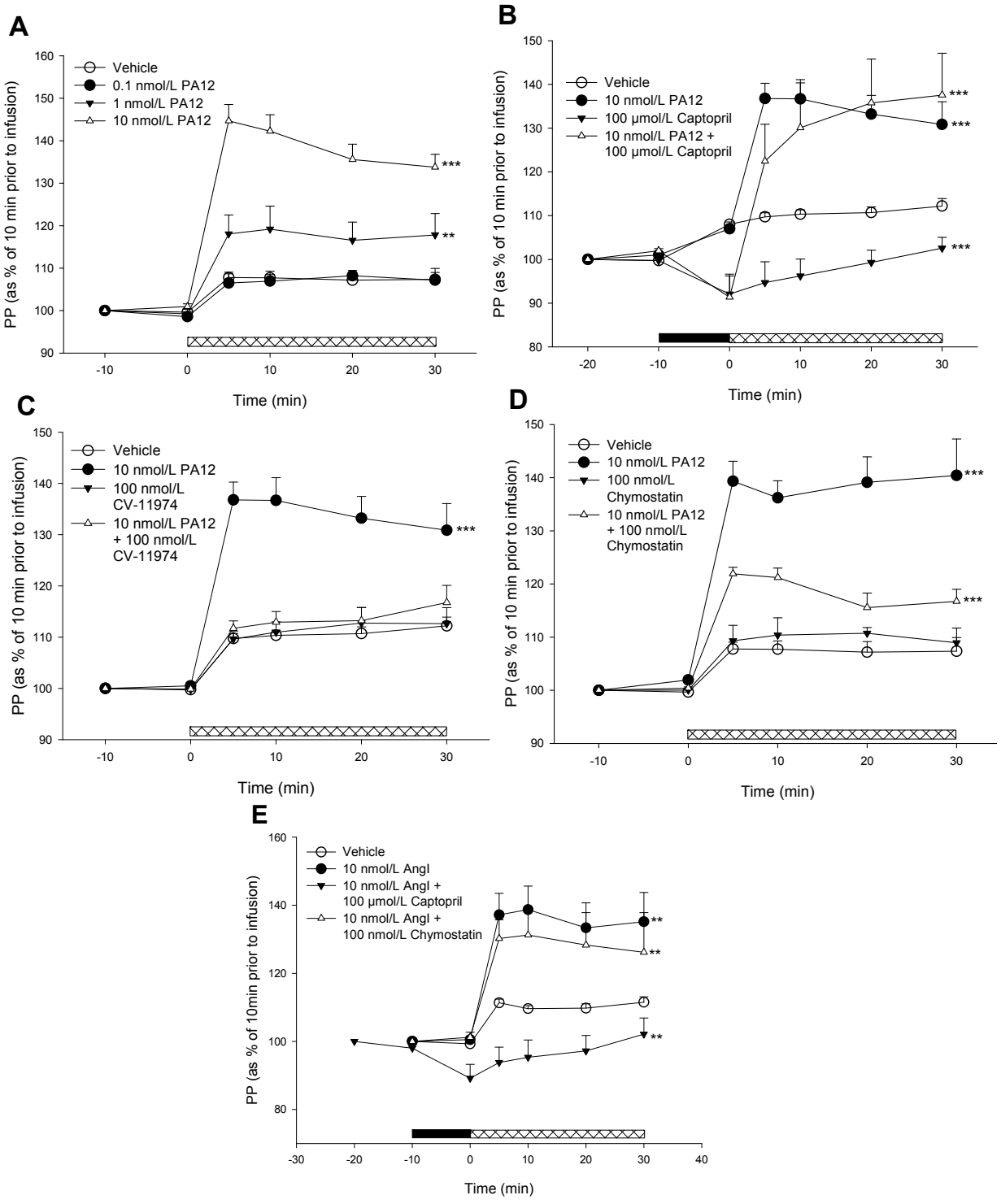
Infusion of 10 nmol/L AngI significantly increased PP ($P<0.05$, n=5), comparable to that of 10 nmol/L PA12 (Fig 2E), and also showed no significant effects on DP or ±dP/dt (ΔDP from 0 to 30 min was 9 ± 1.30 and 9 ± 3.41 % for vehicle and AngI respectively).

Infusion of captopril alone (an ACE1 inhibitor) caused a significant drop in PP compared with vehicle ($P<0.005$, n=5). However, when co-infused with PA12, 100 µmol/L captopril had no effect on PA12-induced increase in PP with levels comparable to that of PA12 infused alone (Figure 8.2B). This lack of response of PA12 to ACE1 inhibition was confirmed by infusion of a second ACE1 inhibitor Ramipril at a dose of 100 µmol/L (Appendix C, Figure C.3). Captopril abolished the AngI-induced elevation in PP significantly below both vehicle and AngI levels (both $P<0.01$, n=5, Figure 8.2E). The gradual increase in PP observed during the co-infusion was equal to that of infusion of captopril alone (see Figure 8.2B). The AT_1R antagonist, CV-11974, completely abolished PA12-induced elevations in PP ($P<0.001$, n=6), suggesting the AT_1 receptor can mediate PA12 activity (Figure 8.2C).

Infusion of the chymase inhibitor chymostatin at the dose employed had no effect on PP or LV contractility. However co-infusion of chymostatin with PA12 significantly attenuated PA12-induced increases in PP by an average of 63.9 ± 1.59 % ($P<0.001$, n=5, Figure 8.2D). In contrast, co-infusion of chymostatin with AngI caused minimal attenuation of AngI-induced increases in PP during infusion, remaining significantly above vehicle ($P<0.003$, Figure 8.2E).

8.3.2 Ischemia-reperfusion

With ischemia-reperfusion, hearts preconditioned with 10 nmol/L PA12 showed significantly elevated PP above vehicle levels throughout the reperfusion period ($P<0.05$, Figure 8.3A). Hearts preconditioned with 10 nmol/L AngI also showed elevated PP above vehicle levels, significant at 120 and 180 minutes (both $P<0.05$, n=5, Figure 8.3D). PA12 and AngI had no significant effect on DP, ±dP/dt, systolic or diastolic pressure during reperfusion.


A
Vehicle
0.1 nmol/L PA12
1 nmol/L PA12
10 nmol/L PA12
PP (as % of 10 min prior to infusion)
Time (min)
B
Vehicle
10 nmol/L PA12
100 µmol/L Captopril
10 nmol/L PA12 +
100 µmol/L Captopril
PP (as % of 10 min prior to infusion)
Time (min)
C
Vehicle
10 nmol/L PA12
100 nmol/L
CV-11974
10 nmol/L PA12
+ 100 nmol/L
CV-11974
PP (as % of 10 min prior to infusion)
Time (min)
D
Vehicle
10 nmol/L PA12
100 nmol/L
Chymostatin
10 nmol/L PA12
+ 100 nmol/L
Chymostatin
PP (as % of 10 min prior to infusion)
Time (min)
E
Vehicle
10 nmol/L AngI
10 nmol/L AngI +
100 µmol/L Captopril
10 nmol/L AngI +
100 nmol/L Chymostatin
PP (as % of 10min prior to infusion)
Time (min)

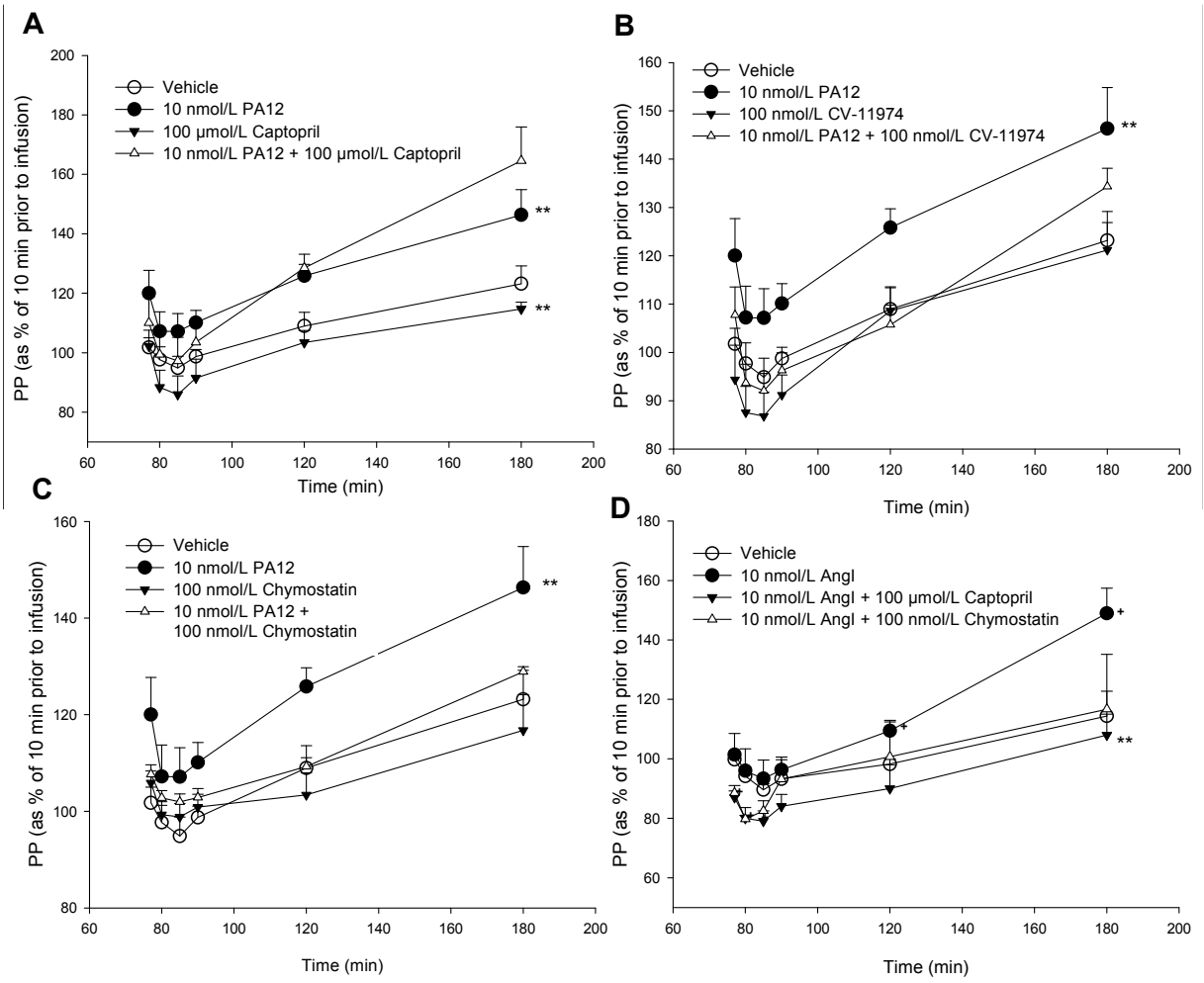


Figure 8.3 Perfusion pressure following global ischemia in isolated hearts preconditioned with 10 nmol/L PA12 (n=8) or 10 nmol/L AngI (n=5). 100 µmol/L captopril had no effect on PA12-induced elevations in PP during reperfusion (panel A), however 100 nmol/L CV-11974 (panel B) and 100 nmol/L chymostatin (panel C) both attenuated the PA12-induced increase. AngI caused minimal elevation of PP immediately following ischemia before rising above control levels ($P<0.05$ at 120 and 180 min, panel D). Captopril significantly attenuated the AngI-induced elevation in PP throughout reperfusion; while chymostatin displayed minimal inhibitive potency, significant for the initial 10 min of reperfusion (panel D). $^{+}$Individual data point comparison using student's t-test $P<0.05$; **Data set comparison using a two-way ANOVA $P<0.05$.

Hearts preconditioned with captopril alone displayed significantly reduced PP throughout reperfusion ($P<0.05$, Figure 8.3A). Captopril attenuated PA12-induced elevations in PP immediately following ischemia, before antagonistic potency was lost and PP increased to equal PA12 alone (Figure 8.3A). In contrast, captopril significantly attenuated AngI-induced elevations in PP during reperfusion ($P<0.05$ compared with AngI alone, Figure 8.3D).

Co-infusion of CV-11974 with PA12 abolished PA12-induced increases in PP during reperfusion ($P<0.005$, Figure 8.3B).

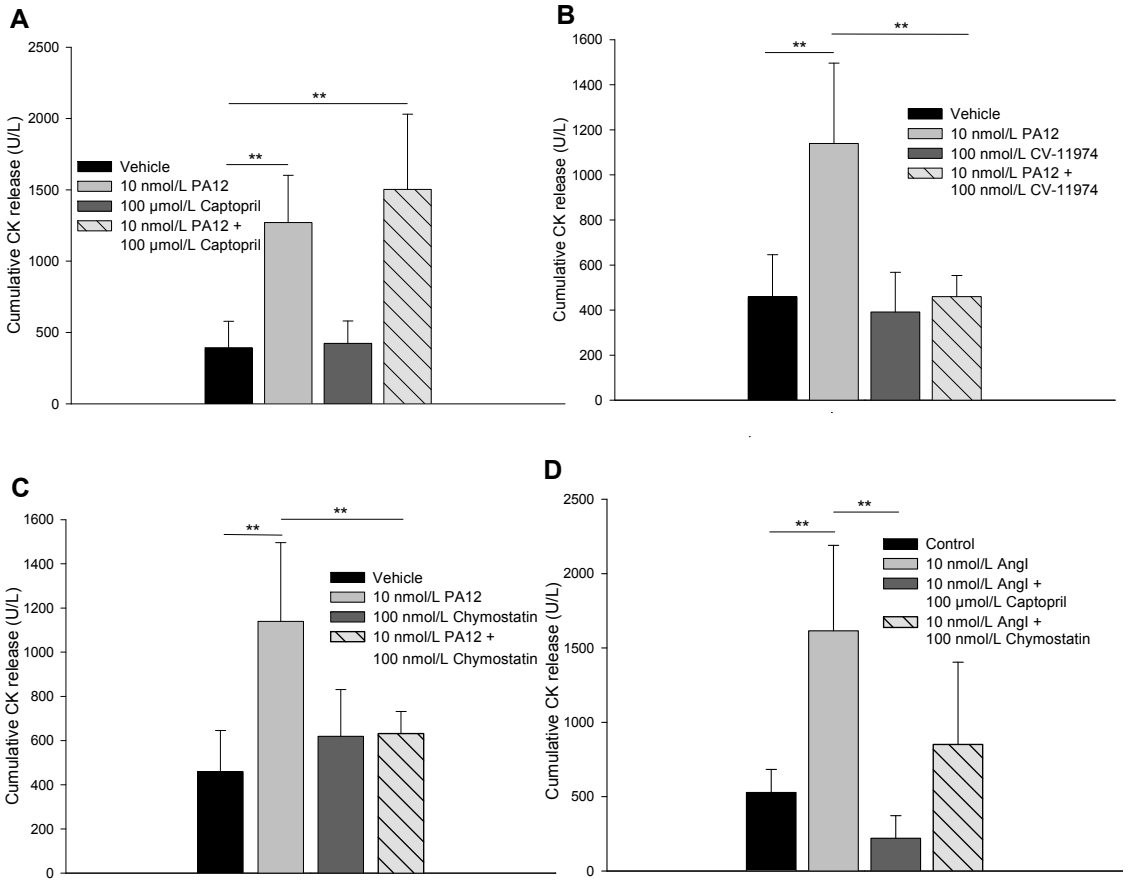


Figure 8.4 Cumulative CK release in PA12 preconditioned hearts was significantly higher compared with vehicle controls, an affect which was not attenuated by 100 µmol/L captopril (panel A), but was significantly abolished by 100 nmol/L CV-11974 (panel B) and 100 nmol/L chymostatin (panel C). Hearts preconditioned with 10 nmol/L AngI produced cumulative CK release significantly higher than vehicle controls (panel D); this increase was significantly attenuated by 100 µmol/L captopril, but not by 100 nmol/L chymostatin. **$P<0.05$ compared with vehicle or PA12, as indicated by bars using a student's t-test.

Chymostatin attenuated the PA12-induced increase in PP following ischemia, maintaining PP below that of hearts preconditioned with PA12 alone ($P<0.05$ at 120 and 180 min, Figure 8.3C). Co-infusion of chymostatin with AngI saw a reduction in PP immediately following ischemia when compared with hearts preconditioned with AngI alone ($P<0.05$ at 77, 80 and 85 min, Figure 8.3D).

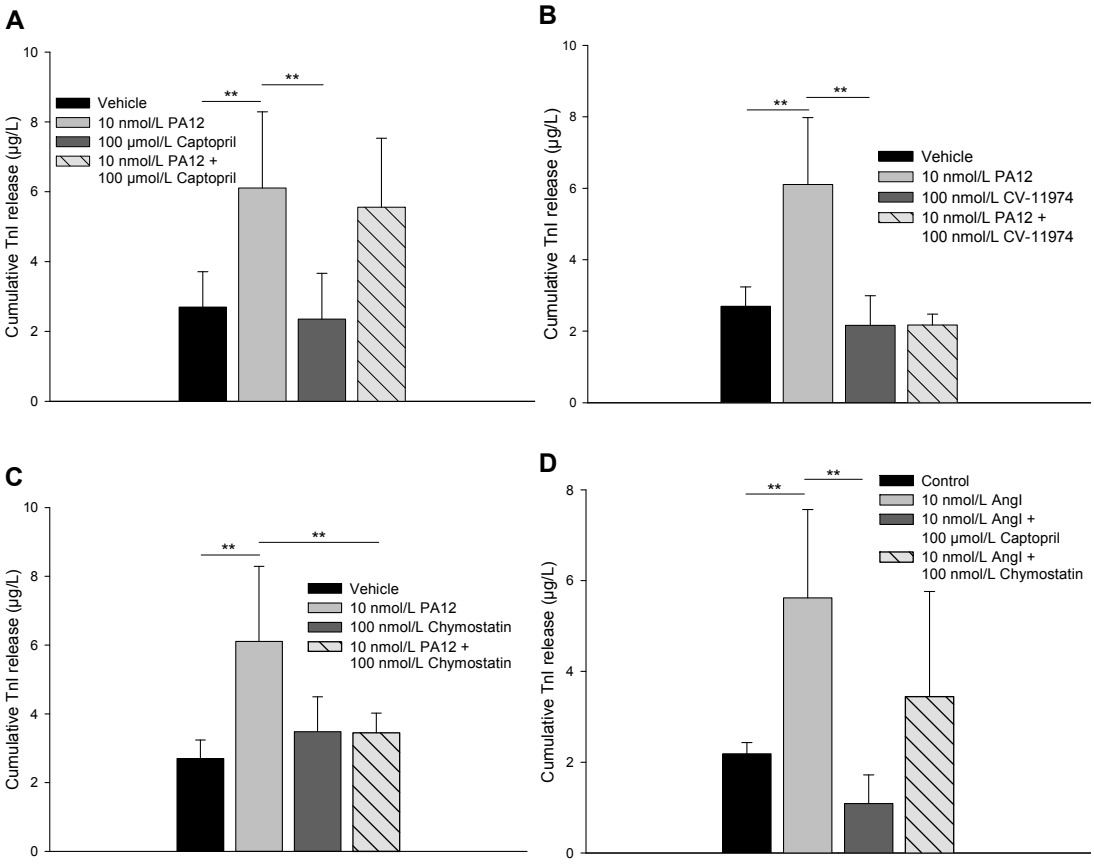


Figure 8.5 Cumulative myocardial TnI release in PA12 preconditioned hearts was significantly higher compared with vehicle controls, an affect which was not attenuated by 100 µmol/L captopril (panel A), but was significantly abolished with 100 nmol/L CV-11974 (panel B) and chymostatin (panel C). Hearts preconditioned with 10 nmol/L AngI produced cumulative TnI release significantly higher than control during reperfusion (panel D); this increase was significantly attenuated by 100 µmol/L captopril, but not by 100 nmol/L chymostatin.**Using a student's t-test $P<0.05$ compared with vehicle or PA12, as indicated by bars.

During reperfusion hearts preconditioned with 10 nmol/L PA12 or 10 nmol/L AngI released significantly more CK and TnI (both $P<0.05$) than control hearts, indicating greater myocardial injury (Figures 4 and 5). The addition of captopril had no effect on PA12-induced CK or TnI release (Figure 8.4A and 8.5A); however, both CV-11974 and chymostatin significantly blocked PA12-induced elevations in both CK and TnI (Figures 4B&C and 5B&C). In contrast to PA12, captopril significantly attenuated AngI-induced elevations in CK and TnI release when compared to hearts preconditioned with AngI alone (both $P<0.05$, Figures 4D & 5D), however chymostatin could not (both $P>0.05$, n=5, Figures 4D & 5D).

8.3.3 Perfusate ANP levels during ischemia-reperfusion

Hearts preconditioned with either 10 nmol/L PA12 or 10 nmol/L AngI showed no significant difference in perfusate ANP concentrations compared with vehicle throughout both infusion and reperfusion. Infusion of 100 μmol/L captopril alone reduced ANP secretion during infusion and significantly during reperfusion ($P<0.05$), as well as attenuating both PA12- and AngI-induced ANP release ($P<0.05$). CV-11974 and chymostatin had no influence on ANP secretion with or without the presence of PA12 or AngI.

8.3.4 PA12 is cleaved into angiotensin II during cardiac passage: dependence on chymase, but not ACE1

An average of 457.2 ± 113.2 pmol/L and 651.3 ± 79.73 pmol/L IR-AngII was measured from perfusate samples collected at 10 minutes into infusion from hearts preconditioned with 10 nmol/L PA12 and 10 nmol/L AngI respectively, both significantly above that of vehicle (8.15 ± 7.75 pmol/L, $P<0.001$, Figure 8.6A & 8.6B). PA12-derived AngII formation was not attenuated by captopril co-infusion (508.0 ± 169.5 pmol/L, P=NS vs. PA12 alone), but was almost completely abolished by chymostatin co-infusion (60.62 ± 8.71 pmol/L, Figure 8.6A), reducing IR-AngII by 87%. In contrast, both captopril and chymostatin significantly inhibited perfusate concentrations of IR-AngII collected from hearts infused with AngI by 92% and 81% respectively ($P<0.001$ vs. AngI alone, Fig 6B). (IR-AngII within the drug-infusion syringe was non-detectable).

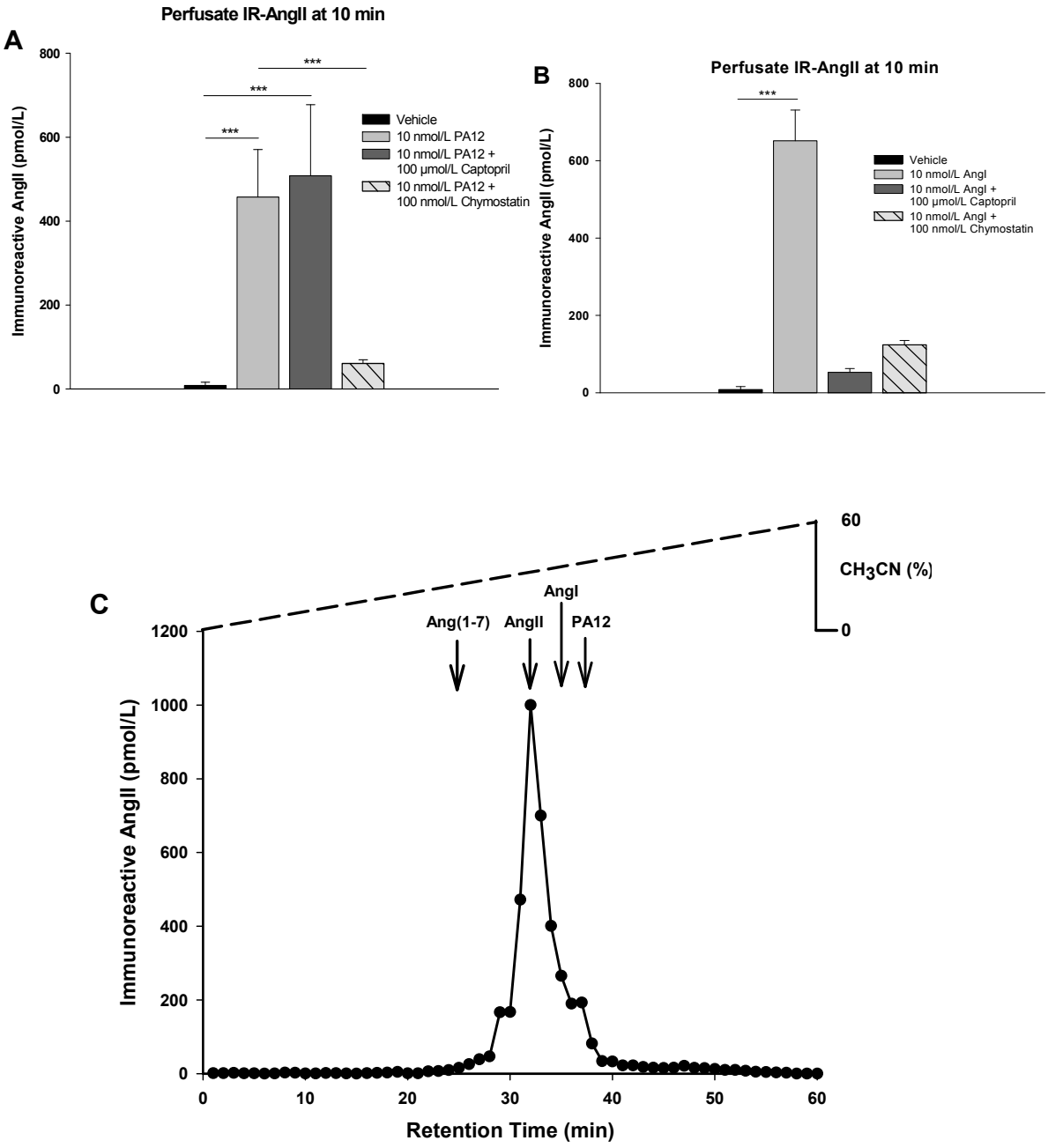


Figure 8.6 Perfusate IR-AngII from rat hearts infused with 10 nmol/L PA12 (A) or 10 nmol/L AngI (B), and effects of supra-added 100 µmol/L captopril and 100 nmol/L chymostatin. (C) Perfusate samples collected from hearts infused with PA12 + captopril were subjected to RP-HPLC (elution gradient 0-60 % CH_3CN over 60 min (1 ml/min)). Eluted fractions were collected and measured for immunoreactive-AngII (IR-AngII) via specific RIA and overlapped with known elution times of angiotensin peptides (indicated by arrows; ***Using a students t-test $P<0.005$).

The presence of authentic AngII within the cardiac perfusate was confirmed by subjecting perfusate samples to RP-HPLC / RIA. Hearts preconditioned with PA12 alone and PA12 with captopril combined produced a peak of immunoreactive AngII that eluted consistent with synthetic standard (Figure 8.6C), indicating captopril was incapable of abolishing the conversion of PA12 to AngII in the heart. In contrast, co-infusion of PA12 with chymostatin significantly attenuated AngII generation (Figure 8.6A).

8.3.5 Chymase converts PA12 to AngII in vitro – HPLC MS/MS analysis

In vitro incubation of PA12 with chymase confirmed chymase is capable of converting PA12 into authentic AngII. Thus, 30 min incubation of PA12 alone in PBS yielded on RP-HPLC / UV a single peak corresponding with PA12 standard (Figure 8.7A, solid line). Incubation of chymase with PA12 resulted in several UV peaks, 2 of which correlated exactly with AngII and PA12 standards (Figure 8.7B). A third minor peak also was observed at 27 min, consistent with Ang(1-7), (Figure 8.7B). The addition of chymostatin abolished all peaks bar PA12 on UV. RIA analysis of UV fractions from HPLC revealed that incubation of PA12 alone contained barely detectable IR-AngII (Figure 8.7A). In contrast, incubation of chymase with PA12 produced significant IR-AngII (Figure 8.7B, dashed line). Addition of chymostatin abolished all IR-AngII (data not shown). MS/MS analysis of the IR / UV AngII peak in Figure 8.7B confirmed that it was authentic AngII, with a confirmed Mr of 1046.4899 (Figure 8.7C). MS/MS analysis of a single UV peak in Figure 8.7A, gave a Mr of 1572.7407, identical with that of complete PA12 (Figure 8.7D).

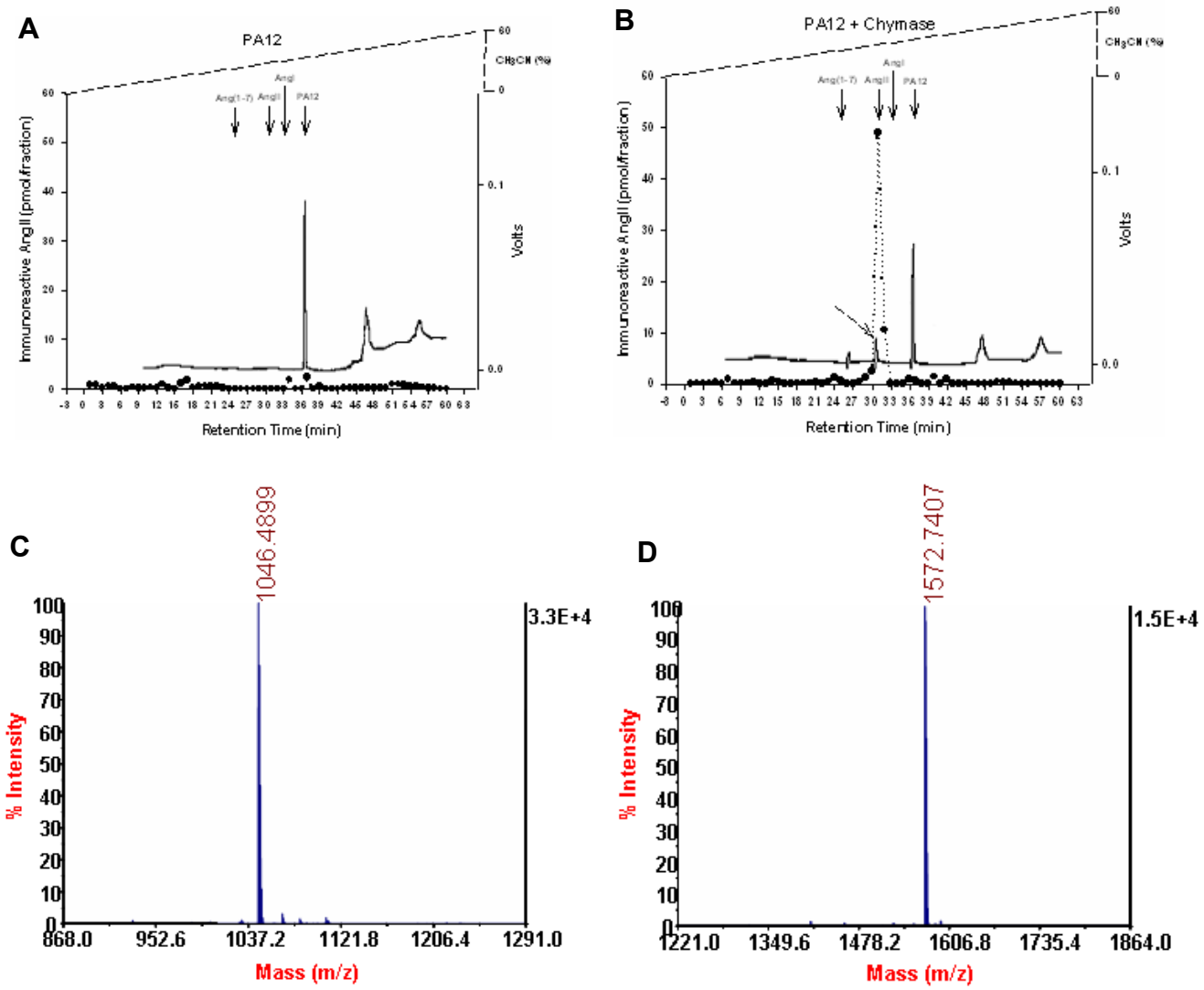


Figure 8.7 RP-HPLC and MS/MS analysis of PA12 incubated with chymase at 37ºC for 30 min in PBS. Each treatment: PA12 alone (panel A), and PA12 + chymase (panel B), underwent RP-HPLC with UV detection (solid line, right-hand axis), and specific AngII RIA for AngII (dotted line, left-hand axis). Arrow indicates AngII peak on the UV profile corresponding to the IR-AngII peak (B). Tandem Mass Spectrometry performed on treatment 'PA12 + chymase' confirmed the presence of both AngII (C) and PA12 (D). Chymostatin abolished all detectable AngII when incubated with PA12 and chymase combined (not shown).

8.4 Discussion

PA12 is a newly isolated peptide, identified as a potential component of the RAS by Nagata and colleagues in late 2006, who reported that PA12 dose-dependently constricted rat aorta segments and significantly elevated blood pressure *in vivo*, via ACE1- and AT_1 receptor-dependent mechanisms (Nagata et al. 2006). There have been no reports describing the direct cardiac actions of PA12, and only a single report has described the conversion of PA12 to AngII by the heart (Trask et al. 2008). Accordingly, we provide in this report the first evidence that:

1) PA12 infusion induces potent, dose-dependent vasoconstriction in rat coronary arteries, without significant effect on LV contractility.
2) PA12 significantly worsens ischemia-reperfusion injury.
3) PA12 actions are blocked by AT_1R and chymase inhibition, but *not* ACE1.
4) Chymase effectively processes PA12 to authentic AngII *ex vivo* and *in vitro*.

The cardiac vasoconstrictive actions of PA12 were approximately equal to that of AngII, and lasting the entire period of infusion and reperfusion. We observed no effects of PA12 upon LV contractility, nor any effect with infusion of 10 nmol/L AngI. This may suggest that PA12 actions are restricted to the vascular surface, and be minimal or absent in cardiac myocytes; or, that its effects were blunted in our single pass Langendorff setup. However, AngI also had no effects upon LV contractility and coupled with previous reports suggesting AngI and AngII exert inotropic effects in atrial but not ventricular myocardium (Holubarsch et al. 1993; Holubarsch et al. 1994), it is possible AngI and PA12 possess limited ability to contract LV tissue.

In a model of ischemia-reperfusion injury, we found preconditioning the heart with PA12 significantly elevated coronary PP following ischemia, and increased both CK and TnI release. PA12 also maintained cardiac atrial natriuretic peptide (ANP) production slightly above that of hearts preconditioned with vehicle during both infusion and reperfusion. Taken together these results indicate PA12 impairs recovery from global ischemia in the rat heart by reducing coronary flow and causing increased myocardial damage. These results were comparative with hearts preconditioned with

AngI, however differed in that AngI exhibited slightly reduced potency immediately following ischemia when compared with PA12.

Captopril, chymostatin and CV-11974 specifically inhibit ACE1, chymase and the AT_1 receptor respectively (Feinstein et al. 1976; Patchett et al. 1980; Naka 1993). Blockade of the AT_1 receptor abolished the PA12-induced vasoconstriction, revealing the AT_1 receptor can mediate the response to PA12, indicating that either PA12 itself binds and activates AT_1R, or PA12 is initially cleaved into fragments capable of binding and activating AT_1R. To examine whether PA12 activity is dependent upon ACE1, captopril (and ramipril) were co-infused directly into the rat heart with PA12. Neither antagonist inhibited PA12-induced vasoconstriction in the isolated rat heart, nor had any effect on CK or TnI levels following ischemia. Analysis of perfusate samples collected after passing through the heart revealed an elevated presence of perfusate AngII from hearts infused with PA12, indicating PA12 was converted to AngII, not by ACE1, but by another cardiac enzyme. The concentrations of captopril and ramipril used in this study were relatively high (100 μmol/L), newly obtained and capable of causing significant vasodilation when infused alone, indicating potency and viability. These results contrast those of Nagata et al., which suggested PA12 constriction of aortic strips and change in mean arterial pressure *in vivo* were dependent on ACE1 (Nagata et al. 2006). Differential tissue expression of chymase and ACE1 may underlie these discrepancies, as well as ex vivo versus in vivo methodological differences, but also the relative activities of chymase and ACE1 in the circulation to produce AngII from PA12 need to be clarified.

The ‘normalized’ results observing PP would suggest that inhibition of ACE may, in fact, potentiate PA12 activity. This fit with the observed minor elevation in perfusate IR-AngII levels from hearts infused with PA12 + captopril compared with infusion of PA12 alone. There is little, if any, literature observing this phenomenon where inhibition of ACE stimulates upregulation of chymase and/or other enzymes capable of generating elevated vasoconstriction and AngII formation; as in all studies observing ACE versus chymase-induced AngII generation and vasoconstriction, AngI has been the substrate, which we and others (de Lannoy et al. 2001; Kokkonen et al. 2003) have observed to have little dependence upon chymase activity. Wei et al. (2003) suggested an interaction between mast cells and Bradykinin (BK) in the

cardiac interstitium, whereby antagonism of the BK_2 receptor significantly reduced mast cell density (Wei et al. 2003). Combining this latter report with knowledge that ACE1 inhibition increases BK_2 activity (Minshall et al. 1997), leads to the hypothesis that ACE inhibition could theoretically increase mast cell density, providing a greater amount of chymase and other ACE-independent AngII-forming enzymes capable of generating AngII from PA12.

Boucher et al. (1974) were the first to show enzyme(s) other than ACE are capable of converting AngI into AngII (Boucher et al. 1974). Indeed enzymes other than ACE1 and ACE2 have been shown to convert angiotensinogen or AngI into smaller active peptides and/or inactive fragments, including carboxypeptidases and chymase (Robertson and Nicholls 1993). ACE1 inhibitors do not completely block AngII production and ACE1-independent pathways are present in many mammalian species (Gondo et al. 1989; Hoit et al. 1995; Urata et al. 1996; Shiota et al. 1997; Padmanabhan et al. 1999). Chymase, a serine protease, is synthesized and stored in granules of mast, endothelial, and mesenchymal cells widely expressed throughout mammalian tissues (Urata et al. 1994; Urata et al. 1996). Indeed, chymase is suggested to be the enzyme primarily responsible for AngII formation in tissue, while ACE1 is the dominant conversion mechanism within the circulation (Miyazaki and Takai 2006; Miyazaki et al. 2006). Accordingly, we employed chymostatin, a chymase inhibitor, to test whether chymase may be responsible for cardiac PA12 conversion to AngII in our heart preparations. Chymostatin significantly inhibited PA12-induced vasoconstriction in the isolated rat heart during both infusion and reperfusion, as well as reducing the perfusate AngII concentrations by ~80%. *In vitro* analysis confirmed that chymase alone is capable of converting PA12 into AngII, and overall these results suggest that the cardiac conversion of PA12 to AngII is at least partially chymase-dependent.

In contrast to PA12, generation of AngII from AngI was shown to be dependent upon ACE1 availability, consistent with prior reports (de Lannoy et al. 2001). The addition of chymostatin caused significant attenuation of AngI-induced AngII generation (as previously reported (Muller et al. 1998)), however exhibited minimal antagonism of AngI-induced vasoconstriction. This indicates chymase can convert AngI to AngII, and suggests that chymase (or other cardiac enzymes) may further cleave AngI or

AngII into AngIII (angiotensin-(2-8)) and/or AngIV (angiotensin-(3-8)), both shown to be potent vasoconstrictors (van Esch et al. 2008). If so, the current study could only observe perfusate AngII levels via RIA, explaining the discrepancy between the non-significant reduction in PP and the significant reduction in AngII production from hearts receiving chymostatin + AngI.

We cannot exclude ACE2-mediated conversion of PA12 to AngII and other related active peptides. For example, our HPLC profiles were consistent with some Ang(1-7) formation, and either chymase or ACE1 may further cleave AngII to AngIII or AngIV. The data also cannot rule out PA12 itself binding to AT_1R, causing the hemodynamic effects observed. However, as chymostatin abolished PA12-induced vasoconstriction and severely reduced generation of AngII, it is likely that AngII is responsible for most of the observed bioactivity.

PA12 is expressed in its highest levels within the small intestine (Nagata et al. 2006), where a close analogue of chymase, chymotrypsin, is also expressed in high amounts and secreted from the pancreas (Goldberg 2000). Bovine α-chymotrypsin is capable of hydrolyzing AngI into AngII, before degrading AngII into biologically inactive fragments (Trong et al. 1987). PA12 may also be converted into active AngII and have a specific role within the small intestine, possibly linking digestive and renin-angiotensin systems. Thus, we extend these reports to identify PA12 as a potentially new precursor peptide specific for tissue-based generation of AngII. AngII blockade (by ACE or AT_1R blockade) is a mainstay of treatment in hypertension, after ischemic cardiac events and in human heart failure. Breakthrough of AngII generation is known to occur over the weeks following initiation of ACE1 treatment and may contribute to alleviation of ACE1-related cardiovascular protection (Robertson and Nicholls 1993). Elucidation of alternative pathways of AngII generation enhances the understanding of cardiovascular pathophysiology and may lay the foundation for improved therapy. Furthermore, the high expression of PA12 within the gut combined with its potent vasoconstrictive properties observed in the heart raises the possibility that PA12 links gut processes with concurrent circulatory responses. It is yet to be reported whether PA12 levels in the gut and circulation correlate with each other and/or the effects of eating.

Whilst it is yet to be established whether ACE2 has any role in PA12 activity, (or whether PA12 is in fact present in humans or other mammalian species), we found this newly discovered peptide PA12 infused in isolated perfused rat hearts caused potent, sustained vasoconstriction and impaired recovery from ischemia. IR-AngII was significantly elevated in perfusate collected from hearts infused with PA12, indicating PA12 is converted into AngII. One enzyme responsible for the conversion of PA12 to AngII appears to be chymase, and the AT_1 receptor was found to mediate PA12 activity, most likely binding PA12-derived AngII. Further studies identifying PA12 in humans and its role in control of the circulation are clearly required.

9. Vascular response to Proangiotensin-12 (PA12) through the rat arterial system

Abstract

Proangiotensin-12 (PA12) is the most recent vasoactive peptide to be identified as a functional component of the renin-angiotensin system (RAS). Recent reports indicate PA12 may provide an alternate substrate to AngI in generating AngII. The vascular response to PA12, and conversion of PA12 to AngII have been reported as being both angiotensin-converting enzyme 1- (ACE1-) and chymase-dependent. The current study employs myography to determine the direct vascular effects of PA12 administered to specific vessels isolated from the rat arterial system including the: right and left common carotid arteries, descending thoracic aorta, abdominal aorta, renal, mesenteric and femoral arteries. The dependence of PA12 upon ACE1 and chymase is revealed through the use of enzyme inhibitors, with AngII applied at the same dose providing a measure of comparison. PA12 caused significant constriction in 5 of 7 the vessels tested (all $P<0.05$), displaying no significant effect on the renal or femoral arteries ($P>0.05$). AngII significantly stimulated constriction in the same arteries (all $P<0.05$), paralleling PA12 vasoactivity. A potency gradient across the animal in response to PA12 was apparent, with vessels in closest proximity to the heart responding with the greatest constriction. Inhibition of ACE1 and chymase significantly attenuated PA12-induced constriction, with chymostatin displaying slightly reduced potency (although not significantly different). In sum, chymase and ACE1 regulated PA12-induced vasoconstriction throughout the rat arterial system. We postulate that ACE1 may primarily regulate RAS activity within the circulation, while chymase may have a greater role within local, tissue-based RAS activity.

9.1 Introduction

The most recent peptide to be identified within the renin-angiotensin system (RAS) is proangiotensin-12 (PA12), which comprises angiotensin I (AngI) with C-terminally extended -Leu^{11}-Tyr^{12}. PA12 was isolated from the rat small intestine, (the organ of highest gene and peptide concentrations, but PA12 is also found throughout the tissues of all major organs as well as being present in the circulation at approximately one third that of angiotensin II (AngII) (Nagata et al. 2006; Jessup et al. 2008). Because of its wide distribution it is desirable to identify the effects of PA12 throughout the systemic and peripheral vasculature. As previously reported, PA12 potently constricts isolated rat aortic strips and rat coronary arteries within an isolated heart preparation (Nagata et al. 2006).

In the current study we sought to document any vasoactive properties of PA12 outside of the heart, employing myography to measure the response to PA12 in 7 different arteries from the rat vascular system: the left and right common carotid arteries (LCCA and RCCA respectively), descending thoracic aorta (DTA), abdominal aorta (AbA), mesenteric artery (Mes), renal artery (Ren) and femoral artery (Fem). The enzyme inhibitors chymostatin and captopril were employed to indicate whether chymase or ACE1 respectively, mediated PA12-induced activity in each vessel.

9.2 Materials and Methods

9.2.1 Myography of rat vessels

Male Sprague Dawley rats weighing 320-420g (60-75 days old) were obtained from Christchurch School of Medicine, Christchurch, NZ. Rats were housed under controlled conditions (21ºC, ~40% humidity, natural day length), and had free access to standard rat chow and water. Rats were anaesthetized by an intraperitoneal (IP) injection of sodium pentobarbital (50mg/kg). Once anaesthetized the head was guillotined and a t-incision made across the belly of the animal exposing the pericardium to the urethra. The rib cage was opened and the ribs cut on either side close to the vertebrae, fully exposing the heart and lungs. The initial ventral incision was continued transverse, across the animal, exposing all arteries to be excised. Sections of the left and right common carotid arteries, descending thoracic aorta, abdominal aorta (posterior to the renal arteries), mesenteric, renal and femoral arteries were all excised and immediately placed into individual, half-filled 20ml vials of ice-cold rat buffer comprising (mM): 123 NaCl, 123 NaCl, 22.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.1 $MgSO_4 \cdot 7H_2O$, 1.5 $CaCl_2 \cdot 2H_2O$ and 11.0 glucose (final pH 7.40). When required, vessels were cut to 2 mm lengths and placed into fresh rat buffer contained within the bath of a Mulvany Myograph (model 410A, JP Trading, Århus, Denmark) maintained at 37 ºC using a flow-through water heating system, and oxygenated with 95% O_2 / 5% CO_2 (see Appendix B.2 for supplementary methods). Wires were inserted through the lumen, whilst avoiding endothelial damage, and attached to jaws on either side of the vessel. The vessel could then be tensioned in the radial plane by moving one jaw away from the other. The bath was drained and replaced with fresh buffer, removing any metabolic agents released during the excision process. Vessels were pretensioned and given at least 1 hr to reach their equilibrated resting tension. Two identical myographs were used, each with 2 sets of jaws with a dam inserted between them enabling experimentation of 4 vessels simultaneously. Both myographs contained buffer from the same stock solution, and the baths were maintained at equal temperatures providing identical media for all 4 vessels, and enabling application of 4 different treatments. Changes in tension were recorded using Chart 4 software (AD Instruments).

One ‘control’ vessel was untouched throughout the experiment to allow measurement of any spontaneous changes in tension. The remaining 3 vessels were administered 10 nM PA12 (obtained from Phoenix Pharmaceuticals, Belmont, CA, USA), followed by a wash out period, removing the media and replacing with fresh buffer at least 3 times. Vessels were then allowed 20 min to resettle at their resting tension before 10 nM AngII (obtained from Sigma-Aldrich, St. Louis, MI, USA) was administered directly into the bath and the response recorded for 25 min. At the end of the experiment each vessel was administered 120 mM KCl to obtain a close to maximal contraction and ensure viability. This process was repeated for each different vessel type.

9.2.2 Antagonist protocol

Vessels dissected from one animal were cut into 2 mm lengths to provide at least 2 smaller sections of the same vessel. These sections were prepared as described above and held within each myograph separated by a dam. One section acted as the control receiving 10 nM PA12 alone, while the other received PA12 combined with 100 nM chymostatin, or preconditioned for 10 min with 100 μM captopril prior to the addition of 10 nM PA12 (both antagonists obtained from Sigma-Aldrich, St. Louis, MI, USA). Running the vessels in pairs enabled simultaneous recording of the effect of the antagonist against its parallel control vessel. This process was repeated for each different vessel type. The doses of antagonists applied were based on a previous report in isolated rat hearts (Prosser et al. 2009).

9.2.3 PA12 peptide stability

In order to determine the stability of PA12 within the rat buffer bath maintained at 37 ºC over the duration of the time period, samples were taken at time 0 (immediately after adding PA12 to the bath), and again at 25 min from the same bath. The bath was maintained under the standardized conditions listed above, but held no vessel. Samples were subjected to Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) using the same C_{18} column and under the same elution gradient (0-60% CH_3CN over 60 min). UV profiles were obtained to determine peptide degradation.

9.2.4 Statistical analysis

All data are presented as mean +S.E.M. Analysis of changes in vessel tone were performed on SPSS using a two-way ANOVA with repeated measures, with Bonferroni's multiple comparison test, post hoc. Individual data point comparisons were made using a Student's T-test. In all statistical tests a value of $P<0.05$ was considered significant.

9.3 Results

9.3.1 Effect of PA12 on vessels throughout the rat arterial system

PA12 potently constricted 5 of the 7 vessels measured (Figure 9.1), the most potent response observed in the left (n=7) and right (n=10) common carotid arteries, increasing tension by 174.5 ± 27.6 (P=0.004) and 181.7 ± 16.8 % (P=0.002) respectively. The descending thoracic aorta (n=10), mesenteric (n=10) and abdominal aorta (n=9) all responded positively to PA12, significantly elevating tension by 128.1 ± 6.4 (P=0.003), 118.9 ± 15.0 (P=0.049) and 115.0 ± 7.6 % (P=0.003) respectively. The renal (n=6) and femoral (n=7) arteries exhibited no significant response to PA12 or AngII (both $P>0.05$), although the renal artery displayed minor constriction towards the end of the time period while the femoral exhibited minor dilation (Figures 9.1F & G).

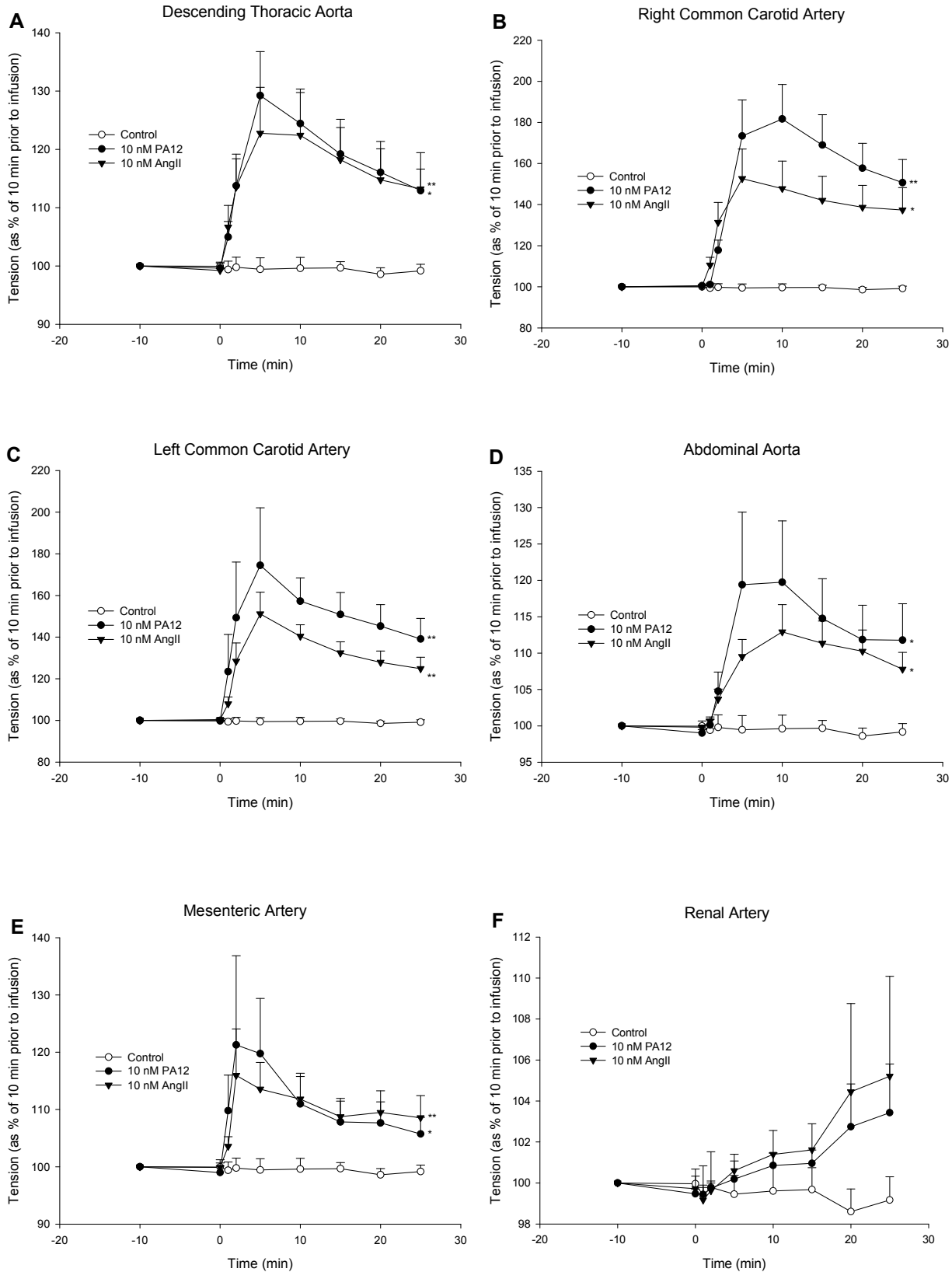
9.3.2 Comparison with angiotensin II

AngII significantly constricted the same 5 vessels as PA12 (all $P<0.05$), and similarly had no significant effect on Fem or Ren arteries ($P>0.05$, Figure 9.1).

Compared with PA12, AngII elicited less vasoconstriction when administered to the RCCA, LCCA, and AbA, although did not significantly differ ($P>0.05$). The DTA and Mes arteries responded equally to both PA12 and AngII indicating approximately equal potency (Figure 9.1).

PA12 constricted the RCCA, LCCA, DTA, AbA, Mes and Ren 90.9, 88.4, 62.5, 15.7, 13.2 and 2.9 % of 120 mM KCl-induced constriction respectively (Figure 9.2). AngII elicited a similar pattern of response to PA12, although displayed slightly reduced potency in some vessels (Figure 9.2). AngII stimulated greater constriction of the

renal artery compared with PA12, and AngII stimulated minor constriction of the femoral artery, while PA12 caused minor dilation (Figure 9.1G & 9.2).



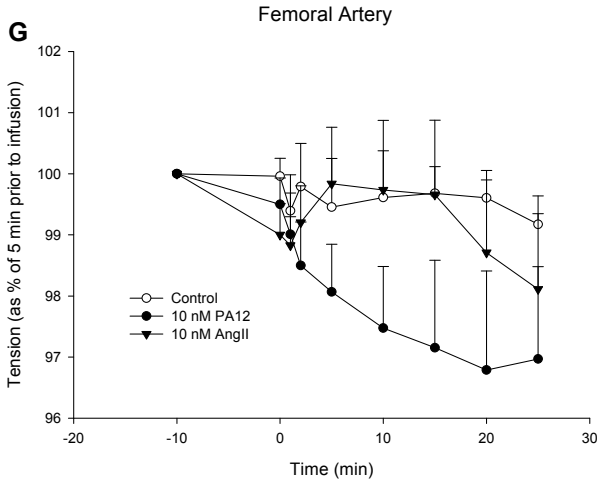


Figure 9.1 PA12 and AngII potently constricted the rat vessels: descending thoracic aorta (A), right and left common carotid arteries (B and C respectively), abdominal aorta (D) and mesenteric artery (E). Both PA12 and AngII had no significant effect on the renal (F) or femoral arteries (G). Using two-way ANOVA with repeated measures: *P<0.05, **P<0.005 compared to control.

9.3.3 Antagonist Studies

Preconditioning the vessels with 100 μM captopril significantly inhibited PA12-induced constriction in all 5 vessels significantly responsive to PA12 (all P<0.05 vs PA12 alone), and abolished the minor PA12-induced vasoconstriction observed in the renal artery (Figure 9.3). The addition of 100 nM chymostatin to 10 nM PA12 also significantly inhibited PA12-induced vasoconstriction in 4 of the 5 vessel types significantly responsive to PA12, but with lower potency; and it abolished the minor PA12-induced constriction of the renal artery (Figure 9.3F). There was evidence of inhibition of PA12-induced vasoconstriction of the DTA by chymostatin, but this was not significant (P=0.674) (Figure 9.3A).

Of note, chymostatin and captopril both attenuated the minor vasodilation observed in the femoral artery in response to PA12 (Figure 9.3G). However, this was not statistically significant as the femoral artery's response to PA12 alone was not significantly different from vehicle levels (P>0.05).

Comparing the two antagonists, captopril exhibited greater inhibitive potency than chymostatin in attenuating PA12-induced activity in all vessels tested and this was most pronounced in the DTA (Figure 9.3A).

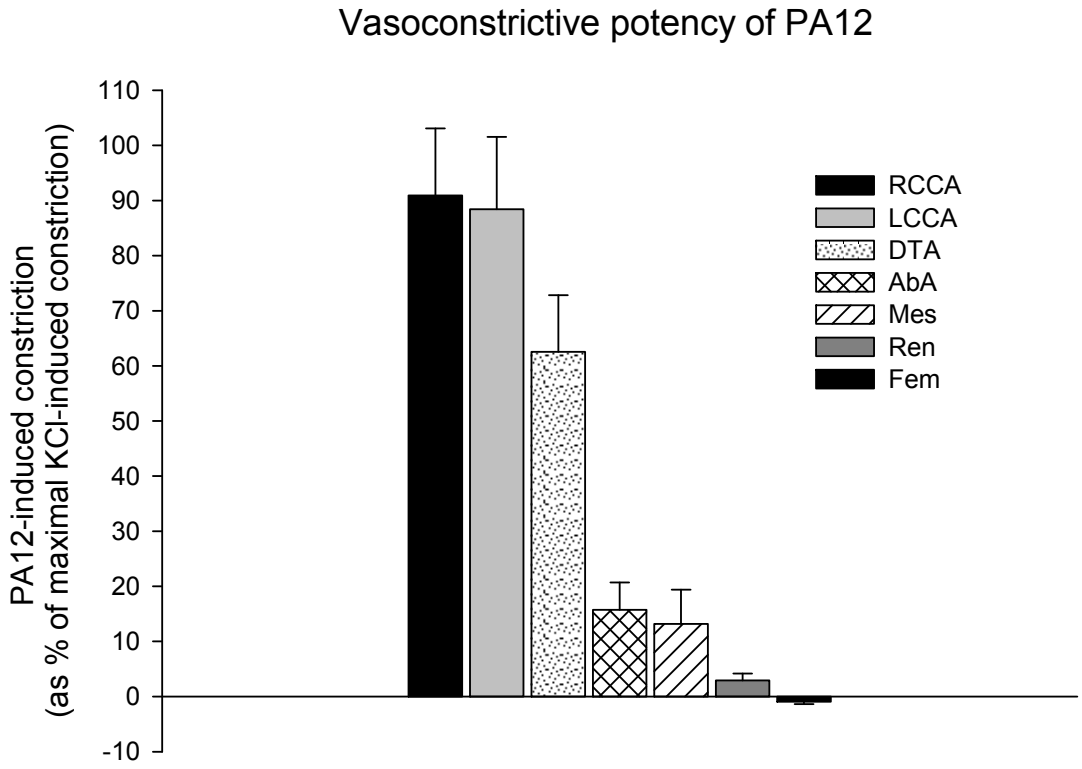
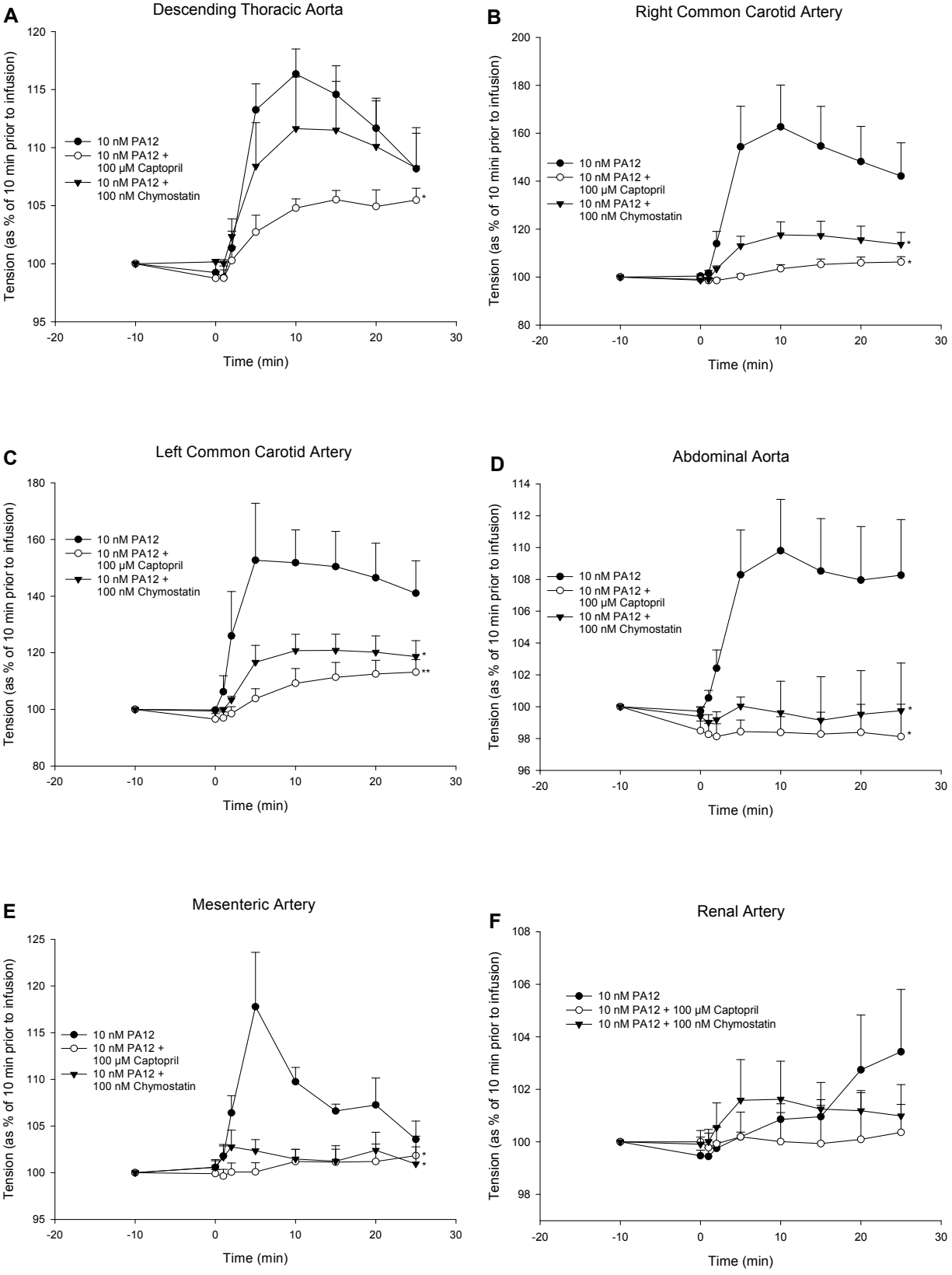


Figure 9.2 The vasoconstrctive potency of PA12 and AngII represented as a % of maximal KCl-induced constriction in each vessel. A potency gradient is clearly apparent based on the vessels' proximity to the heart for both PA12 and AngII.

9.3.4 PA12 Peptide Stability

Samples obtained from a vessel-free bath infused with 10 nM PA12 at times 0 and 25 min were subjected to RP-HPLC. The resulting UV profile displayed equal peak heights from both samples (Figure 9.4) indicating PA12 was stable within the rat buffer at 37 ºC throughout the experimental time period.


A
Descending Thoracic Aorta
Tension (as % of 10 min prior to infusion)
Time (min)
10 nM PA12
10 nM PA12 + 100 μM Captopril
10 nM PA12 + 100 nM Chymostatin
B
Right Common Carotid Artery
Tension (as % of 10 mini prior to infusion)
Time (min)
C
Left Common Carotid Artery
D
Abdominal Aorta
E
Mesenteric Artery
F
Renal Artery

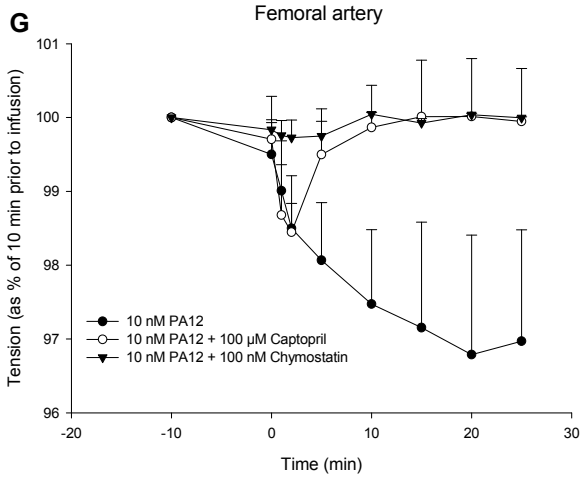


Figure 9.3. The effect of the ACE1 and chymase inhibitors captopril (100 µM) and chymostatin (100 nM) on PA12-induced vasoconstriction in the DTA (A), RCCA (B), LCCA (C), AbA (D) Mes (E), Ren (F) and Fem (G) arteries. Using a two-way ANOVA with repeated measures *P<0.05, **P<0.005 compared to 10 nM PA12 alone.

9.4. Discussion

PA12 caused potent, significant vasoconstriction in 5 of the 7 vessels obtained from the rat arterial system. The vessels with closest proximity to the heart displayed the greatest response to PA12, while potency was reduced and eventually lost in vessels further from the heart. Thus, a potency gradient was observed in response to PA12 with the order of greatest response to the least responsive being: RCCA>LCCA>DTA>Mes>AbA>Ren>Fem. AngII displayed a similar gradient of response as PA12, suggesting both peptides have primary roles within the central arteries immediately surrounding the heart, with less influence on peripheral arteries. To our knowledge, no one study has reported the effects of AngII on isolated vessels from localised points throughout the arterial system.

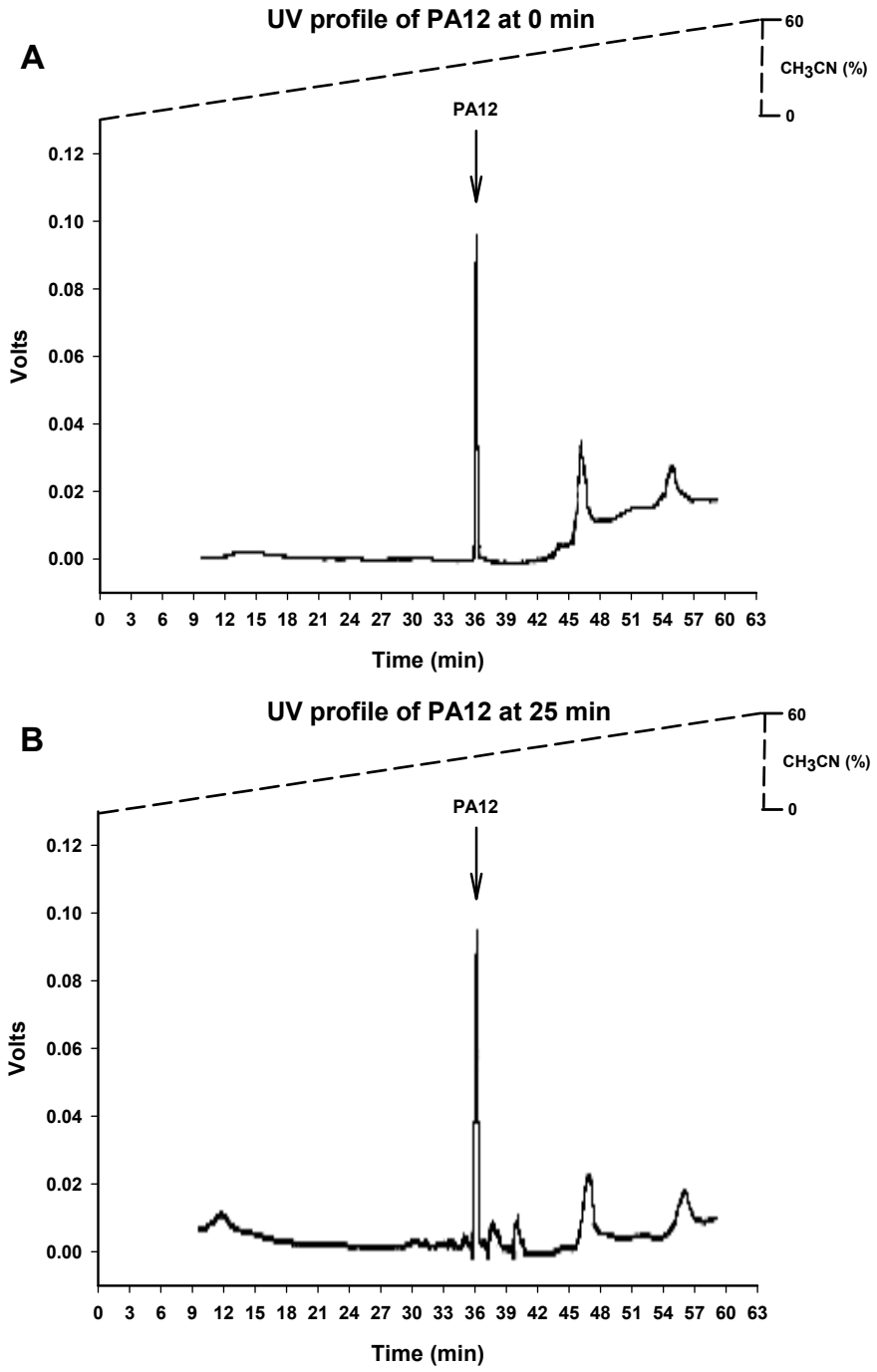


Figure 9.4. UV profile of PA12 in rat buffer at 0 min (A), and after incubation at 37 ºC for 25 min (B). Each sample was subjected to Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) using the same C_{18} column and under the same elution gradient (0-60% CH_3CN over 60 min). Arrow indicates PA12 standard previously undergone RP-HPLC under the same conditions.

However, compiling the current literature it is apparent AngII causes marked vasoconstriction of many arteries including those observed in the current study (Fisher et al. 1968; Gillespie and Rae 1972; Urabe M 1987; Chiba and Tsukada 1989; Merkel et al. 1990; Prins et al. 1992; Caputo et al. 1995). Interestingly, the effect of AngII on the femoral artery has been reported as both constrictive and dilative (Li and Zimmerman 1990), dependent upon the prostaglandins stimulated (Satoh et al. 1984; Yoshida et al. 1991). This provides possible explanation into why no constrictive effects were observed in response to.PA12 or AngII in the current study, and explains the minor reduction in tension observed in response to PA12 in the femoral artery. Whether AngII or PA12 stimulation of prostaglandins within the femoral artery is dependent upon the peptide warrants further research. Both chymostatin and captopril attenuated the minor PA12-induced vasodilation of the femoral artery, suggesting that ACE1 and chymase mediate this response. The renal artery exhibited no significant response to PA12 or AngII, although it did display minor vasoconstriction towards the end of the time period. Previous reports have all indicated AngII constricts the renal artery (Fisher et al. 1968; Cavarape et al. 2003; Zhong et al. 2007), although some have reported that there is a minor and short-lasting constriction in response to AngII (Lupu et al. 1968).

The pattern of response to PA12 over the course of the time period following the bolus infusion was found to be similar in each vessel. PA12 caused immediate constriction rising to its maximum within 5-10 min. Maximal tension was retained for 5-10 min then fell steadily over the course of the remaining recorded time frame. The gradient of both constriction and loss of tension was found to be somewhat vessel-dependent. For example, PA12 caused rapid constriction of the Mes artery, reaching its maximum tension at 5 min before losing 73% of PA12-induced constriction over the following 20 min (Figure 9.1E); compared with RCCA which took longer to reach maximal constriction (at 10 min) but lost just 38% of its initial constriction over the remaining 15 min (Figure 9.1B). The response to AngII was parallel to that of PA12 in all vessels observed, mirroring the rise and fall in tension. The vessel-dependent pattern in response to PA12 and AngII may be indicative of slight variations in the physiological role of each vessel, the distributive pattern of AT1R, and/or the composition of the vessel wall. This may afford a level of autonomy of vessels, despite central control.

The drop in constriction over the course of the time period following the bolus infusion could be indicative of the peptide becoming degraded in the solution over time. However, this theory was tested and disregarded as samples taken at time 0 and 25 min from a water bath infused with 10 nM PA12 under the same standardised conditions found PA12 to be stable over the time period. This indicates the effect of PA12 and AngII are relatively short lasting, revealing the occurrence of tachyphylaxis, and possible loss of intracellular signalling. AngII has been previously reported to provoke tachyphylaxis (Aboulafia et al. 1989), suggested to be mediated by reductions in intracellular PLC/PKC/IP3 activity as well as reduced surface receptor availability (Pfeilschifter 1988; Ullian and Linas 1990; Feng et al. 1998). It was for this reason that vessels were exposed to only a single concentration of agonist.

Our previous report (Prosser et al. 2009) observing PA12 activity in the isolated rat heart concurs with the current findings, where PA12 elicits potent constriction in the coronary arteries. Studies discovering the enzyme mediating PA12 activity and the conversion of PA12 to AngII have given mixed results, with one report indicating ACE1 is responsible for PA12 activity *in vivo* and *ex vivo* (Nagata et al. 2006), whilst our own research reported PA12 to be chymase-dependent *ex vivo* and *in vitro* (Prosser et al. 2009). The current study found inhibition of ACE1 and chymase attenuated constriction in vessels significantly responsive to PA12. Captopril was found to significantly reduce PA12 activity in all 5 vessels significantly responsive to PA12, while chymostatin significantly inhibited PA12 activity in 4 of the vessels, but only showed a non-significant inhibition of PA12 activity in the DTA. ACE1 inhibition elicited slightly greater potency in attenuating PA12-induced constriction in all arteries tested, suggesting ACE1 possesses greater potency than chymase in controlling PA12 activity within the circulation. However, it must be noted that the concentration of captopril was 1000x greater than that of chymostatin, and had equal doses of the 2 antagonists been applied chymostatin may have become equally, or more, potent than captopril.

Our results concur with previous reports indicating PA12 activity is mediated by both ACE1 and chymase activity. This would explain the ACE1-dependent results observed by Nagata et al. (2006) in live rats and aortic strips (Nagata et al. 2006). The

current report supports the ACE1-dependent activity of PA12 in the vascular system, and adds to it by reporting that inhibition of chymase also significantly reduces PA12 activity throughout the rat arterial system. This is in line with our previously reported hypothesis, where combining the literature and our own studies, it appears ACE1 is the enzyme primarily responsible for AngII-generation in the circulation, while chymase may be the dominant AngII-producing enzyme within other tissues including the heart (Urata et al. 1996; Prosser et al. 2009). The tissue-based AngII-generating system is yet to be fully understood, however it is becoming increasingly evident that AngII-generation can occur within the tissue independent of the circulation. Brain, kidney, heart and vascular tissue all contain angiotensinogen, renin and ACE1 as well as their respective mRNAs, providing all the necessary agents required to produce AngII locally (Danser and Schalekamp 1996; Bader and Ganten 2008). Speculation has occurred as to the existence of tissue-based renin and renin mRNA, however it has been reported that renin can be released from mast cells, and cathepsin-D is also capable of converting angiotensinogen to AngI (Katwa et al. 1996; Mackins et al. 2006). PA12 may represent another important agent within the tissue-based RAS, generating ACE1-independent AngII formation within cardiac tissue and potentially other organs as PA12 expression is vastly greater than that of AngI within the tissue of many central organs (Nagata et al. 2006). Taken together, these results, and those of our prior report (Prosser et al. 2009) indicate alternative mechanisms of angiotensin II synthesis within the cardiac tissue and arterial system exist and reinforce the need for novel therapeutic agents to block the tissue-based, ACE1-independent renin-angiotensin system.

It was hypothesized that the mesenteric artery should be highly responsive to PA12, and that chymostatin would abolish its activity. This hypothesis was based upon the report that the rat small intestine expressed the greatest amount of PA12 (Nagata et al. 2006), as well as high levels of chymotrypsin, a potent analogue of chymase (Bacani and Frishman 2006). We found that the mesenteric artery was highly responsive to PA12, and AngII was similarly potent, but the proportional change in tension was less than in carotid arteries. Our hypothesis was supported by the fact that chymostatin showed greater inhibition of PA12-induced vasoconstriction in the mesenteric artery than in any other artery tested. However, captopril displayed equal inhibitive potency, although was 1000 fold greater in concentration than chymostatin. This suggests

PA12 cleavage is not dependent upon chymase availability in the mesenteric artery as ACE1 can mediate PA12 activity.

Based on these results and our previous report, chymase is beginning to emerge as a potentially important enzyme in the local, tissue-based RAS within the heart as well as in the vasculature. PA12 was found to be chymase-dependent within the isolated rat heart with ACE1 inhibition having little effect on PA12 activity (Prosser et al. 2009). Within the circulation both ACE1 and chymase were shown to both be capable of mediating PA12 activity, with inhibition of ACE1 displaying slightly greater inhibitive potency. This was most evident in the DTA of our current report. The notion that the PA12-chymase pathway may be primarily involved in tissue-based, local AngII-generation is supported by the findings of Nagata et al. (2006) that PA12 expression was significantly higher than that of AngI in the tissue of many organs throughout the rat, however AngI expression was greater than PA12 in the aorta and circulation (Nagata et al. 2006).

It must be noted that the chymase inhibitor employed in the current study, chymostatin, is a non-specific chymotryptic inhibitor capable also of inhibiting cathepsin-G, an enzyme known to convert AngI to AngII (Owen and Campbell 1998; Belova 2000; Rykl et al. 2006). Therefore, it cannot be ruled out that cathepsin G is the active enzyme mediating PA12 activity, nor can we positively conclude whether PA12 itself can bind and activate AT_1R or whether it first must be cleaved into AngII or other angiotensin fragments capable of activating the receptor. Thus, future studies employing more specific enzyme inhibitors are required to reveal which enzymes are primarily responsible for converting PA12 to AngII, and structure-activity studies are needed to observe whether PA12 can bind and activate the AT_1 receptor directly.

10. Intracellular pathways stimulated in response to proangiotensin-12 (PA12)

Abstract

PA12 is a newly identified peptide within the renin-angiotensin system (RAS), recently reported to provide an alternate substrate for generation of angiotensin II (AngII) within the rat heart, and stimulate, either directly or indirectly, potent vasoconstrictor activity. PA12 expression has recently been reported to be elevated in spontaneously hypertensive rats, indicating a potential pathological effect, confirmed in a study reporting PA12 to promote and augment ischemic injury in the rat heart. The current study furthers exploration into PA12-induced activity by determining the intracellular proteins stimulated in response to PA12. Langendorff isolated rat hearts were infused with PA12 or angiotensin I (AngI) with or without antagonists for angiotensin-converting enzyme 1 (ACE1), chymase, or the angiotensin II type 1 (AT_1) receptor. Left ventricular heart tissue was removed and subjected to western blotting to determine the phosphorylation, and hence activation, of specific cytokines. PA12 and AngI caused phosphorylation of four cytokines: c-Jun N-terminal kinase (JNK), extracellular-regulated kinase p42/44 (ERK1/2), mitogen-activated protein kinase p38 (p38), and protein kinase C α/β_{II} ($PKC\alpha/\beta_{II}$); but had no effect on PKC isoforms δ/θ. PA12-induced cytokine activity was mediated by the AT_1 receptor. PA12-induced phosphorylation of JNK and $PKC\alpha/\beta_{II}$ were found to be chymase-dependent, while ERK1/2 was both chymase- and ACE1-dependent. Inhibition of chymase and ACE1 did not attenuate PA12-induced phosphorylation of p38. AngI-induced phosphorylation of the cytokines was found to be dependent upon ACE1 availability in all kinases investigated with the exception of JNK. That PA12 induced activation of these cytokines suggests PA12 stimulates cellular growth and differentiation, hypertrophy, apoptosis, inflammation and vasoconstriction within the cardiovascular system. The RAS is known for its potent pathological role in progressing vascular disease and hypertension, mediated by the AT_1 receptor. This report is the first to reveal that PA12 acts through the AT_1R stimulating pathways known to augment cardiovascular disease, however in contrast to AngI, PA12 activity can be independent of ACE1.

10.1 Introduction

The renin-angiotensin system (RAS) has evolved to elevate blood pressure, maintaining it within homeostatic levels via a means of humoral and mechanical mechanisms. Since the initial discovery of renin in 1898 (Phillips and Schmidt-Ott 1999), research into the RAS exploded to uncover a seemingly simple, linear cascade of enzymatic activity cleaving a primary peptide (angiotensinogen) into an inactive secondary (angiotensin I) and highly active tertiary peptide (angiotensin II), circulating within the blood. AngII produces a broad range of effects throughout the mammalian body including arteriolar vasoconstriction, stimulation of aldosterone secretion, increased tubular reabsorption of Na^+ and Cl^- while excreting K^+ which increases water retention, and elicits the release of antidiuretic hormone (ADH) from the pituitary, which stimulates further water absorption at the kidneys, as well as reducing glomular filtration rate (Robertson and Nicholls 1993). However, with the recent discovery of a new enzyme, angiotensin-converting enzyme 2 (ACE2) (Crackower et al. 2002), and the identification and isolation of another potential product of angiotensinogen, proangiotensin-12 (PA12) (Nagata et al. 2006), it appears the RAS is not a simple linear process as once thought.

We recently reported the physiological effects of PA12 in the isolated rat heart and showed that PA12 was converted to AngII during cardiac passage (Prosser et al. 2009). Chymase was the enzyme found to be primarily responsible for mediating PA12 activity, as well as mediating the conversion of PA12 to AngII both *in vitro* and *ex vivo*; while angiotensin-converting enzyme 1 (ACE1) had little influence (Prosser et al. 2009). However, other reports have suggested PA12 activity is also ACE1-dependent in the live rat and in strips of rat aorta (Nagata et al. 2006; Trask et al. 2008). The physiological effects of PA12 are reported to be mediated by the angiotensin II Type 1 receptor (AT_1R), with blockade of this receptor abolishing PA12 activity (Prosser et al. 2009).

In this report we further our study into the actions of PA12, observing the intracellular proteins activated in response to PA12 within the rat left ventricle. Additionally, we explore the effects of specific enzyme inhibitors and receptor blockade on PA12- or AngI-induced intracellular protein activity of the 3 classic mitogen-activated protein

kinase (MAPK) pathways: c-Jun NH_2-terminal kinase (JNK), MAPK p38 (p38) and extracellular-regulated kinase p44/42 (ERK1/2), as well as different isoforms of protein kinase C (PKC).

10.2 Methods

10.2.1 Materials

Male Sprague-Dawley (SD) rats weighing 270-400g (60-75 days old) were obtained from the Christchurch School of Medicine, Christchurch, New Zealand. Rats were housed under controlled temperature (21 ºC), humidity (~40%) and natural day length with free access to standard rat chow and water.

Synthetic rat PA12 was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA), while AngI and the ACE1 antagonist Captopril, as well as the chymase inhibitor Chymostatin were all obtained from Sigma-Aldrich (St Louis, MI, USA). The angiotensin II receptor type 1 (AT_1R) blocker, CV-11974 (Candesartan, 2-ethoxy-1-[[2'-(1*H*-tetorazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid) was a generous gift from Takeda Chemical Industries Ltd (Osaka, Japan). PA12, AngI and captopril were diluted in distilled water, aliquoted, and stored at -20 ºC prior to use. Chymostatin was diluted in DMSO, while CV-11974 was dissolved in 1 M Na_2CO_3 solution, aliquoted, and stored at -20 ºC prior to use.

10.2.2 Langendorff isolated rat heart perfusion

Isolated rat heart perfusion was performed as previously described (Pemberton et al. 2000; Prosser et al. 2009). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The heart was rapidly excised and mounted on the Langendorff apparatus, cannulated above the aortic valve and perfused at 12 ml/min (constant retrograde flow) with perfusion buffer comprising (mmol/L): 123 NaCl, 22.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.1 $MgSO_4 \cdot 7H_2O$, 1.5 $CaCl_2 \cdot 2H_2O$ and 11.0 glucose (final pH 7.40). Buffer was maintained at 37 ºC and oxygenated with 95% O_2/5% CO_2. Hearts were allowed to settle for 30 min before being paced at 320 bpm using an electrode placed on the right atrium and attached to a Digimeter DS2A-Mk. II stimulator. Hearts were allowed a further 30 min to resettle before any experimental

protocol was started; see Appendix B.1 for supplementary information and methods. Drugs were diluted using perfusion buffer to enable infusion of the drug or vehicle over 30 min at 0.5 ml/min using a syringe pump feeding directly into the perfusion line above the heart. This investigation was approved by the University of Otago Animal Ethics committee.

10.2.3 Heart Infusion Protocol

All antagonist agents (100 µmol/L captopril, 100 nmol/L CV-11974, 100 nmol/L chymostatin, and 100 µmol/L and Ramipril) were co-infused with either 10 nmol/L PA12 or 10 nmol/L angiotensin I (AngI). Experiments were run in pairs: one heart infused with both the antagonist and PA12 or AngI, the other simultaneously infused only with PA12 or AngI from the same stock solution and same buffer reservoir. The heart given the antagonist was chosen at random.

The concentrations of 10 nmol/L PA12, 100 µmol/L captopril and 100 µM ramipril were based on prior dose-response studies (see Figure 8.2A and Appendix C, Figures C.2 and C.3, respectively). 100 nmol/L CV-11974 and 100 nmol/L chymostatin were administered based upon manufacturers' recommendations. The dose of 10 nmol/L AngI was chosen to provide a circect comparison with PA12.

10.2.4 Western Blotting

10.2.4.1 Tissue preparation and cellular protein extraction

Upon completion of the 30 min infusion rat hearts were rapidly removed from the perfusion apparatus and a section of the left ventricular free wall was obtained, wrapped in aluminum foil, snap frozen in liquid nitrogen and stored at -80 ºC.

When required, frozen heart tissue was combined with 2 ml cell lysis buffer comprising: Tris (pH 8.0) 10 mM, EDTA (pH 8.0) 1 mM, NaCl 150 mM, IGEPAL CA-630 0.5%, Na_3VO_4 1 mM, and inhibitor cocktail 1 µg/ml, (all obtained from Sigma, St. Louis, MI, USA). The tissue was homogenized in the lysis buffer (using an IKA-Ultra Turrax T25, Janke & Kunkel Labortchnik) then incubated on ice for 30 min before being centrifuged at 15,000 rpm for 5 min at 4ºC. The cytosolic supernatant was removed and stored at -80 ºC. Measurement of viable protein

concentration was performed using Bradford's protein assay (Refer Appendix A for Bradford's protein assay methods).

10.2.4.2 Gel Electrophoresis

Gels and protein samples were prepared as previously described (Sambrook et al. 1989) (refer Appendix A). The proteins to be determined in the current report were between 42 and 82 kD requiring an acrylamide concentration of 10 % (Sambrook et al. 1989). Using 2 BioRad mini-gels a total of 20 wells were available per run. Protein samples comprising 35% protein, laemmli dye and Tris-buffered saline (TBS) were loaded into each well having been incubated at 95 °C for 8 min, and underwent electrophoresis at 100V for 2 hours at room temperature using a BioRad PowerPac-200.

Protein transfer onto nitrocellulose membranes was then accomplished using a BioRad PowerPac-200 run at 80V for 1.5 hrs at 4 °C. Membranes were checked for protein staining using Ponceau S dye before being rinsed in TBS + 1% Tween (TBST) and incubated in 4% milk solution for 1 hour at room temperature using a plate mixer.

10.2.4.3 Antibody Incubation

The primary antibodies specific for phosphorylated: ERK1/2 (p-ERK1/2), SAPK/JNK (p-JNK), p38 (p-p38), PKCα/β_{II} (p-PKCα/β_{II}), and PKCδ/θ (p-PKCδ/θ) were all obtained from Cell Signal Technologies Inc, MA, USA. All primary antibodies were diluted 1:1000, with the exception of β-actin which was diluted at 1:1500 Ab:TBST. Membranes were left to incubate overnight using a platform mixer at 4 ºC.

Membranes were repeatedly rinsed prior to being incubated with secondary antibody (horseradish peroxidase (HRP)–linked anti-rabbit IgG (Cell Signaling Technology, USA), diluted 1:2000 in TBST for 2 hours at room temperature using a platform mixer.

10.2.4.4 Protein Detection

Detection solution was made from scratch combining Luminol, p-courmic acid and H2O2 in 100 mM Tris-HCl solution (refer Appendix A). Membranes were bathed in

detection solution, blotted dry and exposed to x-ray film (Kodak BioMax XAR) or 1-10 minutes. The film was moved directly into an Autotank automatic x-ray film processor (Fischer Industries Inc., Geneva, IL, USA).

The density of the protein bands were quantified using Bio-Rad Quantity One software (Bio-Rad laboratories, Hercules, USA), and reported as intensity / mm^2. β-actin was used as an internal control in all incubations

10.2.5 Statistical Analysis

All data are presented as mean + S.E.M. Original data were log_{10}-transformed to 'normalize' the data, enabling a paired Student's t-test to be performed comparing protein expression of each kinase between the different treatment groups. In all statistical tests a value of $P<0.05$ was considered significant; n=3 for all treatments.

10.3 Results

10.3.1 Effect of PA12, AngI and specific enzyme antagonists on cytokine phosphorylation

ERK1/2: Phosphorylated ERK1/2 (p-ERK1/2) expression was significantly elevated in LV heart tissue infused with PA12 and AngI ($P<0.05$ vs vehicle, Figure 10.1). Co-infusion of the enzyme inhibitors captopril and Chymostatin significantly attenuated the PA12- and AngI-induced phosphorylation of ERK1/2 (all $P<0.05$ vs PA12 and AngI respectively, Figure 10.1).

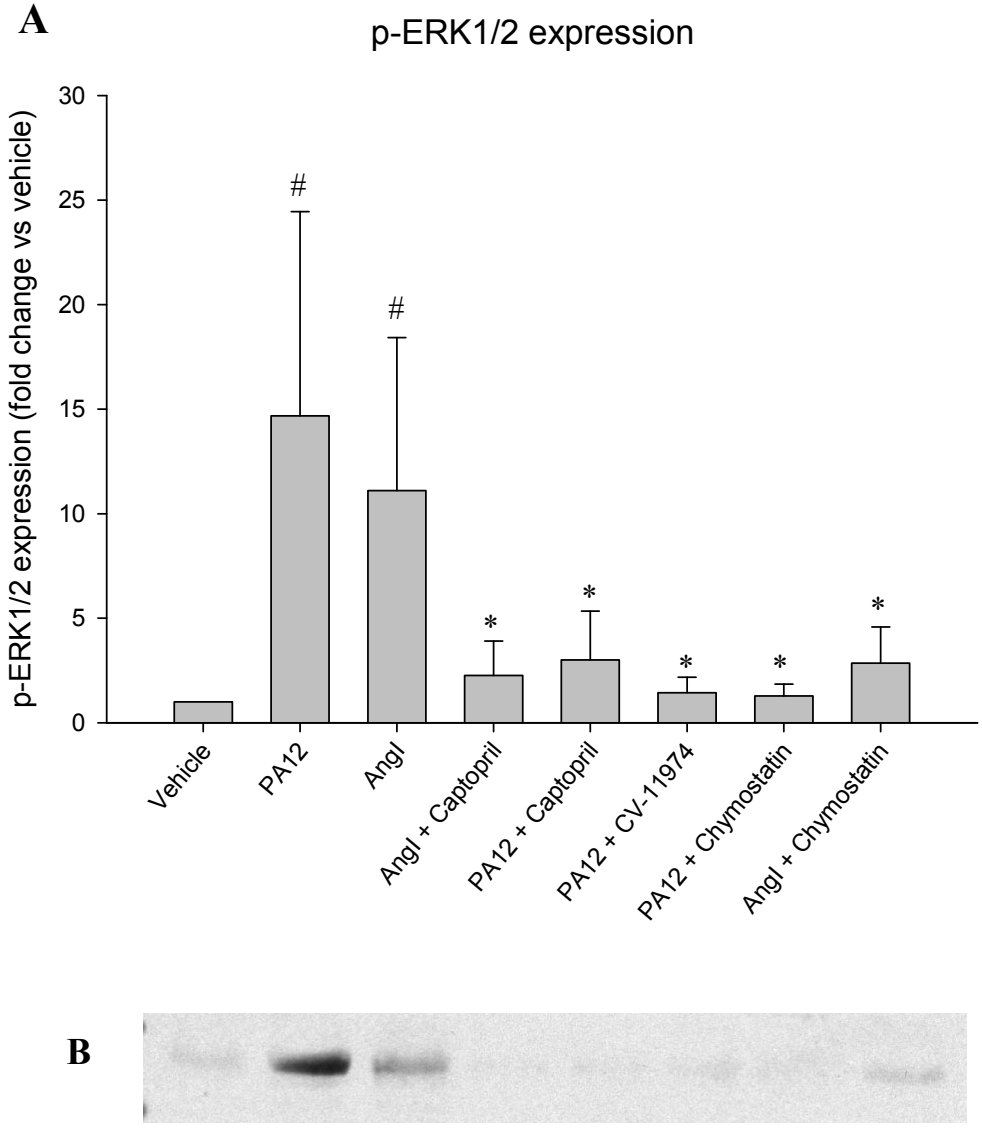


Figure 10.1. The effect of PA12 and AngI with or without specific inhibitors upon phosphorylated ERK1/2 (p-ERK1/2) is evident in the raw, developed film (B). Repeating the experiment in three rat hearts revealed p-ERK1/2 was elevated significantly in cardiomyocytes within the LV of rat hearts in response to infusions of PA12 or AngI (A). The enzyme antagonists captopril and Chymostatin significantly attenuated both PA12- and AngI-induced increases in p-ERK1/2 (A). CV-11974 also abolished PA12-induced p-ERK1/2 expression.

Data in (A) represented as fold change in p-ERK1/2 compared with vehicle, n=3. Using a student's t-test $P<0.05$ for PA12 or AngI vs vehicle (#); $P<0.05$ for PA12 or AngI vs PA12 or AngI + their respective antagonist (*). Western blott (B) displays 42/44kDa.

JNK: Phosphorylated JNK (p-JNK) expression was significantly elevated in response to infusion of PA12 or AngI when compared with vehicle (Figure 10.2). Chymostatin also reduced p-JNK expression, significantly attenuating PA12- and AngI-induced JNK phosphorylation (both $P<0.02$ vs PA12 and AngI respectively, Figure 10.2). However, reductions in both the PA12- and AngI-induced increases in p-JNK expression in the presence of captopril, did not reach significance (both $P>0.05$ vs PA12 and AngI respectively).

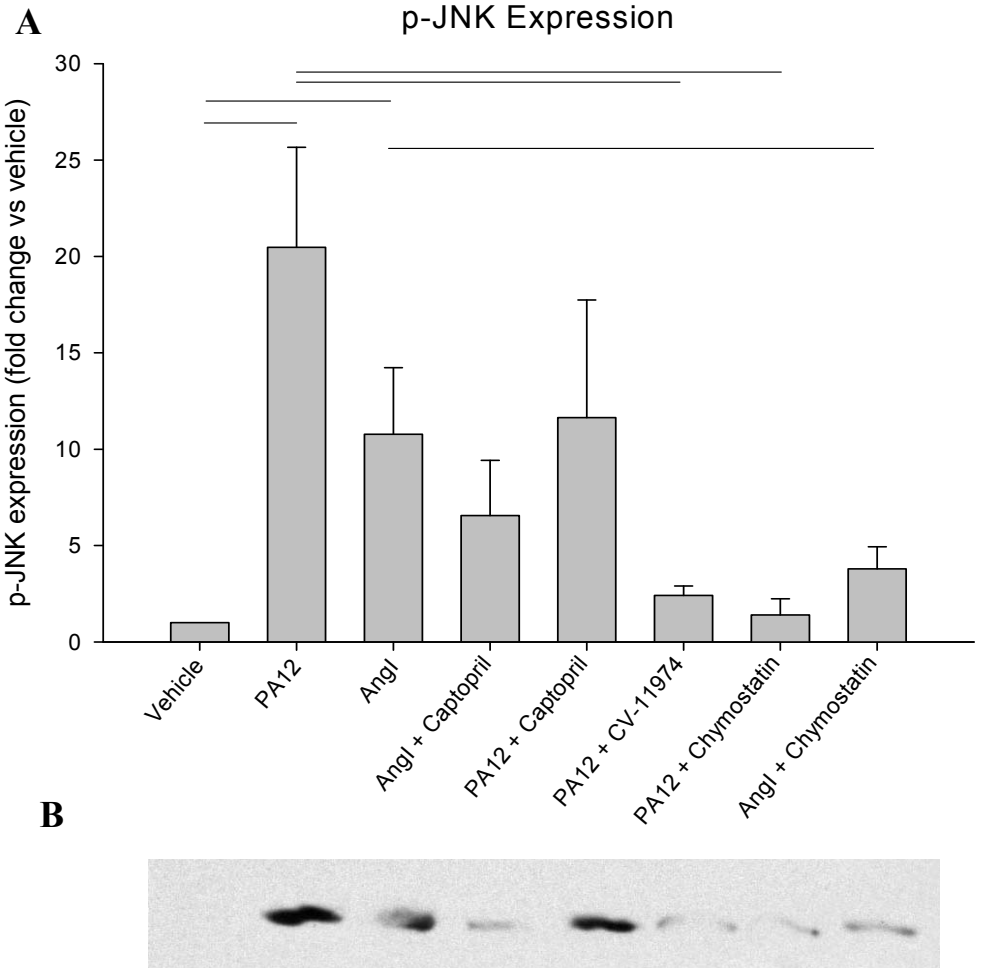


Figure 10.2 Phosphorylated JNK (p-JNK) expression in hearts infused with PA12 or AngI with or without specific antagonists. (B) represents an example of a raw film developed displaying p-JNK expression (46/54 kDa) in LV rat heart tissue from the different treatments in line with the bar graph above (A). PA12 and AngI elevated p-JNK expression that was found to be chymase-dependent and mediated by the AT_1 receptor.
Data represented as fold change compared to vehicle; lines indicate significant differences between treatment groups using a student's t-test ($P<0.05$, $n=3$).

p38: PA12 and AngI significantly elevated phosphorylated p38 (p-p38) expression (Figure 10.3). The ACE1 inhibitor captopril significantly attenuated AngI-induced p-p38 expression ($P<0.05$), but did not significantly inhibit PA12-induced p38 phosphorylation ($P>0.05$). Co-infusion of chymostatin with PA12 and AngI had no inhibitive effect on attenuating p-p38 expression (Figure 10.3).

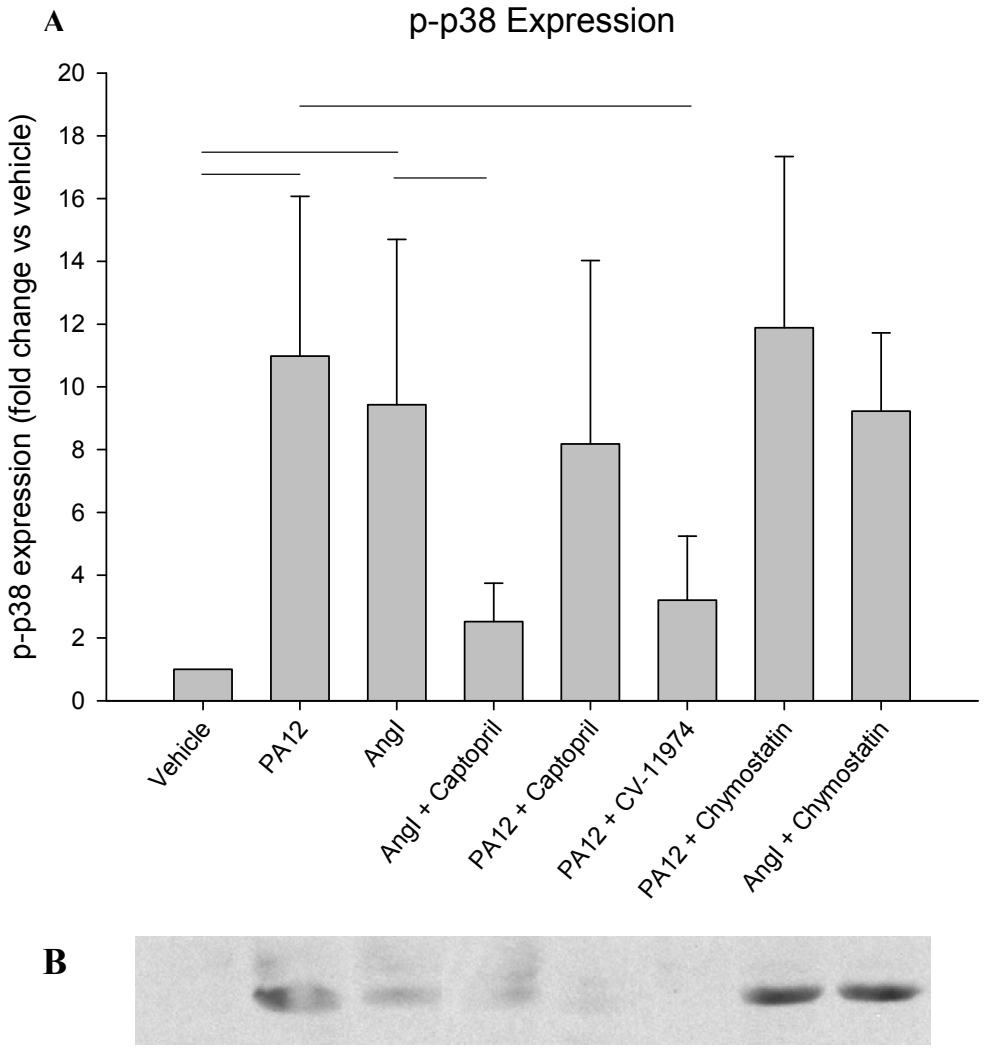


Figure 10.3 Phosphorylated p38 (p-p38) expression in response to PA12 or AngI infusions with or without enzyme inhibitors or AT1R blockade. As clearly shown in the raw developed film displaying 43 kDa (B), PA12 and AngI increased p-p38 expression in the rat heart LV (A), which was found to be mediated by AT1R. Captopril significantly attenuated the AngI-induced increase in p-p38 expression but displayed no inhibitive effect on PA12. Chymostatin had no effect on either peptide's activity.
Data represented as mean + SEM (n=3), fold change in p-p38 expression compared with vehicle. Lines indicate significant differences between treatment groups using student's t-test, $P<0.05$.

PKCα/β: Phosphorylated PKCα/β (p-PKCα/β) expression was significantly elevated in response to infusion of PA12 or AngI in the LV of the rat heart (Figure 10.4). Captopril significantly attenuated the AngI-induced increase in p-PKCα/β ($P<0.05$), however had little influence on PA12 activity ($P>0.05$). Chymostatin had the opposite effect to captopril, significantly inhibiting the PA12-induced elevation in p-PKCα/β expression ($P<0.05$), while having little effect on AngI activity ($P>0.05$).

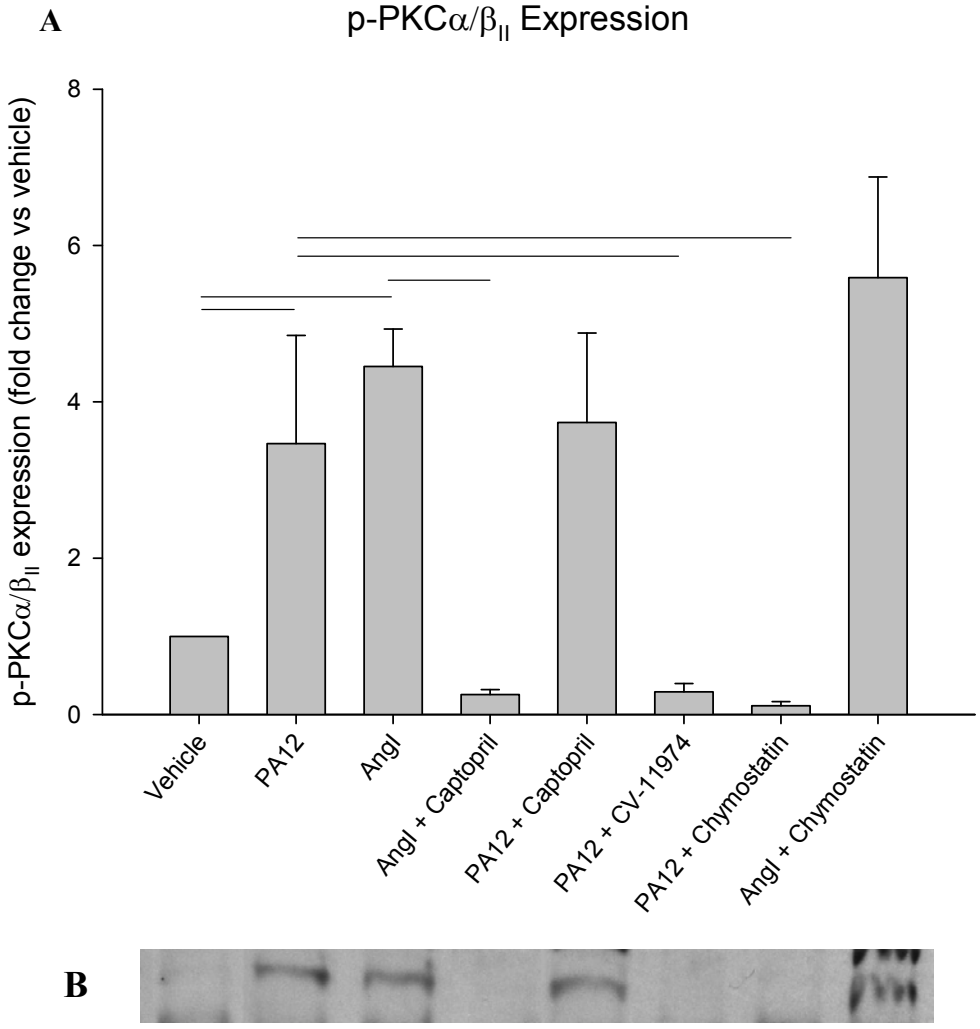


Figure 10.4 Phosphorylated PKCα/β_{II} expression (p-PKCα/β_{II}) in response to PA12 or AngI with or without specific antagonists. As displayed in the raw developed film at 80-82 kDa (B), infusion of PA12 or AngI into isolated rat hearts stimulated an elevation in p-PKCα/βII expression. AngI-induced increase was attenuated with captopril, while the PA12-induced increase was attenuated with chymostatin or CV-11974, indicating PA12 and AngI were dependent upon chymase and ACE1 respectively in phosphorylating PKCα/β_{II} (A). Data represented as fold change in p-PKCα/β_{II} expression compared with vehicle (mean + SEM), n=3. Lines indicate significant differences between treatment groups using student's t-test ($P<0.05$).

PKCδ/θ: Infusion of PA12 or AngI into rat hearts displayed no effect on altering PKCδ/θ phosphorylation (Figure 10.5). Addition of ACE1 and chymase inhibitors or AT1 receptor blockade with PA12 or AngI did not uncover any PA12- or AngI-induced PKCδ/θ phosphorylation.

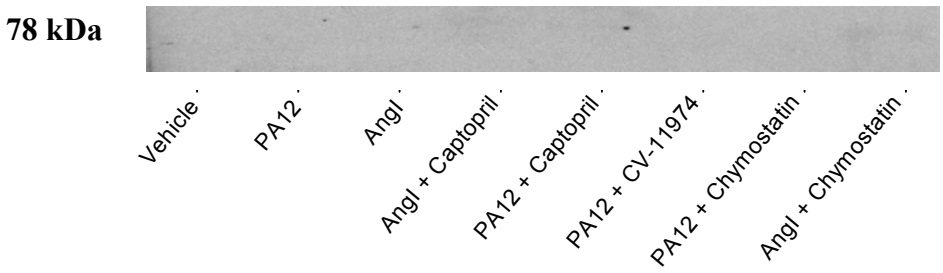


Figure 10.5. Developed film displaying phosphorylated PKCδ/θ expression from hearts treated with PA12 or AngI with or without antagonists. PA12 and AngI had no effect on PKCδ/θ phosphorylation. Co-infusion of captopril, chymostatin or CV-11974 had no effect on altering PA12- or AngI-induced p-PKCδ/θ expression.

10.3.2 Effect of AT_1 receptor blockade on cytokine phosphorylation

Infusion of the AT1 receptor blocker, CV-11974 in combination with PA12 significantly attenuated PA12-induced JNK, ERK1/2, p38 and PKCα/β phosphorylation (Figures 10.1 - 10.4), indicating the AT1 receptor mediated PA12 phosphorylation of the intracellular proteins measured. CV-11974 did not reveal any effect of PA12 on PKCδ/θ phosphorylation (Figure 10.5).

10.4 Discussion

Two reports now reveal PA12 can be converted to AngII by rat cardiac tissue (Trask et al. 2008; Prosser et al. 2009). This conversion is reported to be both ACE1- and chymase-dependent. However, Prosser et al. suggest chymase to be the primary enzyme responsible for mediating PA12 activity, including the conversion of PA12 to AngII, at least within localised rat cardiac tissue (Nagata et al. 2006; Prosser et al. 2009). Whether PA12 itself can bind to the receptor or must initially be cleaved into smaller active peptides, including AngII, is yet to be established, however reports concur that PA12 activity is mediated by the AT1 receptor (Nagata et al. 2006; Prosser et al. 2009).

The AT_1R activates many intracellular pathways (Figure 10.6) stimulating serine/threonine kinases including PKC isoforms, as well as the classic 3 mitogen-activated protein kinase (MAPK) pathways: JNK, p38, and ERK1/2 (Mehta and Griendling 2007). These pathways are known to regulate vasoconstriction, cellular growth, proliferation, migration and differentiation, inflammation and apoptosis, and hence are tightly regulated (Su and Karin 1996; Higuchi et al. 2007; Mehta and Griendling 2007). However, in patients with an overreactive RAS, or chronically elevated AngII, the above pathways can become overstimulated, inducing maladaptive, pathological cardiovascular remodelling and augmenting vascular diseases such as atherosclerosis, and progressing hypertension (Wen et al. 1997; Suzuki et al. 2005; Hunyady and Catt 2006; Otis and Gallo-Payet 2007).

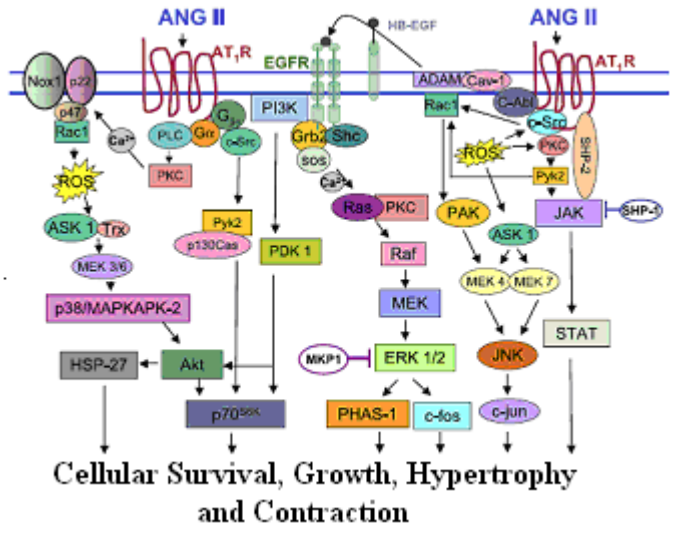


Figure 10.6. AngII binding to AT_1R triggers the activation of many intracellular pathways. The downstream effector agents of these pathways stimulate cellular survival, growth, hypertrophy and contraction. Figure modified from Mehta and Griendling 2007.

The current report explored the intracellular pathways stimulated by PA12, recently identified as a component of the RAS, and proposed as a potential alternate substrate to AngI in the rat (Trask et al. 2008; Prosser et al. 2009). Western blotting was employed to determine the amount of phosphorylation, and hence level of activation, of each of the cytosolic proteins: p38, ERK1/2, JNK, and PKC isoforms α/β_{II} and δ/θ.

We found that infusion of PA12 or AngI into isolated rat hearts stimulated the phosphorylation of all 3 classic MAPK pathways: ERK1/2, JNK, and p38, as well as phosphorylating PKCα/β_{II} in the left ventricle of the rat, indicating elevated activity of these cytokines. Increased activity of the same kinases in response to PA12 or AngI also supports the theory that PA12 can act as an alternate peptide to AngI in stimulating cardiac RAS activity. Furthermore, activation of the intracellular proteins responsive to PA12 were all found to be dependent upon AT_1 receptor binding, supporting our previous report that the AT_1 receptor mediates PA12-induced physiological effects within the rat heart (Prosser et al. 2009).

10.4.1 PA12 activation of ERK1/2

PA12 and AngI significantly elevated ERK1/2 phosphorylation, mediated by the AT_1 receptor. This is consistent with our prior report indicating PA12 to be AT_1R-dependent, and is in agreement with prior reports indicating AngI-derived AngII-induced activation of the AT_1R stimulates ERK1/2 (Mazak et al. 2004). ERK1/2 is widely reported as a stimulating pro-mitogenic, anti-apoptotic and vasoconstrictive intracellular pathways within cardiomyocytes (Baines and Molkentin 2005; Mehta and Griendling 2007). As previously reported, PA12 potently constricted rat coronary arteries and aortic strips (Nagata et al. 2006; Prosser et al. 2009). The current findings suggests this observed constriction may, at least in part, be mediated by PA12-induced phosphorylation of ERK1/2. The constrictive effects of ERK1/2 are reportedly caused by ERK1/2-induced increases in Ca^{2+} availability within the cell (Touyz et al. 2001).

AT_1R-induced ERK1/2 activity has also been reported to: inhibit apoptosis (Barki-Harrington et al. 2003), promote cellular growth and protein synthesis (Rocic et al. 2003), as well as stimulate cell differentiation and migration through activation of c-Fos and c-Jun (Touyz 2004) (Figure 10.6); suggesting that PA12-induced ERK1/2 activity may also stimulate these pro-mitogenic effects. Blockade of the AT_1 receptor has been shown to significantly attenuate cardiovascular remodelling (van Eickels et al. 1999; Azuma et al. 2000; Tamura et al. 2000; Bai et al. 2004), while more specific inhibition of AngII-induced ERK1/2 activity attenuates maladaptive vascular remodelling in spontaneously hypertensive rats (Touyz et al. 2002). Taken together these studies indicate that inhibition of PA12 activity may also attenuate pathological cardiovascular remodeling.

Inhibition of the proteolytic enzymes ACE1 and chymase effectively inhibited PA12-induced ERK1/2 activity, either via attenuating the conversion of PA12 to its active, effector peptide(s), or through captopril or chymostatin stimulating intracellular proteins antagonising ERK1/2 activity. This is yet to be determined, however our findings do reveal that administering ACE1 or chymase inhibitors, or blocking the AT_1 receptor, may significantly attenuate pathological effects stimulated by the PA12-ERK1/2 pathway.

10.4.2 PA12 activation of JNK and p38

MAPK p38 and JNK share many characteristics as both kinases are activated in response to cellular stress such as increased ROS expression within the cell (Ohtsu et al. 2005; Chen and Mehta 2006; Liu et al. 2008). JNK and p38 influence vascular remodeling, primarily promoting apoptosis and inflammation (Force et al. 1996; Ohtsu et al. 2005). AngII is known to stimulate ROS production (Griendling et al. 1994) and stimulate JNK/p38 proteins (Mehta and Griendling 2007). In this report we observed the effect of PA12 on JNK and p38 activity. PA12 and AngI significantly elevated JNK and p38 phosphorylation indicating activation of these proteins and subsequent downstream JNK/p38-activated pathways. JNK has been reported to activate many pro-apoptotic effector proteins including bad, bim, bid, bmf, cyt-c and c-Jun, as well as stimulating PKCδ causing the activation of further cell-death

pathways (Baines and Molkentin 2005; Mehta and Griendling 2007). However, p38 has been reported to possess protective mechanisms including attenuating apoptosis, enhancing cardiomyocyte survival (Kang et al. 2000), and stimulating downstream proteins Akt and heat shock protein 27 (HSP-27) which stabilizes intracellular proteins and stimulates protein synthesis (Stokoe et al. 1992; Taniyama et al. 2004). In contrast, JNK has been reported to phosphorylate just one potential cell survival protein, Bcl-2 (Fan et al. 2000) which is capable of stimulating both pro- and anti-apoptotic effects (Chao and Korsmeyer 1998). p38 is also suggested to stimulate cyclooxygenase-2, activating inflammatory prostaglandins, causing vascular damage (Scheuren et al. 2002). Taken together PA12-induced activation of JNK and p38 potentially stimulates pro-apoptotic, inflammatory factors promoting deleterious cellular effects and potentially progressing cardiovascular disease (Figure 10.7). AngII-induced activation of JNK is reported to progress CHF (Mehta and Griendling 2007; Liu et al. 2008). However another study indicated activation of p38 negatively regulated AngII-induced ERK1/2 activity, supporting AT_1R as a regulator of vascular remodelling (Kintscher et al. 2003) (Figure 10.7). In the diseased state stimulation of AT_1R-induced pathways can become unbalanced and unregulated resulting in AT_1R promoting pathogenesis, for example JNK activity is significantly elevated while ERK1/2 activity is unchanged in a rat model of hypertension (Vogel et al. 2001).

10.4.3 PA12 activation of PKC

There are 11 isoforms of PKC identified to date, grouped into three main groups based on their factors required for activation. The classical group containing α, β_I, β_{II} and γ require Ca^{2+} and diacylglycerol (DAG) for activation, while the novel isoforms δ, ε, η and θ require DAG alone. The third atypical PKC group does not require Ca^{2+} or DAG to stimulate their activity (Mellor and Parker 1998). The current report observed the effect of PA12 on stimulating two of the PKC groups: PKCα/β_{II} and PKCδ/θ, which are reported to cause contrasting cellular effects as described below.

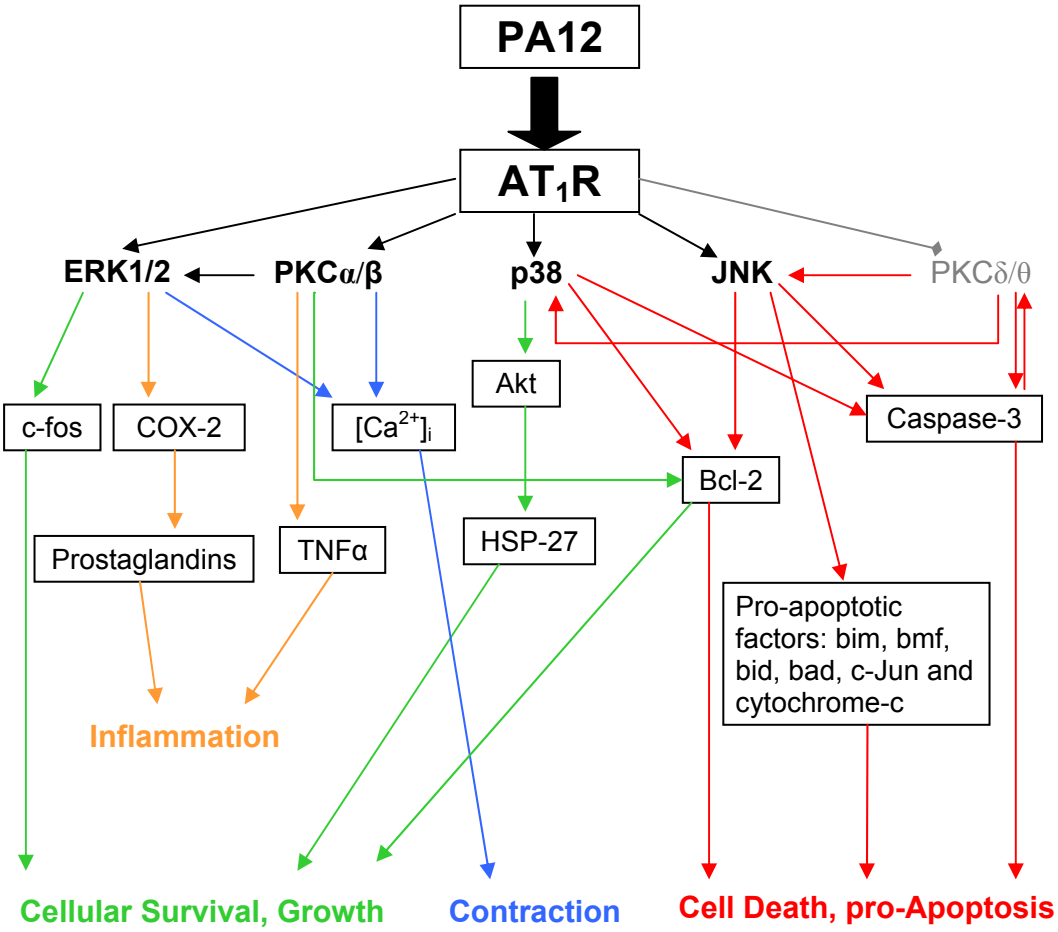


Figure 10.10.7. Schematic overview detailing the activity of the cyotosolic proteins observed in the current study. PA12-induced activation of the AT_1R can stimulate cellular survival and growth (green pathways), inflammation (orange pathways), contraction (blue pathways) and cell death (red pathways), indicating the receptors broad regulatory influence on the vasculature. During the diseased state the AT_1R can become overstimulated resulting in unregulated activation of these pathways causing maladaptive vascular remodeling, hypertension, and endothelial dysfunction; ultimately progressing atherosclerosis and cardiovascular disease. (PA12 did not phosphorylate $PKC_{\delta/\theta}$)

PKCα/β_{II} are reported to promote cell survival and stimulate vascular constriction (Cross et al. 2000; Mehta and Griendling 2007). PKCα/β-induced cell survival is promoted through inhibiting apoptosis (potentially via stimulation of Bcl-2 (Ito et al. 1997; Ruvolo et al. 1998), as well as promoting cellular growth through activating the ERK1/2 pathway (Braz et al. 2002; Greco et al. 2003; Vijayan et al. 2004). However, PKCα expression is elevated in many cancers, indicating that chronic activation of PKCα can cause unregulated, pathological cellular growth (Lord and Pongracz 1995). Activation of PKC also stimulates sustained vasoconstriction, with AngII-induced PKC activity suggested to play a potential pathological role in the progression of hypertension and promote ROS production (Figure 10.6) (Baines et al. 1996; Touyz et al. 2001). In contrast, the PKC isoforms δ/θ are reported as pro-cell death, pro-apoptotic isoforms in both cardiac and non-cardiac cells (Heidkamp et al. 2001; Brodie and Blumberg 2003), and progress cardiac injury during times of stress. For example, PKCδ was reported to exacerbate ischemic injury in rat cardiomyocytes, while inhibition of PKCδ attenuated the injury (Chen et al. 2001). PKCδ/θ's pro-apoptotic effects are suggested to be stimulated through activation of downstream kinases including JNK and p38, as well as activating caspase-3 (Heidkamp et al. 2001). One report indicated caspase-3 can also activate PKCδ, potentially providing a positive feedback loop for PKCδ-induced apoptosis (Datta et al. 1997; Basu and Akkaraju 1999) (Figure 10.7).

Results from the current study support previous reports indicating that activation of the AT_1 receptor stimulates PKCα/β activation (Higuchi et al. 2007; Mehta and Griendling 2007) as PA12 and AngI significantly elevated p-PKCα/β_{II} expression, which was abolished by the AT_1R blocker CV-11974. PA12 and AngI displayed no effect on phosphorylating PKCδ/θ, suggesting PA12 and AngI stimulate PKC isoforms promoting cellular growth and inhibiting apoptosis. PA12-induced activation of PKCα/β_{II} also conforms with the physiological response to PA12 as a potent chymase-dependent, ACE1-independent vasoconstrictor (Prosser et al. 2009), as PKC is known to stimulate Ca^{2+}-dependent vasoconstriction (Mehta and Griendling 2007). The response to the enzyme inhibitors employed in the current report also correlate with the vasoconstrictive activity of PA12 previously reported; indicating that AngI is dependent upon ACE1 availability, while PA12 is chymase-dependent, with ACE1

having little influence on PA12's constrictive potency (Prosser et al. 2009), or PKCα/β_{II} activation as evidenced in the current study.

Chymase-dependent PA12-induced PKCα/β stimulation may also develop a positive feedback system if unregulated, as both the RAS and chymase are reported to be elevated during times of vascular stress, promoting hypertension and the resultant vascular diseases (Shiota et al. 1993; Shiota et al. 1997; Takai et al. 1997a; Takai et al. 1998; Guo et al. 2001; Ju et al. 2001). Combining these findings with reports indicating PKC increases mast cell number and can trigger mast cell degranulation (Peng and Beaven 2005; Palaniyandi et al. 2008) suggests PA12 may be involved in initiating a positive feedback mechanism within the cardiovasculature, progressing pathological remodeling and cardiac dysfunction.

It must be noted that the chymase inhibitor used in the current study, chymostatin obtained from Sigma Aldrich (St Louis, MI, USA), is a non-specific chymotryptic inhibitor, capable of inhibiting chymases as well as cathepsin-G. It would be of interest to employ inhibitors with greater specificity in future studies, in order to reveal whether chymase or cathepsin-G is the primary enzyme in converting PA12 to AngII in rat cardiac tissue.

Inhibition of the RAS is commonly used to ameliorate hypertension and inhibit pathological vascular remodelling through the use of ACE1, renin and/or AT_1R antagonists (Yang et al. 1996; Kim et al. 1998; Al Khalaf et al. 2009). The current report indicates that PA12 is an active component of the RAS and the PA12-chymase pathway may potentially provide an alternate therapeutic target in ameliorating RAS activity, and thus attenuate RAS-induced cardiovascular disease.

10.4.4 Summary

In summary, PA12 and AngI were found to stimulate ERK1/2, JNK, p38 and PKCα/β_{II} phosphorylation. These MAPKs are known to regulate cellular growth, apoptosis, cell differentiation, hypertrophy, inflammation and muscle contraction. Our results concur with our previous report observing the physiological effects of these peptides on the isolated rat heart (Prosser et al. 2009). PA12-induced stimulation of the intracellular cytokines JNK, ERK1/2 and PKCα/β_{II} were found to be dependent upon chymase, while ERK1/2 was the only intracellular kinase to be inhibited by captopril when co-infused with PA12, significantly attenuating the PA12-induced increase in p-ERK1/2. All PA12-induced cytokine activity was found to be mediated by the AT_1 receptor, indicating PA12 can potentially stimulate both pro-apoptotic, cell death pathways (p38 and JNK), as well as anti-apoptotic, cell survival, hypertrophic pathways (ERK1/2, p38 and PKCα/β_{II}) as displayed in Figure 10.7. The RAS is known for its pathological role in progressing vascular disease and hypertension, mediated by the AT_1 receptor. This report is the first to reveal that PA12 acts through the AT_1R, stimulating pathways known to augment cardiovascular disease, but differs from AngI in that its activity is suggested to be independent of ACE1.

11. Concluding Summary and Discussion

There have been few reports determining the hemodynamic and humoral functions of URP and PA12 within the cardiovasculature, while studies observing the function of UII have reported highly contradictory findings. All three peptides are low molecular weight proteins, with wide tissue expression, including the cardiovascular system, and are reported to be elevated in patients suffering cardiovascular disease, indicating a potential pathological role in the mammalian cardiovascular system. It is, therefore, of importance to define the role of these three peptides within the mammalian cardiovascular system in both the healthy and diseased states in order to enable therapeutic manipulation of their effects, be they pathogenic or beneficial. The current thesis approached this task in a logical, methodical manner, with experiments building upon previous ones to produce a solid foundation for continuing investigations into the role and effects of UII, URP and PA12 in the cardiovascular system, as well as reveal some of the mechanisms regulating their responses.

Initial studies identified UII to be highly species specific (while the URP sequence is the same across all known species), as non-native species-forms of UII either severely attenuated, or stimulated opposite effects when compared to application of its native form. This specificity was evident in both salmon and rat coronary arteries, indicating that species-dependence was conserved over evolution. The results from this study also provided some explanation into the contrasting results reported in response to UII, as commonly the species form of UII administered was not native to the animal subject. UII and URP were found to stimulate potent vasodilation within the rat and salmon coronary vasculature, in contrast to many reports suggesting UII to be a potent vasoconstrictor. The essential difference being that the current study used UII native to the appropriate species (i.e. administering rat UII into the rat, and salmon UII into salmon), providing the same vasodilatory effect in both species, indicating UII's form-function to be highly conserved. UII/URP-induced nitric oxide production and and elevated cyclooxygenase activity were suggested to mediate the vascular response to UII and URP, stimulating relaxation of VSMCs.

Infusion of PA12 to rat hearts caused potent, sustained vasoconstriction of the coronary arteries but had little influence on altering left ventricular contractile parameters. This study was the first to report the direct effects of PA12 on an isolated mammalian heart, and its effects were shown to be similar to that of angiotensin I (AngI), although displaying reduced potency. As the amino acid sequence of PA12 is AngI-Leu^{11}-Tyr^{12}-C' the vasoconstrictive effects, although novel and important to report, were not surprising. Again as with AngI, PA12 activity was shown to be mediated by the AT_1 receptor. The enzyme mediating AngI activity is primarily ACE1 (Robertson and Nicholls 1993), but inhibition of ACE1 failed to attenuate PA12-induced vasoactivity, while significantly attenuating AngI-induced vasoconstriction. The enzyme found to be responsible in mediating PA12 activity was chymase, a carboxypeptidase capable of cleaving angiotensin peptides into smaller active and/or inactive fragments.

Chymase is contained with mast cells and displays high species-specificity, with rat chymase initially reported only to degrade angiotensins into inactive fragments. However, with the identification of a novel chymase isoform within rat VSMCs, it was evident that chymase could, in fact, convert AngI to the potent, effector peptide AngII. We found that chymase mediates PA12 activity in the rat heart and was responsible for converting PA12 to AngII both in vitro and within rat cardiac tissue. These studies have opened up a potentially novel, alternate pathway within the RAS, independent of both AngI and ACE1. This new PA12-chymase pathway is an important discovery as it a) presents a novel AngII-generating pathway within the cardiac tissue, and b) may provide an alternate target in reducing RAS activity, helping to ameliorate hypertension, atherosclerosis, and other cardiovascular diseases of which the RAS is implicated.

As UII, URP and PA12 are elevated in patients suffering cardiovascular diseases and in spontaneously hypertensive rats (Douglas et al. 2002; Richards et al. 2002; Lapp et al. 2004; Jessup et al. 2008; Mori et al. 2009), it was necessary to investigate the role of UII, URP and PA12 in a model of heart disease. Using a rat heart model of ischemia-reperfusion, UII, URP and PA12 were administered prior to ischemia, in

order to simulate patients expressing elevated circulating levels of these peptides prior to an ischemic event.

UII and URP were both found to significantly alleviate ischemic injury during reperfusion, dilating the coronary arteries, reducing the release of biomarkers of myocardial damage, and reducing ANP release. Only UII had mild negative inotropic effects, potentially reducing cardiac energy requirements following ischemia. A further study simulating administering UII or URP to a patient following a cardiac ischemic event indicated that despite endothelial damage sustained during ischemia, UII and URP maintained their vasodilative, cardioprotective effects. These studies suggest that patients with elevated circulating UII levels should have a better outcome following ischemia, a finding supported by some reports (Khan et al. 2006; Zoccali et al. 2006), with contrasting studies indicating beneficial effects of UII receptor blockade (Tzanidis et al. 2001; Tzanidis et al. 2003; Bousette et al. 2006a; Bousette et al. 2006b). The current thesis examined blockade of the UII receptor in a model of ischemia-reperfusion employing the UII receptor blocker Palosuran. We reported that Palosuran itself acted as a mild agonist of the UII receptor, suggesting that not only is UII species specific, but receptor differences between species may also alter ligand binding, activation and the resultant effects. This was previously reported for another UII receptor blocker, SB-710411, in monkeys and rats where it antagonised UII-induced activity at the rat UII receptor, but functioned as an agonist at the monkey UII receptor (Behm et al. 2004). This indicates that a) synthetically manufactured receptor blockers may need to be designed for each individual species, and b) the use of these UII receptor blocking agents employed in studies observing UII's pathogenic effects may, in fact, be reporting false results due to the receptor blocker itself stimulating activity.

In contrast to UII and URP, infusion of PA12 prior to ischemia worsened recovery during reperfusion, causing greater myocardial damage and constricting the coronary arteries. As observed in healthy hearts, PA12 activity was mediated by chymase activity, with inhibition of ACE1 having no influence. This finding is of great importance as currently millions of hypertensive patients as well as others suffering from the detrimental effects of an overactive RAS are administered ACE1 inhibiting drugs. However, it is noted by cardiologists that ACE1 inhibition may not be

sufficient in alleviating hypertension, while causing adverse side effects in some patients (Yusuf et al. 2008; Sealey and Laragh 2009), and thus a 'perfect treatment' in reducing RAS activity is yet to be established. The chymase-activated PA12 pathway provides a novel target in alleviating RAS activity, as inhibition of chymase has been reported to prevent cardiac fibrosis and dysfunction in rats and dogs (Matsumoto et al. 2003; Kanemitsu et al. 2005), and to significantly attenuate pathological cardiac remodelling, aiding myocardial function in a rat model of hypertension-induced heart failure (Palaniyandi et al. 2008).

However, the findings obtained from observing the effect of PA12 on the rat arterial system suggests that ACE1 can also alter PA12 activity within the circulation. PA12 activity was found to be both ACE1 and chymase-dependent, with inhibition of ACE1 attenuating PA12-induced vasoconstriction with greater potency than chymase. This suggests that ACE1 may be primarily responsible for mediating PA12 (and AngI) activity within the circulation, while chymase plays a greater role in tissue-based, local RAS activity, including regulating PA12-derived generation of AngII.

To further explore the pathways stimulated by PA12, western blotting was employed to ascertain whether the 3 classical MAPK pathways (ERK1/2, JNK and p38), as well as PKC isoforms were activated. PA12-induced effects are suggested to be entirely mediated through the AT_1 receptor. The intracellular pathways stimulated by the AT_1 receptor are well defined, providing the basis to suggest that PA12 may also stimulate these same cytosolic pathways. Indeed analysis of heart tissue infused with PA12 revealed that PA12 stimulated ERK1/2, p38, JNK and PKC activity, as in AngI-infused heart tissue. These pathways are known regulators of cellular proliferation, growth, differentiation, apoptosis, inflammation and vasoconstriction (Mehta and Griendling 2007). Therefore, it could be postulated that PA12 induces pro-mitogenic, pro-apoptotic, hypertrophic, constrictive and inflammatory effects. The PA12-chymase pathway may provide an alternate target in inhibiting RAS activity and attenuate hypertension, atherosclerosis and other cardiovascular diseases promoted by the RAS.

The contrasting effects of UII and PA12 also suggests that *in vivo* UII and PA12 may be antagonistic. For example, UII stimulation of PGE_2 activity has been shown to

attenuate AngII-induced oxidative stress, and inhibit hypertension (Jia et al. 2008). Furthermore, AngII is reported to decrease NO synthase expression, limiting UII-induced NO production (Ramseyer and Garvin 2008). Due to the intracellular pathways activated by UII and AngII, there are possible circumstances where UII and AngII may work in additive, or synergistic ways, rather than opposing each other's actions. These pathways and their effects require defining, specifically with regard to the development and progression of cardiovascular disease.

The current thesis has provided much needed information extending the knowledge of the UII/URP and PA12 systems, and has provided key reasons explaining the large amount of discrepancy in the literature concerning the cardioprotective versus pathological effects of UII. In our hands UII and URP were found to provide significant cardioprotection following ischemia provided the native form was administered. In contrast, PA12 was found to augment ischemic injury and cause further myocardial injury. The key finding that PA12 elicits its effects independent of ACE1, and rather, is dependent upon the presence of chymase in cardiac tissue illustrates a potentially new target for alleviating RAS-induced hypertension at the local, tissue level, and may provide some explanation into the reason why ACE1 inhibitor therapy loses potency over time. Therefore, the current thesis provides a solid foundation for further intracellular, molecular and *in vivo* work to be performed on both stimulating UII/URP production and inhibiting PA12 activity in order to provide cardioprotection for at risk patients and attenuate cardiovascular disease.

REFERENCES

Abdelrahman, A. M. and Pang, C. C. Y. (2002). "Involvement of the nitric oxide/L-arginine and sympathetic nervous systems on the vasodepressor action of human urotensin II in anesthetized rats." Life Sci. **71**(7): 819-825.

Aboulafia, J., Oshiro, M. E., Feres, T., Shimuta, S. I. and Paiva, A. C. (1989). "Angiotensin II desensitization and Ca2+ and Na+ fluxes in vascular smooth muscle cells." Pflugers Arch. **415**(2): 230-234.

Agnisola, C., Mustafa, T. and Hansen, J. K. (1996). "Autoregulatory index, adrenergic responses, an interaction between adrenoreceptors and prostacyclin in the coronary vessels of rainbow trout." J Exp Biol **275**: 239-248.

Agnisola, C. (2005). "Role of nitric oxide in the control of coronary resistance in teleosts." Comp Biochem Physiol A **142**(2): 178-187.

Akasu, M., Urata, H., Kinoshita, A., Sasaguri, M., Ideishi, M., et al. (1998). "Differences in tissue angiotensin II-forming pathways by species and organs in vitro." Hypertension **32**: 514-520.

Al Khalaf, M. M., Thalib, L. and Doi, S. A. (2009). "Cardiovascular outcomes in high-risk patients without heart failure treated with ARBs: a systematic review and meta-analysis." Am J Cardiovasc Drugs **9**(1): 29-43.

Albertin, G., Casale, V., Ziolkowska, A., Spinazzi, R., Malendowicz, L. K., et al. (2006). "Urotensin-II and UII-receptor expression and function in the rat adrenal cortex." Int J Mol Med **17**(6): 1111-1115.

American Heart Association (2009). Heart Disease and Stroke Statistics - 2009 Update. A.H.A. Texas.

Ames, R. S., Sarau, H. M., Chambers, J. K., Willette, R. N., Aiyar, N. V., et al. (1999). "Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14." Nature **401**: 282-286.

Anderson, L. (2005). "Candidate-based proteomics in the search for biomarkers of cardiovascular disease." J. Physiol. **563**: 23-60.

Andersson, S. E., Edvinsson, M. L. and Edvinsson, L. (2003). "Cutaneous vascular reactivity is reduced in aging and in heart failure: association with inflammation." Clin Sci (Lond). **105**(6): 699-707.

Arnold-Reed, D. E. and Belmont, R. J. (1989). "Steroidogenic role of the caudal neurosecretory system in the flounder, Platichthys flesus." Gen. Comp. Endocrinol **76**(2): 267-273.

Azuma, M., Takahashi, K., Fukuda, T., Ohyabu, Y., Yamamoto, I., et al. (2000). "Taurine attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac myocytes." Eur J Pharmacol **403**(3): 181-188.

Bacani, C. and Frishman, W. H. (2006). "Chymase: a new pharmacologic target in cardiovascular disease." Cardiol Rev. **14**(4): 187-193.

Bader, M. (2002). "Role of the local renin-angiotensin system in cardiac damage: a minireview focussing on transgenic animal models." J Mol Cell Cardiol. **34**(11): 1455-1462.

Bader, M. and Ganten, D. (2008). "Update on tissue renin-angiotensin systems." J Mol Med. **86**(6): 615-621.

Bai, H., Wu, L. L., Xing, D. Q., Liu, J. and Zhao, Y. L. (2004). "Angiotensin II induced upregulation of G alpha q/11, phospholipase C beta 3 and extracellular signal-regulated kinase 1/2 via angiotensin II type 1 receptor." Chin Med J (Engl) **117**(1): 88-93.

Baines, C. P. and Molkentin, J. D. (2005). "STRESS signaling pathways that modulate cardiac myocyte apoptosis." J Mol Cell Cardiol. **38**: 47-62.

Baines, R. J., Brown, C., Ng, L. L. and Boarder, M. R. (1996). "Angiotensin II-stimulated phospholipase C responses of two vascular smooth muscle-derived cell lines. Role of cyclic GMP." Hypertension **28**: 772-778.

Balcells, E., Meng, Q. C., Johnson, W. H., Oparil, S. and Dell'Italia, L. J. (1997). "Angiotensin II formation from ACE and chymase in human and animal hearts: methods and species considerations." Am J Physiol **273**(4 Pt 2): H1769-1774.

Barki-Harrington, L., Luttrell, L. M. and Rockman, H. A. (2003). "Dual inhibition of beta-adrenergic and angiotensin II receptors by a single antagonist: a functional role for receptor-receptor interaction in vivo." Circulation **108**(13): 1611-1618.

Basu, A. and Akkaraju, G. R. (1999). "Regulation of caspase activation and cis-Diamminedichloroplatinum(II)-induced cell death by protein kinase C." Biochemistry **38**: 4245-4251.

Bauersachs, J. and Fraccarollo, D. (2008). "More NO-no more ROS: combined selective mineralocorticoid receptor blockade and angiotensin-converting enzyme inhibition for vascular protection." Hypertension **51**(3): 624-625.

Behm, D. J., Harrison, S. M., Ao, Z., Maniscalco, K., Pickering, S. J., et al. (2003). "Deletion of the UT receptor gene results in the selective loss of urotensin-II contractile activity in aortae isolated from UT receptor knockout mice." Br J Pharmacol **139**: 464-472.

Behm, D. J., Herold, C. L., Camarda, V., Aiyar, N. V. and Douglas, S. A. (2004). "Differential agonistic and antagonistic effects of the urotensin-II ligand SB-710411 at rodent and primate UT receptors." Eur J Pharmacol **492**(2-3): 113-116.

Behm, D. J., McAtee, J. J., Dodson, J. W., Neeb, M. J., Fries, H. E., et al. (2008). "Palosuran inhibits binding to primate UT receptors in cell membranes but demonstrates differential activity in intact cells and vascular tissues." Br J Pharmacol. **155**(3): 374-386.

Belova, L. A. (2000). "Angiotensin II-generating enzymes." Biochemistry **65**(12): 1337-1345.

Bern, H. A. and Lederis, K. (1969). "A reference preparation for the study of active substances in the caudal neurosecretory system of teleosts." J Endocrinol. **45**(1): Suppl:xi-xii.

Bern, H. A., Pearson, D., Larson, B. A. and Nishioka, R. S. (1985). "Neurohormones from fish tails: the caudal neurosecretory system. I. "Urophysiology" and the caudal neurosecretory system of fishes." Recent Prog Horm Res. **41**: 533-552.

Bern, H. A. (2008). "From fish tails to human brain. Preface." Peptides **29**(5): 649-650.

Böhm, F. and Pernow, J. (2002). "Urotensin II evokes potent vasoconstriction in humans *in vivo*." British Journal of Pharmacology **135**: 25-27.

Bolli, R., Shinmura, K., Tang, X. L., Kodani, E., Xuan, Y. T., et al. (2002). "Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning." Cardiovasc Res. **55**(3): 506-519.

Bond, H., Winter, M. J., Warne, J. M., McCrohan, C. R. and Balment, R. J. (2002). "Plasma concentrations of arginine vasotocin and urotensin II are reduced following transfer of the euryhaline flounder (Platichthys flesus) from seawater to fresh water." Gen Comp Endocrinol. **125**(1): 113-120.

Border, W. A. and Noble, N. A. (1994). "Transforming growth factor ß in tissue fibrosis." New. Engl. J. Med. **331**: 1286-1300.

Borland, J. A., Chester, A. H., Morrison, K. A. and Yacoub, M. H. (1998). "Alternative pathways of angiotensin II production in the human saphenous vein." Br J Pharmacol. **125**(3): 423-428.

Borland, J. A., Kelsall, C., Yacoub, M. H. and Chester, A. H. (2005). "Expression, localisation and function of ACE and chymase in normal and atherosclerotic human coronary arteries." Vasc Pharmacol. **42**: 99-108.

Bottrill, F. E., Douglas, S. A., Hiley, C. R. and White, R. (2000). "Human urotensin-II is an endothelium-dependent vasodilator in rat small arteries." Br J Pharmacol **130**: 1865-1870.

Bouchard, J. F., Dumont, E. and Lamontagne, D. (1994). "Evidence that prostaglandins I2, E2 and D2 may activate ATP sensitive potassium channels in the isolated rat heart." Cardiovasc Res. **28**(6): 901-905.

Boucher, R., Asselin, J. and Genest, J. (1974). "A new enzyme leading to the direct formation of angiotensin II." Circ Res **34**(35(suppl I)): I-203-I-212.

Bousette, N., Patel, L., Douglas, S. A., Ohlstein, E. H. and Giaid, A. (2004). "Increased expression of urotensin II and its cognate receptor GPR14 in atherosclerotic lesions of the human aorta." Atherosclerosis **176**(1): 117-123.

Bousette, N. and Giaid, A. (2006). "Urotensin-II and cardiovascular diseases." Curr Hypertens Rep. **8**(6): 479-483.

Bousette, N., Hu, F., Ohlstein, E. H., Dhanak, D., Douglas, S. A., et al. (2006a). "Urotensin-II blockade with SB-611812 attenuates cardiac dysfunction in a rat model of coronary artery ligation." J Mol Cell Cardiol. **41**(2): 285-295.

Bousette, N., Pottinger, J., Ramli, W., Ohlstein, E. H., Dhanak, D., et al. (2006b). "Urotensin-II receptor blockade with SB-611812 attenuates cardiac remodeling in experimental ischemic heart disease." Peptides **27**(11): 2919-2926.

Brailoiu, E., Jiang, X., Brailoiu, G. C., Yang, J., Chang, J. K., et al. (2008). "State-dependent calcium mobilization by urotensin-II in cultured human endothelial cells." Peptides **29**(5): 271-276.

Braz, J. C., Bueno, O. F., De Windt, L. J. and Molkentin, J. D. (2002). "PKC alpha regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2)." J Cell Biol. **156**(5): 905-919.

Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., et al. (1973). "Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone." Science **179**(68): 77-79.

Brilla, C. G., Reams, G. P., Maisch, B. and Weber, K. T. (1993). "Renin-angiotensin system and myocardial fibrosis in hypertension: regulation of the myocardial collagen matrix." Eur Heart J. **14**(Suppl J): 57-61.

Brkovic, A., Hattenberger, A., Kostenis, E., Klabunde, T., Flohr, S., et al. (2003). "Functional and Binding Characterizations of Urotensin II-Related Peptides in Human and Rat Urotensin II-Receptor Assay." J Pharmacol Exp Ther **306**: 1200-1209.

Brodie, C. and Blumberg, P. M. (2003). "Regulation of cell apoptosis by protein kinase c delta." Apoptosis **8**(1): 19-27.

Brooks, A. J., Wooh, J. W., Tunny, K. A. and Waters, M. J. (2008). "Growth hormone receptor; mechanism of action." Int J Biochem Cell Biol. **40**(10): 1984-1989.

Cai, H. and Harrison, D. G. (2000). "Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress." Circ Res. **87**(10): 840-844.

Caputo, L., Benessiano, J., Boulanger, C. M. and Lévy, B. I. (1995). "Angiotensin II increases cGMP content via endothelial angiotensin II AT1 subtype receptors in the rat carotid artery." Arterioscler Thromb Vasc Biol. **15**(10): 1646-1651.

Case, D. B., Wallace, J. M., Keim, H. J., Sealey, J. E. and Laragh, J. H. (1976). "Usefulness and limitations of saralasin, a partial competitive agonist of angiotensin II, for evaluating the renin and sodium factors in hypertensive petients." Am J Med **60**(6): 825-836.

Caughey, G. H., Raymond, W. W. and Wolters, P. J. (2000). "Angiotensin II generation by mast cell alpha- and beta-chymases." Biochim Biophys Acta. **1480**: 245-257.

Cavarape, A., Bauer, J., Bartoli, E., Endlich, K. and Parekh, N. (2003). "Effects of angiotensin II, arginine vasopressin and tromboxane A2 in renal vascular bed: role of rho-kinase." Nephrol Dial Transplant. **18**(9): 1764-1769.

Chan, D. K. O. (1975). "Cardiovascular and Renal effects of Urotensins I and II in the eel, *Anguilla rostrata*." Gen Comp Endocrinol **27**: 52-61.

Chandrasekharan, U. M., Sanker, S., Glynias, M. J., Karnik, S. S. and Husain, A. (1996). "Angiotensin II-forming activity in a reconstructed ancestral chymase." Science **271**: 502-505.

Chao, D. T. and Korsmeyer, S. J. (1998). "BCL-2 family: regulators of cell death." Annu Rev Immunol. **16**: 395-419.

Charles, C. J., Rademaker, M. T., Richards, A. M. and Yandle, T. G. (2005). "Urotensin II: Evidence for cardiac, hepatic and renal production." Peptides **26**(11): 2211-2214.

Chatenet, D., Dubessy, C., Leprince, J., Boularan, C., Carlier, L., et al. (2004). "Structure-activity relationships and structural conformation of a novel urotensin II-related peptide." Peptides **25**(10): 1819-1830.

Chen, J. and Mehta, J. L. (2006). "Interaction of oxidized low-density lipoprotein and the renin-angiotensin system in coronary artery disease." Curr Hypertens Rep. **8**(2): 139-143.

Chen, L., Hahn, H., Wu, G., Chen, C. H., Liron, T., et al. (2001). "Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC." Proc Natl Acad Sci USA **98**(20): 11114-11119.

Chen, Y.-L., Liu, J.-C., Loh, S.-H., Chen, C.-H., Hong, C.-Y., et al. (2008). "Involvement of reactive oxygen species in urotensin II-induced proliferation of cardiac fibroblasts." Eur J Pharmacol **593**(1-3): 24-29.

Cheung, B. M., Leung, R., Man, Y. B. and Wong, L. Y. (2004). "Plasma concentration of urotensin II is raised in hypertension." J Hypertens **22**(7): 1341-1344.

Chiba, S. and Tsukada, M. (1989). "Pharmacological features of vascular responses of isolated rat common carotid arteries to vasoactive substances revealed by the cannula inserting method." Br J Pharmacol. **95**(1): 177-182.

Cipollone, F., Fazia, M., Iezzi, A., Ciabattoni, G., Pini, B., et al. (2004a). "Balance between PGD synthase and PGE synthase is a major determinant of atherosclerotic plaque instability in humans." Arterioscler Thromb Vasc Biol. **24**(7): 1259-1265.

Cipollone, F., Fazia, M., Mincione, G., Iezzi, A., Pini, B., et al. (2004b). "Increased expression of transforming growth factor-beta1 as a stabilizing factor in human atherosclerotic plaques." Stroke **35**(10): 2253-2257.

Clark, S. D., Nothacker, H.-P., Wang, Z., Saito, Y., Leslie, F. M., et al. (2001). "The urotensin II receptor is expressed in the cholinergic mesopontine tegmentum of the rat." Brain Res **923**: 120-127.

Clozel, M., Binkert, C., Birker-Robaczewska, M., Boukhadra, C., Ding, S. S., et al. (2004). "Pharmacology of the urotensin-II receprtor antagonist palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt): first demonstration of a pathophysiological role of the urotensin System." J Pharmacol Exp Ther **311**(1): 204-212.

Conlon, J. M., O'Harte, F., Smith, D. D., Tonon, M. C. and Vaudry, H. (1992). "Isolation and primary structure of urotensin II from the brain of a tetrapod, the frog Rana ridibunda." Biochem Biophys Res Commun **188**(2): 578-583.

Conlon, J. M., Yano, K., Waugh, D. and Hazon, N. (1996). "Distribution and molecular forms of Urotensin II and its role in cardiovascular regulation in vertebrates." J Exp Zool **275**: 226-238.

Conlon, J. M., Tostivint, H. and Vaudry, H. (1997). "Somatostatin- and urotensin II-related peptides: molecular diversity and evolutionary perspectives." Regul Pept **69**(2): 95-103.

Conner, A. C., Simms, J., Hay, D. L., Mahmoud, K., Howitt, S. G., et al. (2004). "Heterodimers and family-B GPCRs: RAMPs, CGRP and adrenomedullin." Biochem Soc Trans **32**(5): 843-846.

Cornish, E. J., Goldie, R. G., Krstew, E. V. and Miller, R. C. (1983). "Effect of indomethacin on responses of sheep isolated coronary artery to arachidonic acid." Clin Exp Pharmacol Physiol. **10**(2): 171-175.

Coulouarn, Y., Lihrmann, I., Jegou, S., Anouar, Y., Tostivint, H., et al. (1998). "Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord." Proc. Natl. Acad. Sci. **95**: 15803-15808.

Coulouarn, Y., Jegou, S., Tostivint, H., Vaudry, H. and Lihrmann, I. (1999). "Cloning, sequence analysis and tissue distribution of the mouse and rat urotensin II precursors." FEBS Letters **457**(1): 28-32.

Coy, D. H., Rossowski, W. J., Cheng, B.-L. and Taylor, J. E. (2002). "Structural requirements at the N-terminus of urotensin II octapeptides." Peptides **23**: 2259-2264.

Crackower, M. A., Sarao, R., Oudit, G. Y., Yagil, C., Kozieradzki, I., et al. (2002). "Angiotensin-converting enzyme 2 is an essential regulator of heart function." Nature **417**: 822-828.

Cross, T. G., Scheel-Toellner, D., Henriquez, N. V., Deacon, E., Salmon, M., et al. (2000). "Serine/Threoniine protein kinases and apoptosis." Exp Cell Res. **256**: 34-41.

Dai, H. Y., Kang, W. Q., Wang, X., Yu, X. J., Li, Z. H., et al. (2007). "The involvement of transforming growth factor-beta1 secretion in urotensin II-induced collagen synthesis in neonatal cardiac fibroblasts." Regul Pept. **140**(1-2): 88-93.

Dales, N. A., Gould, A. E., Brown, J. A., Calderwood, E. F., Guan, B., et al. (2002). "Substrate-based desig of the first class of angiotensin-converting enzyme-related carboxypeptidase (ACE2) inhibitors." JACS **124**: 11852-11853.

Danser, A. H. and Schalekamp, M. A. (1996). "Is there an internal cardiac renin-angiotensin system?" Heart **76**: 28-32.

Datta, R., Kojima, H., Yoshida, K. and Kufe, D. (1997). "Caspase-3-mediated cleavage of protein kinase C θ in induction of apoptosis." J Biol Chem **272**: 20317-20320.

Dawn, B. and Bolli, R. (2002). "Role of nitric oxide in myocardial preconditioning." Ann N Y Acad Sci. **962**: 18-41.

de Lannoy, L. M., Schuijt, M. P., Saxena, P. R., Schalekamp, M. A. and Danser, A. H. (2001). "Angiotensin converting enzyme is the main contributor to angiotensin I-II conversion in the interstitium of the isolated perfused rat heart." J Hypertens **19**: 959-965.

de Lecea, L. and Bourgin, P. (2008). "Neuropeptide interactions and REM sleep: a role for Urotensin II?" Peptides **29**(5): 845-851.

Deeb, R. S., Shen, H., Gamss, C., Gavrilova, T., Summers, B. D., et al. (2006). "Inducible nitric oxide synthase mediates prostaglandin h2 synthase nitration and suppresses eicosanoid production." Am J Pathol. **168**(1): 349-362.

Deeb, R. S., Upmacis, R. K., Lamon, B. D., Gross, S. S. and Hajjar, D. P. (2008). "Maintaining equilibrium by selective targeting of cyclooxygenase pathways: promising offensives against vascular injury." Hypertension **51**(1): 1-7.

Der Sarkissian, S., Grobe, J. L., Yuan, L., Narielwala, D. R., Walter, G. A., et al. (2008). "Cardiac overexpression of angiotensin converting enzyme 2 protects the heart from ischemia-induced pathophysiology." Hypertension **51**(3): 712-718.

Deuchar, G. A., Morecroft, I., Dempsie, Y., Herold, N., Nilsen, M., et al. (2006). "The in vivo effects of human urotensin II in the rabbit and rat pulmonary circulation: Effects of experimental pulmonary hypertension." Eur J Pharmacol **537**: 135-142.

Dias-Peixoto, M. F., Santos, R. A., Gomes, E. R., Alves, M. N., Almeida, P. W., et al. (2008). "Molecular mechanisms involved in the angiotensin-(1-7)/Mas signaling pathway in cardiomyocytes." Hypertension **52**(3): 542-548.

Djordjevic, T., BelAiba, R. S., Bonello, S., Pfeilschifter, J., Hess, J., et al. (2005). "Human urotensin II is a novel activator of NADPH oxidase in human pulmonary artery smooth muscle cells." Arterioscler Thromb Vasc Biol. **25**(3): 519-525.

do Rego, J. C., Leprince, J., Scalbert, E., Vaudry, H. and Costentin, J. (2008). "Behavioral actions of urotensin-II." Peptides **29**(5): 838-844.

Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., et al. (2000). "A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9." Circ Res **87**(5): E1-9.

Douglas, S. A. and Ohlstein, E. H. (2000). "Human Urotensin-II, the Most Potent Mammalian Vasoconstrictor Identified To Date, as a Therapeutic Target for the Management of Cardiovascular Disease." Trends Cardiovasc Med **10**(6): 229-237.

Douglas, S. A., Sulpizio, A. C., Piercy, V., Sarau, H. M., Ames, R. S., et al. (2000). "Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey." Br J Pharmacol **131**: 1262-1274.

Douglas, S. A., Tayara, L., Ohlstein, E. H., Halawa, N. and Giaid, A. (2002). "Congestive heart failure and expression of myocardial urotensin II." Lancet **359**(9322): 1990-1997.

Douglas, S. A., Dhanak, D. and Johns, D. G. (2004). "From 'gills to pills': urotensin-II as a regulator of mammalian cardiorenal function." Trends Pharmacol Sci **25**(2): 76-85.

Dschietzig, T., Richter, C., Bartsch, C., Bohme, C., Heinze, D., et al. (2001). "Flow-Induced Pressure Differentially Regulates Endothelin-1, Urotensin II, Adrenomedullin, and Relaxin in Pulmonary Vascular Endothelium." Biochem Biophys Res Commun **289**(1): 245-251.

Dschietzig, T., Bartsch, C., Pregla, R., Zurbrugg, H. R., Armbruster, F. P., et al. (2002). "Plasma levels and cardiovascular gene expression of urotensin-II in human heart failure." Regul Pept **110**(1): 33-38.

Dubessy, C., Cartier, D., Lectez, B., Bucharles, C., Chartrel, N., et al. (2008). "Characterization of urotensin II, distribution of urotensin II, urotensin II-related peptide and UT receptor mRNAs in mouse: evidence of urotensin II at the neuromuscular junction." J Neurochem. **107**(2): 361-374.

Egginger, J. G. and Calas, A. (2005). "A novel hypothalamic neuroendocrine peptide: URP (urotensin-II-related peptide)?" C R Biol **328**(8): 724-731.

Fan, M., Goodwin, M., Vu, T., Brantley-Finley, C., Gaarde, W. A., et al. (2000). "Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade." J Biol Chem. **275**(39): 29980-29985.

Feinstein, G., Mealemud, C. J. and Janoff, A. (1976). "The inhibition of human leucocyte elastase and chymotrypsin-like protease by elastinal and chymostatin." Biochem Biophys Acta **429**(3): 925-932.

Feng, X., Zhang, J., Barak, L. S., Meyer, T., Caron, M. G., et al. (1998). "Visualization of dynamic trafficking of a protein kinase C betaII/green fluorescent protein conjugate reveals differences in G protein-coupled receptor activation and desensitization." J Biol Chem. **273**(17): 10755-10762.

Ferrario, C. M., Richmond, R. S., Smith, R., Levy, P., Strawn, W. B., et al. (2004). "Renin-angiotensin system as a therapeutic target in managing atherosclerosis." Am J Ther. **11**(1): 44-53.

Ferrario, C. M. (2006). "Role of angiotensin II in cardiovascular disease therapeutic implications of more than a century of research." J Renin Angiotensin Aldosterone Syst. **7**(1): 3-14.

Fisher, J. W., Samuels, A. and Langston, J. (1968). "Effects of angiotensin, norepinephrine and renal artery constriction on erythropoietin production." Ann N Y Acad Sci. **149**(1): 308-317.

Flohr, S., Kurz, M., Kostenis, E., Brkovich, A., Fournier, A., et al. (2002). "Identification of nonpeptidic urotensin II receptor antagonists by virtual screening based on a pharmacophore model derived from structure-activity relationships and nuclear magnetic resonance studies on urotensin II." J Med Chem **45**(9): 1799-1805.

Fontes-Sousa, A. P., Pires, A. L., Monteiro-Cardoso, V. F. and Leite-Moreira, A. F. (2008). "Urotensin II-induced increase in myocardial distensibility is modulated by angiotensin II and endothelin-1." Physiol Res **In Press**.

Force, T., Pombo, C. M., Avruch, J. A., Bonventre, J. V. and Kyriakis, J. M. (1996). "Stress-activated protein kinases in cardiovascular disease." Circ Res **78**: 947-953.

Forster, M. E. (1981). "Effects of catecholamines on the hearts and ventral aortas of the eels Anguilla australis schmidtii and Anguilla dieffenbachii." Comp Biochem Physiol C. **70**(1): 85-90.

Gamboa, A., Shibao, C., Diedrich, A., Choi, L., Pohar, B., et al. (2007). "Contribution of endothelial nitric oxide to blood pressure in humans." Hypertension **49**(1): 170-177.

Gardiner, S. M., March, J. E., Kemp, P. A., Davenport, A. P. and Bennett, T. (2001). "Depressor and regionally-selective vasodilator effects of human and rat urotensin II in conscious rats." Br J Pharmacol **132**: 1625-1629.

Gardiner, S. M., March, J. E., Kemp, P. A. and Bennett, T. (2004). "Bolus injection of human UII in conscious rats evokes a biphasic haemodynamic response." Br J Pharmacol **143**(3): 422-430.

Gardiner, S. M., March, J. E., Kemp, P. A., Maguire, J. J., Kuc, R. E., et al. (2006). "Regional heterogeneity in the haemodynamic responses to urotensin II infusion in relation to UT receptor localisation." Br J Pharmacol **147**(6): 612-621.

Gendron, G., Gobeil, F. J., Bélanger, S., Gagnon, S., Regoli, D., et al. (2005). "Urotensin II-induced hypotensive responses in Wistar-Kyoto (Wky) and spontaneously hypertensive (Shr) rats." Peptides **26**: 1468-1474.

Gibson, A., Wallace, P. and Bern, H. A. (1986). "Cardiovascular effects of urotensin II in anaethetized and pithed rats." Gen Comp Endocrinol **64**: 435-439.

Gibson, A. (1987). "Complex effects of Gillichthys urotensin II on rat aortic strips." Br J Pharmacol **91**: 205-212.

Gibson, A., Conyers, S. and Bern, H. A. (1988). "The influence of urotensin II on calcium influx in rat aorta." J Pharm Pharmacol **40**: 893-895.

Giles, T. D. (1992). "Importance of long-acting angiotensin-converting enzyme inhibitors for congestive heart failure." Am J Cardiol. **70**(10): 98C-101C.

Gillespie, J. S. and Rae, R. M. (1972). "Constrictor and compliance responses of some arteries to nerve or drug stimulation." J Physiol. **223**(1): 109-130.

Goldberg, D. M. (2000). "Proteases in the evaluation of pancreatic function and pancreatic disease." Clinica Chemica Acta **291**: 201-221.

Gondo, M., Maruta, H. and Arakawa, K. (1989). "Direct formation of angitensin II wihout renin or converting enzyme in the ischemic dog heart." Jpn. Heart J. **30**: 219-229.

Gong, H., Wang, Y.-X., Zhu, Y.-Z., Wang, W.-W., Wang, M.-J., et al. (2004). "Cellular distribution of GPR14 and the positive inotropic role of urotensin II in the myocardium in adult rat." J Appl Physiol **97**(6): 2228-2235.

Gray, G. A., Jones, M. R. and Sharif, I. (2001). "Human urotensin II increases coronary perfusion pressure in the isolated rat heart: Potentiation by nitric oxide synthase and cyclooxygenase inhibition." Life Sci **69**(2): 175-180.

Greco, S., Muscella, A., Elia, M. G., Salvatore, P., Storelli, C., et al. (2003). "Angiotensin II activates extracellular signal regulated kinases via protein kinase C and epidermal growth factor receptor in breast cancer cells." J Cell Physiol. **196**(2): 370-377.

Griendling, K. K., Minieri, C. A., Ollerenshaw, J. D. and Alexander, R. W. (1994). "Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells." Circ Res. **74**(6): 1141-1148.

Gruson, D., Rousseau, M. F., Ahn, S. A., van Linden, F. and Ketelslegers, J. M. (2005). "Circulating urotensin II levels in moderate to severe congestive heart failure: Its relations with myocardial function and well established neurohormonal markers." Peptides **27**(6): 1527-1531.

Guerrini, R., Camarda, V., Marzola, E., Arduin, M., Calo, G., et al. (2005). "Structure-activity relationship study on human urotensin II." J Pept Sci **11**(2): 85-90.

Guo, C., Ju, H., Leung, D., Massaeli, H., Shi, M., et al. (2001). "A novel vascular smooth muscle chymase is upregulated in hypertensive rats." J Clin Invest **107**(6): 703-715.

Hadi, H. A., Carr, C. S. and Al Suwaidi, J. (2005). "Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome." Vasc Health Risk Manag. **1**(3): 183-198.

Haluska, B. A., Jeffriess, L., Downey, M. and Carlier, S. G. M., T.H. (2008). "Influence of cardiovascular risk factors on total arterial compliance." J Am Soc Echocardiogr. **21**(2): 123-128.

Harris, G. S., Lust, R. M. and Katwa, L. C. (2007). "Hemodynamic effects of chronic urotensin II administration in animals with and without aorto-caval fistula." Peptides **28**(8): 1483-1489.

Hassan, G. S., Chouiali, F., Saito, T., Hu, F., Douglas, S. A., et al. (2003). "Effect of human urotensin-II infusion on hemodynamics and cardiac function." Can J Physiol Pharmacol **81**: 125-128.

Hassan, G. S., Douglas, S. A., Ohlstein, E. H. and Giaid, A. (2005). "Expression of urotensin-II in human coronary atherosclerosis." Peptides **26**(12): 2564-2572.

Hazon, N., Bjenning, C. and Conlon, J. M. (1993). "Cardiovascular actions of dogfish urotensin II in the dogfish *Scyliorhinus canicula*." Am J Physiol **265**: R573-R576.

Hearse D.J. and Sutherland F.J. (2000). "Experimental models for the study of cardiovascular function and disease." Pharmacol Res. **41**(6): 597-603

Heidkamp, M. C., Bayer, A. L., Martin, J. L. and Samarel, A. M. (2001). "Differential activation of mitogen-activated protein kinase cascades and apoptosis by protein kinase C epsilon and delta in neonatal rat ventricular myocytes." Circ Res. **89**(10): 882-890.

Hein, S., Arnon, E., Kostin, S., Schönburg, M., Elsässer, A., et al. (2003). "Progression from compensated hypertrophy to failure in the pressure-overloaded human heart: structural deterioration and compensatory mechanisms." Circulation **107**(7): 984-991.

Heller, J., Schepke, M., Neef, M., Woitas, R., Rabe, C., et al. (2002). "Increased urotensin II plasma levels in patients with cirrhosis and portal hypertension." J Hepatol **37**: 762-772.

Heringlake, M., Kox, T., Uzun, O., Will, B., Bahlmann, L., et al. (2004). "The relationship between urotensin II plasma immunoreactivity and left ventricular filling pressures in coronary artery disease." Regul Pept **121**(1-3): 129-136.

Higashi, Y., Sasaki, S., Nakagawa, K., Matsuura, H., Oshima, T., et al. (2002). "Endothelial function and oxidative stress in renovascular hypertension." N Engl J Med. **346**(25): 1954-1962.

Higuchi, S., Ohtsu, H., Suzuki, H., Shirai, H., Frank, G., et al. (2007). "Angiotensin II signal transduction through the AT_1 receptor: novel insights into mechanisms and pathophysiology." Clin. Sci. **112**: 417-428.

Hillier, C., Berry, C., Petrie, M. C., O'Dwyer, P. J., Hamilton, C., et al. (2001). "Effects of urotensin II in human arteries and veins of varying caliber." Circulation **103**: 1378-1381.

Hoit, B. D., Shao, Y., Kinoshita, A., Gabel, M., Husain, A., et al. (1995). "Effects of angiotensin II generated by an angiotensin converting enzyme-independent

pathway on left ventricular performance in the conscoius baboon." J Clin Invest **95**: 1519-1527.

Hollenberg, N. K. (2000). "The renin-angiotensin system and cardiovascular disease." Blood Press Suppl. **1**: 5-8.

Holubarsch, C., Hasenfuss, G., Schmidt-Schweda, S., Knorr, A., Pieske, B., et al. (1993). "Angiotensin I and II exert inotropic effects in atrial but not in ventricular human myocardium. An in vitro study under physiological experimental conditions." Circulation **88**(3): 1228-1237.

Holubarsch, C., Schmidt-Schweda, S., Knorr, A., Duis, J., Pieske, B., et al. (1994). "Functional significance of angiotensin receptors in human myocardium. Significant differences between atrial and ventricular myocardium." Eur Heart J **15 Suppl D**: 88-91.

Hood, S. G., Watson, A. M. D. and May, C. N. (2005). "Cardiac actions of central but not peripheral urotensin II are prevented by ß-adrenoceptor blockade." Peptides **26**: 1248-1256.

Horie, S., Tsurumaki, Y., Someya, A., Hirabayashi, T., Saito, T., et al. (2005). "Involvement of cyclooxygenase-dependent pathway in contraction of isolated ileum by urotensin II." Peptides **26**: 323-329.

Hubert, C., Houoy, A. M., Corvol, P. and Soubrier, F. (1991). "Structuer of the angiotensin I-converting enzyme gene. Two alternate promoters correspond to evolutionary steps of a duplicated gene." J Biol Chem **266**: 15377-15383.

Hunyady, L. and Catt, K. J. (2006). "Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II." Mol Endocrinol. **20**(5): 953-970.

Inagami, T. (1998). "A memorial to Robert Tiegerstedt." Hypertension **32**: 953-957.

Ishihata, A., Ogaki, T., Aita, T. and Katano, Y. (2005a). "Urotensin II-stimulated production of prostaglandins and nitric oxide in coronary arteries of aged rats." J Pharmacol Sci **97**: 182P.

Ishihata, A., Ogaki, T., Aita, T. and Katano, Y. (2005b). "Role of prostaglandins in urotensin II-induced vasodilation in the coronary arteries of aged rats." Eur J Pharmacol **523**(1-3): 119-126.

Ishihata, A., Sakai, M. and Katano, Y. (2006). "Vascular contractile effect of urotensin II in young and aged rats: Influence of aging and contribution of endothelial nitric oxide." Peptides **27**(1): 80-86.

Ito, T., Deng, X., Carr, B. and May, W. S. (1997). "Bcl-2 phosphorylation required for anti-apoptosis function." J Biol Chem **272**: 11671-11673.

Itoh, H., Itoh, Y., Rivier, J. and Lederis, K. (1987). "Contraction of major artery segments of rat by fish neuropeptide urotensin II." Am J Physiol **252**(2): R361-366.

Itoh, H., McMaster, D. and Lederis, K. (1988). "Functional receptors for fish neuropeptide urotensin II in major rat arteries." Eur J Pharmacol **149**(1-2): 61-66.

Jégou, S., Cartier, D., Dubessy, C., Gonzalez, B. J., Chatenet, D., et al. (2006). "Localization of the urotensin II receptor in the rat central nervous system." J Comp Neurol **495**(1): 21-36.

Jessup, J. A., Trask, A. J., Chappell, M. C., Nagata, S., Kato, J., et al. (2008). "Localisation of the novel angiotensin peptide, Angiotensin-12 [Ang-(1-12)], in heart and kidney of hypertensive and normotensive rats." Am J Physiol Heart Circ Physiol **294**(6): H2614-2618.

Jia, Z., Guo, X., Zhang, H., Wang, M. H., Dong, Z., et al. (2008). "Microsomal prostaglandin synthase-1-derived prostaglandin E2 protects against

angiotensin II-induced hypertension via inhibition of oxidative stress." Hypertension **52**(5): 952-959.

Jin, D., Takai, S., Yamada, M., Sakaguchi, M., Yao, Y., et al. (2001). "Possible roles of cardiac chymase after myocardial infarction in hamster hearts." Jpn J Pharmacol. **86**(2): 203-214.

Jin, D., Ueda, H., Takai, S., Okamoto, Y., Muramatsu, M., et al. (2005). "Effect of chymase inhibition on the arteriovenous fistula stenosis in dogs." J Am Soc Nephrol. **16**(4): 1024-1034.

Ju, H., Gros, R., You, X., Tsang, S., Husain, M., et al. (2001). "Conditional and targeted overexpression of vascular chymase causes hypertension in transgenic mice." Proc Natl Acad Sci USA **98**(13): 7469-7474.

Kaartinen, M., Penttilä, A. and Kovanen, P. T. (1994). "Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture." Circulation **90**(4): 1669-1678.

Kanemitsu, H., Takai, S., Tsuneyoshi, H., Nishina, T., Yoshikawa, K., et al. (2005). "Chymase inhibition prevents cardiac fibrosis and dysfunction after myocardial infarction in rats." Hypertens Res **29**(1): 57-64.

Kang, Y. J., Zhou, Z. X., Wang, G. W., Buridi, A. and Klein, J. B. (2000). "Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of p38 mitogen-activated protein kinases." J Biol Chem. **275**(18): 13690-13698.

Karlson, U., Pejler, G., Fröman, G. and Hellman, L. (2002). "Rat mast cell protease 4 is a ß-chymase with unusually stringent substrate recognition profile." J Biol Chem **277**(21): 18579-18585.

Katano, Y., Ishihata, A., Aita, T., Ogaki, T. and Horie, T. (2000). "Vasodilator effect of urotensin II, one of the most potent vasoconstricting factors, on rat coronary arteries." Eur J Pharmacol **402**(1-2): 209-211.

Katovich, M. J., Grobe, J. L. and Raizada, M. K. (2008). "Angiotensin-(1-7) as an antihypertensive, antifibrotic target." Curr Hypertens Rep. **10**(3): 227-232.

Katugampola, S. D., Kuc, R. E., Maguire, J. J. and Davenport, A. P. (2002). "G-protein-coupled receptors in human atherosclerosis: comparison of vasoconstrictors (endothelin and thromboxane) with recently de-orphanized (urotensin-II, apelin and ghrelin) receptors." Clin Sci (Lond). **103**(Suppl 48): 171S-175S.

Katwa, L. C., Tyagi, S. C., Campbell, S. E., Lee, S. J., Cicila, G. T., et al. (1996). "Valvular interstitial cells express angiotensinogen and cathepsin D, and generate angiotensin peptides." Int J Biochem Cell Biol. **28**(7): 807-821.

Katz, D. (1997). "Mechanisms and implications of endothelial dysfunction in congestive heart failure." Curr Opin Cardiol. **12**(3): 259-264.

Khan, S. Q., Bhandari, S. S., Quinn, P., Davies, J. E. and Ng, L. L. (2006). "Urotensin II is raised in acute myocardial infarction and low levels predict risk of adverse clinical outcome in humans." Int J Cardiol **117**(3): 323-328.

Kim, S., Izumi, Y., Yano, M., Hamaguchi, A., Miura, K., et al. (1998). "Angiotensin blockade inhibits activation of mitogen-activated protein kinases in rat balloon-injured artery." Circulation **97**: 1731-1737.

Kinney, W. A., Almond Jr, H. R., Qi, J., Smith, C. E., Santulli, R. J., et al. (2002). "Structure-function analysis of Urotensin II and its use in the construction of a ligand-receptor working model." Angew. Chem. Int. Ed. **41**(16): 2940-2944.

Kintscher, U., Bruemmer, D., Blaschke, F., Unger, T. and Law, R. E. (2003). "p38 MAP kinase negatively regulates angiotensin II-mediated effects on cell cycle molecules in human coronary smooth muscle cells." Biochem Biophys Res Commun. **305**(3): 552-556.

Kirimura, K., Takai, S., Jin, D., Muramatsu, M., Kishi, K., et al. (2005). "Role of chymase-dependent angiotensin II formation in regulating blood pressure in spontaneously hypertensive rats." Hypertens Res **28**: 457-464.

Kishi, K., Jin, D., Takai, S., Muramatsu, M., Katayama, H., et al. (2006). "Role of chymase-dependent angiotensin II formation in monocrotaline-induced pulmonary hypertensive rats." Pediatr Res **60**(1): 77-82.

Kokkonen, J. O., Lindstedt, K. A. and Kovanen, P. T. (2003). "Role for chymase in heart failure: Angiotensin II-dependent or -independent mechanisms?" Circulation **107**: 2522-2524.

Kriebel, R. M. (1980). "Ultrastructural changes in the urophysis of Mollienesia sphenops following adaptation to seawater." Cell Tissue Res. **207**(1): 135-142.

Kunori, Y., Muroga, Y., Iidaka, M., Mitsuhashi, H., Kamimura, T., et al. (2005). "Species differences in angiotensin II generation and degredation by mast cell chymases." J Recept Signal Transduct Res **25**(1): 35-44.

Labarrère, P., Chatenet, D., Leprince, J., Marionneau, C., Loirand, G., et al. (2003). "Structure-activity relationships of human urotensin II and related analogues on rat aorta ring contraction." J Enz Inh Med Chem **18**: 77-88.

Lacza, Z. and Busija, D. W. (2006). "Urotensin-II is a nitric oxide-dependent vasodilator in the pial arteries of the newborn pig." Life Sci **78**(23): 2763-2766.

Lamarre, N. S. and Tallarida, R. J. (2008). "A quantitative study to assess synergistic interactions between urotensin II and angiotensin II." Eur J Pharmacol **586**: 350-351.

Lamontagne, D., Pohl, U. and Busse, R. (1992). "Mechanical deformation of vessel wall and shear stress determine the basal release of endothelium-derived relaxing factor in the intact rabbit coronary vascular bed." Circ Res. **70**(1): 123-130.

Lancien, F., Leprince, J., Mimassi, N., Mabin, D., Vaudry, H., et al. (2004). "Central effects of native urotensin II on motor activity, ventilatory movements, and heart rate in the trout Oncorhynchus mykiss." Brain Res **1023**(2): 167-174.

Langham, R. G., Kelly, D. J., Gow, R. M., Zhang, Y., Dowling, J. K., et al. (2004). "Increased expression of urotensin II and urotensin II receptor in human diabetic nephropathy." Am J Kidney Dis **44**(5): 826-831.

Lapp, H., Boerrigter, G., Costello-Boerrigter, L. C., Jaekel, K., Scheffold, T., et al. (2004). "Elevated plasma human urotensin-II-like immunoreactivity in ischemic cardiomyopathy." Int J Cardiol **94**(1): 93-97.

Lassègue, B., Sorescu, D., Szöcs, K., Yin, Q., Akers, M., et al. (2001). "Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways." Circ Res. **88**(9): 888-894.

Lavi, S., Yang, E. H., Prasad, A., Mathew, V., Barsness, G. W., et al. (2008). "The interaction between coronary endothelial dysfunction, local oxidative stress, and endogenous nitric oxide in humans." Hypertension **51**(1): 127-133.

Le Mével, J. C., Olson, K. R., Conklin, D., Waugh, D., Smith, D. D., et al. (1996). "Cardiovascular actions of trout urotensin II in the conscious trout, *Oncorhynchus mykiss*." Am J Physiol Regulatory Integrative Comp Physiol **271**: R1335-R1343.

Lee, M. A., Böhm, M., Paul, M. and Ganten, D. (1993). "Tissue renin-angiotensin systems. Their role in cardiovascular disease." Circulation **87**((5 Suppl)): IV7-13.

Lewis, L. K. (1998). Identity, measurement and metabolism of 'new' vasoactive components. Dunedin, University of Otago.

Li, G., Li, R. K., Mickle, D. A., Weisel, R. D., Merante, F., et al. (1998). "Elevated insulin-like growth factor-I and transforming growth factor-beta 1 and their

receptors in patients with idiopathic hypertrophic obstructive cardiomyopathy. A possible mechanism." Circulation. 1998 Nov 10;98(19 Suppl):II144-9 **98**(19 Suppl II): 144-149.

Li, L., Yuan, W.-J. and Su, D.-F. (2004). "Effects of rat urotensin II on coronary flow and myocardial eNOS protein expression in isolated rat heart." Acta Pharmacol Sin **25**(11): 1444-1449.

Li, T. and Zimmerman, B. G. (1990). "In vivo comparison of renal and femoral vascular sensitivity and local angiotensin generation." Hypertension **15**(2): 204-209.

Lihrmann, I., Bern, H. A. and Vaudry, H. (2006). Urotensin II and urotensin II-related peptide. Handbook of Biologically Active Peptides. A. J. Kastin. New York, Elsevier**:** 815-823.

Lim, M., Honisett, S., Sparkes, C. D., Komesaroff, P., Kompa, A. R., et al. (2004). "Differential effect of urotensin II on vascular tone in normal subjects and patients with chronic heart failure." Circulation **109**: 1212-1214.

Lin, L., Ding, W.-H., Jiang, W., Zhang, Y.-G., Qi, Y.-F., et al. (2004). "Urotensin-II activates L-arginine/nitric oxide pathway in isolated rat aortic adventitia." Peptides **25**: 1977-1984.

Lin, X. and Peter, R. E. (2001). "Somatostatins and their receptors in fish." Comp Biochem Physiol B **129**(2-3): 543-550.

Lin, Y., Tsuchihashi, T., Matsumura, K., Fukuhara, M., Ohya, Y., et al. (2003). "Central cardiovascular action of urotensin II in spontaneously hypertensive rats." Hypertens Res **26**: 839-845.

Liu, D., Gao, L., Roy, S. K., Cornish, K. G. and Zucker, I. H. (2008). "Role of oxidant stress on AT1 receptor expression in neurons of rabbits with heart failure and in cultured neurons." Circ Res. **103**(2): 186-193.

Liu, Q., Pong, S.-S., Zeng, Z., Zhang, Q., Howard, A. D., et al. (1999). "Identification of urotensin II as the endogenous ligand for the orphan G-Protein-Coupled Receptor GPR14." Biochem Biophys Res Commun **266**: 174-178.

Lord, J. M. and Pongracz, J. (1995). "Protein kinase C: A family of isoenzymes with distinct roles in pathogenesis." J Clin Pathol Mol Pathol **48**: M57-M64.

Lovren, F., Pan, Y., Quan, A., Teoh, H., Wang, G., et al. (2008). "Angiotensin converting enzyme-2 confers endothelial protection and attenuates atherosclerosis." Am J Physiol Heart Circ Physiol. **295**(4): H1377-1384.

Lu, W., Abdel-Razik, A. E., Ashton, N. and Balment, R. J. (2008). "Urotensin II: Lessons from comparative studies for general endocrinology." Gen Comp Endocrinol **157**: 14-20.

Lupu, A. N., Kaufman, J. J. and Maxwell, M. H. (1968). "Renal artery constriction: physiological determinants of pressure gradients." Ann Surg. **167**(2): 246-250.

Maassen Van Den Brink, A., de Vries, R., Saxena, P. R., Schalekamp, M. A. and Danser, A. H. (1999). "Vasoconstriction by in situ formed angiotensin II: role of ACE and chymase." Cardiovasc Res. **44**(2): 407-415.

Mackins, C. J., Kano, S., Seyedi, N., Schäfer, U., Reid, A. C., et al. (2006). "Cardiac mast cell-derived renin promotes local angiotensin formation, norepinephrine release, and arrhythmias in ischemia/reperfusion." J Clin Invest. **116**(4): 1063-1070.

MacLean, M. R., Alexander, D., Stirrat, A., Gallagher, M., Douglas, S. A., et al. (2000). "Contractile responses to human urotensin-II in rat and human pulmonary arteries: effect of endothelial factors and chronic hypoxia in the rat." Br J Pharmacol **130**(2): 201-204.

Maguire, J. J., Kuc, R. E. and Davenport, A. P. (2000). "Orphan-receptor ligan human urotensin II: receptor localization in human tissues and comparison of vasoconstrictor responses with endothelin-1." Br J Pharmacol **131**: 441-446.

Maguire, J. J., Kuc, R. E., Wiley, K. E., Kleinz, M. J. and Davenport, A. P. (2004). "Cellular distribution of immunoreactive urotensin-II in human tissues with evidence of increased expression in atherosclerosis and a greater constrictor response of small compared to large coronary arteries." Peptides **25**(10): 1767-1774.

Maguire, J. J., Kuc, R. E., Kleinz, M. J. and Davenport, A. P. (2008). "Immunocytochemical localization of the urotensin-II receptor, UT, to rat and human tissues: relevance to function." Peptides **29**(5): 735-742.

Malagon, M. M., Molina, M., Gahete, M. D., Duran-Prado, M., Martinez-Fuentes, A. J., et al. (2008). "Urotensin II and urotensin II-related peptide activate somatostatin receptor subtypes 2 and 5." Peptides **29**(5): 711-720.

Mallamaci, F., Cutrupi, S., Pizzini, P., Tripepi, G. and Zoccali, C. (2006). "Urotensin II and Biomarkers of Endothelial Activation and Atherosclerosis in End-Stage Renal Disease." Am J Hypertens. **19**(5): 505-510.

Marchese, A., Heiber, M., Nguyen, T., Heng, H. H., Saldivia, V. R., et al. (1995). "Cloning and chromosomal mapping of three novel genes, GPR9, GPR10, and GPR14, encoding receptors related to interleukin 8, neuropeptide Y, and somatostatin receptors." Genomics **29**(2): 335-344.

Masson, R., Nicklin, S. A., Craig, M. A., McBride, M., Gilday, K., et al. (2009). "Onset of experimental severe cardiac fibrosis is mediated by overexpression of Angiotensin-converting enzyme 2." Hypertension **53**(4): 694-700.

Matsumoto, T., Wada, A., Tsutamoto, T., Ohnishi, M., Isono, T., et al. (2003). "Chymase inhibition prevents cardiac fibrosis and improves diastolic dysfunction in the progression of heart failure." Circulation **107**: 2555-2558.

Matsushita, M., Shichiri, M., Imai, T., Iwashina, M., Tanaka, H., et al. (2001). "Co-expression of urotensin II and its receptor (GPR14) in human cardiovascular and renal tissues." J Hypertens **19**: 2185-2190.

Matsushita, M., Shichiri, M., Fukai, N., Ozawa, N., Yoshimoto, T., et al. (2003). "Urotensin II is an autocrine/paracrine growth factor for the porcine renal epithelial cell line, LLCPK1." Endocrinology **144**(5): 1825-1831.

Mazak, I., Fiebeler, A., Muller, D. N., Park, J. K., Shagdarsuren, E., et al. (2004). "Aldosterone potentiates angiotensin II-induced signaling in vascular smooth muscle cells." Circulation **109**(22): 2792-2800.

Mehta, P. K. and Griendling, K. K. (2007). "Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system." Am J Physiol Cell Physiol **292**: C82-C97.

Meigs, J. B., Larson, M. G., D'Agostino, R. B., Levy, D., Clouse, M. E., et al. (2002). "Coronary artery calcification in type 2 diabetes and insulin resistance: the framingham offspring study." Diabetes Care. **25**(8): 1313-1319.

Mellor, H. and Parker, P. J. (1998). "The extended protein kinase C superfamily." Biochem. J. **332**: 281-292.

MERCATOR (1992). "Does the new angiotensin converting enzyme inhibitor cilazapril prevent restenosis after percutaneous transluminal coronary angioplasty? Results of the MERCATOR study: a multicenter, randomized, double-blind placebo-controlled trial. Multicenter European Research Trial with Cilazapril after Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis (MERCATOR) Study Group." Circulation **86**(1): 100-110.

Mercure, C., Yogi, A., Callera, G. E., Aranha, A. B., Bader, M., et al. (2008). "Angiotensin(1-7) blunts hypertensive cardiac remodeling by a direct effect on the heart." Circ Res. **102**(11): 1319-1326.

Merkel, L. A., Rivera, L. M., Bilder, G. E. and Perrone, M. H. (1990). "Differential alteration of vascular reactivity in rabbit aorta with modest elevation of serum cholesterol." Circ Res. **67**(3): 550-555.

Minshall, R. D., Tan, F., Fumiaki, N., Rabito, S. F., Becker, R. P., et al. (1997). "Potentiation of the Actions of Bradykinin by Angiotensin I–Converting Enzyme Inhibitors." Circ Res **81**: 848-856.

Miyazaki, M., Wada, T., Shiota, N. and Takai, S. (1999). "Effect of an angiotensin II receptor antagonist, candesartan cilexetil, on canine intima hyperplasia after balloon injury." J Hum Hypertens. **13**(Suppl 1): S21-25.

Miyazaki, M. and Takai, S. (2006). "Tissue angiotensin II generating system by angiotensin-converting enzyme and chymase." J Pharmacol Sci **100**: 391-397.

Miyazaki, M., Takai, S., Jin, D. and Muramatsu, M. (2006). "Pathological roles of angiotensin II produced by mast cell chymase and the effects of chymase inhibition in animal models." Pharmacol Ther **112**(3): 668-676.

Mori, M., Sugo, T., Abe, M., Shimomura, Y., Kurihara, M., et al. (1999). "Urotensin II Is the Endogenous Ligand of a G-Protein-Coupled Orphan Receptor, SENR (GPR14)." Biochem Biophys Res Commun **265**(1): 123-129.

Mori, M. and Fujino, M. (2004). "Urotensin II-related peptide, the endogenous ligand for the urotensin II receptor in the rat brain." Peptides **25**: 1815-1818.

Mori, N., Hirose, T., Nakayama, T., Ito, O., Kanazawa, M., et al. (2009). "Increased expression of urotensin II-related peptide and its receptor in kidney with hypertension or renal failure." Peptides **30**(2): 400-408.

Muller, D. N., Fischli, W., Clozel, J. P., Hilgers, K. F., Bohlender, J., et al. (1998). "Local angiotensin II generation in the rat heart: role of renin uptake." Circ Res **82**(1): 13-20.

Musini, V. M., Fortin, P. M., Bassett, K. and Wright, J. M. (2009). "Blood pressure lowering efficacy of renin inhibitors for primary hypertension: a Cochrane systematic review." J Hum Hypertens. **In Press**.

Nagata, S., Kato, J., Sasaki, K., Minamino, N., Eto, T., et al. (2006). "Isolation and identification of proangiotensin-12, a possible component of the renin-angiotensin system." Biochem Biophys Res Commun **350**(4): 1026-1031.

Naka, T. (1993). "Angiotensin II receptor antagonist activities and mode of action of benzimidazole-7-carboxylic acid derivatives." Nippon Risho. **51**(6): 1575-1579.

Ng, L. L., Loke, I., O'Brien, R. J., Squire, I. B. and Davies, J. E. (2002). "Plasma urotensin in human systolic heart failure." Circulation **106**(23): 2877-2880.

Nicholls, M. and Espiner, E. A. (1976). "A sensitive, rapid radioimmunoassay for angiotensin II." NZ Med J. **83**: 399-403.

Nicholls, M. G., Charles, C. J., Crozier, I. G., Espiner, E. A., Ikram, H., et al. (1994). "Blockade of the renin-angiotensin system." J Hypertens Suppl. **12**(10): S95-103.

Nickenig, G. and Harrison, D. G. (2002). "The AT(1)-type angiotensin receptor in oxidative stress and atherogenesis: part I: oxidative stress and atherogenesis." Circulation **105**(3): 393-396.

Nishikimi, T., Maeda, N. and Matsuoka, H. (2006). "The role of natriuretic peptides in cardioprotection." Cardiovasc Res. **69**(2): 318-328.

Nishimoto, M., Takai, S., Sawada, Y., Yuda, A., Kondo, K., et al. (2001). "Chymase-dependent angiotensin II formation in the saphenous vein versus the internal thoracic artery." J Thorac Cardiovasc Surg. **121**(4): 729-734.

Nishimoto, M., Takai, S., Fukumoto, H., Tsunemi, K., Yuda, A., et al. (2002). "Increased local angiotensin II formation in aneurysmal aorta." Life Sci. **71**(18): 2195-2205.

Nothacker, H. P., Wang, Z., McNeill, A. M., Saito, Y., Merten, S., et al. (1999). "Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction." Nat Cell Bio **1**(6): 383-385.

Nothacker, H. P. and Clark, S. (2005). "From heart to mind. The urotensin II system and its evolving neurophysiological role." FEBS J. **272**(22): 5694-5702.

Ohsako, S., Ishida, I., Ichikawa, T. and Deguchi, T. (1986). "Cloning and sequence analysis of cDNAs encoding precursors of urotensin II-alpha and -gamma." J Neurosci. **6**(9): 2730-2735.

Ohtsu, H., Mifune, M., Frank, G. D., Saito, S., Inagami, T., et al. (2005). "Signal-crosstalk between Rho/ROCK and c-Jun NH2-terminal kinase mediates migration of vascular smooth muscle cells stimulated by angiotensin II." Arterioscler Thromb Vasc Biol. **25**(9): 1831-1836.

Okunishi, H., Miyazaki, M. and Toda, N. (1984). "Evidence for a putatively new angiotensin II-generating enzyme in the vascular wall." J Hypertens **2**(3): 277-284.

Okunishi, H., Oka, Y., Shiota, N., Kawamoto, T., Song, K., et al. (1993). "Marked species-difference in the vascular angiotensin II-forming pathways: humans versus rodents." Jpn J Pharmacol. **62**(2): 207-210.

Onan, D., Hannan, R. D. and Thomas, W. G. (2004). "Urotensin II: the old kid in town." Trends Endocrinol Metab **15**(4): 175-182.

Ong, K. L., Lam, K. S. L. and Cheung, B. M. (2005). "Urotensin II: Its function in health and its role in disease." Cardiovasc Drugs Ther **19**(1): 65-75.

Ono, T., Kawaguchi, Y., Kudo, M., Kushikata, T., Hashiba, E., et al. (2008). "Urotensin II evokes neurotransmitter release from rat cerebrocortical slices." Neurosci Lett. **440**(3): 275-279.

Opgaard, O. S., Nothacker, H.-P., Ehlert, F. J. and Krause, D. N. (2000). "Human urotensin II mediates vasoconstriction via an increase in inositol phosphates." Eur J Pharmacol **406**(2): 265-271.

Otani, H. (2009). "The Role of Nitric Oxide in Myocardial Repair and Remodeling." Antioxid Redox Signal. **In Press**.

Otis, M. and Gallo-Payet, N. (2007). "Role of MAPKs in angiotensin II-induced steroidogenesis in rat glomerulosa cells." Mol Cell Endocrinol. **265**: 126-130.

Oudit, G. Y., Crackower, M. A., Backx, P. H. and Penninger, J. M. (2003). "The role of ACE2 in cardiovascular physiology." Cardiovasc Med **13**: 93-101.

Owen, C. A. and Campbell, E. J. (1998). "Angiotensin II generation at the cell surface of activated neutrophils: novel cathepsin G-mediated catalytic activity that is resistant to inhibition." J Immunol. **160**(3): 1436-1443.

Ozer, O., Davutoglu, V., Ercan, S., Akcay, M., Sari, I., et al. (2009). "Plasma urotensin II as a marker for severity of rheumatic valve disease." Tohoku J Exp Med. **218**(1): 57-62.

Padmanabhan, N., Jardine, A. G., McGarth, J. C. and Connell, J. M. (1999). "Angiotensin-converting enzyme-independent contraction to angiotensin I in human resistance arteries." Circulation **99**(22): 2914-2920.

Palaniyandi, S. S., Inagaki, K. and Mochly-Rosen, D. (2008). "Mast cells and epsilonPKC: a role in cardiac remodeling in hypertension-induced heart failure." J Mol Cell Cardiol. **45**(6): 779-786.

Palmer, B. R., Jarvis, M. D., Pilbrow, A. P., Ellis, K. L., Frampton, C. M., et al. (2008). "Angiotensin-converting enzyme 2 A1075G polymorphism is associated with survival in an acute coronary syndromes cohort." Am Heart J. **156**(4): 752-758.

Papadopoulos, P., Bousette, N. and Giaid, A. (2008). "Urotensin-II and cardiovascular remodeling." Peptides **29**(5): 764-769.

Patchett, A. A., Harria, E., Tristam, E. W., Wyvratt, M. J., Wu, M. T., et al. (1980). "A new class of angiotensin-converting enzyme inhibitors." Nature **288**: 280-283.

Pearson, D., Shively, J. E., Clark, B. R., Geschwind, I. I., Barkley, M., et al. (1980). "Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes." Proc. Natl. Acad. Sci. **77**(8): 5021-5024.

Pelletier, G., Lihrmann, I., Dubessy, C., Luu-The, V., Vaudry, H., et al. (2005). "Androgenic down-regulation of urotensin II precursor, urotensin II-related peptide precursor and androgen receptor mRNA in the mouse spinal cord." Neuroscience **132**(3): 689-696.

Pemberton, C. J., Johnson, M. L., Yandle, T. G. and Espiner, E. A. (2000). "Deconvolution analysis of cardiac natriuretic peptides during acute volume overload." Hypertension **36**: 355-359.

Pemberton, C. J., Raudsepp, S. D., Yandle, T. G., Cameron, V. A. and Richards, A. M. (2005). "Plasma cardiotrophin-1 is elevated in human hypertension and stimulated by ventricular stretch." Cardiovasc Res **68**(1): 109-117.

Peng, Z. and Beaven, M. A. (2005). "An essential role for phospholipase D in the activation of protein kinase C and degranulation in mast cells." J Immunol. **174**(9): 5201-5208.

Pfeilschifter, J. (1988). "Protein kinase C from rat renal mesangial cells: its role in homologous desensitization of angiotensin II-induced polyphosphoinositide hydrolysis." Biochim Biophys Acta. **969**(3): 263-270.

Phillips, M. I. and Schmidt-Ott, K. M. (1999). "The Discovery of Renin 100 Years Ago." News Physiol Sci. **14**: 271-274.

Potter, L. R., Yoder, A. R., Flora, D. R., Antos, L. K. and Dickey, D. M. (2009). "Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications." Handb Exp Pharmacol. **191**: 341-366.

Prins, B. A., Weber, M. A. and Purdy, R. E. (1992). "Norepinephrine amplifies angiotensin II-induced vasoconstriction in rabbit femoral artery." J Pharmacol Exp Ther. **262**(1): 198-203.

Prosser, H. C., Forster, M. E., Richards, A. M. and Pemberton, C. J. (2008). "Urotensin II and urotensin II-related peptide (URP) in cardiac ischemia-reperfusion injury." Peptides **29**(5): 770-777.

Prosser, H. C., Forster, M. E., Richards, A. M. and Pemberton, C. J. (2009). "Cardiac chymase converts rat proAngiotensin-12 (PA12) to angiotensin II: effects of PA12 upon cardiac haemodynamics." Cardiovasc Res **82**(1): 40-50.

Prosser, H. C. G., Leprince, J., Vaudry, H., Richards, A. M., Forster, M. E., et al. (2006). "Cardiovascular effects of native and non-native UII and URP on rat and salmon hearts." Peptides **27**: 3261-3268.

Proulx, C. D., Holleran, B. J., Lavigne, P., Escher, E., Guillemette, G., et al. (2008). "Biological properties and functional determinants of the urotensin II receptor." Peptides **29**(5): 691-699.

Qi, J.-s., Minor, L. K., Smith, C., Hu, B., Yang, J., et al. (2005). "Characterization of functional urotensin II receptors in human skeletal muscle myoblasts: comparison with angiotensin II receptors." Peptides **26**: 683-690.

Rad, A. (2006). Renin-Angiotensin-Aldosterone System. Renin-angiotensin-aldosterone_system.png#file, Wikipedia.org. **1,050×585**.

Rakowski, E., Hassan, G. S., Dhanak, D., Ohlstein, E. H., Douglas, S. A., et al. (2005). "A role for urotensin II in restenosis following balloon angioplasty: use of a selective UT receptor blocker." J Mol Cell Cardiol. **39**(5): 785-791.

Ramseyer, V. D. and Garvin, J. L. (2008). "Angiotensin II decreases nitric oxide synthase 3 expression via nitric oxide and superoxide in the thick ascending limb." Hypertension **53**(2): 313-318.

Ravani, P., Tripepi, G., Pecchini, P., Mallamaci, F., Malberti, F., et al. (2008). "Urotensin II is an inverse predictor of death and fatal cardiovascular events in chronic kidney disease." Kidney Int. **73**(1): 95-101.

Richards, A. M., Nicholls, M. G., Lainchbury, J. G., Fisher, S. and Yandle, T. G. (2002). "Plasma urotensin II in heart failure." Lancet **360**(9332): 545-546.

Richards, A. M. and Charles, C. (2004). "Urotensin II in the cardiovascular system." Peptides **25**: 1795-1802.

Robertson, J. and Nicholls, M. (1993). The renin-angiotensin system. New Jersey, Gower Medical Publishing.

Rocic, P., Jo, H. and Lucchesi, P. A. (2003). "A role for PYK2 in ANG II-dependent regulation of the PHAS-1-eIF4E complex by multiple signaling cascades in vascular smooth muscle." Am J Physiol Cell Physiol. **285**(6): C1437-1444.

Rossowski, W. J., Cheng, B.-L., Taylor, J. E., Datta, R. and Coy, D. H. (2002). "Human urotensin II-induced aorta ring contractions are mediated by protein kinase C, tyrosine kinases and Rho-kinase: inhibition by somatostatin receptor antagonists." Eur J Pharmacol **438**(3): 159-170.

Ruiz-Ortega, M., Lorenzo, O., Rupérez, M., Esteban, V., Suzuki, Y., et al. (2001). "Role of the renin-angiotensin system in vascular diseases: expanding the field." Hypertension **38**: 1382-1387.

Russell, F. D., Molenaar, P. and O'Brien, D. M. (2001). "Cardiostimulant effects of urotensin-II in human heart *in vitro*." Br J Pharmacol **132**: 5-9.

Russell, F. D., Meyers, D., Galbraith, A. J., Bett, N., Toth, I., et al. (2003). "Elevated plasma levels of human urotensin-II immunoreactivity in congestive heart failure." Am J Physiol Heart Circ Physiol **285**(4): H1576-1581.

Russell, F. D. (2004). "Emerging roles of urotensin-II in cardiovascular disease." Pharmacol Ther **103**(3): 223-243.

Russell, F. D., Kearns, P., Toth, I. and Molenaar, P. (2004). "Urotensin-II-converting enzyme activity of furin and trypsin in human cells in vitro." J Pharmacol Exp Ther. **310**(1): 209-214.

Russell, F. D. and Molenaar, P. (2004). "Investigation of signaling pathways that mediate the inotropic effect of urotensin-II in human heart." Cardiovasc Res **63**(4): 673-681.

Ruvolo, P., Deng, X., B.K., C. and May, W. S. (1998). "A functional role for mitochondrial protein kinase C_{alpha} in Bcl2 phosphorylation and suppression of apoptosis." J Biol Chem **273**: 25436-25442.

Rykl, J., Thiemann, J., Kurzawski, S., Pohl, T., Gobom, J., et al. (2006). "Renal cathepsin G and angiotensin II generation." J Hypertens. **24**(9): 1797-1807.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual. New York, Cold Spring Harbor Laboratory Press.

Sanker, S., Chandrasekharan, U. M., Wilk, D., Glynias, M. J., Karnik, S. S., et al. (1997). "Distinct multisite synergistic interactions determine substrate specificities of human chymase and rat chymase-1 for angiotensin II formation and degradation." J Biol Chem **272**(5): 2963-2968.

Sanoski, C. A. (2009). "Aliskiren: an oral direct renin inhibitor for the treatment of hypertension." Pharmacotherapy. **29**(2): 193-212.

Satoh, H., Hosono, M. and Satoh, S. (1984). "Distinctive effect of angiotensin II on prostaglandin production in dog renal and femoral arteries." Prostaglandins **27**(6): 807-820.

Sauzeau, V., Le Mellionnec, E., Bertoglio, J., Scalbert, E., Pacaud, P., et al. (2001). "Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase." Circ Res **88**(11): 1102-1104.

Scheuren, N., Jacobs, M., Ertl, G. and Schorb, W. (2002). "Cyclooxygenase-2 in myocardium stimulation by angiotensin-II in cultured cardiac fibroblasts and role at acute myocardial infarction." J Mol Cell Cardiol. **34**(1): 29-37.

Schiffrin, E. L. (2008a). "Oxidative stress, nitric oxide synthase, and superoxide dismutase: a matter of imbalance underlies endothelial dysfunction in the human coronary circulation." Hypertension **51**(1): 31-32.

Schiffrin, E. L. (2008b). "The flame that lights the fire: oxidative stress, inflammation, and renal damage in angiotensin II-induced hypertension." Hypertension **52**(2): 205-206.

Sealey, J. E. and Laragh, J. H. (2009). "Aliskiren fails to lower blood pressure in patients who have either low PRA levels or whose PRA falls insufficiently or reactively rises." Am J Hypertens. **22**(1): 112-121.

Serova, O. N., Shevchenko, L. V., Elfimov, A. I., Kotov, A. V. and Torshin, V. I. (2004). "Water and salt consumption and suppression of Angiotensin-induced thirst in rats after carotid glomectomy." Bull Exp Biol Med. **138**(5): 437-439.

Shenouda, A., Douglas, S. A., Ohlstein, E. H. and Giaid, A. (2002). "Localization of urotensin-II immunoreactivity in normal human kidneys and renal carcinoma." J Histochem Cytochem. **50**(7): 885-889.

Shi, L., Ding, W., Li, D., Wang, Z., Jiang, H., et al. (2006). "Proliferation and anti-apoptotic effects of human urotensin II on human endothelial cells." Atherosclerosis **188**(2): 260-264.

Shiota, N., Okunishi, H., Fukamizu, A., Sakonjo, H., Kikumori, M., et al. (1993). "Activation of two angiotensin-generating systems in the balloon-injured artery." FEBS Lett. **323**(3): 239-242.

Shiota, N., Fukamizu, A., Takai, S., Okunishi, H., Murakami, K., et al. (1997). "Activation of angiotensin II-forming chymase in the cardiomyopathic hamster heart." J Hypertens **15**: 431-440.

Shiraishi, Y., Watanabe, T., Suguro, T., Nagashima, M., Kato, R., et al. (2008). "Chronic urotensin II infusion enhances macrophage foam cell formation and atherosclerosis in apolipoprotein E-knockout mice." J Hypertens. **26**(10): 1955-1965.

Silvestre, R. A., Egido, E. M., Hernández, R., Leprince, J., Chatenet, D., et al. (2004). "Urotensin-II is present in pancreatic extracts and inhibits insulin release in the perfused rat pancreas." Eur J Endocrinol **151**(6): 803-809.

Singh, L. K., Pang, X., Alexacos, N., Letourneau, R. and Theoharides, T. C. (1999). "Acute immobilization stress triggers skin mast cell degranulation via corticotropin releasing hormone, neurotensin, and substance P: A link to neurogenic skin disorders." Brain Behav Immun. **13**(3): 225-239.

Song, W., Abdel-Razik, A. E., Lu, W., Ao, Z., Johns, D. G., et al. (2006). "Urotensin II and renal function in the rat." Kidney Int. **69**(8): 1360-1368.

Sowers, J. R. (2002). "Hypertension, angiotensin II, and oxidative stress." N Engl J Med. **346**(25): 1999-2001.

Spinazzi, R., Albertin, G., Nico, B., Guidolin, D., Di Liddo, R., et al. (2006). "Urotensin-II and its receptor (UT-R) are expressed in rat brain endothelial

cells, and urotensin-II via UT-R stimulates angiogenesis in vivo and in vitro." Int J Mol Med. **18**(6): 1107-1112.

Stirrat, A., Gallagher, M., Douglas, S. A., Ohlstein, E. H., Berry, C., et al. (2001). "Potent vasodilator responses to human urotensin-II in human pulmonary and abdominal resistance arteries." Am J Physiol Heart Circ Physiol **280**(2): H925-928.

Stokoe, D., Engel, K., Campbell, D. G., Cohen, P. and Gaestel, M. (1992). "Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins." FEBS Lett. **313**(3): 307-313.

Strittmatter, S. M., Thiele, E. A., De Souza, E. B. and Snyder, S. H. (1985). "Angiotensin-converting enzyme in the testis and epididymis: differential development and pituitary regulation of isozymes." Endocrinology **117**(4): 1374-1379.

Su, B. and Karin, M. (1996). "Mitogen-acivated protein kinase cascades and regulation of gene expression." Curr Opinion Immunol. **8**: 402-411.

Sugo, T., Murakami, Y., Shimomura, Y., Harada, M., Abe, M., et al. (2003). "Identification of urotensin II-related peptide as the urotensin II-immunoreactive molecule in the rat brain." Biochem Biophys Res Commun **310**(3): 860-868.

Sugo, T. and Mori, M. (2008). "Another ligand fishing for G protein-coupled receptor 14. Discovery of urotensin II-related peptide in the rat brain." Peptides **29**(5): 809-812.

Suguro, T., Watanabe, T., Ban, Y., Kodate, S., Misaki, A., et al. (2007). "Increased human urotensin II levels are correlated with carotid atherosclerosis in essential hypertension." Am J Hypertens. **20**: 211-217.

Sutherland, F.J. and Hearse D.J. (2000). "The isolated blood and perfusion fluid perfused heart." Pharmacol Res. **41**(6): 613-627

Suzuki, H., Motley, E. D., Frank, G. D., Utsunomiya, H. and Eguchi, S. (2005). "Recent progress in signal transduction research of the angiotensin II type-1 receptor: protein kinases, vascular dysfunction and structural requirement." Curr Med Chem Cardiovasc Hematol Agents **3**(4): 305-322.

Takai, S., Shiota, N., Yamamoto, D., Okunishi, H. and Miyazaki, M. (1996). "Purification and characterization of angiotensin II-generating chymase from hamster cheek pouch." Life Sci. **58**(7): 591-597.

Takai, S., Shiota, N., Kobayashi, S., Matsumura, E. and Miyazaki, M. (1997a). "Induction of chymase that forms angiotensin II in the monkey atherosclerotic aorta." FEBS Lett. **412**(1): 86-90.

Takai, S., Shiota, N., Sakaguchi, M., Muraguchi, H., Matsumura, E., et al. (1997b). "Characterization of chymase from human vascular tissues." Clin Chim Acta. **265**(1): 13-20.

Takai, S., Shiota, N., Jin, D. and Miyazaki, M. (1998). "Functional role of chymase in angiotensin II formation in human vascular tissue." J Cardiovasc Pharmacol. **32**(5): 826-833.

Takai, S., Sakaguchi, M., Jin, D., Yamada, M., Kirimura, K., et al. (2001). "Different angiotensin II-forming pathways in human and rat vascular tissues." Clin Chim Acta. **305**: 191-195.

Takai, S., Jin, D., Sakaguchi, M., Katayama, S., Muramatsu, M., et al. (2003). "A novel chymase inhibitor, 4-[1-([bis-(4-methyl-phenyl)-methyl]-carbamoyl)3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yloxy]-benzoic acid (BCEAB), suppressed cardiac fibrosis in cardiomyopathic hamsters." J Pharmacol Exp Ther. **305**(1): 17-23.

Tamura, K., Okazaki, M., Tamura, M., Isozumi, K., Tasaki, H., et al. (2003). "Urotensin II-induced activation of extracellular signal-regulated kinase in cultured vascular smooth muscle cells: Involvement of cell adhesion-mediated integrin signaling." Life Sci **72**(9): 1049-1060.

Tamura, T., Said, S., Harris, J., Lu, W. and Gerdes, A. M. (2000). "Reverse remodeling of cardiac myocyte hypertrophy in hypertension and failure by targeting of the renin-angiotensin system." Circulation **102**(2): 253-259.

Taniyama, Y., Ushio-Fukai, M., Hitomi, H., Rocic, P., Kingsley, M. J., et al. (2004). "Role of p38 MAPK and MAPKAPK-2 in angiotensin II-induced Akt activation in vascular smooth muscle cells." Am J Physiol Cell Physiol. **287**(2): C494-499.

Tian, L., Li, C., Qi, J., Fu, P., Yu, X., et al. (2008). "Diabetes-induced upregulation of urotensin II and its receptor plays an important role in TGF-B1-mediated renal fibrosis and dysfunction." Am J Physiol Endocrinol Metab **295**: E1234-1242.

Tölle, M. and van der Giet, M. (2008). "Cardiorenovascular effects of urotensin II and the relevance of the UT receptor." Peptides **29**(5): 743-763.

Tostivint, H., Joly, L., Lihrmann, I., Parmentier, C., Morisson, M., et al. (2006). "Comparative genomics provides evidence for close evolutionary relationships between the urotensin II and somatostatin gene families." Proc. Natl. Acad. Sci. USA **103**: 2237-2242.

Totsune, K., Takahashi, K., Arihara, Z., Sone, M., Satoh, F., et al. (2001). "Role of urotensin II in patients on dialysis." Lancet **358**: 810-811.

Totsune, K., Takahashi, K., Arihara, Z., Sone, M., Murakami, O., et al. (2004). "Elevated plasma levels of immunoreactive urotensin II and its increased urinary excretion in patients with Type 2 diabetes mellitus: association with progress of diabetic nephropathy." Peptides **25**(10): 1809-1814.

Touyz, R. M., He, G., Wu, X. H., Park, J. B., Mabrouk, M. E., et al. (2001). "Src is an important mediator of extracellular signal-regulated kinase 1/2-dependent growth signaling by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients." Hypertension **38**(1): 56-64.

Touyz, R. M., Deschepper, C., Park, J. B., He, G., Chen, X., et al. (2002). "Inhibition of mitogen-activated protein/extracellular signal-regulated kinase improves endothelial function and attenuates Ang II-induced contractility of mesenteric resistance arteries from spontaneously hypertensive rats." J Hypertens. **20**(6): 1127-1134.

Touyz, R. M. (2004). "Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease." Braz J Med Biol Res. **37**(8): 1263-1273.

Trask, A. J., Jessup, J. A., Chappell, M. C. and Ferrario, C. M. (2008). "Angiotensin-(1-12) is an alternate substrate for angiotensin peptide production in the heart." Am J Physiol Heart Circ Physiol **294**(5): H2242-2247.

Triggle, C. R., Hollenberg, M., Anderson, T. J., Ding, H., Jiang, Y., et al. (2003). "The endothelium in health and disease--a target for therapeutic intervention." J Smooth Muscle Res. **39**(6): 249-267.

Trong, H. L., Neurath, H. and Woodbury, R. G. (1987). "Substrate specificity of the chymotrypsin-like protease secretory granules isolated from rat mast cells." Proc. Nat. Acad. Sci. **84**: 364-367.

Troughton, R. W. and Richards, A. M. (2006). "BNP for clinical monitoring of heart failure." Heart Fail Clin. **2**(3): 333-343.

Tsunemi, K., Takai, S., Nishimoto, M., Yuda, A., Hasegawa, S., et al. (2002). "Possible roles of angiotensin II-forming enzymes, angiotensin converting enzyme and chymase-like enzyme, in the human aneurysmal aorta." Hypertens Res. **25**(6): 817-822.

Tzanidis, A., Hannan, R. D., Walter G. Thomas, Done, O., See, F., et al. (2001). "Urotensin II stimulates collagen synthesis by cardiac fibroblasts and hypertrophic signalling in cardiomyocytes via G(alpha)q- and ras-dependent pathways." J Am Coll Cardiol **37**(2, Supplement 1): A164.

Tzanidis, A., Hannan, R. D., Thomas, W. G., Onan, D., Autelitano, D. J., et al. (2003). "Direct actions of urotensin II on heart: Implications for cardiac fibrosis and hypertrophy." Circ Res **93**: 246-253.

Uchide, T., Fujimori, Y., Fukushima, U., Uechi, M., Sasaki, T., et al. (2006). "cDNA cloning of hamster angiotensin-converting enzyme and mRNA expression." DNA Seq. **17**(5): 319-325.

Ullian, M. E. and Linas, S. L. (1990). "Angiotensin II surface receptor coupling to inositol trisphosphate formation in vascular smooth muscle cells." J Biol Chem. **265**(1): 195-200.

Urabe M, S. C., Lee TJ. (1987). "Pre- and postsynaptic effects of angiotensins in the femoral artery of spontaneously hypertensive and Wistar-Kyoto rats." Blood Vessels. **24**(1-2): 1-10.

Urata, H., Healy, B., Stewart, R. W., Bumpus, F. M. and Husain, A. (1990a). "Angiotensin II-forming pathways in normal and failing human hearts." Circ Res **66**(4): 883-890.

Urata, H., Kinoshita, A., Misono, K. S., Bumpus, F. M. and Husain, A. (1990b). "Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart." J Biol Chem **265**(36): 22348-22357.

Urata, H., Boehm, K. D., Philip, A., Kinoshita, A., Gabrovsek, J., et al. (1993). "Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart." J Clin Invest **91**: 1269-1281.

Urata, H., Strobel, F. and Ganten, D. (1994). "Widespread distribution of human chymase." J. Hypertens. Suppl. **12**: S17-S22.

Urata, H., Nishimura, H. and Ganten, D. (1996). "Chymase-dependent angiotensin II forming system in humans." AJH **9**: 277-284.

Urata, H. (2000). "Pathological involvement of chymase-dependent angiotensin II formation in the development of cardiovascular disease." JRAAS **1**(2): 35-37.

van Eickels, M., Grohé, C., Löbbert, K., Stimpel, M. and Vetter, H. (1999). "Angiotensin converting enzyme inhibitors block mitogenic signalling pathways in rat cardiac fibroblasts." Naunyn Schmiedebergs Arch Pharmacol. **359**(5): 394-399.

van Esch, J. H., Oosterveer, C. R., Batenburg, W. W., R., v. V. and Jan Danser, A. H. (2008). "Effects of angiotensin II and its metabolites in the rat coronary vascular bed: is angiotensin III the preferred ligand of the angiotensin AT2 receptor?" Eur J Pharmacol **588**: 286-293.

Varagic, J. and Frohlich, E. D. (2002). "Local cardiac renin-angiotensin system: hypertension and cardiac failure." J Mol Cell Cardiol. **34**(11): 1435-1442.

Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., et al. (2002). "Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase." J Biol Chem **277**(17): 14838-14843.

Vijayan, K., Szotek, E. L., Martin, J. L. and Samarel, A. M. (2004). "Protein kinase C-alpha-induced hypertrophy of neonatal rat ventricular myocytes." Am J Physiol Heart Circ Physiol. **287**(6): H2777-2789.

Vivet, F., Callard, P. and Gamoudi, A. (1987). "Immunolocalization of angiotensin 1 converting enzyme in the human male genital tract by the avidin-biotin-complex method." Histochemistry **86**(5): 499-502.

Vogel, V., Bokemeyer, D., Heller, J. and Kramer, H. J. (2001). "Cardiac hypertrophy in the Prague-hypertensive rat is associated with enhanced JNK2 but not ERK tissue activity." Kidney Blood Press Res. **24**(1): 52-56.

Wang, H., Mehta, J. L., Chen, K., Zhang, X. and Li, D. (2004). "Human urotensin II modulates collagen synthesis and the expression of MMP-1 in human endothelial cells." J Cardiovasc Pharmacol. **44**(5): 577-581.

Wang, Y. X., Ding, Y. J., Zhu, Y. Z., Shi, Y., Yao, T., et al. (2007). "Role of PKC in the novel synergistic action of urotensin II and angiotensin II and in urotensin II-induced vasoconstriction." Am J Physiol Heart Circ Physiol. **292**(1): H348-359.

Watanabe, T., Katagiri, T., Pakala, R. and Benedict, C. R. (2001a). "Synergistic effect of urotensin II with serotonin on vascular smooth muscle cell proliferation." J Am Coll Cardiol **41**(6, Supplement 1): 306.

Watanabe, T., Pakala, R., Katagiri, T. and Benedict, C. R. (2001b). "Synergistic effect of Urotensin II with mildly oxidized LDL on DNA synthesis in vascular smooth muscle cells." Circulation **104**: 16-18.

Watanabe, T., Suguro, T., Kanome, T., Sakamoto, Y.-i., Kodate, S., et al. (2005). "Human urotensin II accelerates foam cell formation in human monocyte-derived macrophages." Hypertension **46**: 738-744.

Watanabe, T., Kanome, T., Miyazaki, A. and Katagiri, T. (2006). "Human Urotensin II as a link between Hypertension and Coronary Artery Disease." Hypertens Res **29**(6): 375-387.

Watson, A. M., McKinley, M. J. and May, C. N. (2008). "Effect of central urotensin II on heart rate, blood pressure and brain Fos immunoreactivity in conscious rats." Neuroscience **155**(1): 241-249.

Watson, A. M. D., Lambert, G. W., Smith, K. J. and May, C. N. (2003). "Urotensin II acts centrally to increase epinephrine and ACTH release and cause potent inotropic and chronotropic actions." Hypertension **42**: 373-379.

Watson, A. M. D. and May, C. N. (2004). "Urotensin II, a novel peptide in central and peripheral cardiovascular control." Peptides **25**(10): 1759-1766.

Waugh, D. and Conlon, J. M. (1993). "Purification and Characterization of Urotensin II from the Brain of a Teleost (Trout, Oncorhynchus mykiss) and an Elasmobranch (Skate, Raja rhina)." Gen Comp Endocrinol **92**(3): 419-427.

Waugh, D., Youson, J., Mims, S. D., Sower, S. and Conlon, J. M. (1995). "Urotensin II from the River Lamprey (Lampetra fluviatilis), the Sea Lamprey (Petromyzon marinus), and the Paddlefish (Polyodon spathula)." Gen Comp Endocrinol **99**(3): 323-332.

Wei, C. C., Lucchesi, P. A., Tallaj, J., Bradley, W. E., Powell, P. C., et al. (2003). "Cardiac interstitial bradykinin and mast cells modulate pattern of LV remodeling in volume overload in rats." Am J Physiol Heart Circ Physiol. **285**(2): H784-792.

Wen, Y., Scott, S., Liu, Y., Gonzales, N. and Nadler, J. L. (1997). "Evidence that angiotensin II and lipoxygenase products activate c-Jun NH2-terminal kinase." Circ Res. **81**(5): 651-655.

Whiting, P., Nava, S., Mozley, L., Eastham, H. and Poat, J. (1991). "Expression of angiotensin converting enzyme mRNA in rat brain." Brain Res Mol Brain Res. **11**(1): 93-96.

Wintroub, B. U., Schechter, N. B., Lazarus, G. S., Kaempfer, C. E. and Schwartz, L. B. (1984). "Angiotensin I conversion by human and rat chyotryptic proteinases." J Invest Dermatol **83**(5): 336-339.

Wollert, K. C. and Drexler, H. (1999). "The renin-angiotensin system and experimental heart failure." Cardiovasc Res. **43**(4): 838-849.

Xia, W., Mruk, D. D. and Cheng, C. Y. (2007). "C-type natriuretic peptide regulates blood-testis barrier dynamics in adult rat testes." Proc Natl Acad Sci USA **104**(10): 3841-3846.

Yamamoto, D., Shiota, N., Takai, S., Ishida, T., Okunishi, H., et al. (1998). "Three-dimensional molecular modeling explains why catalytic function for

angiotensin-I is different between human and rat chymases." Biochem Biophys Res Commun. **242**(1): 158-163.

Yandle, T. G., Espiner, E. A., Nicholls, M. and Duff, H. (1986). "Radioimmunoassay and characterisation of atrial natriuretic peptide in human plasma." J Clin Endo Metab. **63**: 72-79.

Yang, X., Zhu, Q., Fong, J., Gu, X., Hicks, G. L., et al. (1996). "Enalaprilat, an angiotensin-converting enzyme inhibitor, enhances functional preservation during long-term cardiac preservation. Possible involvement of bradykinin and PKC." J Mol Cell Cardiol. **28**(7): 1445-1452.

Yano, K., Vaudry, H. and Conlon, J. M. (1994). "Spasmogenic actions of frog urotensin II on the bladder and ileum of the frog, Rana catesbeiana." Gen Comp Endocrinol **96**(3): 412-419.

Yano, K., Hicks, J. W., Vaudry, H. and Conlon, J. M. (1995). "Cardiovascular Actions of Frog Urotensin II in the Frog, Rana catesbeiana." Gen Comp Endocrinol **97**(1): 103-110.

Yayama, K. and Okamoto, H. (2008). "Angiotensin II-induced vasodilation via type 2 receptor: Role of bradykinin and nitric oxide." Int. Immunopharmacol. **8**: 312-318.

Yoshida, K., Yamazaki, M. and Toda, N. (1991). "Different modulation by cyclooxygenase inhibitors of the response to angiotensin II in monkey arteries and veins." Jpn J Pharmacol. **55**(4): 469-475.

Yoshimoto, T., Matsushita, M. and Hirata, Y. (2004). "Role of urotensin II in peripheral tissue as an autocrine/paracrine growth factor." Peptides **25**(10): 1775-1781.

Yusuf, S., Sleight, P., Pogue, J., Bosch, J., Davies, R., et al. (2000). "Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in

high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators." N Engl J Med. **342**(3): 145-153.

Yusuf, S., Pfeffer, M. A., Swedberg, K., Granger, C. B., Held, P., et al. (2003). "Effects of candesartan in patients with chronic heart failure and preserved left-ventricular ejection fraction: the CHARM-Preserved Trial." Lancet **362**: 777-781.

Yusuf, S., Teo, K. K., Pogue, J., Dyal, L., Copland, I., et al. (2008). "Telmisartan, ramipril, or both in patients at high risk for vascular events." N Engl J Med. **358**(15): 1547-1559.

Zhang, A. Y., Chen, Y.-F., Zhang, D. X., Yi, F.-X., Qi, J., et al. (2003). "Urotensin II is a nitric oxide-dependent vasodilator and natriuretic peptide in the rat kidney." Am J Physiol Renal Physiol **285**(4): F792-798.

Zhang, Y., Li, J., Cao, J., Chen, J., Yang, J., et al. (2002). "Effect of chronic hypoxia on contents of urotensin II and its functional receptors in rat myocardium." Heart Vessels **16**(2): 64-68.

Zhang, Y. G., Li, Y. G., Liu, B. G., Wei, R. H., Wang, D. M., et al. (2007). "Urotensin II accelerates cardiac fibrosis and hypertrophy of rats induced by isoproterenol." Acta Pharmacol Sin. **28**(1): 36-43.

Zhang, Y. G., Li, J., Li, Y. G. and Wei, R. H. (2008). "Urotensin II induces phenotypic differentiation, migration, and collagen synthesis of adventitial fibroblasts from rat aorta." J Hypertens. **26**(6): 1119-1126.

Zhong, J. C., Huang, Y., Yung, L. M., Lau, C. W., Leung, F. P., et al. (2007). "The novel peptide apelin regulates intrarenal artery tone in diabetic mice." Regul Pept. **144**(1-3): 109-114.

Zhou, P., Wu, S., Yu, C., Wang, H., Tang, C., et al. (2003). "Effects of urotensin II on isolated rat hearts under normal perfusion and ischaemia reperfusion." Sheng Li Xue Bao **55**(4): 442-448.

Zhu, Y. Z., Wang, Z. J., Zhu, Y. C., Zhang, L., Oakley, R. M. E., et al. (2004). "Urotensin II causes fatal circulatory collapse in anesthesized monkeys in vivo: a "vasoconstrictor" with a unique hemodynamic profile." Am J Physiol Heart Circ Physiol **286**(3): H830-836.

Zoccali, C., Mallamaci, F., Tripepi, G., Cutrupi, S., Pizzini, P., et al. (2006). "Urotensin II is an inverse predictor of incident cardiovascular events in end-stage renal disease." Kidney Int. **69**(7): 1253-1258.

Zoccali, C., Mallamaci, F., Benedetto, F. A., Tripepi, G., Pizzini, P., et al. (2008). "Urotensin II and cardiomyopathy in end-stage renal disease." Hypertension **51**(2): 326-333.

Zou, Y., Nagai, R. and Yamazaki, T. (2001). "Urotensin II induces hypertrophic responses in cultured cardiomyocytes from neonatal rats." FEBS Lett **508**(1): 57-60.

APPENDIX A

WESTERN BLOTTING PROTOCOL

A.1 Bradford's protein assay

A stock solution of Bradford reagent was made by dissolving 0.2 g Coomassie Brilliant Blue G-250 (BDH Laboratories, England) into 200 ml 85% (w/v) phosphoric acid and mixing the dye until completely dissolved. The solution was then made up to 1 litre with distilled water, and filtered through 0.45µM filter paper (Lewis 1998).

Bovine Serum Albumin (BSA) was made to 1 mg/ml with distilled water and 0.1% sodium azide added. 2 ml distilled water was pipetted into each tube and the appropriate measure of BSA pipetted into its correlate tube to produce known protein concentrations of 0.5, 1, 1.5, 2, 2.5, 4, 6 and 8 µg/µL. 10µL of 'unknown' cytosolic samples were added to their correlate labelled tubes. Two tubes had no protein added to provide blank controls. 2 ml Bradford's reagent was then added to all tubes, which were vortexed and allowed to incubate for 5 min at room temperature.

The spectrophotometer (Helios Epsilon visible spectrometer, Thermo Spectronic, Cambridge, UK) was allowed to warm to running temperature and stabilize before being programmed to read the single absorbance wavelength of 595 nm.

Aliquots of each tube were placed into quartz cuvettes for precise measurement within the spectrophotometer. The spectrophotometer was zeroed using the two blank tubes prior to measuring the standards and samples.

A.2 Calculation of results

The protein concentration of the known BSA standards were plotted against their measured absorbance to produce a standard curve. Using the regression equation of the standard curve, the samples containing unknown concentrations of viable protein were calculated.

A.3 Gel Electrophoresis

1. Prior to beginning gel electrophoresis stock solutions were prepared/obtained as follows:

Separating buffer (4x):	1.5 M Tris (90.8 g / 500 ml ddH_2O) 0.4 % SDS (2.0 g / 500 ml ddH_2O) pH adjusted to 8.8
Stacking buffer (1x):	0.139 M Tris (8.4 g / 500 ml ddH_2O) 0.11 % SDS (0.55 g / 500 ml ddH_2O) pH adjusted to 6.8
Acrylamide solution:	Purchased from Sigma as ready-made 29:1 acrylamide:Bisacrylamide solution
AMPS (10%):	Freshly prepared each time with Ammoniumperoxidisulphate (AMPS) diluted in ddH_2O
TEMED:	N'-N'-N'-N'-Tetramethylethylenediamine purchased from Sigma as ready-made solution
Laemmli dye (1x):	50 mM Tris·Cl (pH 6.8) 100 mM dithiothreitol 2 % SDS 0.1 % bromophenol blue 10 % glycerol
Running buffer (10x):	144 g glycine 30 g Tris 10 g SDS Make up to 1L ddH_2O, pH 8.3

% Gel	10 %
dd H_2O	6.25 ml
Separating gel buffer	3.75 ml
Acrylamide solution (1:29)	5.0 ml
10% AMPS	75 µl
TEMED	7.5 µl

Seperating gel ingredients comprising 10% acrylamide solution

2. The glass plates were clamped into their vice and separating gel poured between them. 1 cm of isopropanol was poured on top of the gel to ensure the gel set with a level surface. The gel polymerized after 30-60 min enabling the isopropanol to be poured off and the gel rinsed with ddH_2O several times prior to adding the stacking gel.

The percentage of acrylamide used was based on the weight of the proteins to be determined. The proteins to be determined in the current report were between 42 and 82 kD requiring an acrylamide concentration of 10 % (Sambrook et al. 1989).

Gel Size	2x BioRad Minigels, 0.75 mm thick
Stacking gel buffer	4.25 ml
Acrylamide solution	0.75 ml
10 % AMPS	25 µl
TEMED	2.5 µl

Stacking gel composition sufficient for 2 BioRad Minigels

3. The stacking gel (see Table 10.2 for ingredients) was added on top of the separating gel and combs inserted with their tips 2 mm above the separating gel layer. It was ensured that no air bubbles were present anywhere within the gel.

A.4 Loading protein samples

4. Using 2 BioRad minigels a total of 20 wells could be loaded per run. Thus, 20x 1 ml centrifuge vials were numbered and a total of 17 µL protein solution was added to each, comprising 4 µL Laemmli dye, 35 µg protein sample, and the difference made up to 17 µL with Tris-buffered solution (TBS), comprising 2.42 g Tris-HCl + 8.0 g NaCl dissolved in 1 L ddH_2O (pH 7.6).

5. Once all agents were added each vial was centrifuged for 1 min at 5000 rpm, vortexed, and incubated at 95 ºC for 8 min before being placed on ice and centrifuged for 3 min at 5000 rpm ensuring all liquid was collected at the bottom of each vial. Two vials of 17 µL of protein marker (purchased from Cell Signal Technology) also underwent the same process. All samples were then loaded into the wells under the 'wet condition' with the running buffer submerging the wells prior to loading.

6. Gel electrophoresis was conducted at a constant 100 V for 2 hours at room temperature until the dye reached the bottom of the plates.

A.5 Protein transfer and staining

Transfer buffer (1x):	14.4 g glycine 3.0 g Tris 200 ml 100% methanol Made up to 1L with ddH_2O
Ponceau S dye (10x):	0.2 g Ponceau S (Sigma) 3.0 g Trichloroacetic acid 3.0 g Sulfosalicylic acid Made up to 10 ml with dd H_2O
TBST (1x):	Dissolve 2.42 g Tris-HCl + 8.0 g NaCl into 800 ml ddH_2O and pH at 7.6. Make up to 1 L with ddH_2O and gradually add 1 ml Tween-20 under constant stirring.

7. The transfer 'sandwich' was prepared as detailed below:

Positive electrode (+)

**********	** Sponge
----------	--- Whatman paper
————————	— Nitrocellulose membrane
==========	== Gel membrane

**********	

Negative electrode (-)

All layers were maintained submerged in transfer buffer prior to use.

8. Protein transfer was achieved using a BioRad PowerPac-200 at a constant 80V for 1.5 hours at 4 ºC.

9. The nitrocellulose membrane was removed and placed into a bath of Ponceau S dye (1x) where the transferred protein bands appeared under gentle agitation. Ponceau S was salvaged and the membrane was then rinsed repeatedly in TBST until the run-off became clear and no dye was present on the membrane. The membranes were then incubated in 4 % skim milk in TBST for 1 hour at room temperature using a plate mixer.

A.6 Antibody incubation

10. Following the milk blocking incubation, membranes were incubated in primary antibody diluted in TBST. The primary antibodies specific for phosphorylated: ERK1/2 (p-ERK1/2), SAPK/JNK (p-JNK), p38 (p-p38), PKCα/β_{II} (p-PKCα/β_{II}), and PKCδ/θ (p-PKCδ/θ) were used (all obtained from Cell Signal Technologies Inc, MA, USA). All primary antibodies were diluted 1:1000, with the exception of β-actin which was diluted at 1:1500 Ab:TBST. The membranes were left to incubate overnight using a platform mixer at 4 ºC.

11. The next day, membranes were washed 3 times (5-10 min per wash) with TBST using a platform mixer.

12. The membranes were then incubated with secondary antibody (anti-rabbit IgG, horse radish peroxidase (HRP) –linked antibody, (Cell Signaling Technology, USA) diluted in TBST alone (1:2000). The membrane was incubated with the secondary Ab for 1-2 hours at room temperature using a platform mixer.

13. The membranes were then washed as above - 3 times in TBST for 5-10 min each wash.

A.7 Protein Detection System

Homemade detection solution was made immediately prior to use, comprising of stock chemiluminescence solution combined with H_2O_2 in a 1:1 ratio.

Chemiluminescence Solution:	100 µL 250 mM Luminol (in DMSO)
	44 µL 90 mM *p*-coumaric acid (in DMSO)
	10 ml 100 mM Tris-HCl, pH 8.5
Oxidation solution:	6.1 µL of 30 % H_2O_2
	10 ml 100 mM Tris-HCl, pH 8.5

14. The membranes were bathed in the detection solution for 5-10 min, ensuring the protein-primary antibody-secondary antibody-HRP-bound surface was facing upwards, and that the solution completely covered the membrane. The membranes were removed and placed on glass plates within a film cassette and carefully blotted dry. Plastic wrap was placed over the membranes and all bubbles trapped between the layers removed using clean tissues.

15. In a dark room under red light only, Kodak BioMax XAR film was cut to shape and placed over the plastic wrap-covered membranes, and sealed within the film cassette. The film was left for the appropriate amount of time

depending upon the primary Ab being measured, and the effectiveness of the detection solution (30 sec - 15 min).

16. Again under red light, the cassette was opened and the film moved directly into an Autotank automatic x-ray film processor (Fischer Industries Inc., Geneva, IL, USA), developing the visible bands where the HRP-linked antibodies were present.

17. The density of the protein bands were quantified using Bio-Rad Quantity One software (Bio-Rad laboratories, Hercules, USA), and reported as intensity (INT) / mm^2. β-actin was used as an internal control in all incubations to ensure gel electrophoresis and antibody incubation were successful.

APPENDIX B

THE LANGENDORFF ISOLATED, PERFUSED HEART APPARATUS AND THE MULVANY MYOGRAPH.

B.1 The Langendorff isolated rat heart system

The Langendorff isolated perfused heart technique is described in detail by Sutherland & Hearse (Sutherland and Hearse 2000; Hearse and Sutherland 2000). In order to measure the specific cardiac effects of new peptides or drugs, including their potential role in cardiovascular disease, experimental animal models are required. These models must balance their reproducibility and quality of data with its representation of the natural *in vivo* setting and clinical relevance of the model. The Langendorff isolated, perfused heart model provides a good compromise to this balance, showing good reproducibility, providing physiological, biochemical and pharmacological characteristics to be measured and recorded easily (Figures B.1 and B.2). The apparatus delivers perfusate either under 'constant flow' or 'constant pressure,' depending upon the requirements for the experiment. Constant flow delivers perfusate to the heart at a rate controlled by the researcher and this flow does not alter despite the heart undergoing hemodynamic changes. This is the method the current thesis employed (Figure B.1). Constant pressure requires a more complex system whereby perfusate flow is adjusted accordingly to maintain constant pressure within the heart. The perfusate expelled from the heart is easily collected into vials containing aprotinin and triton-X for analysis of specific factors at a later date. It must be noted that this model is constantly deteriorating as it has been removed from the body and this must be taken into consideration, especially for longer experiments. Once attached to the Langendorff apparatus, the hearts can also be manipulated to simulate cardiac dysfunction, most commonly ischemia, as it is simple to induce. Ischemia can be initiated either globally by halting perfusion through the heart (as conducted in Chapters 5 and 6 of the current thesis), or locally by surgical ligation of an artery. These techniques combined with easy infusion of any desired agents enable simple and quick measure of their role in heart disease and dysfunction.

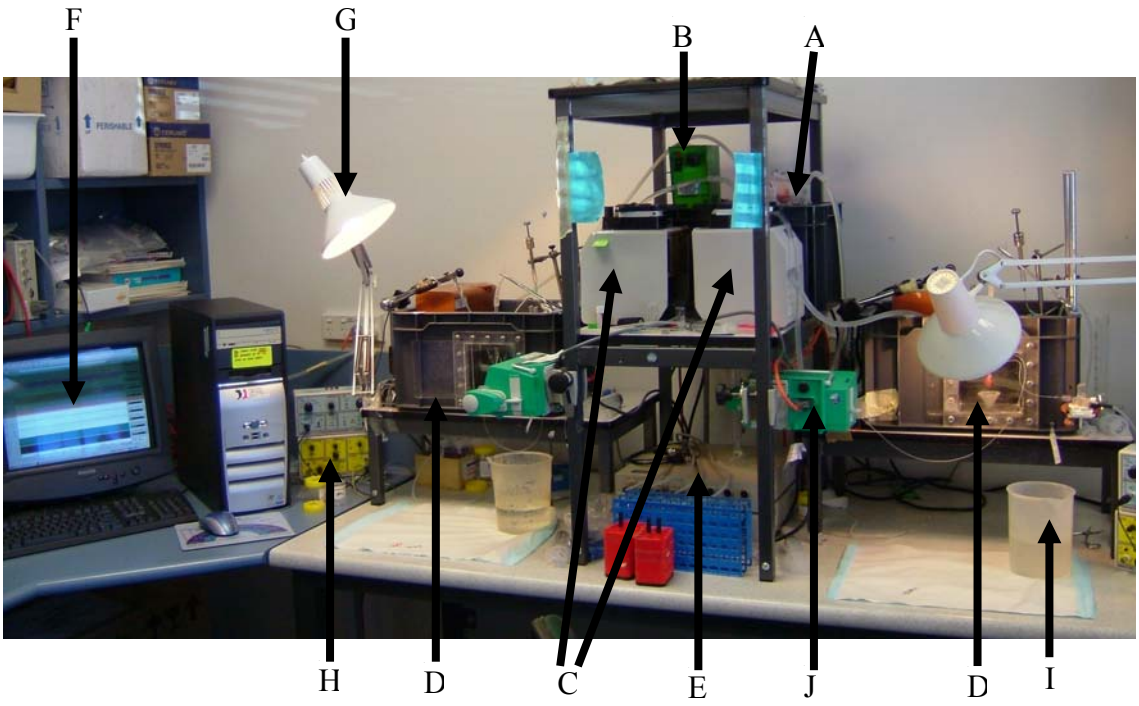


Figure B.1. Two constant flow Langendorff isolated heart systems side by side feeding from the same perfusion solution (A), maintained at 37°C by a water heater within a water bath (B). The two perfusion lines feeding from the buffer reservoir are attached to individual peristaltic pumps (C) before trailing into waterbaths containing the isolated rat hearts (D). Cardiac parameters are recorded via pressure transducers supplying data to an ADInstrumants powerlab (E) that conveys the data to Chart 5 software on a single PC in real time (F). Lamps are placed in from of the hearts to maintain them at 37°C (G) and stimulators (H) maintain a constant heart rate via pacing electrodes. The constant flow system provides a single pass of perfusing solution through the heart with the perfusate collected (I) and either discarded or held for further analysis. Syringe pumps (J) provide a method of infusing drugs or agents at a specific rate over time.

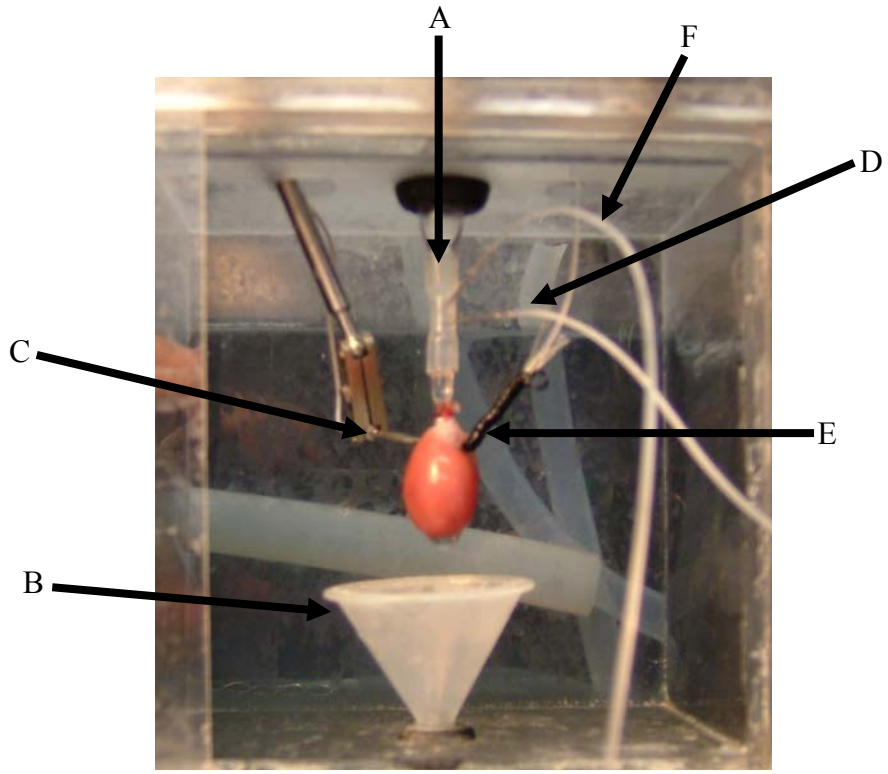


Figure B.2. The isolated, perfused rat heart. The heart is perfused with modified Krebs buffer through the perfusion line (A) in a single pass system with perfusate collected after passing through the heart (B). Heart rate is maintained at a constant rate using a pacing electrode (C), while coronary artery pressure is measured using a cannula inserted into the perfusion line (D), which is attached to a pressure transducer. Left ventricular contractility is determined through the insertion of an ethanol-filled balloon (E) attached to a pressure transducer. Infusion of agents is achieved by inserting a line directly into the perfusion line above the heart (F).

B.2 The Mulvany myograph

The Langendorff isolated, perfused heart system provided an ideal method for determining the role of UII, URP, PA12, and their respective antagonists on the rat heart as it provided a measure of both coronary artery activity and LV contractility. However, in order to provide a comparative measure of the vascular effects of UII and URP between salmon and rat hearts, myography had to be employed as attaching a salmon heart to the Langendorff system does not provide a measure of coronary artery

activity. The Mulvany myograph technique also enabled direct measurement of drug effects on specific vessels isolated from throughout the rat (Figure B.3).

The Mulvany myograph technique provides a measure of vessel activity via an increase or decrease in tension in response to infusion of vasoactive agents. Vessels are removed from the animal, stored in ice-cold buffer, before being cut transversely to 2 mm lengths when required. Two wires are threaded through the lumen of the artery before each wire is attached to opposing jaws within a bath of solution specific for the vessel type, fastening the vessel between the two jaws. This bath is maintained at the temperature required via a simple flow-through water system pumped from a separate waterbath maintained at the desired temperature. The jaws are spread apart using micromanipulators, stretching the vessel to provide some tension as it would have experienced was it still within the live animal. Vessels are then allowed at least 1 hour to settle to their resting tension. Each myograph bath can hold two vessels and a dam can be placed between the two, enabling vessels to be run in pairs providing a direct control for drug effects. A lid is placed over the bath, maintining the bath at temperature and enabling the bath to be gassed with 95% O_2 / 5% CO_2. Agents are administered directly into the bath where diffusion occurs rapidly and drug effects are recorded on Chart 4 software via an ADInstruments powerlab.

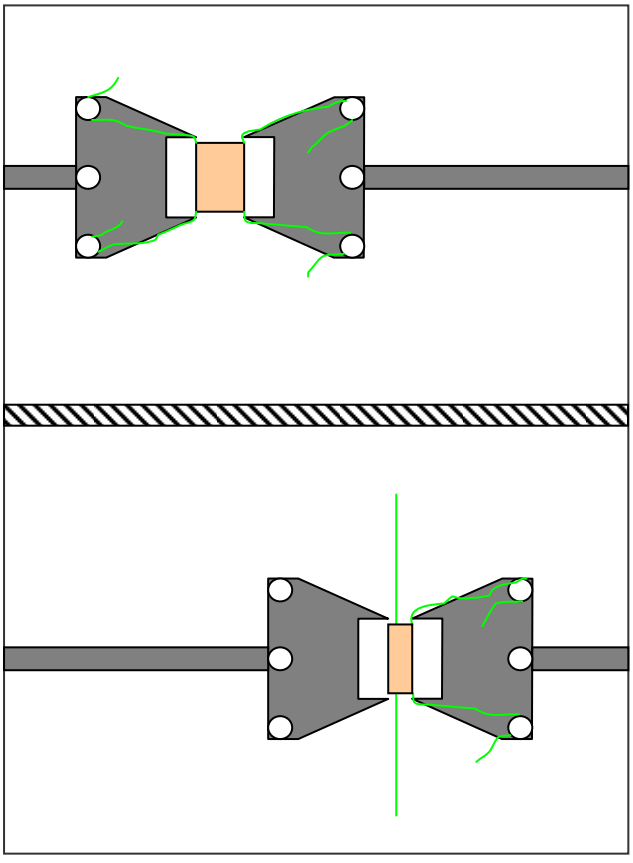


Figure B.3. Schematic representation of the two vessels held between the two sets of jaws (grey) within the bath of the myograph. The vessel (red) is held between the jaws by wires (green) fastened to screws (open circles). The two vessels can be divided by a dam (striped bar) enabling the effects of drug and control to be measured simultaneously.
The top vessel is fully attached and stretched to achieve a resting level of activity. The lower vessel has only one wire attached, with the other threaded through the lumen.

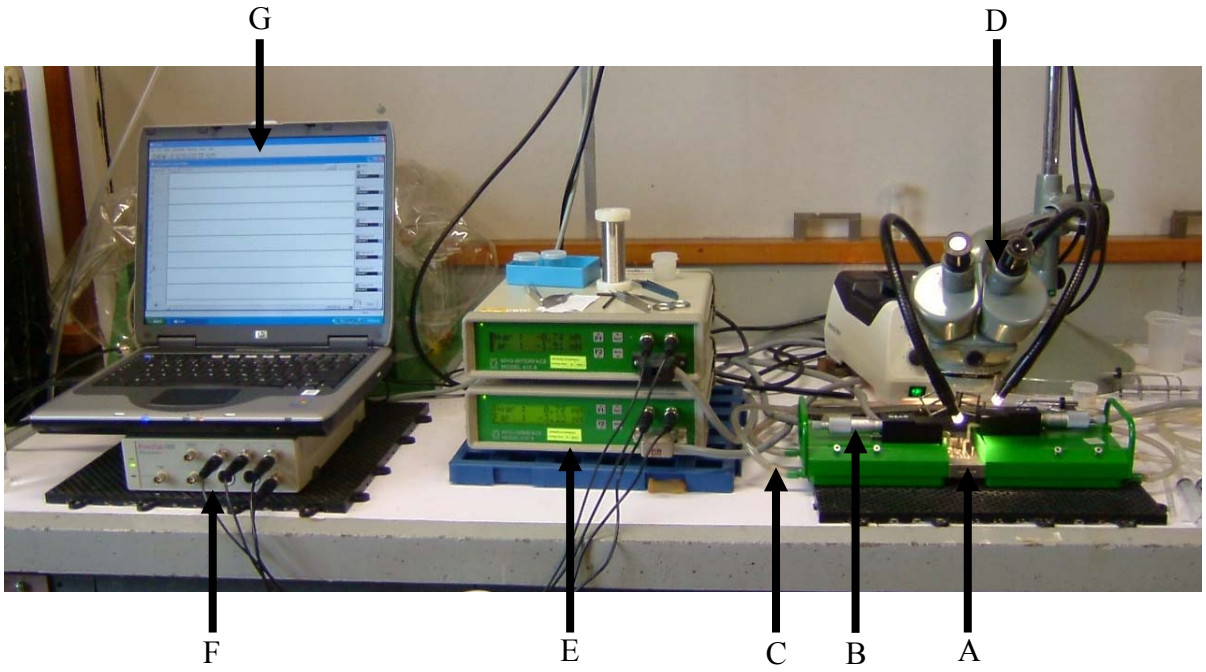


Figure B.4. The Mulvany myograph system. Vessels are held within a 10 ml bath of Krebs buffer (A) (see Figure B.3. for enlargement of the bath). Vessels are stretched or relaxed using micromanipulators (B), and the bath is maintained at the desired temperature using a flow-through recirculating water system (C) sourced from a water bath (out of view). Vessels are attached to the jaws under a dissection microscope (D) and changes in vessel tension are recorded via the myograph data interpretor (E) and amplified using a Powerlab (F) before being viewed and recorded on Chart 4 software (G).

APPENDIX C

GRAPHS OMITTED FROM PUBLISHED PAPERS

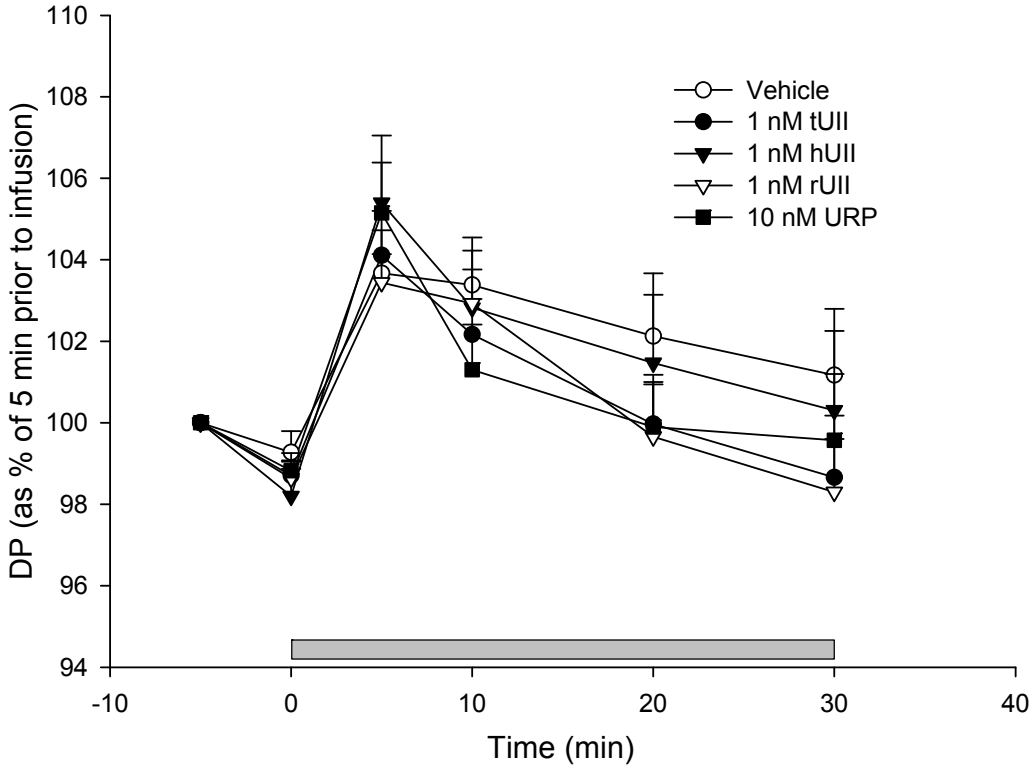


Figure C.1. The effect of URP, native (rUII) and non-native UII on left ventricular contractility (DP) in the isolated, perfused rat heart. UII or URP had no significant effect on DP compared with vehicle (P>0.05). Grey bar indicates infusion period.

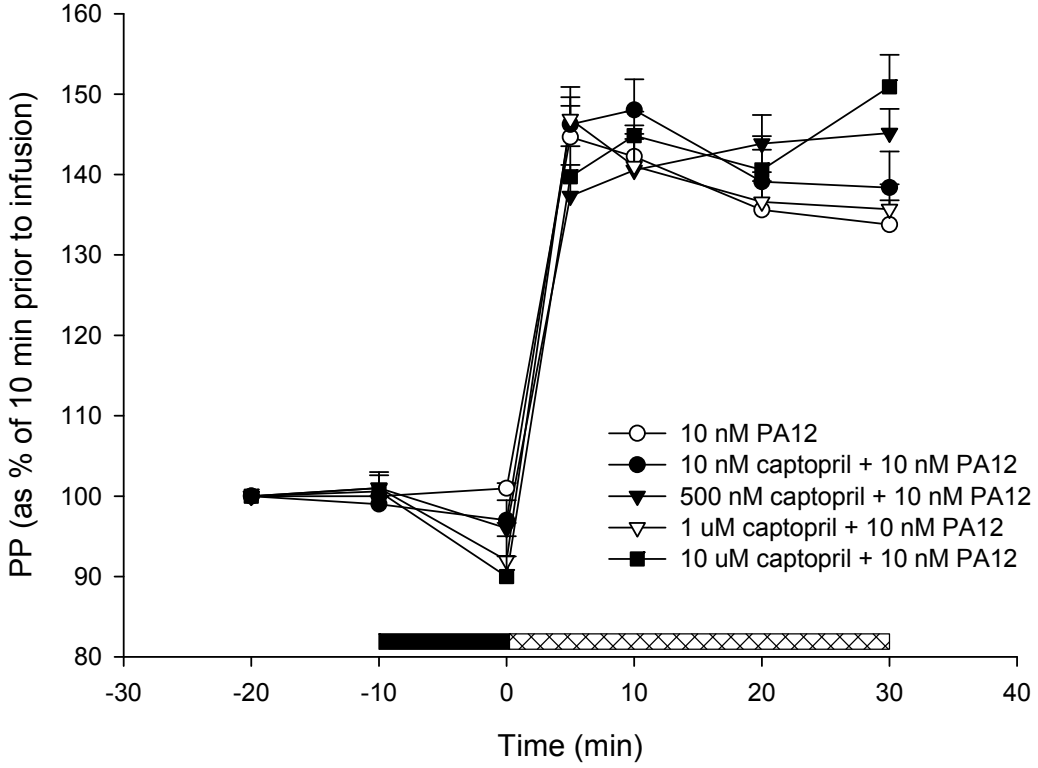


Figure C.2. Dose response of the ACE1 antagonist captopril on PA12-induced elevation in PP of the isolated, perfused rat heart. Captopril was infused alone for an initial 10 min (solid black bar) before 10 nM PA12 was co-infused for a following 30 min (thatched bar). Captopril had no significant effect on altering PA12-induced activity at any dose administered.

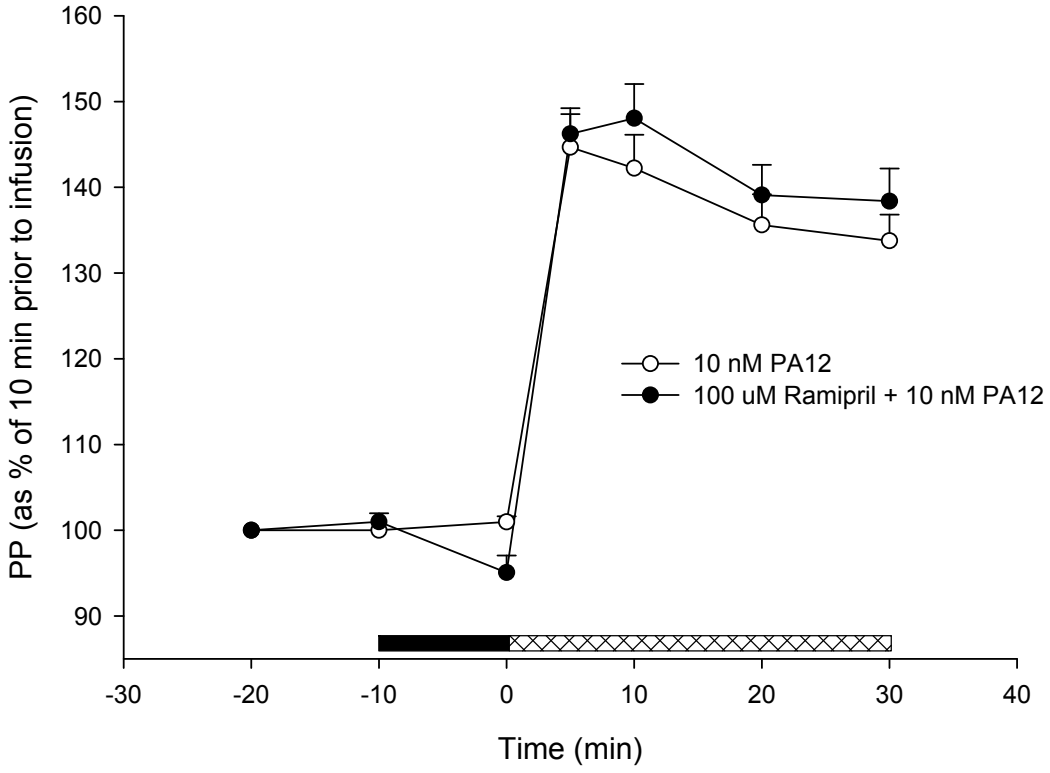


Figure C.3. The ACE1 inhibitor ramipril infused alone for 10 min (solid black bar) reduced PP but had no effect on altering PA12-induced activity on PP in the isolated, perfused rat heart when 10 nM PA12 and 10 μM Ramipril were co-infused for 30 min (thatched bar).

APPENDIX D

MEASUREMENT OF ANGII AND ANP

D.1 Angiotensin II

Perfusate samples collected after passing through the heart were analysed for immunoreactive AngII (IR-AngII). 1 ml of each perfusate sample was passed through individual Bond Elut columns containing carbon$_{18}$ cartridges. Protein bound to the columns was eluted using 100 % ethanol and collected into glass tubes containing 500 IU Aprotinin/0.1% Triton X-100. Samples were dreid down under air held in a waterbath maintained at 37°C. Dried samples were reconstituted with 2 ml AngII-specific buffer, thus diluting each sample 2 fold.
100 µL of each sample was subjected to RIA in duplicates. The AngII assay was an in-house assay with a sensitivity of 1.4 pmol/L; interassay variability of 11.5%, and an intra-assay variability of 4.8%.

D.2 Rat ANP

Perfusate samples collected after passing through the heart were analysed for immunoreactive rat ANP (IR-ANP). 10 ml of each perfusate sample was passed through individual SepPak columns containing carbon$_{18}$ cartridges. Protein bound to the columns was eluted and collected into plastic tubes containing 0.1% Triton X-100. Samples were dreid down under air held in a waterbath maintained at 37°C. Dried samples were reconstituted with 1 ml K^+-buffer, thus concentrating each sample 10 fold.
50 µL of each sample was subjected to RIA in duplicates. The rat ANP assay was an in-house assay with a sensitivity of 1.2 pmol/L; interassay variability of 6.8%, and an intra-assay variability of 3.6%.