Investigation of the Role of Angiotensin 1-9 in Cardiomyocyte Hypertrophy

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Author's Declaration

I declare that this thesis has been written entirely by myself and is a record of work performed by myself with the exception of Home Office licensed procedures (Dr. Lorraine M. Work, Dr. Laura Denby and Dr. Delyth Graham), echocardiography (Dr. Kirsten Gilday). This thesis has not been submitted previously for a higher degree. The research was carried out in the Division of Cardiovascular and Medical Sciences, University of Glasgow, under the supervision of Dr. Stuart A. Nicklin and Prof. Andrew H. Baker.

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List of Abbreviations/Definitions

3-NT	3-nitrotyrosine
AAV	Adeno associated virus
ACE 2	Angiotensin converting enzyme 2
ACE	Angiotensin converting enzyme
Ad	Adenovirus
ANF	Atrial natriuretic factor
Ang1-7	Angiotensin 1-7
Ang1-9	Angiotensin 1-9
AngI	Angiotensin I
AngII	Angiotensin II
AngIII	Angiotensin III
AngIV	Angiotensin IV
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AP-1	Activator protein 1
APES	3-aminopropyltriethoxysaline
ApoE-/-	Apolipoprotein E knockout
APS	Ammonium persulphate
ARAP1	AT1R-associated protein
AT1R	Angiotensin type 1 receptor
AT2R	Angiotensin type 2 receptor
ATBP50	AT2R interacting or binding protein
ATIP1	AT2R interacting or binding protein
ATRAP	AT1R-associated protein
AU	Arbitrary units
AWTd	Anterior wall thickness in diastole
AWTs	Anterior wall thickness in systole
BCA	Bicinchoninic acid
β-gal	β-galactosidase
β-MHC	β isoforms of myosin heavy chain
BiP	Binding immunoglobulin protein

BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
cAMP	3’-5’-cyclic adenosine monophosphate
CCD	Charged-coupled device
cDNA	Comlplementary DNA
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CHO	Chinese hamster ovary cell
CHO-K1	Chinese hamster ovary cell subclone K1
CMVIEP	Cytomegalovirus immediate early promoter
CNP	C-type natriuretic peptide
CNS	Central nervous system
CO	Cardiac output
CT-1	Cardiotropin 1
CVD	Cardiovascular disease
DAG	*sn*-1,2 diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
DOCA	Deoxycorticosterone acetate
dsDNA	Double stranded DNA
ECHO	Echocardiography
ECL	Enhanced Chemiluminescence
EDD	End diastolic diameter
EDTA	Ethylenediaminetetraacetic acid
EDV	End diastolic volume
EF	Ejection fraction
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
Elk-1	Ets-like gene-1
eNOS	Endotelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ESV	End systolic volume

FAK	Focal adhesion kinases
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FS	Fractional shortening
GABA	Gamma-aminobutyric acid
GATA-4	Transcription factors GATA binding protein 4
GDP	Guanosine diphosphate
gp130	Glycoprotein 130
gp-39	Glycoprotein-39 precursor
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
H9c2	Rat neonatal cardiomyocyte cell line
HeLa	Human epithelial cervical cancer cell line
HF	Heart failure
HR	Heart rate
HRP	Horseradish peroxidase
Hsp90	Heat shock protein 90
ICAM-1	Intracellular adhesion molecule 1
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGF-IR	Insulin-like growth factor receptor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP_3	Inositol-1,4,5-trisphosphate
IRAP	Insulin-regulated aminopeptidase
ISWT	Change in interventricular septal wall thickness
JAK	Janus kinase
JAM-1	Junctional adhesion molecule-1
JNK	c-Jun N-terminal kinases
junB	Transcription factor jun-B
LB	Luria-Bertani
L-NAME	N-(G)-nitro-L-arginine methyl ester
LVAWd	Left ventricular anterior wall in diastole

LVEDD	Left ventricular end diastolic diameter
LVEDV	Left ventricular end diastolic volume
LVESD	Left ventricular end systolic diameter
LVESV	Left ventricular end systolic volume
LVIDd	Left ventricular internal diameter in diastole
LVMI	Left ventricular mass index
LVPWd	Left ventricular posterior wall in diastole
MAP	Mitogen-activated protein
$Mas^{-/-}$	Mas knockout
MCP-1	Monocyte chemoattractant protein
ME	Mean
MEF-2	Myocyte enhancer factor 2C
MEK	MAP/ERK kinase
MEKK	MAP/ERK kinase kinase
MMP	Matrix metalloproteinases
MMS2	Ubiquitin-conjugating enzyme like protein MMS2
mRNA	Messenger ribonucleic acid
MTSG	Mitochondrial tumor suppressor gene
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NFAT	Nuclear factor of activated T-cells
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NP-40	Nonyl phenoxylpolyethoxylethanol
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NTS	Nucleus tractus solitarii
OCT	Optimal cutting temperature compound
oxLDL	Oxidative low density lipoprotein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDGFR	Platelet-derived growth factor receptor
PE	Phenylephrine
PFA	Paraformaldehyde
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3 kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A_2
PLB	Phospholamban
PLC	Phospholipase C
PLZF	Promyelocytic zinc finger protein
PTX	Pertussis toxin
PWTd	Posterior wall thickness in diastole
qPCR	Quantitative real-time polymerase chain reaction
Rac-1	Ras-related C3 botulinum toxin substrate 1
RACK	Receptor for Activated C-Kinase
RAd	Recombinat adenovirus
RAS	Renin-angiotensin system
RhoA	Ras homolog gene A
ROS	Reactive oxygen species
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulphate
SE	Standard error
SERCA2a	Sarcoplasmic reticulum Ca(2+) ATPase
SHP-1	Src homology 2 domain containing phosphatase 1
SHR	Spontaneously hypertensive rat
SHRSP	Stroke prone spontaneously hypertensive rat
SLK	Serine/threonine kinase Ste20-related kinase
SRP	Signal recognition particle
ssDNA	Single stranded DNA
STAT	Signal transducer and activator of transcription
TBE	Tris/Borate/EDTA
TBS-T	Tris buffered saline-tween
TE	Tris-EDTA

TEMED	Tetramethylethylenediamine
TGFβ1	Transforming growth factor beta 1
TIMP-2	Tissue inhibitors of metalloproteinases-2
TNFalpha	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
TRPC	Transient receptor potential channel
VCAM-1	Vascular cell adhesion molecule 1
WKY	Wistar Kyoto rat

Summary

The renin angiotensin system (RAS) is an important regulator of blood pressure, volume and electrolytes. Angiotensin II (AngII) is the main modulator of RAS effects, in tissues such as heart, blood vessels, brain, kidney, adrenal cortex and adipose tissue, via the angiotensin type 1 receptor (AT1R) or the angiotensin type 2 receptor (AT2R). The characteristic actions of AngII are mediated by the AT1R and over-activity of AngII and the AT1R play a fundamental role in the pathophysiology of hypertension and heart failure. The AT2R is expressed in embryonic tissue and in disease states and may antagonize AT1R-mediated pathophysiological signalling, however this is still controversial. The recent discovery of a novel RAS enzyme angiotensin converting enzyme 2 (ACE 2) expressed in brain, testis, kidney and cardiomyocytes, changed the classical view of the RAS. ACE 2 cleaves AngI and AngII to form the novel peptides Ang1-9 and Ang1-7, respectively. It has been demonstrated that by activating the Mas receptor, Ang1-7 antagonizes many of the pathophysiological actions of AngII. Ang1-9 has been described as an inhibitor of ACE, and as a substrate to form Ang1-7. The work in this thesis assessed the effects of the novel RAS peptides Ang1-7 and Ang1-9 in cardiac hypertrophy *in vitro* and *in vivo* in the stroke prone spontaneously hypertensive rat (SHRSP), an experimental model of hypertension. Further work generated gene transfer vectors for over-expression of Ang1-7 and Ang1-9 to facilitate delivery of these peptides to specific cell types.

First, rat H9c2 cardiomyocytes were stimulated with AngII and cardiomyocyte hypertrophy was confirmed by increased cell size, elevated gene expression of the hypertrophy marker brain natriuretic peptide (BNP) and reorganization of α-actin filaments following exposure to AngII. Addition of Ang1-7 or Ang1-9 to AngII-stimulated H9c2 cardiomyocytes blocked the increase in cell size and in BNP gene expression as well as α-actin filament reorganization. Similar effects were observed in adult rabbit primary left ventricle cardiomyocytes, where AngII induced a significant increase in cell width which led to increased cell volume, an effect blocked by both Ang1-7 and Ang1-9. Inhibition of ACE with captopril did not affect the antihypertrophic effect of Ang1-9, supporting the hypothesis that conversion to

Ang1-7 was not necessary for the antihypertrophic effects of Ang1-9. Next, the receptor signalling mechanisms for Ang1-7 and Ang1-9 were studied by focusing on Mas, the AT1R and the AT2R. Addition of the Mas antagonist A779 to H9c2 and rabbit primary cardiomyocytes blocked the antihypertrophic effect of Ang1-7 without affecting Ang1-9, demonstrating an independent role for Ang1-9. To investigate the role of the AT1R we used arg-vasopressin to induce hypertrophy and blocked the AT1R with losartan. Addition of losartan had no effect on the antihypertrophic effect of Ang1-7 or Ang1-9, indicating that neither of these peptides signalled through the AT1R and that both peptides were able to block hypertrophy induced by different stimuli. However, addition of PD123,319, an AT2R antagonist, blocked the antihypertrophic effect of Ang1-9, while Ang1-7 was unaffected, indicating that Ang1-9 may signal through the AT2R.

In vivo delivery of Ang1-9 to the SHRSP was achieved by the use of osmotic minipumps. Blood pressure was monitored via radiotelemetry from 11 weeks of age until the end of the experiment. During the study three echocardiograms were performed (at 11 weeks, 15 weeks and 17 weeks of age). After sacrifice of the rats, aorta and heart were collected for further study. After 4 weeks of Ang1-9 delivery in SHRSP no significant difference in blood pressure was observed between groups. Echocardiography showed no difference in cardiac hypertrophy between control and Ang1-9 infused groups. However, co-infusion of Ang1-9 with PD123,319 produced a significant increase in left ventricular mass index. There was a significant increase in cardiac output in SHRSP infused with Ang1-9 compared to control, an effect that was abolished by co-infusion with the AT2R antagonist. Furthermore, histological assessment of the heart showed reduced perivascular fibrosis in Ang1-9-infused rats when compared to controls. Interestingly, co-infusion of PD123,319 increased interstitial fibrosis, suggesting that the AT2R might have an intrinsic basal effect in the SHRSP. In addition, pressure myography showed a significant increase in basal nitric oxide bioavailability in the aorta of Ang1-9-infused rats when compared with controls, an effect that was abolished by the co-infusion of PD123,319, suggesting Ang1-9 effects were mediated by the AT2R.

The development of gene transfer approaches has facilitated research in cardiovascular disease. Thus, in order to develop tools to study the effects of Ang1-7

and Ang1-9 in specific cells, adenovirus vectors were generated which utilized a fusion protein expression cassette. Expression of the fusion protein was confirmed by western immunoblotting. Over-expression of Ang1-7 or Ang1-9 via the recombinant adenovirus was able to block AngII-induced hypertrophy. To confirm this effect was a result of peptide secretion from transduced cells a conditioned media assay was performed in which HeLa cells were transduced with RAdAng1-7 or RAdAng1-9 and 48 hours later conditioned media was transferred to AngII-stimulated H9c2 cells. Conditioned media from RAdAng1-7 or RAdAng1-9 transduced HeLa cells inhibited AngII-induced hypertrophy.

In summary, Ang1-9 and Ang1-7 inhibit cardiomyocyte hypertrophy *in vitro* induced by AngII or arg-vasopressin. Importantly we ascribe a direct biological role for Ang1-9 as a RAS hormone, signalling via the AT2R *in vitro* and *in vivo*. Furthermore, Ang1-9 increased cardiac output, improved endothelial function and reduced cardiac fibrosis in an experimental model of hypertension supporting direct biological effects of Ang1-9. These results have implications for our understanding of RAS function and identification of new therapeutic targets in cardiovascular disease.

C H A P T E R 1

Introduction

1.1 The renin-angiotensin system

The renin-angiotensin system (RAS) is one of the most important processes by which the body regulates blood pressure, volume and electrolytes (Keidar *et al.*, 2007). The relationship between hypertension and renal disease has long been known and in 1898 Tigerstedt and Bergman discovered renin and described it as a pressor compound (Tigerstedt, 1898). Many efforts were made to establish the pathway between the kidney and the development of hypertension, mainly through attempting to identify direct effects on arterial pressure through kidney function. By the end of the 1930's the presence of a pressor agent in the venous blood of the ischemic kidney was described (Fasciolo *et al.*, 1938). Two studies confirmed these findings (Goldblatt, 1937, Harrison, 1937), and a pressor protein (originally called hypertensin) was then isolated from the blood (Braun-Menéndez, 1939) and described as a potent short duration pressor protein, completely different from renin. To further investigate the relationship between renin and this newly discovered hypertension protein, semi-purified kidney extract was incubated with plasma and a similar compound to hypertensin was generated and isolated (Braun-Menéndez, 1939). The description of renin as a protease led to the search for its substrate which was found to be formed in the liver and was subsequently named angiotensinogen (Leloir, 1942). Finally, the octapeptide pressor protein angiotensin II (AngII) was described for the first time (Bumpus *et al.*, 1957) and this led to major studies which have contributed to our in depth knowledge of the RAS.

1.2 The classical RAS

The renin-angiotensin cascade system initiates via the release of renin from the juxtaglomerular complex in the kidney (Figure 1-1), a segment of the distal tubule that passes through the angle between the afferent and efferent arteriole. The epithelial cells of the distal tubule proximal to arterioles termed the macula densa detect changes in sodium chloride and promote the release of renin from the granular cells. Alternatively, renin is also released when hypoxia or a decrease in arterial blood pressure is sensed or by the action of the sympathetic

nervous system (β_1 adrenergic receptor stimulation) (Guyton, 2007, Rhoedes and Tanner, 1997). Once secreted, renin cleaves the 12 amino acid peptide angiotensinogen. Angiotensinogen is a glycoprotein constitutively produced mainly in the liver and cannot be stored (Deschepper, 1994). The cleavage of angiotensinogen between the leucine and valine amino acids (11^{th} and 12^{th} amino acids) forms the decapeptide angiotensin I (AngI). AngI is then converted into AngII through the cleavage of the N-terminal amino acids via angiotensin converting enzyme (ACE), first discovered in the 1950's (Skeggs *et al.*, 1956). ACE is an ecto-peptidase attached to the luminal side of the endothelial membrane which as well as forming AngII also inactivates bradykinin (Erdos et al., 1999, Yang et al., 1970) The ACE gene is located on chromosome 17 and encodes for two different isoforms, the somatic and the germinal form (Soubrier *et al.*, 1988, Rigat *et al.*, 1992). The somatic form is expressed in endothelium, mainly in the lung but also in kidney, intestine, placenta and choroid plexus; while the germinal form is expressed mainly in testes. Both ACE isoforms are bound to the membrane and hydrolyze circulating peptides, such as AngI (Turner and Hooper, 2002, Guyton, 2007, (Erdos and Skidgel, 1987) (Figure 1-2).

1.2.1 Angiotensin II

AngII is the main active peptide of the RAS with a half life of 30 seconds in circulation and 15 to 30 minutes in tissue. Normal concentration in arterial blood of AngII has been described as 4.7 ± 1.2 pg/mL (Vilas-Boas et al., 2009). Once formed AngII acts in heart, blood vessels, brain, kidney, adrenal cortex and adipose tissue. There are two receptors known to mediate most of the effects of AngII. The angiotensin type 1 receptor (AT1R) and angiotensin type 2 receptor (AT2R) are seven transmembrane G-protein coupled receptors (GPCR) which interact with several G-proteins to activate cell signalling pathways causing vasoconstriction, central nervous system stimulation, cell proliferation, hypertrophy, inflammation, extracellular matrix remodelling and aldosterone release (Mehta and Griendling, 2007).

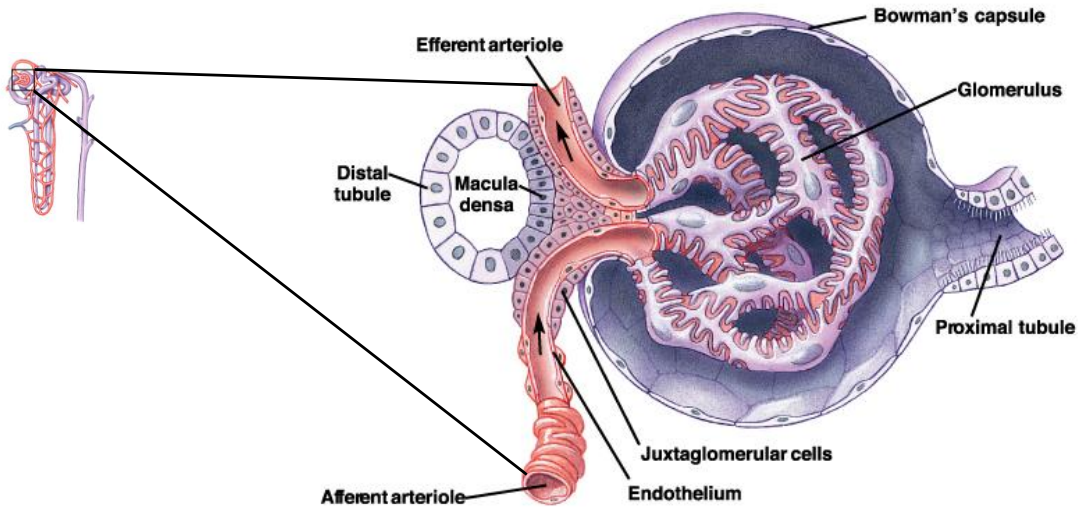


Figure 1-1. The juxtaglomerular complex of the kidney.

The juxtaglomerular complex is situated outside the nephron's glomerulus. It is composed of a group of specialized cells. The macula densa, which is a specialized epithelium sited in major density in the distal tubule of the nephron, is in close contact with the granular cells or juxtaglomerular cells (smooth muscle cells from the afferent and efferent arterioles before entering Bowman's capsule) (Diagram taken from (Lopez-Franco, 2004)).

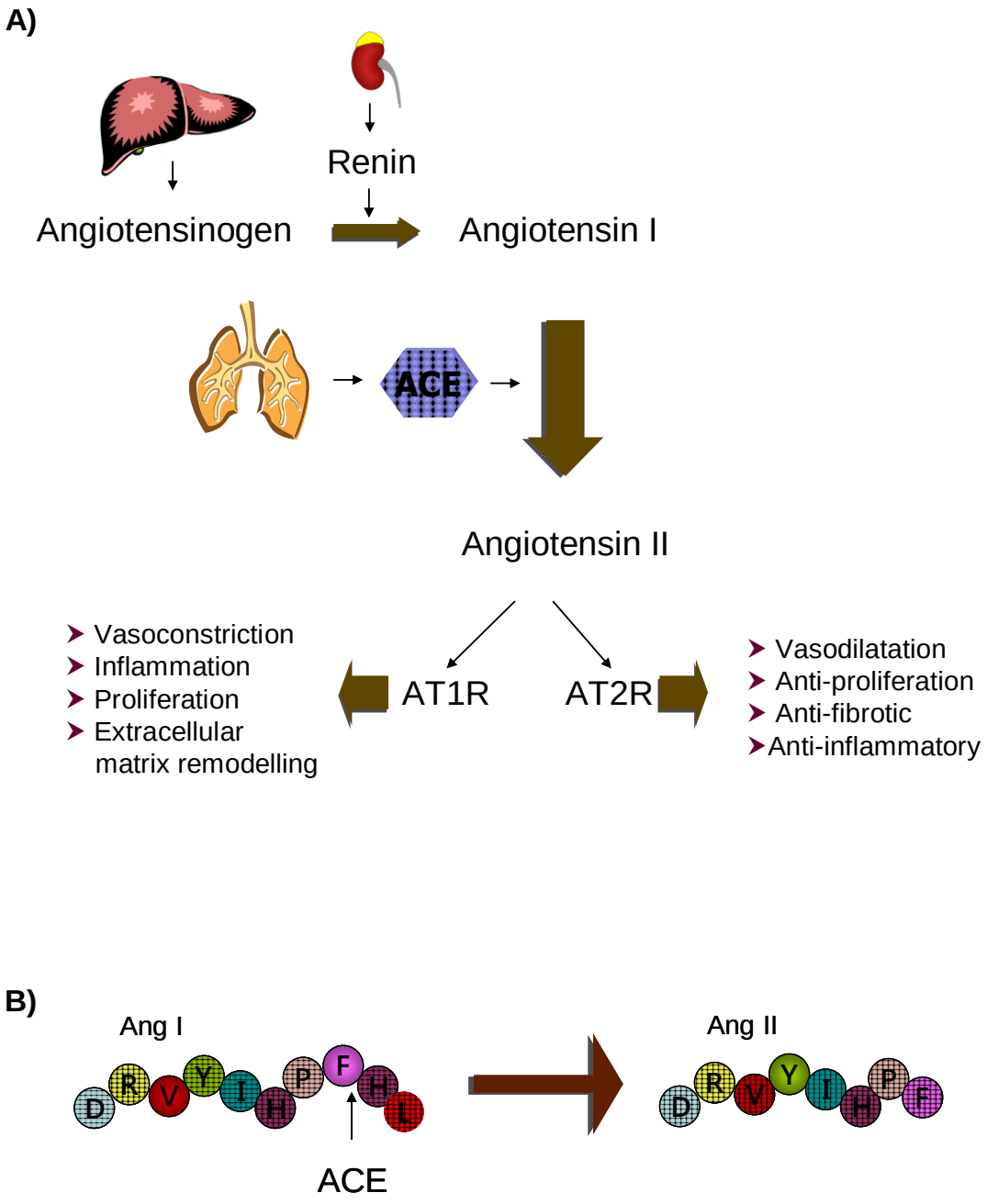


Figure 1-2. The classical renin-angiotensin system.

(A) Angiotensinogen (a 12 amino acid peptide) is released from the liver and cleaved by renin (released from the glanular cells of the juxtaglomerular complex) forming AngI. AngI is cleaved by ACE expressed on endothelial cells in the lung and is transformed into AngII. AngII is the main active peptide of the RAS and acts through binding to the AT1R or the AT2R. The AT1R is responsible for the vasoconstriction, pro-proliferation, pro-inflammation and extracellular matrix remodelling actions of AngII, while AT2R seems to mediate opposite actions to the AT1R. (B) Schematic of amino acids sequence of AngI and AngII. ACE cleaves the last two amino acids of AngI to transform it to AngII.

1.2.1.1 The angiotensin type 1 receptor

The AT1R is a 40 kDa receptor that is distributed in most organs. It regulates the physiological and pathological actions of AngII and is responsible for the characteristic actions of AngII. Once AngII bind to the transmembrane domain of the AT1R, the receptor undergoes rapid desensitization and endocytosis (Griendling *et al.*, 1987). Desensitization of the AT1R involves phosphorylation of the receptor by G-protein receptor kinases (Mehta and Griendling, 2007). Once desensitized, internalization of the AT1R occurs via clathrin-coated vesicles mediated by β-arrestin (Gaborik *et al.*, 2001). β-arrestins promotes activation of mitogen-activated protein kinases (MEK) and c-Jun terminal kinases (Kim *et al.*, 1997).

There is also evidence that the AT1R is internalized by caveolae (non-clathrin coated vesicles) (Ishizaka *et al.*, 1998). These vesicles are associated with caveolin and are colocalized with signalling molecules such as Src (Rothberg *et al.*, 1992). Caveolae are abundant in smooth muscle cells, fibroblasts and endothelial cells (Rothberg et al., 1992, Zuo et al., 2005, Peters et al., 1985). In smooth muscle cells caveolin-1 seems to be important for the activation of Ras-related C3 botulinum toxin substrate 1 (Rac-1) (Zuo *et al.*, 2005). Approximately 25% of these internalized receptors are recycled to the cell membrane for reactivation via agonist binding (Griendling *et al.*, 1987). To regulate AT1R internalization studies have described the presence of novel accessory proteins that bind to GPCR. Tsurumi *et al.* (2006) demonstrated that overexpression of the AT1R-associated protein (ATRAP) reduced expression of the AT1R in mouse cardiomyocytes by inhibiting cell surface expression (Tsurumi *et al.*, 2006) while Guo *et al.* (2001) showed that ARAP1 (AT1R-associated protein 1) promoted recycling of the AT1R to the cell membrane following internalization (Guo *et al.*, 2001).

In recent years cell signalling through dimerization between membrane receptors has been identified (Lyngso *et al.*, 2009). Homodimers of AT1R linked by the intracellular factor XIIIA transglutaminase have been reported (AbdAlla *et al.*, 2004). Homodimerization of the AT1R with the B2 bradykinin receptor and the AT2R and Mas receptor have also been shown to influence receptor activation (Canals et al., 2006, Castro et al., 2006, AbdAlla et al., 2005, AbdAlla et al., 2000,

Kostenis et al., 2005) and signalling responses. These influences depend upon each individual receptor subunit, e.g. AT1R heterodimerization with the B2 bradykinin receptor enhances AngII signalling in rat renal mesangial cells, while AT1R and Mas or AT2R homodimerization decreases AngII signalling (AbdAlla *et al.*, 2005, Castro *et al.*, 2005). Furthermore, AngII also acts by transactivating other receptors such as the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) or insulin-like growth factor receptor (IGF-IR) (Saito and Berk, 2001). Transactivation of the EGFR by AngII leads to activation of the Ras-extracellular signal-regulated kinase (ERK) pathway (Yang et al., 2005, Ohtsu et al., 2008, Lautrette et al., 2005), stimulates Akt and p38 and results in an increase in *c-fos* expression (Suzuki *et al.*, 2005) contributing to cell growth. PDGFR transactivation by AngII induces activation of phospholipase C (PLC), protein kinase C (PKC) and ERK in cardiomyocytes and smooth muscle cells (Kuma *et al.*, 2007, Wang *et al.*, 2008). IGF-IR transactivation by AT1R activates Src and inositol triphosphate (IP_3) kinases (Zahradka *et al.*, 2004). *In vivo* experiments, however, show less clear effects of AngII transactivation of the IGF-IR as opposite effects of IGF-IR transactivation on vascular tone have been observed (Nguyen and White, 2005, Cao *et al.*, 2006).

1.2.1.2 The angiotensin type 2 receptor

The AT2R is a 41 kDa 7 transmembrane receptor consisting of 363 amino acids (Mukoyama *et al.*, 1993) and is located on the X chromosome (Mukoyama *et al.*, 1993)(Koike *et al.*, 1995). Although AngII binds with a similar affinity to both the AT1R and the AT2R, nucleotide homology between them is only approximately 32% (Mukoyama *et al.*, 1993). Both receptors are ubiquitously abundant but distributed differently. The AT2R is more highly expressed in foetal tissues than the AT1R (Shanmugam *et al.*, 1996)(Grady *et al.*, 1991). Furthermore, expression of the AT2R decreases rapidly after birth, resulting in the AT1R having a higher density in tissues in adults (Shanmugam *et al.*, 1996). Furthermore, the AT2R is induced in pathological conditions (Akishita, 1997, Nio et al., 1995), for example in humans it has been shown that AT2R is 3.5-fold upregulated in dilated cardiac myocytes in cardiomyopathy (Porrello et al., 2009b, Kim and Iwao, 2000, Mehta and Griendling, 2007).

The AT2R is a 7 transmembrane GPCR, but unlike the AT1R, the AT2R has some unusual characteristics. For example, it is not internalized once bound to its ligand and it can demonstrate constitutive activation, as well as ligand-independent activation (Schluter and Wenzel, 2008, Funke-Kaiser *et al.*, 2009) (Miura and Karnik, 2000). These non-classical characteristics support the concept that the AT2R can also act as a non-GPCR and has meant that the cellular pathways activated by the AT2R have been difficult to elucidate.

There is evidence that the AT2R couples to differential signalling networks. For example, the AT2R couples to the $G_{\alpha s}$-subunit in COS-7 cells (Feng *et al.*, 2002), which reinforces the concept of AT2R being a non-classical GPCR through not coupling to the classical $G_{\alpha q}$-subunits. A second interaction described is with the Src homology 2 domain containing phosphatase 1 (SHP-1). SHP-1 is a tyrosine phosphatase described to have a protective role in endothelial oxidative stress by inhibiting nicotinamide adenine dinucleotide phosphate (NAD(P)H) through blocking the regulatory subunit p38 of phosphoinositide 3 kinase (PI3K) and Rac1(Krotz *et al.*, 2005). More recently some studies have shown an anti-apoptotic effect mediated by SHP-1 in vascular, mast and PC12W cells (Geraldes et al., 2009, Inoue et al., 2009, Cui et al., 2002). In accordance with this, antagonism of SHP-1 with small interfering RNA targeting SHP-1, in an acute myocardial ischemia model in mice reduced infarct size and promoted angiogenesis (Sugano *et al.*, 2005). AT2R interaction with SHP-1 occurs via activation of the $G_{\alpha s}$-subunit (Cui *et al.*, 2001, Zhang *et al.*, 2008). Through AT2R stimulation, SHP-1 inhibits mitogen-activated protein (MAP) kinase activity in smooth muscle cells increasing apoptosis and proliferation. The AT2R has also been described to interact with the ubiquitin-conjugating enzyme like protein MMS2 in neuronal cells (Mogi *et al.*, 2007), a protein involved in the deoxyribonucleic acid (DNA) repair system (Broomfield *et al.*, 1998). AT2R association with MMS2 has been demonstrated in neurones, where activation of the AT2R upregulates MMS2 expression leading to neuronal differentiation (Li *et al.*, 2007).

One class of proteins described to directly interact with the AT2R are the accessory proteins which bind to the carboxyl terminus of the AT2R and modulate receptor trafficking and signalling (Bockaert *et al.*, 2004). The interaction of the AT2R with

these accessory proteins could explain the differential functions of the receptor. The AT2R interacting or binding protein (ATIP1) (Nouet *et al.*, 2004), also called AT2R binding protein (ATBP50) (Wruck *et al.*, 2005) or mitochondrial tumour suppressor gene (MTSG) (Seibold *et al.*, 2003) has been shown to mediate trans-inactivation of tyrosine kinases when associated with the AT2R (Mogi *et al.*, 2007). Overexpression of the AT2R has also been shown to inhibit ERK2 through interaction with the ATIP1 in CHO-AT2R transfected cells (Nouet *et al.*, 2004). Also, AT2R stimulation translocates ATIP1 protein to the nucleus promoting neuronal differentiation (Funke-Kaiser et al., 2009, Mogi et al., 2007). In two studies stimulation of the AT2R promotes the association and nuclear translocation of ATIP with SHP-1, leading to neuronal differentiation (Mogi *et al.*, 2007). Promyelocytic zinc finger protein (PLZF) is a transcription factor expressed robustly in the heart and it has been shown that stimulating the AT2R with AngII promotes PLZF interaction with the AT2R in eukaryotic transfected cells (Senbonmatsu *et al.*, 2003). Once bound, the AT2R/PLZF complex was internalized and translocated to the nucleus where PLZF crossed the nuclear membrane and increased the expression of the p85α-PI3K gene. Furthermore, upregulation of the p70(S6) kinase, whose role is important in protein synthesis, was also observed (Senbonmatsu *et al.*, 2003).

Like the AT1R, AT2R homodimers have also been reported (Miura et al., 2005, AbdAlla et al., 2001). Promotion of apoptosis in murine fibroblasts in the absence of AngII stimulation has been reported following homodimerization of the AT2R (Miura *et al.*, 2005) while heterodimerization of the AT2R and the AT1R leads to inhibition of AT1R activation (AbdAlla *et al.*, 2001). The bradykinin B_2 receptor has been also reported to associate with the AT2R, leading to inhibition of fibrosis and promotion of vasodilatation through B_2 receptor-mediated activation of endothelial nitric oxide synthase (eNOS) (Kurisu et al., 2003, Yayama and Okamoto, 2008, Munk et al., 2007, Abadir et al., 2006). A recently observed characteristic of the AT2R is that, unlike the AT1R, activation can be ligand-independent (Miura *et al.*, 2005). Several studies have shown that overexpression of AT2R in different cell types (e.g. neonatal cardiomyocytes, endothelial cells, and COS-1 cells) can induce apoptosis, growth and regulation of gene expression in the absence of ligand-mediated activation (Porrello et al., 2009b, Mogi et al., 2007).

1.2.2 The cellular actions of AngII

Most of AngII's cellular responses are mediated by the AT1R, due predominantly to activation of the $G_{\alpha q/11}$ protein subunit (de Gasparo *et al.*, 1995, Wang *et al.*, 1995). AngII, through $G_{\alpha q/11,}$ activates PLC which in turn forms IP_3 and diacylglycerol (DAG) (Bai *et al.*, 2004). These second messengers have direct effects on Ca^{2+} handling in many cell types (Heineke and Molkentin, 2006, Liu *et al.*, 2008). AngII-mediated induction of IP_3 and DAG has also been shown to activate PKC (Chintalgattu and Katwa, 2009, Qin et al., 2001, Liao et al., 1997). The effects of AngII on Ca^{2+} handling and PKC-ζ activation have been reported to result in contraction in vascular smooth muscle cells or hypertrophy in cardiac myocytes (Liao *et al.*, 1997, Liu *et al.*, 2008). AngII also activates the Ras/Raf/MEK/ERK pathway in cardiac myocytes, vascular smooth muscle cells and fibroblasts via the AT1R, promoting hypertrophy, proliferation, cell migration and fibrosis (Aplin et al., 2007, Liao et al., 1997, Min et al., 2005, Olson et al., 2008).

Beside the ERK pathway, AngII also promotes the phosphorylation of stress-kinases like c-Jun kinases (JNK) and p38 in monocytes, macrophages and smooth muscle cells (Kim and Iwao, 2000), promoting expression of atrial natriuretic peptide, skeletal α-actin and transforming growth factor beta 1 (TGF-β1) and triggering inflammatory responses (Force *et al.*, 1996). AT1R activation also leads to stimulation of cytosolic phospholipase A_2 ($cPLA_2$) and formation of arachidonic acid resulting in oxidation of NAD(P)H and vasoconstriction in vascular smooth muscle cells (Rao *et al.*, 1994). Phospholipase D is also activated by AngII in vascular smooth muscle cells, generating choline and phosphatidylic acid. Phosphatidylic acid transforms into DAG which activates PKC which in turn phosphorylates several proteins associated with growth and contraction (Kim and Iwao, 2000)(Touyz and Schiffrin, 2000).

The Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway play important roles in the signalling of several cellular functions, for example, inhibiting proliferation, growth, haematopoiesis, and immune response (Berk and Corson, 1997, Hefti *et al.*, 1997). Once JAK is activated, it induces dimerization of STAT proteins, and STAT then translocate to the nucleus to mediate

transcription of the early growth response genes (*c-fos* and *c-myc*) (Aaronson and Horvath, 2002). AngII induces activation of JAK through the AT1R in astrocytes supporting the concept that AngII mediates cell growth, migration and remodelling (Kandalam and Clark, 2009, Mehta and Griendling, 2007).

AngII can lead to phosphorylation of several intracellular proteins by activating the c-Src tyrosine kinase in smooth muscle cells (Taniyama *et al.*, 2003). This activation can also be mediated by the AT1R when coupled to the β G-protein subunit, resulting in activation of several pathways such as Ras, JAK/STAT, focal adhesion kinases (FAK) and PLC (Mehta and Griendling, 2007, Kodama et al., 1998). By regulating FAK activation AngII promotes cell adhesion to extracellular matrix and activation of cytoskeletal proteins to allow remodelling of cell shape and movement in cardiomyocytes (Mehta and Griendling, 2007, Kodama et al., 1998).

Reactive oxygen species (ROS) are molecules produced as intermediates in the process of oxygen transformation to water (Harman, 1956). Physiologically these molecules act as important second messengers in cell signalling as they diffuse between membranes and act on downstream molecules to produce transient effects (Cakir and Ballinger, 2005, Sedeek *et al.*, 2009). AngII stimulates ROS production by activating NAD(P)H oxidase, an enzyme involved in the production of ROS in many cell types, including vascular smooth muscle cells, endothelial cells and fibroblasts (Griendling et al., 1994, Pagano et al., 1998, Rajagopalan et al., 1996). The major targets of ROS are ion channels, matrix metalloproteinases, MAP kinases, tyrosine kinases and transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) leading to inflammatory, anti-oxidant and growth responses (Mehta and Griendling, 2007, Choudhary et al., 2008, Touyz, 2004).

AngII can also regulates extracellular matrix remodelling by increasing synthesis of collagen and fibronectin, promoting the accumulation of proteoglycans and increasing expression of TGF-β via the AT1R in cardiac fibroblast (Ju and Dixon, 1996, Huang et al., 2008, Tiede et al., 2003, Brilla et al., 1993, Schroder et al., 2006).

1.3 The counter-regulatory RAS

The counter-regulatory RAS has been identified through the concept of a local RAS which was based on two fundamental discoveries, first expression of all components of the RAS in specific tissues. For example in the heart renin and angiotensinogen have been shown to be expressed, although the levels are very low (Paul *et al.*, 1993). ACE has also been detected in the heart of rats and humans (Hirsch *et al.*, 1991)(Paul *et al.*, 1993, Burrell *et al.*, 2005). The presence of AngI and AngII in cardiomyocytes was demonstrated by van Kats *et al*. (1998), who by measuring plasma and tissue levels of infused radiolabeled angiotensin peptides they were able to show cardiac synthesis of both peptides (van Kats *et al.*, 1998). Both the AT1R and the AT2R have also been shown to be expressed in cardiac cells (Booz and Baker, 1996). All these findings suggested that generation and consequently AngII-mediated signalling could be initiated directly in individual tissues without the requirement for systemic, circulating RAS components. The second discovery was the identification of new components of the RAS expressed in specific tissues, predominantly the enzyme Angiotensin converting enzyme 2 (ACE 2). These findings resulted in the theory of a "local and tissue-specific" RAS. Since the recognition of this local tissue-specific RAS many studies have been described in order to elucidate the actions and mechanisms of their components and to attempt to segregate these actions from those mediated by circulating AngII produced via the classical pathway. This has led to the characterization of the physiological and pathophysiological action of the RAS in different organs. Local tissue-specific RAS has been described in the heart, blood vessels, kidney, adrenal gland, nervous system, reproductive system, skin, digestive system, lymphatic and adipose tissue and in foetal development. (Figure 1-3).

1.3.1 Angiotensin Converting Enzyme 2

ACE 2 is a zinc metalloproteinase that acts as a carboxypeptidase (Warner *et al.*, 2004). The gene maps to the X chromosome and has 42% homology with ACE in the catalytic site. ACE 2 is a membrane-bound enzyme expressed mainly in cardiac blood vessels and tubular epithelia of the kidney and testis (Tipnis *et al.*, 2000).

The main action of ACE 2 is the cleavage of the COO-H terminal amino acid from AngI and AngII generating the new peptides angiotensin 1-9 (Ang1-9) (from AngI) and angiotensin 1-7 (Ang1-7) (from AngII) (Figure 1-3) (Chappel and Ferrario, 2006, Donoghue et al., 2000, Trask et al., 2007, Vickers et al., 2002). ACE 2 also cleaves des-Arg(9)-bradykinin but not bradykinin and is not affected by ACE inhibitors (Donoghue *et al.*, 2000).

1.3.2 Angiotensin peptide metabolites

1.3.2.1 Angiotensin III (2-8)

Angiotensin III (AngIII) peptide is generated from the cleavage of the AngII N-terminal amino acid by aminopeptidase A (Zini *et al.*, 1996) (Figure 1-3). This peptide has been shown to signal through the AT1R, however, its affinity for the AT1R is 10 times lower than AngII (Pendleton *et al.*, 1989). AngIII has been shown to have similar actions to AngII in the brain. Furthermore AngIII actions in the brain are similar to AngII actions, for example infusion of AngIII to rat's brains stimulated release of vasopressin (Yamaguchi et al., 1979, Reaux et al., 2001, Hohle et al., 1995), suggesting that AngIII is an active metabolite of the RAS in the brain.

1.3.2.2 Angiotensin IV (3-8)

Angiotensin IV (AngIV) is formed from the cleavage of AngIII at the N-terminus amino acid by aminopeptidase N and was considered originally an inactive peptide. However, it has been shown that this peptide binds with high affinity to the insulin-regulated aminopeptidase (IRAP) (Swanson *et al.*, 1992, Albiston *et al.*, 2001) (Figure 1-3). The distribution of the IRAP receptor is tissue specific being mainly expressed in different areas of the brain (Chai *et al.*, 2000), in the heart, adrenal cortex, kidney and vascular smooth muscle cells (de Gasparo *et al.*, 2000). AngIV has been shown to have a role in the cognitive and sensory functions of the brain such as memory and locomotor behaviour (Wright et al., 1993, Braszko et al., 1988).

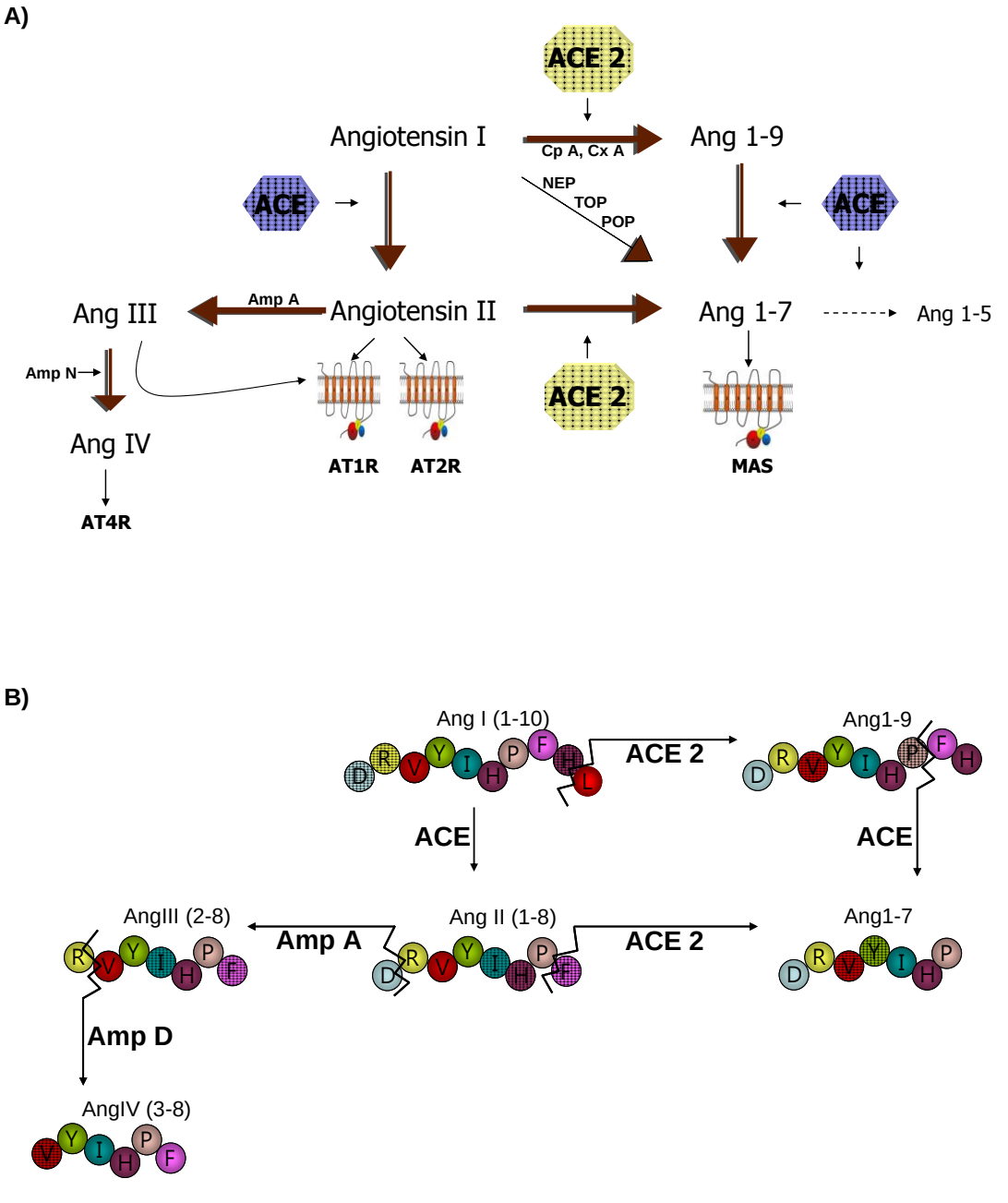


Figure 1-3 The tissue-specific renin-angiotensin system.

(A) AngI and AngII are cleaved by ACE 2 to form Ang1-9 and Ang1-7, respectively. Ang1-9 is further cleaved by ACE to form Ang1-7. AngI can also be cleaved by cathepsin A (CpA) and carboxypeptidase A (Cx A) to form Ang1-9; or by prolyl endopeptidase (POP), neutral endopeptidase (NEP) and thimet oligopeptidase (TOP) to form Ang1-7. Ang1-7 is an active peptide that can antagonise AngII signalling whose effects are mediated by the Mas receptor. Ang1-7 is further cleaved by ACE to form the inactive peptide metabolite Ang1-5. AngII is also converted to AngIII through the cleavage of the N-terminus amino acid by Aminopeptidase A (Amp A). AngIII mediates actions through the AT1R. AngIII is further cleaved by Aminopeptidase N and transformed into AngIV. AngIV binds to the insulin-regulated aminopeptidase (IRAP). (B) Schematic of angiotensin peptides and cleavage sites.

1.3.2.3 Angiotensin 1-7

Ang1-7 is one of the novel peptides of the local tissue-specific RAS and is the result of several pathways. Ang1-7 is a seven amino acid peptide first described to be generated in the endothelium (Santos *et al.*, 1992). Ang1-7 is mainly formed by the cleavage of the terminal amino acid of AngII (phenylalanine) by ACE 2 (Garabelli *et al.*, 2008). Alternatively AngI can be converted into Ang1-7, via either cleavage by ACE 2, to form Ang1-9 which is then hydrolyzed by ACE transforming it into Ang1-7, or by a direct conversion by the action of prolyl endopeptidase, neutral endopeptidase or thimet oligopeptidase (Welches *et al.*, 1993, Keidar *et al.*, 2007, Trask *et al.*, 2007) (Figure 1-3). The half life of Ang1-7 in the circulation is approximately 10 seconds before it is cleaved by ACE to form the inactive metabolite Ang1-5 (Yamada et al., 1998, Chappell et al., 1998). In normal conditions the circulating levels of Ang1-7 are much lower than AngII (20.1 pg/mL and 47 pg/mL, respectively) (Yamada *et al.*, 1998, Vilas-Boas *et al.*, 2009). Ang1-7 acts through engaging the orphan Mas proto-oncogene (Santos *et al.*, 2003). The interest in Ang1-7 comes from the fact that it is able to antagonize many of the effects of AngII. Roks *et al.* demonstrated for the first time that in human blood vessels Ang1-7 inhibited AngII-induced vasoconstriction (Roks *et al.*, 1999). Ang1-7 has also been shown to mediate vasodilatation, antiproliferative, anti-inflammatory and cardioprotective effects.

Ang1-7 was originally proposed to mediate its effects via the classical AngII receptors in peripheral arterioles (Kono *et al.*, 1986). However, Santos *et al.* generated Mas-knockout mice and showed through a radioligand binding assay in rat kidneys a lack of Ang1-7 binding (Santos *et al.*, 2003). In addition chinese hamster ovary (CHO) cells were transfected with Mas cDNA and fluorescent labelled Ang1-7 was shown to bind to the receptor, confirming that Ang1-7 bound to this orphan G-protein coupled receptor Mas (Santos *et al.*, 2003). The gene encoding the Mas receptor has been mapped to the distal half of chromosome 6q and encodes for a 325 amino acid protein (Alenina *et al.*, 2008). Its distribution is tissue-specific and it is expressed largely in the brain, but is also found in the testis, heart and kidney (Metzger *et al.*, 1995). Several studies have been carried out to demonstrate that the effects of Ang1-7 are mediated via Mas (see later).

As described for the AT1R and the AT2R, the existence of crosstalk between Mas and other receptors has been shown (Castro et al., 2005, Kostenis et al., 2005, Soares de Moura et al., 2004). Castro *et al.* showed differences in perfusion pressure when stimulating Mas with Ang1-7 in the presence or absence of AT1R and AT2R antagonists, suggesting crosstalk between the three receptors (Castro *et al.*, 2005). In $Mas^{-/-}$ mice the changes in perfusion pressure were not seen. In CHO-K1 cells overexpression of Mas and AT1R demonstrated an interaction between the two receptors (Kostenis *et al.*, 2005). In the presence of both receptors the initial AngII-induced production of inositol phosphate and intracellular Ca^{2+} mobilization decreased approximately 50%, suggesting that Mas acted as an antagonist of the AT1R. However, it has also been suggested that Mas expression upregulates AT1R expression and activates PKC (Canals *et al.*, 2006). Furthermore, an interaction between Mas and the bradykinin B_2 receptor has also been shown as relaxation of mesenteric arteries in $Mas^{-/-}$ mice is abolished both basally and when stimulated with bradykinin (Peiro *et al.*, 2007). Soares de Moura *et al.* demonstrated that both Mas and the AT2R enhanced the vasodilator effect of the bradykinin B_2 receptor (Soares de Moura *et al.*, 2004).

1.3.2.4 Angiotensin 1-9

Ang1-9 is a 9 amino acid peptide that results from the metabolism of AngI by ACE 2 as ACE 2 cleaves the terminal amino acid leucine of AngI and transforms it into Ang1-9 (Donoghue *et al.*, 2000, Li *et al.*, 2005c). Although ACE 2 is the main enzyme to form Ang1-9, it has also been reported that following incubation of AngI with human heart homogenates Ang1-9 was also formed through the enzymatic activity of carboxypeptidase A (Kokkonen *et al.*, 1997). This was also confirmed in $ACE^{-/-}$ and ACE $2^{-/-}$ null mice where Ang1-9 was the main product of AngI following its cleavage by carboxypeptidase A (Garabelli *et al.*, 2008). These reports also show that formation of Ang1-9 from AngI is the main pathway in cardiomyocytes (Kokkonen *et al.*, 1997, Garabelli *et al.*, 2008). Furthermore Jackman *et al.* showed that the conversion of AngI to Ang1-9 can also occur through the action of cathepsin A in human heart extracts (Jackman *et al.*, 2002). Once Ang1-9 is formed ACE cleaves the 2 last amino acids (phenylalanine and histidine) generating the active peptide Ang1-7 (Figure 1-3). Very little is known about Ang1-

9. Ang1-9 has been described to be a competitive inhibitor of ACE because it is an ACE substrate (Snyder and Wintroub, 1986). Ang1-9 has also been shown to potentiate bradykinin B_2 receptor activity, by enhancing the release of arachidonic acid in human atria and ventricles induced by a B_2 agonist 3-fold (Erdos *et al.*, 2002). It also enhances intracellular Ca^{2+} concentration and nitric oxide (NO) synthesis triggered by exposure of endothelial cells to bradykinin by 5-fold (Erdos *et al.*, 2002). Importantly, it was also shown that Ang1-9 was an active peptide and that it was a more potent in potentiating the effects of bradykinin than Ang1-7 (Erdos *et al.*, 2002, Jackman *et al.*, 2002). However, a direct receptor-mediated role for Ang1-9 is unclear and the importance of Ang1-9 not well understood, notwithstanding the fact that it is an indirect pathway by which angiotensin I is transformed into Ang1-7.

1.4 The role of AngII and the RAS in cardiovascular disease

Cardiovascular diseases (CVD) are the number one cause of death in the world, with an estimated 17.5 million people dying of CVD in 2006 (30% of all deaths) (World-Health-Organization, 2009). Coronary heart disease and stroke cause the highest mortality, accounting for the deaths of 7.6 million people and 5.7 million people worldwide, respectively.

Through the years the RAS has been the main target for treatment of CVDs. Clinical trials blocking the RAS have shown reduction in target organ damage in the heart, blood vessels and kidney (Dahlof et al., 2002, Mancini et al., 1996, Jandeleit-Dahm et al., 2005). Drugs to block the RAS are classified into three basic groups. The first group is the β-blockers which inhibit renin release from the kidney and therefore AngII production. The second group is the ACE inhibitors. These drugs also block AngII generation via ACE cleavage; however, it is important to mention that these drugs do not block AngII formation. The third group is the angiotensin receptor blockers which block AngII engagement to the AT1R by binding to this receptor.

AngII mediates effects on almost all cardiovascular cells, including cardiomyocytes, fibroblasts, smooth muscle cells, endothelial cells and macrophages often inducing

identical signalling pathways, but producing differential phenotypes depending on the cell type stimulated (Mehta and Griendling, 2007). For example, by inducing activation of the MEK pathway, AngII promotes hypertrophy in cardiomyocytes, proliferation and cell migration in smooth muscle cells and remodelling in fibroblasts (Mehta and Griendling, 2007). These differential actions are a major contributor to the development of many CVDs.

1.4.1 Atherosclerosis

Atherosclerosis is the most common underlying cause for many CVD, including coronary heart disease (CHD), stroke and thrombosis. The risk factors associated with atherosclerosis (hyperlipidemia, hypertension, obesity, smoking and high levels of cholesterol) are common in the populations of developed countries; in fact statistics show that 17 out of every 1000 people have atherosclerosis (Fauci, 2008, Tedgui and Mallat, 2006).

The theory of atheroma plaque formation was proposed by Russell Ross in the 1970´s (Ross and Glomset, 1976a, Ross and Glomset, 1976b), who described an initial endothelial lesion and as a consequence the adherence of platelets. This theory was further modified in 1993 when inflammatory factors in the plaque were discovered (Tedgui and Mallat, 2006). As a definition atherosclerosis is the inflammatory thickening of the artery, leading to diminished lumen size of the vessel. This thickening occurs due to a lesion in the wall of the arteries usually initially due to endothelial damage. The process of plaque development is then influenced by inflammatory factors such as cytokines produced by macrophages and leukocytes, smooth muscle cells, matrix, and lipids (cholesterol). These lesions initiate as fatty streaks and develop into larger lesions that eventually can lead to total occlusion of the vessel, blocking the blood supply, potentially triggering a myocardial infarction if the blockage is in a coronary artery or a stroke if in a cerebral vessel (Tedgui and Mallat, 2006, Robbins, 1998, Fauci, 2008).

The later modified theory of atherosclerosis proposed it as a chronic inflammatory disease, whereby an initial injury to the endothelium, caused by several factors including hypertension, hyperglycaemia, toxins from smoking, infection, oxidative

(ox) low density lipoprotein (LDL) and infection (Robbins, 1998). This lesion causes dysfunction of the endothelium, characterized by higher permeability and cell division, and a higher adhesion of monocytes due to the expression of adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Libby, 1995). The lesion in the endothelium also triggers the accumulation of lipids to the subintimal layer forming a fatty streak (Robbins, 1998). Monocytes migrate to the intima and transform into macrophages where they ingest LDL and convert into foam cells (Ghosh *et al.*, 2009). Concurrently, macrophages and T lymphocytes release inflammatory mediators such as interleukin-1-β (IL-1-β) and tumour necrosis factor alpha (TNF-α). These two proinflammatory factors promote the proliferation and migration of smooth muscle cells and also contribute to changes in the endothelium overlying the plaque (Bevilacqua *et al.*, 1984). The changes mediated by IL-1-β and TNF-α also transform the anticoagulant surface of the endothelium to a prothrombotic state by reducing the production of tissue plasminogen activator and protein-S, and stimulating higher production of matrix metalloproteinases, endothelin-1, platelet activating factor, ICAM-1 and VCAM-1 (Isoda and Ohsuzu, 2006, Robbins, 1998, DeGraba, 2004, Ohsuzu, 2004, Tedgui and Mallat, 2006). The natural development of the plaque leads to erosion and rupture which triggers thrombosis and acute blockage. AngII induces increased production of ROS in the endothelium of the atheromatous wall leading to activation of apoptosis pathways and promotion of thrombosis (Dimmeler *et al.*, 1997). In vascular smooth muscle cells, AngII induces expression of plasminogen-activator inhibitor type 1, inhibiting fribrinolysis and promoting thrombosis (Feener *et al.*, 1995).

The involvement of AngII in the pathogenesis of atherosclerosis is clear. AngII has been shown to be localized in the atheroma plaque (Schieffer *et al.*, 2000) and AngII has also been shown to induce endothelial dysfunction, smooth muscle cell proliferation and migration, and activation of pro-inflammatory pathways (Mehta and Griendling, 2007). In endothelial cells the main action of AngII is related to the stimulation of oxidative stress (Watanabe *et al.*, 2005). NO bioavailability is significantly impaired by the action of AngII (Imanishi *et al.*, 2006) as it has been shown that AngII promotes the production of ROS by either increasing NAD(P)H oxidase activity or by uncoupling eNOS (Dimmeler *et al.*, 1997)(Yan *et al.*, 2003a,

Doughan *et al.*, 2008). AngII also, promotes the uptake of oxLDL by the endothelium which leads to decreased production of NO (Li *et al.*, 1999).

In vascular smooth muscle cells AngII promotes migration and proliferation through the activation of the Akt and ERK1/2 pathway (Ohtsu et al., 2008, Bunkenburg et al., 1992, Kyaw et al., 2004, Min et al., 2005, Yang et al., 2005). In similarity to endothelial cells, AngII induces ROS generation via activation of NAD(P)H oxidase but it has been suggested that ROS production from endothelial cells may act as second messengers to promote vascular smooth muscle cell hypertrophy or hyperplasia (Griendling *et al.*, 1994).

AngII also directly promotes inflammation during atheroma plaque formation (Ferrario and Strawn, 2006). Through the AT1R, AngII activates NF-κB (Ruiz-Ortega *et al.*, 2000b) the key pro-inflammatory transcription factor, leading to increased expression of E-selectin, monocyte chemoattractant protein (MCP-1), VCAM-1 and ICAM-1 in endothelial and smooth muscle cells, increasing leukocyte adhesion to the site of rupture in the atheromatous plaque (Moreno et al., 2009, Ruiz-Ortega et al., 2000b). It has also been demonstrated that AngII mediates direct effects on the atherosclerotic plaque by increasing production of IL-6 (Kandalam and Clark, 2009)(Schuett et al., 2009, Rojas et al., 2009), IL-8 (Souza et al., 2009, Moreno et al., 2009) and IL-18 (Naka et al., 2008, Sahar et al., 2005, Doi et al., 2008). IL-6 increases leukocyte adhesion to the vessel wall and promotes the release of pro-inflammatory cytokines as well as activating matrix metalloproteinanses. By increasing production of IL-8 and IL-18 in vascular smooth muscle cells, AngII induced chemotaxis of neutrophils and activation of PKC, and transcription factors NF-kB and AP-1 (Sahar et al., 2005, Moreno et al., 2009).

Conversely, Kaschina *et al.* showed that stimulation of AT2R with the oral agonist compound 21 in post-myocardial infarction rats diminished the expression of the inflammatory factors monocyte chemoattractant protein-1, myeloperoxidase, and the inflammatory cytokines IL-6, IL-1β and IL-2 (Kaschina *et al.*, 2008). Furthermore, reductions in scar size and improvement in systolic and diastolic function were also demonstrated. This evidence suggests that AngII, via activating the AT2R, also has anti-inflammatory actions. The AT2R has also been implicated as a positive

regulator of atherosclerotic lesion formation in apolipoprotein E knockout ($ApoE^{-/-}$) mouse, where loss of the AT2R gene augmented atherosclerotic lesion development (Sales *et al.*, 2005). Also, the AT2R has been reported to have a positive action in the adipose tissue of the atherosclerotic plaque, improving adipocyte function and differentiation (Iwai *et al.*, 2009). Furthermore, the AT2R has also been shown to be expressed in human atherosclerotic plaques (Johansson *et al.*, 2008). The role of ACE 2 in atherosclerosis has also been studied. In a rabbit atherosclerosis model, adenovirus-mediated overexpression of ACE 2 resulted in reduction of macrophage numbers, lipid deposits and collagen which led to higher plaque stability (Dong *et al.*, 2008). Conversely, to date there have been no study on the role of Ang1-7 in atherosclerosis published.

1.4.2 Stroke

Stroke is the interruption of blood flow to the brain due to rupture of a blood vessel (hemorrhagic stroke) or vessel occlusion by a clot (ischaemic stoke), and can cause brain tissue damage and death. Ischemic stroke is the major cause of stroke and the primary risk factors are hypertension and hyperlipidemia. Symptoms of stroke depend on the area affected, usually with an acute presentation. The most common symptoms of a stroke are sudden weakness or numbness of the face, arm or leg, most often on one side of the body, difficulty to walk, loss of balance or coordination, dizziness, vomiting, difficulty to speak or understand speech, to see with one or both eyes, severe headache, confusion, fainting and unconsciousness (Andreoli, 2001, Fauci, 2008).

AngII has been shown to promote the pathogenesis of intracerebral haemorrhage via increasing levels of oxidative stress and matrix metalloproteinase 9 via the AT1R (Wakisaka *et al.*, 2009). However, neuroprotection in stroke has also been reported as an action of the AT2R. The AT2R is upregulated in the peri-infarcted zone 2 days after an ischemic event (Li *et al.*, 2005b). This expression seems to be distributed through the brain but limited at the cellular level to neurons in mouse models of ischemia, caused by cerebral artery occlusion (Li *et al.*, 2005b). Deletion of the AT2R in murine models of stroke has been shown to increase the area of ischemia and decrease neuronal survival and neurite growth (Iwai *et al.*, 2004, Li *et al.*,

2005a)(McCarthy *et al.*, 2009). Furthermore, in AT2R knockout mice there is increased activity of NAD(P)H oxidase and increased superoxide production suggesting suppression of oxidative stress is an AT2R pathway in ischaemia protection (Iwai *et al.*, 2004). In AT2R knockout mice cognitive function due to ischaemia is also impaired (Iwai *et al.*, 2004, Mogi *et al.*, 2007, McCarthy *et al.*, 2009). All these studies indicate a beneficial action of AT2R in protection of neuronal tissue in stroke and it may therefore be an important therapeutic target.

1.4.3 Hypertension

Hypertension has been identified as one of the most important risk factors for CVD (Robbins, 1998, Raizada and Der Sarkissian, 2006). The British Heart Foundation, in accordance with the European Hypertension Society and the World Health Organization-International Society of Hypertension, established the definition of hypertension in 2004: systolic blood pressure of 140 mmHg or higher; or diastolic blood pressure of 90 mmHg or higher. However, diagnosis of hypertension is complicated and several grades of high blood pressure have been defined to help guide intervention. Grade 1 or mild hypertension is defined as systolic blood pressure from 140 to 159 mmHg and diastolic blood pressure from 90 to 99 mmHg. Grade 2 or moderate hypertension is defined as systolic blood pressure from 160 to 179 mmHg and diastolic blood pressure from 100 to 109 mmHg, and grade 3 is severe systolic blood pressure above 180 mmHg and diastolic blood pressure above 110 mmHg (Table 1) (Williams *et al.*, 2004)(Mancia et al., 2007, World-Health-Organization, 1999). According to the World Health Organization around 11% of all disease in developed countries is triggered by initial development of hypertension, including 50% of all CHD and 75% of all stroke events. Furthermore the INTERHEART study showed that around 22-25% of myocardial infarctions in Europe were due to hypertension and that history of hypertension doubled the risk of developing a myocardial infarction (World-Health-Organization, 2009, Yusuf et al., 2004). British surveys of 2006 showed that around 30% of women and men over 16 years of age had hypertension (Allender, 2008), while the American Heart Association statistics of 2006 showed that 30% of the adult population had high blood pressure (Lloyd-Jones *et al.*, 2009).

In a very simplistic way the cause of hypertension can be divided into an increase in blood volume or an increase in peripheral resistance. In every contraction cycle the heart pumps 70 mLs of blood into the arterial system. This perfusion puts pressure on the arterial wall, so blood pressure is the result of the cardiac output multiplied by the peripheral resistance. Peripheral resistance is defined by the vessel's radius, length and wall smoothness. The ejection of blood from the heart is periodic, meaning that the pressure in arteries is pulsatil; the highest pressure is detected when a bolus of blood is going through the artery and the lowest after it has passed. These different pressures are known as systolic (highest) and diastolic (lowest) blood pressure (Guyton, 2007). Blood pressure is controlled by several systems which are interconnected and grouped into two basic responses, 1) acute control of blood pressure or baroreceptor reflex and 2) long term control of blood pressure. Baroreceptors, chemoreceptors, ischaemia of the central nervous system and the release of hormones are the first response when blood pressure drops. These four mechanisms activate within seconds and remain activated, resulting in adaptation to the new blood pressure. Over time this results in a permanent resetting of sensitivity, leading to basal blood pressure elevation (Guyton, 2007, Rhoedes, 1997, Lifton et al., 2001). The arterial baroreceptor reflex arc consists of terminal axons of afferent fibers that are in the adventitia of the aortic arch, carotid sinuses which are part of the internal carotid arteries.

Depolarization of such sensors happens when the wall vessel is stretched. Signals travel through the glosso-pharyngeal and vagus cranial nerves from carotid sinus and aortic arch baroreceptors respectively to the nucleus tractus solitarii (NTS), caudal ventrolateral medulla and rotral ventrolateral medulla. From this central nucleus efferent fibers are projected to the intermediolateral cell column where sympathetic preganglionic neurons project to organs such as heart and blood vessels. Baroreceptor stimulation results in vasodilatation of peripheral vessels and a decrease in heart rate and cardiac output. If the blood pressure drops, the firing rate of baroreceptors also drops causing vasoconstriction (Guyton, 2007). The adaptation of this reflex to new levels of blood pressure results in transient loss of sensitivity to the response. However if this reset is chronic (48 hours after high blood pressure) the baroreceptor reflex sensitivity is impaired (Moreira *et al.*, 1992).

Category	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)
Blood Pressure		
Optimal	< 120	< 80
Normal	< 130	< 85
High Normal	130 - 139	85 - 89
Hypertension		
Grade 1 (mild)	140 – 159	90 – 99
Grade 2 (moderate)	160 – 179	100 – 109
Grade 3 (severe)	≥ 180	≥ 110
Isolated Systolic Hypertension		
Grade 1	140 - 159	< 90
Grade 2	≥ 160	< 90

Table 1. Blood pressure classification.

The classification of blood pressure according to systolic and diastolic blood pressure measurements. Measurements should be taken on three different occasions. Blood pressure is considered normal when blood pressure is between 120/80 and 139/89 mmHg. Blood pressure above 140/90 mmHg is considered hypertension, which depending on the systolic and diastolic blood pressure measurements can be divided into mild, moderate and severe. Table was been reproduced from Williams *et al.* (Williams *et al.*, 2004).

Chemoreceptors in the wall of the aorta and carotid arteries sense the drop in levels of oxygen or augmentation in carbon dioxide levels and fire triggering sympathetic drive to motoneurons which constricts the vessel. A similar reaction is triggered when chemoreceptors in the medulla oblongata sense augmentation in carbon dioxide. In addition, when ischaemia is sensed in the brain stem, specifically in the NTS, by inadequate perfusion an intense activation of the sympathetic neurones takes place. This ischemic response leads to increased blood pressure to supra-physiological levels to the extent of totally occluding peripheral vessels. This sympathetic nerve activity has been thought to mediate the development of essential hypertension (Grassi, 2004).

The hormonal mechanisms controlling blood pressure include noradrenaline-adrenaline, vasopressin, the RAS and natriuretic peptides. Noradrenaline-adrenaline is liberated by autonomic nervous system stimulation of the adrenal medulla, and enters the blood stream and causes peripheral vasoconstriction for around three minutes before being metabolized. By the same action of the sympathetic nervous system vasopressin is liberated from the posterior pituitary gland. Besides the antidiuretic and antinatriuretic effect, vasopressin also has a direct vasoconstrictor effect on arterial walls causing intense vasoconstriction. AngII has 6 basic functions: 1) it triggers the release of aldosterone from the suprarenal cortex; 2) it increases the kidney's reabsorption of sodium; 3) it mediates vasoconstriction; 4) induces an increase in water ingestion; 5) it increases activation of the sympathetic nervous system and; 6) it promotes vasopressin release. Over-activity of the RAS has been described as one of the most frequent cause of hypertension. Natriuretic peptides are hormones produced in the heart and secreted by myocytes in response to high cardiac filling pressures. There are three types: atrial natriuretic peptide (ANP) produced mainly in myocytes of the atria, brain natriuretic peptide (BNP) produced mainly in ventricular myocytes and C-type natriuretic peptide (CNP) produced in the brain. Once in the blood stream ANP and BNP cause natriuresis, diuresis and vasodilatation while inhibiting aldosterone synthesis and renin secretion. In the last decade it has been shown that ANP and BNP also have direct effects on cardiomyocyte growth (discussed later) (Rhoedes, 1997, Guyton, 2007)(Pocock, 2004, Nishikimi *et al.*, 2006).

The main action of AngII is in maintaining blood pressure by promoting vasoconstriction and releasing aldosterone, which leads to retention of sodium and water to maintain the blood volume (Wolf *et al.*, 1999). Based on this, by definition AngII is a pro-hypertensive peptide and this pro-hypertensive action is mediated by the AT1R (Wolf *et al.*, 2003). The literature that supports AngII as hypertensive peptide is vast. As examples, AngII has been shown to induce vasoconstrictive signalling via the AT1R by increasing formation of arachidonic acid and formation of the nitrite tyrosine residues forming 3-nitrotyrosine (3NT) (Wattanapitayakul *et al.*, 2000). In hypertensive rat aortas, treatment with losartan (an AT1R blocker) abolished the AngII-induced vasoconstriction (Cosentino *et al.*, 2005). By inducing the release of aldosterone from the adrenal glands, AngII increases the retention of sodium and excretion of potassium which leads to increases in blood volume (Guyton, 2007). Furthermore, in vascular smooth muscle cells it has been described that aldosterone promotes the formation of ROS and potentiates AngII-induced MAP kinase activation (Mazak *et al.*, 2004). Also, the reduced blood pressure in AT1R knockout mice has been shown to be a direct effect of AngII signalling through the AT1R (Audoly *et al.*, 2000).

AngII has also been described to have a role in central nervous system blood pressure control. A decreased baroreceptor reflex sensitivity was shown when AngII is intravenously infused in rabbits (Guo and Abboud, 1984). Interestingly, AngII is unlikely to cross the blood barrier, which suggested production of AngII in the brain. Several studies have described the presence of high density AngII-containing neurones which express the AT1R in the NTS (Healy et al., 1989, Gutkind et al., 1988, Paton and Kasparov, 1999). In addition to this and supporting the theory of local brain production of AngII, in the spontaneously hypertensive rat levels of AngII were described to be elevated only in the brain (Ferguson and Washburn, 1998, Campbell et al., 1995, Morishita et al., 1995). Furthermore, Paton *et al.* showed an increase in NO levels in the NTS when locally infusing AngII, which was mediated through the AT1R, (Paton *et al.*, 2001a). AngII-induced release of NO promoted gamma-aminobutyric acid (GABA) release from the NTS which led to depression of the baroreceptor reflex (Wang *et al.*, 2006b). Moreover, blockade of eNOS in the NTS with an adenovirus expressing a dominant negative mutant form of

eNOS, abolished the AngII-induced baroreceptor reflex inhibition (Paton *et al.*, 2001b), showing a clear role of AngII in the neurogenic control of blood pressure.

Recently it has been shown that an inflammatory process in the brainstem may participate in the development of essential hypertension (Waki et al., 2009, Waki et al., 2007). Inflammatory processes can obstruct blood flow resulting in localized hypoxia leading to activation of the central nervous system ischemic reflex. Consistent with this, Waki *et al.* demonstrated an up-regulation in junctional adhesion molecule-1 (JAM-1), glycoprotein-39 precursor (gp39), ICAM-1, VCAM-1 and MCP-1 expression in NTS of spontaneously hypertensive rat (SHR) compared to Wistar Kioto (WKY) rats (Waki *et al.*, 2007, Waki *et al.*, 2008). AngII is a high pro-inflammatory molecule and it has been shown that blockade of AT1R in SHR reduces macrophage infiltration and ICAM-1 expression in the microvasculature of SHR brains when compared to control rats (Ando *et al.*, 2004). Similar to what has been observed with AngII, infusion of AngIII into the supraoptic and the paraventricular nucleus stimulated release of vasopressin (Yamaguchi et al., 1979, Reaux et al., 2001, Hohle et al., 1995). In addition, inhibition of aminopeptidase A in mice brains blocked the AngII-induced vasopressin release (Reaux et al., 2000, Zini et al., 1996). Furthermore, in hypertensive animal models, AngIII has shown to induce a similar increase in blood pressure to AngII by increasing sympathetic nerve activity and inhibiting the baroreceptor reflex at the NTS level (Wright et al., 1989, Abhold et al., 1987). All these results suggest AngIII as a regulator of central blood pressure.

One of the first recognized actions of AT2R stimulation was the regulation of vascular tone. Strong evidence *in vivo* has shown that AT2R heterodimerization with the B_2 bradykinin receptor induce vasodilatation in hypertensive rodent models through the production of eNOS (Yayama and Okamoto, 2008). Similarly, this heterodimer has been shown to promote the formation of NO and cyclic guanosine monophosphate (cGMP) (Abadir *et al.*, 2006). However, there is also evidence for AT2R activation directly triggering vasodilatation (Lemarie and Schiffrin, 2009). Ritter *et al.* showed increased production of eNOS in cardiomyocytes and aortic endothelial cells due to stimulation of AT2R with AngII (Ritter *et al.*, 2003). ENOS activation was mediated by the calcineurin/nuclear factor of activated T-cells

(NFAT) pathway. Furthermore, stimulation of the AT2R in denuded rat aorta stimulated inducible nitric oxide synthase (iNOS) mediated vasodilatation (Lee *et al.*, 2008). AT2R activation has also been shown to inhibit AT1R-induced membrane translocation of Ras homolog gene A (RhoA) and myosin light chain phosphorylation in smooth muscle cells, leading to vasodilatation in the stroke prone spontaneously hypertensive rat (Guilluy *et al.*, 2008). Furthermore, it has been shown that inhibition of RhoA was due to AT2R-dependent activation of the SHP-1 pathway and the serine/threonine kinase Ste20-related kinase (SLK) (Guilluy *et al.*, 2008).

In the kidney AT2R activation also regulates pressor responses leading to induction of vasodilatation (Siragy, 2009). Studies with AT2R knockout mice have shown an increase in blood pressure as well as a decrease in NO, cGMP and bradykinin production. Overexpression of the AT2R in mice increased vasodilatation (Ichiki *et al.*, 1995, Tsutsumi *et al.*, 1999). A phenomenon that appeared to be related to blocking nitric oxide formation or B_2 receptor activation (Siragy *et al.*, 1999). Pro-inflammatory effects in the kidney have also been allocated to AngIII. An increase in TGF-β and MCP-1 expression and fibronectin production was observed when mesangial cells were incubated with AngIII (Ruiz-Ortega *et al.*, 1998). Furthermore, AngIII was also able to increase activation of NF-κB in mesangial cells (Ruiz-Ortega *et al.*, 2000a).

Due to the cleavage of AngII and the consequent generation of Ang1-7, ACE 2 was thought to participate in the regulation of blood pressure, but its actual role is controversial. In transgenic SHRSP overexpression of ACE 2 in vascular smooth muscle cells led to increased levels of Ang1-7 and a reduction in blood pressure, as well as attenuated AngII-mediated vasoconstriction (Rentzsch *et al.*, 2008). It has also been reported that lentivirus-mediated overexpression of ACE 2 in the heart diminished blood pressure, cardiac hypertrophy (evaluated by body:heart weight and left ventricular wall thickness) and perivascular and interstitial fibrosis (Huentelman *et al.*, 2005, Diez-Freire *et al.*, 2006).

Ang1-7 triggers vasodilatation by activating several pathways, the most studied is the NO production pathway. Benter *et al.* showed that co-treatment of SHR with N-

(G)-nitro-L-arginine methyl ester (L-NAME) (a NO synthesis inhibitor) and Ang1-7 prevented the development of severe hypertension and heart damage (Benter *et al.*, 2006). In addition, Zhi *et al.* compared the effect of Ang1-7 in different blood vessels and showed that Ang1-7 induced endothelium-dependent vasorelaxation mediated by the NO pathway (Zhi *et al.*, 2004). Independently, Sampaio *et al.* demonstrated the role of Ang1-7 in regulating the phosphorylation of eNOS through increasing eNOS activation in human aortic endothelial cells when stimulated with Ang1-7 (Sampaio *et al.*, 2007b). Furthermore, Heitsch *et al.* measured NO in Ang1-7-stimulated bovine aortic endothelial cells and showed an increase in its release (Heitsch *et al.*, 2001). These studies showed a direct effect of Ang1-7 over NO production. Other pathways that have been considered for Ang1-7-mediated vasodilatation are: the release of prostaglandins; an antagonistic role blocking the actions of AngII by acting as a competitor at the AT1R; or affecting bradykinin (Abbas et al., 1997, Keidar et al., 2007, Paula et al., 1999, Benter et al., 1995). Zhu *et al.* showed that administration of Ang1-7 to rats significantly reduced baseline levels of PKC and ERK1/2 and the levels triggered by AngII stimulation (Zhu *et al.*, 2002). In aortic injury models, Tallant *et al.* demonstrated that Ang1-7 inhibited vascular smooth muscle cell growth by promoting the release of prostacyclin (PGI_2) to augment cyclic adenosine monophosphate (cAMP) and activate protein kinases (Tallant and Clark, 2003). This reduction of activation was notable when the MEK were stimulated with AngII. In this same context Gallagher *et al.* showed the effects of Ang1-7 in lung cancer cells, proving it diminished growth and reduced activation of ERK1/2 in this cell line (Gallagher and Tallant, 2004). This has also been observed in rat kidney cells where Ang1-7 inhibits AngII-mediated activation of MEK (Su *et al.*, 2006).

The role of Mas signalling in the development on oxidative stress has also been confirmed by the use of knockout mice (Ferrario *et al.*, 1998). These mice have impaired endothelial function and diminished production of NO due to decreased eNOS expression, leading to impaired vessel relaxation and increased blood pressure following Ang1-7 infusion (Dias-Peixoto et al., 2008, Peiro et al., 2007, Xu et al., 2008). AngIV has also been shown to have an effect in blood vessels. In vascular endothelium AngIV induced NO production by increasing eNOS activity leading to

vessel dilatation; however AngIV signalling through the IRAP was not tested (Cheng *et al.*, 1994, Li *et al.*, 1997, Kramar *et al.*, 1998).

1.4.4 Cardiac dysfunction and heart failure

Heart failure (HF) is defined as the inability of the heart to pump adequate amounts of blood to meet the requirements for tissue metabolism due to an abnormality in cardiac function. Many disorders result in HF, hypertension being one of the most common. There are several forms of HF for example, it can be systolic or diastolic, of high output or low output, left or right sided and finally chronic or acute. When HF is systolic the heart does not contract strongly enough which diminishes the ejection fraction due to an inadequate ventricular emptying leading to cardiac dilatation and high ventricular diastolic pressure. In diastolic HF there is an abnormal relaxation of the myocardium; this can be due to transitory ischaemia or thickening of the myocardial wall. Even though hypertrophic cardiomyopathy results in diastolic HF, it can also be present in the systolic form. HF is classified as high output when there are elevated metabolic demands and the heart is unable to achieve them; while a low output heart failure the heart is unable to meet metabolic demand at rest or at times of high metabolic demand. Left or right HF depends on whether the right or the left side of the heart is affected (Andreoli, 2001, Fauci, 2008).

Signs and symptoms that accompany HF are dyspnoea which is worsened in a recumbent position and after several hours of sleep (paroxysmal nocturnal dyspnoea), fatigue due to diminished blood flow to muscles, peripheral oedema which worsens during the day, distended neck veins and congested liver. At auscultation increased heart rate can be detected, crackles in the lung field can be heard; also a third heart sound or gallop can be detected. Electrocardiograms show no specific signs and a chest radiograph usually shows enlargement of the heart and signs of pulmonary oedema (Fauci, 2008, Andreoli, 2001).

HF is also characterized intracellularly by re-expression of foetal gene program, cardiomyocyte hypertrophy and fibrosis, and activation of apoptotic pathways (Lips *et al.*, 2003). Deleterious effects in cardiomyocytes and fibroblasts mediated by

AngII in HF are similar to those described during the development of cardiac hypertrophy (see later).

There are very few studies investigating the effects if the AT2R during HF, although AT2R stimulation has been shown to inhibit inotropy (Masaki et al., 1998, Castro-Chaves et al., 2008, Nakayama et al., 2005). These results may suggest that AT2R has a protective role in HF, although as yet there is no direct evidence.

Ang1-7 is produced in the heart via either AngI or from AngII (Zisman et al., 2003, Wei et al., 2002). Several authors have described Ang1-7 as a regulator of the cardiac conduction system (Ferreira *et al.*, 2001, De Mello *et al.*, 2007, Castro-Chaves *et al.*, 2009, Stewart *et al.*, 2009). It has been described that in induced ischaemia in rat hearts Ang1-7 reduces the incidence of arrhythmias after reperfusion compared to AngII perfused hearts (Ferreira *et al.*, 2001), as well as inducing vasodilatation in postural tachycardia syndrome (Stewart *et al.*, 2009). De Mello *et al.* showed that in cardiomyocytes exposed to Ang1-7 the cardiac action potential was reduced and there was an increase in conduction velocity due to the activation of the sodium pump, assigning Ang1-7 antiarrhythmic properties (De Mello *et al.*, 2007). Interestingly, when cardiomyocytes were treated with higher doses of Ang1-7 the action potential was not only not decreased, but augmented as well as the early-after depolarisations leading to promotion of arrhythmias. In rabbit papillary muscle Ang1-7 has been shown to reduce muscle contraction (Castro-Chaves *et al.*, 2009). Relaxation was related to the integrity of endocardial endothelium, suggesting that Ang1-7 has negative inotropic effects mediated through endothelium. However, the effects of Ang1-7 were completely abolished when cardiac papillary muscle was incubated with the Mas antagonist A779.

1.4.5 Cardiac Hypertrophy

Hypertrophy is defined as “the enlargement or overgrowth of an organ due to an increase in size of its constituent cells”(Dorn *et al.*, 2003). By definition cardiac hypertrophy is an increase in size of the walls or interventricular septum of the heart (Carreno *et al.*, 2006). The heart has the ability to increase its productivity when there is an increase on physiological demands by augmenting the capacity of single

cardiomyocytes. This increase in heart size is termed physiological hypertrophy, as it is an initial compensatory response to stress and examples of these are cardiac growth in athletes or pregnancy (Chien, 1999, Carreno *et al.*, 2006). However, if the stress signal continues compensatory hypertrophy switches to pathological hypertrophy with implications for cardiac function. Cardiac hypertrophy is one of the main risk factors for ischaemic heart disease, arrhythmias, HF and sudden death (Komuro, 2001, Frey and Olson, 2003). The mechanisms by which physiological and pathological hypertrophy differentiate are still unclear. Pathological hypertrophy is accompanied by fibrosis, abnormalities in contraction and electrophysiology, changes in energy consumption and altered gene expression profiles (Wang, 2001). Based on phenotype, pathological cardiac hypertrophy can be classified into two types: concentric or eccentric. Concentric hypertrophy is the result of an increase in the thickness of the heart walls (predominantly the left ventricular wall) and has been associated with chronic pressure overload. Eccentric hypertrophy is defined as the thinning of the heart wall and consequent dilatation of the heart cavities, usually due to volume overload (Dorn et al., 2003, Carreno et al., 2006, Chien, 1999) (Figure 1-4). At a cellular level hypertrophy of the cardiomyocyte is established by four major characteristics, increases in cell size and protein synthesis, reorganization of sarcomeric units and re-entrance to the foetal gene programme (Chien, 1999, Carreno *et al.*, 2006, Rajabi *et al.*, 2007). Increases in cell size (by measuring cell area or volume) and reorganization of sarcomeres at the cellular level differentiates concentric and eccentric hypertrophy. Concentric hypertrophy induces an increase in cell width through addition of sarcomeres in parallel, whereas eccentric hypertrophy adds sarcomeres in series resulting in an increase in cell length (Satoh et al., 1996, Hwang et al., 2006, Frey et al., 2004).

Although studies have demonstrated mitotic activity in cardiomyocytes (Beltrami *et al.*, 2001, Datwyler *et al.*, 2003, Engel *et al.*, 2005, Novoyatleva *et al.*, 2009), generally cardiomyocytes are considered non-dividing cells. When stress signals are applied to cardiomyocytes the normal mitotic pathway is not activated, instead a pattern of foetal metabolism, resulting in expression of molecular hypertrophy markers occurs. Hypertrophy results from the binding of different kinds of stimuli (such as AngII, endothelin-1 or growth factors) to specific receptors in cardiomyocytes which triggers signalling pathways that activate or inhibit gene

expression. Such genes include atrial natriuretic factor (ANF) or ANP, BNP, β isoforms of myosin heavy chain (β-MHC), skeletal α-actin, *fos*, *c-jun*, *myc* and *junB*.

The hypertrophy-inducing factors in cardiomyocytes can be broadly divided into two groups, mechanical stretch and neurohumoral factors, although the actual mechanism of how cardiomyocytes transform mechanical stimuli to a hypertrophic response is still unclear. There are several studies that show the involvement of ion channels in the response of cardiomyocytes to stretch, especially the transient receptor potential channel (TRPC) family (Sharif-Naeini *et al.*, 2009, Stiber *et al.*, 2009).

These mechanoreceptors are activated directly by tension and then may interact with G-protein coupled receptors such as the AT1R (Yamazaki and Yazaki, 1999, Nishimura *et al.*, 2008). It has been described that coactivation of the AT1R and TRPC can be AngII-independent (Zuo et al., 2005, Onohara et al., 2006). Signalling through these receptors is thought to be via the interaction with the cytoskeleton. Cytoskeletal re-arrangement transforms and activates signalling proteins, such as calcineurin, which then activate developmental cell signalling pathways (Bush *et al.*, 2006, Kuwahara *et al.*, 2006, Zobel *et al.*, 2007). Mechanical stretch of cardiomyocytes has also been demonstrated to release preformed AngII from cytosolic granules (Cingolani *et al.*, 2005).

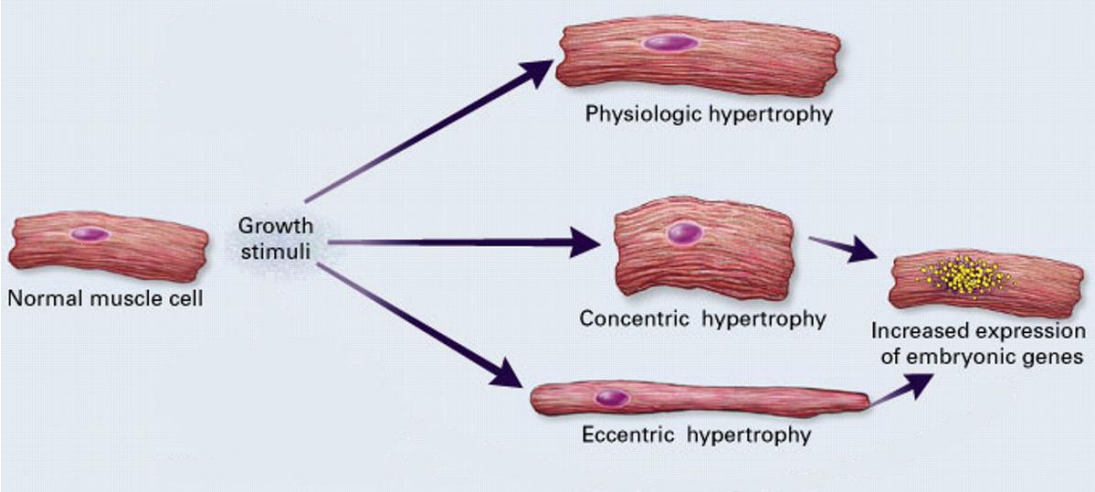


Figure 1-4. Type of cardiac hypertrophy.

Schematic showing the different types of hypertrophy that can develop in cardiomyocytes. The proportionate increase of cell width and length lead to physiological hypertrophy. Increases in width only result in concentric hypertrophy, while increases in length result in eccentric hypertrophy. Both concentric and eccentric hypertrophy are developed as a result of pathological stimuli and lead to re-expression of the foetal gene program. [Diagram modified from (Hunter and Chien, 1999) Copyright © [1999] Massachusetts Medical Society. All rights reserved].

Neurohumoral factor-mediated stimulation of cardiomyocyte hypertrophy is complex and includes molecules acting in an endocrine, paracrine and/or autocrine manner leading to activation of different pathways. Growth factors including TGF-β, fibroblast growth factor (FGF) and insulin-like growth factor (IGF) have been studied in cardiac hypertrophy (Molkentin and Dorn, 2001). IGF is a single chain peptide that induces cell proliferation or differentiation through binding to the tyrosine kinase transmembrane receptor IGFR (Ren *et al.*, 1999). IGF induces hypertrophy in skeletal muscle by increasing myotubule diameter through phosphorylation and activation of Akt (Morissette *et al.*, 2009). In myocardium IGF increases transcription of myocyte enhancer factor 2C (MEF-2C) [a key transcription factor that stimulates activation of genes that promote cardiac hypertrophy (Xu *et al.*, 2006)], including ANP, BNP and skeletal alpha-actin genes (Munoz et al., 2009, Chu et al., 2008). TGF-β_1 is a polypeptide that regulates cell growth, proliferation and differentiation by binding to the transmembrane receptors TGFβIR and TGFβIIR (Moustakas *et al.*, 2002, ten Dijke and Arthur, 2007). TGF-β_1 is present throughout development of the heart and also promotes cardiac differentiation of embryonic stem cells (Gwak et al., 2009, Lim et al., 2007). TGF-β_1 has also been implicated in cardiac fibrosis and hypertrophy in the heart (Khan and Sheppard, 2006). TGF-β_1 interaction with its receptors leads to phosphorylation of the conserved transcription factor family Smad (Schiller *et al.*, 2004). In the heart Smad2 predominantly mediates TGF-β_1 actions (Pokharel *et al.*, 2002) and Smad proteins then bind to specific nucleotides sequences in the regulatory regions of genes essential for matrix remodelling, such as collagen and gelatinase A and B (Greene *et al.*, 2003, Tsuchida *et al.*, 2003). TGF-β_1 expression is increased in the scar tissue in rats which have undergone myocardial infarction suggesting that TGF-β_1 regulates scar remodelling via matrix deposition (Hao *et al.*, 1999, Ellmers *et al.*, 2008). Expression of TGF-β_1 in myocardial infarction is of special importance as it has also been associated with decompensated hypertrophy and HF (Li *et al.*, 2009b). There is also evidence of a crosstalk between TGF-β_1 and AngII signalling in the heart. Several studies have shown that AngII regulates messenger ribonucleic acid (mRNA) expression of TGF-β_1 in cardiac and vascular tissue (Kupfahl *et al.*, 2000), and furthermore secretion of TGF-β_1 from cardiofibroblasts mediates AngII-induced hypertrophy in cardiomyocytes (Gray *et al.*, 1998).

Cytokines also form part of the neurohumoral signalling network. Although cytokines have been strongly related to the development of the atherosclerotic plaque, there is also evidence of a direct effect of cytokines [especially those from the IL-6 family including cardiotropin 1 (CT-1)] in cardiac hypertrophy. CT-1 activates the IL-6 receptor and in conjunction with the glycoprotein 130 (gp130) is a potent prohypertrophic agent through increasing cell length and expression of hypertrophy markers (Wang, 2001)(Calabro *et al.*, 2009). Fredj *et al.* demonstrated that IL-6 also increased the expression of ANP and β-MHC in co-cultures of murine cardiomyocytes and cardiofibroblasts (Fredj *et al.*, 2005). Other cytokines involved in cardiac hypertrophy are IL-1 and TNF-α. (for more detail see section 1.4.5.1).

The final remaining group of hypertrophic stimuli include agonists which bind to GPCR. Among these the most studied are arg-vasopressin, endothelin-1, norepinephrine and AngII. GPCRs are one of the largest family of receptors. These are 7-transmembrane-spanning receptors which bind to G-proteins interconnected to intracellular signalling pathways. G-proteins are localized on the inner side of the cell membrane and are responsible for signal transduction. They are present in an inactive form consisting of 3 subunits, α [bound to guanosine diphosphate (GDP)], β and γ (Marinissen and Gutkind, 2001). G-proteins are freely scattered in the cell membrane, enabling them to interact with several receptors. Once an agonist is bound to the transmembrane receptor it induces a conformational change in the receptor enabling it to bind the αβγ complex. When the protein complex attaches to the receptor the GDP bound to the α-subunit is exchanged for guanosine triphosphate (GTP). This change causes a disassociation of the α-GTP from the βγ-subunits and the release of two new active complexes. The G-protein returns to its inactive form through interchanging GTP for GDP in the α-subunit and re-forming the αβγ complex. (Rang, 2007, Kroeze et al., 2003, Fredholm et al., 2007) (Figure 1-5). As one GPCR can activate several G-protein complexes, the specificity of the signal is given by the molecular variation of the α-subunit. In the heart there are 4 main classes of α-subunits, $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$ (Fredholm *et al.*, 2007). These variants show specificity in the receptors they bind and the pathways they activate.

Although, many of the signalling pathways that individual G-protein subunits are coupled to have been identified, many are still not well understood, particularly in

cardiomyocyte hypertrophy. For example, some studies of the $G_{\alpha i}$-subunit have shown that it inhibits adenylyl cyclase, and consequently cAMP decreases, resulting in impaired Ca^{2+} handling leading to hypertrophy and alterations in cardiac rhythm (McCloskey et al., 2008, Ruan et al., 2007). However, activation of the $G_{\alpha i}$-subunit stimulates Akt and inhibits cardiomyocyte hypertrophy (Moore *et al.*, 2007). Furthermore, $G_{\alpha s}$ has been proposed to activate adenylyl cyclase, while $G_{\alpha i}$ inhibits it, activating potassium channels and cGMP phosphodiesterases (Rang, 2007). $G_{\alpha 12/13}$ regulates the Na^+/H^+ exchanger, and $G_{\alpha q/11}$ activates PLC β and increases IP3 and DAG (Jalili *et al.*, 1999b, Rang, 2007).

1.4.5.1 Cell signalling in Hypertrophy

To stimulate hypertrophy AngII acts mainly through the $G_{\alpha q/11}$-subunit (Sadoshima and Izumo, 1993, Bai *et al.*, 2004). The initial response of a G-protein coupled receptor to activation by an agonist is a transient increase in cytoplasmic Ca^{2+} (Thomas *et al.*, 1996, Emkey and Rankl, 2009) which leads to downstream signalling effects through specific pathways and molecules.

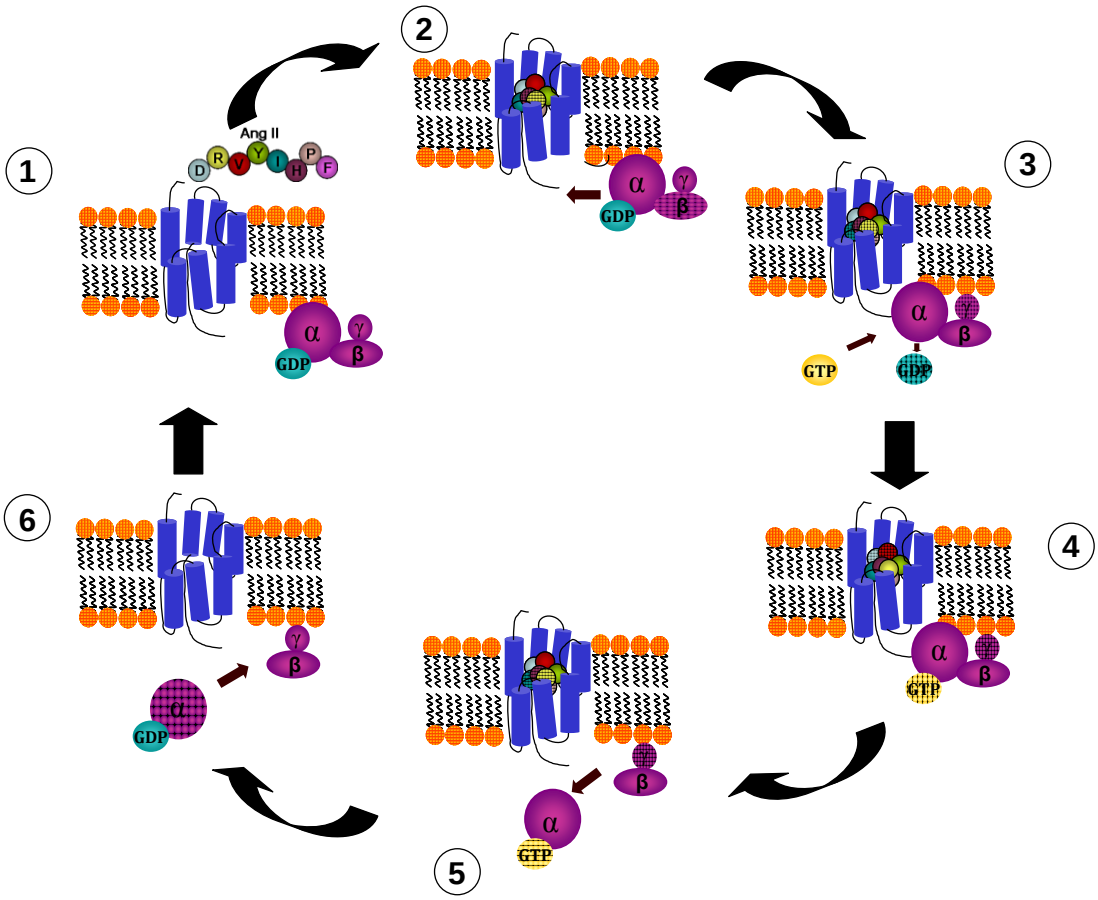


Figure 1-5. G-protein coupled receptor activation.

(1-2) Binding of an agonist to a G-protein coupled receptor results in conformational changes that bind the receptor to the G-protein subunits α, β and/or γ. (3-4) Once a G-protein is bound to the receptor the α-subunit interchanges GDP for GTP, (5) this causes disassociation of the α-subunit. (6) G-proteins are inactivated via the interchange of GTP for GDP in the α-subunit and reassociation with subunits β and γ. Imaged modified from:

http://www.scientificblogging.com/adaptive_complexity/blog/surprise_gprotein_coupled_receptor_aficionado

The PLC/PKC Pathway

Cardiomyocyte hypertrophy induced via $G_{\alpha q}$ is classically through activation of the PLC pathway (Figure 1-6). PLC is a membrane bound enzyme that cleaves the cell membrane component phosphatidylinositol bisphosphate (PIP_2) (Jalili *et al.*, 1999a). This phospholipid is then split into IP_3 and DAG second messengers. IP_3 is water soluble and is released to the cytosol where it act as a second messenger and binds to its receptor present in the endoplasmic reticulum and sarcoplasmic reticulum, opening Ca^{2+} channels and increasing cytoplasmic Ca^{2+} (Putney *et al.*, 1986, Taylor *et al.*, 2009). The increase in cytoplasmic Ca^{2+} promotes migration of PKC from the cytoplasm to the cell membrane by binding to the C2 domain of PKC (Disatnik *et al.*, 1994). Once in the membrane DAG binds to the C1 domain of PKC and activates the enzyme (Jalili *et al.*, 1999a, Kim and Iwao, 2000, Rang, 2007). There are several isoforms of PKC and they are grouped into three categories: the classical PKC are activated by Ca^{2+} and DAG, novel PKC require DAG but not Ca^{2+} to be activated and the atypical PKC which are independent of both DAG and Ca^{2+} (Palaniyandi et al., 2009).

Not all PKC isoforms are translocated to the membrane as this process depends on the isoform of the Receptor for Activated C-Kinase (RACK) PKC binds. RACKs are specific for each PKC subtype and help determine their compartmentalization, so while PKCα, βII and ζ are translocated from the cytosol to the membrane, PKCϵ translocates from the nucleus to myofibrils (Jalili et al., 1999b). This family of enzymes is implicated in regulating transcription factor activity, activating immune responses as well as cell growth, among other functions (Churchill et al., 2008, Malhotra et al., 2001). In cardiomyocyte hypertrophy it has been demonstrated that PKC isoforms cause an increase in the transcription factors c-jun and fos as well as activating MEK pathways through activation of Raf (Liao et al., 1997, Mehta and Griendling, 2007)(Dostal et al., 1997). Recently it has also been observed that the distribution of PKC isoforms is different, for example the Ca^{2+}-dependent isoforms (α, βI and βII) are mainly found in the ventricle while PKC δ and ζ are found mainly in the atria. However, different isoforms are also activated through different stimuli and induce different features of hypertrophy (Churchill et al., 2008). Furthermore, studies have shown that PKCα increases α-actin mRNA levels and ANP secretion

(Church et al., 1994); conversely other studies have shown that PKC ε activation leads to increased levels of β-MHC and skeletal α-actin while no induction of ANP was observed (Takeishi et al., 2000). Although its role is not completely understood it is clear that PKC activity is integrally linked to the development of cardiac hypertrophy.

The MAPK pathway

As well as G-protein coupled receptors, an alternative class of small G-proteins exist which reside in the cytosol and act independently of agonist-mediated receptor activation. The Ras superfamily represents this group and their activation by AngII leads to phosphorylation of the MAPK pathway. This pathway has four branches of downstream activation: ERK 1 and 2, JNKs, p38 and ERK 5 (Figure 1-7) (Molkentin and Dorn, 2001).

The ERK1/2 branch initiates with the translocation of Raf1 to the cell membrane which is promoted by Ras, and is related to activation of $G_{\alpha q}$ (Molkentin and Dorn, 2001). Also there is evidence of Raf1 activation through PKC (Liao *et al.*, 1997)(Liebmann, 2001). Raf phosphorylates MEK1/2 and activates ERK1/2 by dual phosphorylation of the threonine and tyrosine residues (Thr183 and Tyr185) (Muslin, 2005, Rang, 2007, Lorenz *et al.*, 2009a). It has been shown that this dual phosphorylation of ERK1/2 is essential for its activation (Muslin, 2005). ERK1/2 then, phosphorylates different substrates in the cytoplasm and nucleus including the transcription factors GATA binding protein 4 (GATA-4) and Elk-1 which are integrally involved in cardiac hypertrophy (Pikkarainen *et al.*, 2004, Aplin *et al.*, 2007). GATA-4 has been shown to bind to motifs in promoter regions of hypertrophic genes such as α-MHC, ANP, BNP and myosin light chain-3 (Grepin et al., 1994, McGrew et al., 1996, Thuerauf et al., 1994), whereas Elk-1 activates transcription of the early gene c-fos (Babu *et al.*, 2000).

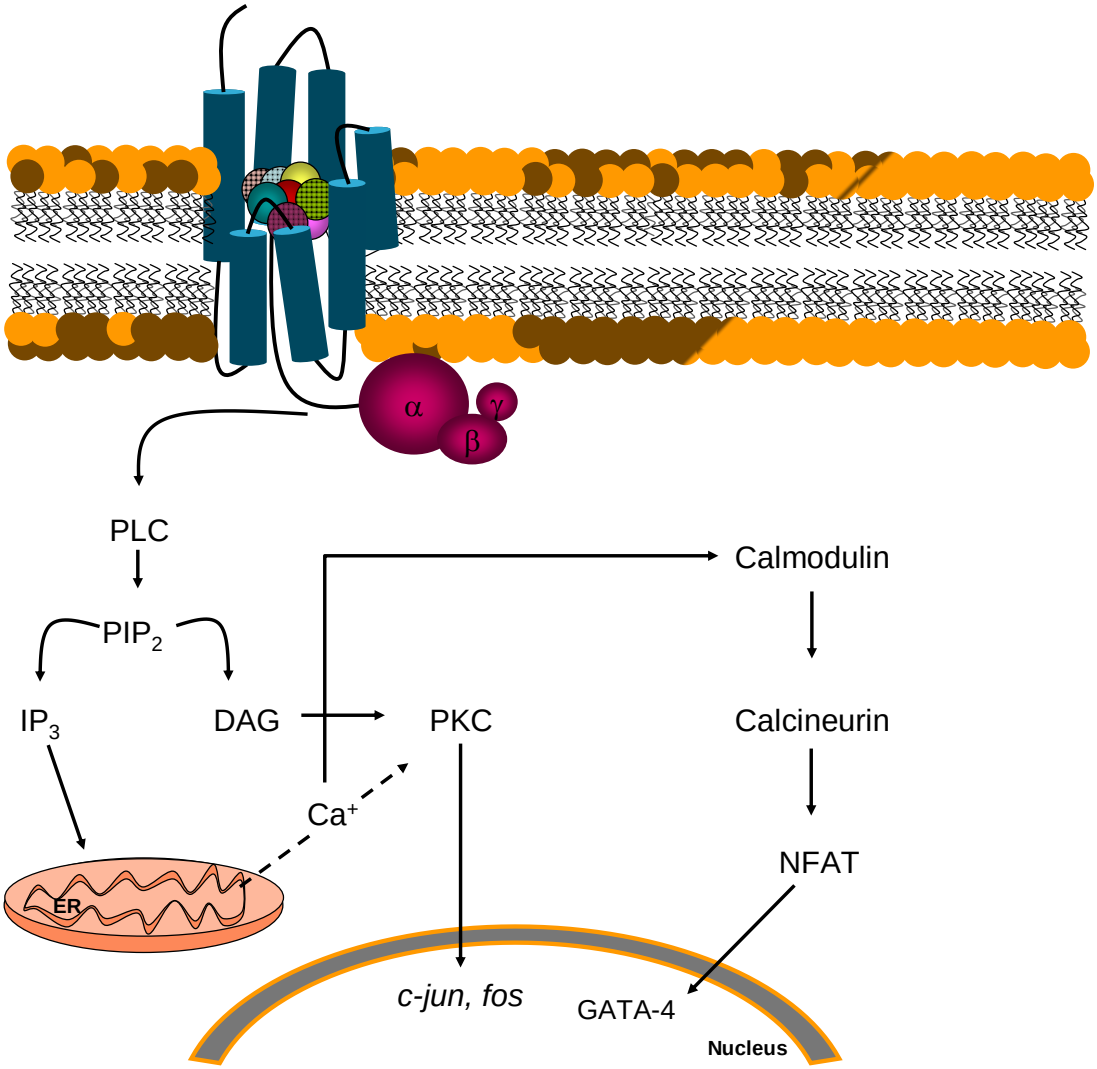


Figure 1-6. Intracellular PLC/PKC and calcium cell signalling pathways in cardiac hypertrophy

Schematic showing the PLC/PKC intracellular signalling pathway that leads to cardiomyocyte hypertrophy. AngII binds to the AT1-GPCR which leads to activation of PLC. PLC promotes membrane translocation of PKC. Also PLC increases intracellular Ca^{2+} levels activating calmodulin/calcineurin/NFAT signalling pathway (taken from Heineke, J and Molkentin, J. Molecular Cell Biology. 2006).

ERK1/2 stays in the cytoplasm by interacting with G-protein coupled receptor-β-arrestin complexes (Aplin et al., 2007). Interestingly Alpin *et al.* showed that when ERK1/2 is activated by this pathway and retained in the cytoplasm, it does not phosphorylate any of the transcription factors already described (Elk-1, c-fos), and does not cause cardiomyocyte hypertrophy, showing a differential phenotype between activation of ERK1/2 by the $G_{\alpha q}$-subunit and the receptor-β-arrestin complex (Aplin *et al.*, 2007). The mechanisms that target ERK1/2 to the nucleus are unclear. Recently, Lorenz *et al* showed a direct interaction of ERK1/2 with the $G_{\beta\gamma}$-subunit, which led to an autophosphorylation of ERK1/2 in the threonine residue 188 (Thr188) (Lorenz *et al.*, 2009a). This led to translocation of ERK1/2 to the nucleus and promotion of cardiac hypertrophy. Importantly, in order for the $G_{\beta\gamma}$-subunit to interact with ERK1/2, the complex Raf/MEK/ERK1/2 has to be formed.

JNK are kinases that phosphorylate c-Jun. The initiation of the pathway for JNK is less characterised. Ras phosphorylates mitogen-activated protein kinase kinase kinase (MEKK) which in turn MEK, in this case MEK 4/7 (Molkentin and Dorn, 2001). MEK 4 /7 then activates JNK at a dual motif formed by threonine, proline and tyrosine amino acids (Ito *et al.*, 1999). Of the 3 isoforms of JNK, JNK3 is expressed mainly in brain while JNK1 and 2 are expressed ubiquitously, including in the heart (Ito *et al.*, 1999, Molkentin and Dorn, 2001, Liang and Molkentin, 2003). Activated JNK phosphorylates AP-1 which is stimulated by c-Jun and c-fos proteins (Liang and Molkentin, 2003) and AP-1 increases transcription of genes, for example ANP, skeletal α-actin and TGF-β1 (Kim and Iwao, 2000). Conversely, JNK also has an antihypertrophic effect by inhibiting NFAT, demonstrating an overlapping effect of this kinase (Liang and Molkentin, 2003). This illustrates the complexity of JNK signalling as it is part of 2 counter-regulatory signalling pathways.

P38 is a stress-stimulated kinase activated through MEKK (Molkentin and Dorn, 2001). Activated MEK 3/6 phosphorylates p38 in the motif formed by threonine-glycine-tyrosine (Park *et al.*, 2001, Tanno *et al.*, 2003) leading to its activation. There are four isoforms of this kinase, α, β, γ and δ, but p38α is the most abundant isoform in heart tissue (Gum et al., 1998, Wilson et al., 1996). Once activated, p38 regulates several transcription factors including MEF2, MAPK2 and 3, Elk-1, among others (Molkentin and Dorn, 2001, Muslin, 2008). Although p38 is a stress-

stimulated kinase, it has been demonstrated that AngII increases p38 phosphorylation in neonatal rat ventricular cardiomyocytes (Li *et al.*, 2009a) and transgenic hypertensive mice (Pellieux *et al.*, 2000).

Calcium mediated pathways

The Ca^{2+}/calmodulin/calcineurin/NFAT pathways have also been implicated in cardiac hypertrophy (Figure 1-6). Calcineurin is a serine/threonine protein-phosphatase consisting of a catalytic subunit of 59 to 61 kDa (CnA) and a regulatory subunit of 19 kDa (CnB) (Klee *et al.*, 1998). Calcineurin becomes activated when the $Ca2^{+}$-binding adaptor protein calmodulin, saturated with Ca^{2+}, binds to it (Heineke and Molkentin, 2006). Calcineurin, then, binds directly to the transcription factor NFAT in the cytoplasm and promotes its translocation to the nucleus, where it regulates expression of hypertrophy-related genes such as GATA-4, MEF-2, ANP and BNP (Diez *et al.*, 2001, Heineke and Molkentin, 2006, Liu *et al.*, 2008).

1.4.5.2 Regulation of hypertrophy-associated cell signalling pathways by the RAS

Within the development of cardiac hypertrophy the role of individual RAS components in cell signalling pathways in the heart have been studied. ACE 2 knockout mice developed severe cardiac dysfunction characterized by decreased myocardial contractility, but however with no signs of cardiac hypertrophy or fibrosis (Crackower *et al.*, 2002). However, in an independent study in ACE 2 knockout mice no effect on cardiac function or size was observed (Gurley *et al.*, 2006). In myocardial infarction the loss of ACE 2 promotes fibroblast proliferation by activating ERK1/2 and JNK1/2, as well as inflammation by increasing neoutrophil infiltration to the infarct zone, leading to an increase in inflammatory components such as cytokines including IL-6, and a deficiency in MMP2 which leads to disruption of the extracellular matrix (Kassiri *et al.*, 2009). It was also found that in these mice the lack of ACE 2 induced the upregulation of hypoxic genes and levels of AngII were increased. In a rat model of myocardial infarction, lentiviral overexpression of ACE 2 also had a protective role by rescuing cardiac function through improved cardiac output, ejection fraction and contractility,

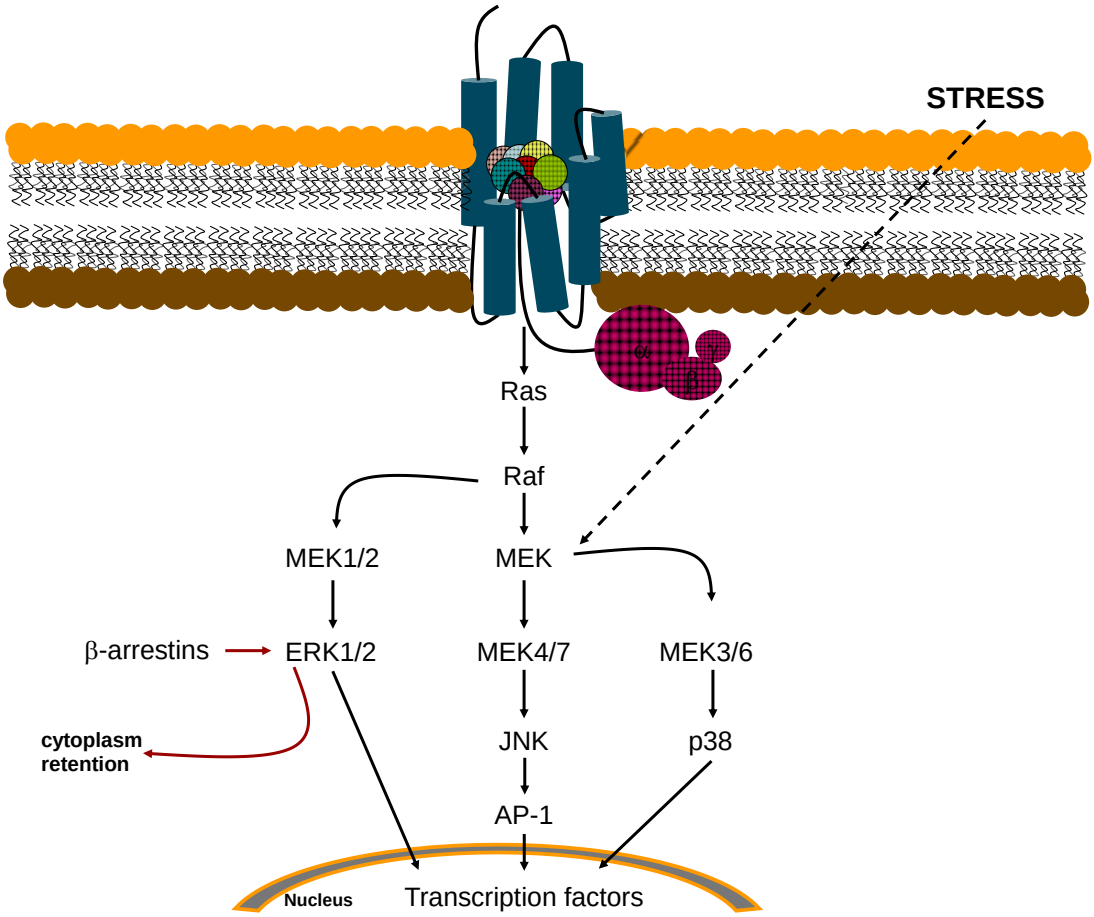


Figure 1-7. Intracellular MAPK signalling pathway in cardiac hypertrophy

Schematic showing the MAPK signalling pathway that leads to cardiomyocyte hypertrophy. GPCR activation leads to activation of Ras/Raf. Raf activates 3 different branches, 1. MEK1/2 that phosphorylates ERK1/2. ERK1/2 is translocated to the nucleus where it activates different transcription factors. ERK1/2 association with B-arrestins leads to ERK1/2 retention in the cytoplasm. 2. Raf phosphorylates MEK which activates MEK4/7-JNK pathway. 3. MEK is also phosphorylated by stress, in this case MEK phosphorylates MEK3/6 which further phosphorylates p38.

as well as reducing left ventricular wall thinning (Der Sarkissian *et al.*, 2008). Furthermore, transfection of fibroblasts with an ACE 2 expression plasmid attenuated collagen and cytokine production (Grobe *et al.*, 2007a). In similarity to these findings, ACE 2 knockout mice develop defects in conduction and contraction (Crackower *et al.*, 2002). These studies suggest a protective role for ACE 2 in heart failure. However studies have also shown a negative role. Donoghue *et al.* showed that cardiac specific overexpression of ACE 2 in transgenic mice caused severe and progressive malfunction of rhythm and conduction leading to ventricular tachycardia and sudden death (Donoghue *et al.*, 2003). This group also showed that ACE 2 overexpressing transgenic mice had mild interstitial fibrosis. Masson *et al.* also showed cardiac fibrosis and deficits in ejection fraction and fractional shortening in SHRSP overexpressing ACE 2 mediated by adeno-associated virus serotype 6 delivery (Masson *et al.*, 2009). In this study ACE 2 increased the expression of collagen type IIIα1, fibronectin and lysyl oxidase genes, suggesting a profibrotic action of ACE 2. In accordance expression of genes involved in cardiac maintenance (apelin, myosin heavy chain II and GATA6) were diminished. Therefore further research is required to address the true role of ACE 2 in cardiovascular physiology and pathophysiology.

In the heart it has been shown that Ang1-7 regulates many growth cell signalling pathways (Ferreira and Santos, 2005). In Sprague-Dawley rats coinfusion of AngII and Ang1-7 blocks the cardiac phosphorylation of ERK1/2 and Rho kinases induced by AngII alone and furthermore the effects of Ang1-7 were abolished in the presence of the Mas antagonist A779 (Giani *et al.*, 2007). Interestingly, Ang1-7 also activated STAT3 and STAT5a/b in the heart, however this effect was not abolished by A779, but was by an AT1R antagonist suggesting its involvement. Ang1-7 has also been shown to have a role in recovery following ischaemia/reperfusion injury (Zhang et al., 2008, Al-Maghrebi et al., 2009, Castro et al., 2006). In rat models of myocardial infarction, treatment with Ang1-7 has been demonstrated to prevent left ventricular deterioration (Loot *et al.*, 2002). In this same study Ang1-7 blocked the deterioration in aorta relaxation by preserving endothelial function. Al-Maghrebi *et al* treated diabetic rats with Ang1-7 before inducing ischemia and demonstrated recovery of left ventricular function after ischemia an effect that was abolished by the co-infusion of A779 (Al-Maghrebi *et al.*, 2009). At the molecular level Ang1-7

decreased the levels of NF-κB and the expression of genes needed for activation of NF-κB; including toll-like receptor 2, IL-1 receptor associated kinase 1 and inhibitor of kappa B kinase (Al-Maghrebi *et al.*, 2009). Furthermore an inhibitory effect for Ang1-7 in inflammatory pathways was demonstrated as it reduced expression of complement component C3, IL-1β, IL-6 and caspase 1.

One of the most studied actions of Ang1-7 is in cardiac remodelling. Many authors have confirmed that Ang1-7 has some effect in at least one of the components of cardiac remodelling. Grobe *et al.* demonstrated reduced myocyte hypertrophy, interstitial fibrosis and TGF-β levels in response to Ang1-7 in an Ang II induced rat model of hypertension and cardiac remodelling (Grobe *et al.*, 2007b). These effects of Ang1-7 were also blocked in the presence of A779, and were independent of effects on blood pressure. Studies with transgenic mice overexpressing Ang1-7 in the heart have shown that in the presence of AngII hypertrophy and fibrosis of the left ventricle is reduced, as well as expression of the hypertrophy markers ANP and BNP, and TGF-β1 (Mercure *et al.*, 2008). In these mice Ang1-7 had no effect on AngII-induced ERK1/2 phosphorylation but reduced AngII-induced phosphorylation of c-Src and p38 kinases. One important finding was the fact that Ang1-7, both in the presence or absence of AngII, increased the levels of SHP-1 protein (Mercure *et al.*, 2008). Dias-Peixoto *et al.* investigated the molecular mechanisms of the actions of Ang1-7 in cardiac hypertrophy (Dias-Peixoto *et al.*, 2008). An increase in NO phosphorylation due to Ang1-7-mediated activation of the PI3K/Akt signalling pathway was observed and these actions were blocked by co-treatment with A779, confirming the importance of Mas. Ang1-7 also promoted the phosphorylation of eNOS at Ser177 which augmented enzyme activity. Furthermore, this study showed that in cardiomyocytes of Mas knockout mice a lack of nitric oxide production due to decreased Akt activity, led to decreased phosphorylation of eNOS at Ser177 and therefore decreased activation (Dias-Peixoto *et al.*, 2008). Caveolin-3 expression, which inhibits basal activity of eNOS, was also increased; and heat shock protein 90 (Hsp90) (a scaffold protein which recruits Akt) was also decreased. Furthermore in normal cardiomyocytes Ang1-7 was not able to produce changes in Ca^{2+} transient parameters but curiously in Mas knockout ($Mas^{-/-}$) cardiomyocytes Ang1-7 showed a smaller peak in the intracellular Ca^{2+} transient and slower Ca^{2+} kinetics, which was explained by a decreased expression of the sarcoplasmic reticulum Ca^{2+} ATPase

(SERCA2a) (Dias-Peixoto *et al.*, 2008). This suggests that Ang1-7 modulates Ca^{2+} handling by increasing nitric oxide and eNOS activity leading to activation of SERCA2a.

In cardiac fibrosis, exposure of AngII-stimulated cardiac fibroblasts to Ang1-7 inhibited collagen synthesis and expression of the growth factors endothelin-1 and leukaemia inhibitory factor (Iwata *et al.*, 2005). It has also been described that Ang1-7 normalises the decreased levels of matrix metalloproteinases (MMP) in AngII-stimulated cardiac fibroblasts and myocytes (Pan *et al.*, 2008). In addition, Grobe *et al.* showed that Ang1-7 was able to prevent interstitial fibrosis by decreasing collagen deposition in the deoxycorticosterone acetate (DOCA) salt hypertensive rat model (Grobe *et al.*, 2006). In this model Ang1-7 did not prevent the increase in ventricular mass or myocyte diameter or have any effect on blood pressure. In nephrectomised mice Ang1-7 prevented left ventricular remodelling and preservation of cardiac function, and also diminished interstitial fibrosis by reducing the levels of TGF-β and increasing MMP2 and 9 (Li *et al.*, 2009c). Ang1-7 also decreased the expression levels of inflammatory cytokines and suppressed oxidative damage (Li *et al.*, 2009c).

In the cardiovascular system $Mas^{-/-}$ mice have been widely used and the results of these studies have contributed to the knowledge of Ang1-7 actions (Lemos *et al.*, 2005). $Mas^{-/-}$ mice have been used to show the involvement of Mas in heart function. For example, Santos *et al.* characterized the phenotype of $Mas^{-/-}$ mice and showed lower global ventricular function (Santos *et al.*, 2006). Lack of Mas caused a decrease in systolic tension and heart rate, as well as left ventricular posterior wall thickness and decreased fractional shortening. At a molecular level, $Mas^{-/-}$ mice have increased expression of type I and III collagen and fibronectin, while type IV collagen expression is decreased. In accordance with these findings, Castro et al showed a decrease in cardiac contractility in $Mas^{-/-}$ mice (Castro *et al.*, 2006).

The RAS is integral to the normal physiology of the cardiovascular system and furthermore its dysregulation is important in the development of many CVDs. A clear role for AngII acting via the AT1R to activate downstream cell signalling pathways has been established. These downstream effects are pleiotropic and occur

in many different cells and tissues including smooth muscle cells, endothelial cells, fibroblast and cardiomyocytes. However, there still remain many unanswered questions on the integrated function of the RAS. The true role of the AT2R and whether it does antagonise the AT1R signalling is controversial. Other peptide metabolites of the RAS also play a role in CVD development. This is particularly evident for Ang1-7 which appears to antagonize the effects of AngII in many cells and tissues in CVD models via the receptor Mas. The role of other angiotensin metabolites, e.g. Ang1-9, AngIV, in the development of CVD phenotypes is less clear.

1.5 Aims

Investigate Ang1-9 and Ang1-7 function in cardiomyocyte hypertrophy in rat neonatal (H9c2) and primary adult rabbit left ventricular cardiomyocytes *in vitro*.

Assess the effects of Ang1-7 and Ang1-9 *in vivo* in the stroke prone spontaneously hypertensive rat model.

Develop vectors for expression of RAS peptides that will be useful for probing RAS pathways *in vitro* following delivery to individual cells and tissues. Assess the effect of gene transfer from these vectors on cardiac hypertrophy in an *in vitro* model.

C H A P T E R 2

Materials and Methods

2.1 Chemicals

All chemicals unless otherwise indicated were obtained from Sigma Chemical Company (Dorset, UK). Angiotensin peptides were purchased from Sigma or Phoenix Pharmaceuticals (Karlsruhe, Germany). Pharmacological receptor antagonists were purchased from Sigma (Losartan, Captopril, PD123,319) or Bachem (Rhein, Germany) (A779).

2.2 Tissue culture

All tissue culture reagents were purchased from Lonza, (Braine-L'Alleud, Belgium) unless otherwise indicated. H9c2, an immortalized cardiomyocyte cell line derived from neonatal rat cardiomyocytes and HeLa cells (an immortalized cell line derived from cervical cancer) cell lines were purchased from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK). The 293 cell line (adenovirus type 5 transformed human embryonic kidney cells) was purchased from Microbix Biosystems (Toronto, Canada). Adult rabbit heart tissue was a gift from Dr. Anthony Workman (BHF Glasgow Cardiovascular Centre, University of Glasgow, UK).

2.3 Cell culture

All tissue culture involving primary, transformed, genetically manipulated or other animal tissue was performed under containment level 2, in sterile conditions, using a vertical laminar flow hood. H9c2 cardiomyocytes, HeLa and 293 cell lines were cultured in a humidified chamber at 37ºC in 5% CO_2/95% air in Dulbecco´s Modified Eagle's medium supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM L-glutamine and 1 IU penicillin, 100 µg/mL streptomycin (Invitrogen, Paisley, UK). H9c2 cells were passaged by trypsin-ethylenediamine tetra-acetic acid (trypsin-EDTA) when the culture reached 50% confluence. HeLa and 293 cells were passaged upon reaching 90% confluence. Briefly, cells were washed twice with sterile Dulbecco´s calcium and magnesium free phosphate buffered saline (PBS),

and incubated with 2 ml of trypsin- EDTA (H9c2 and HeLa cells) or citric acid (293 cells) at 37°C for approximately 5 minutes. Once the majority of the cells were detached the action of the trypsin-EDTA were blocked with an equal volume of 100% FCS. Cells were harvested by centrifugation at 500 g and re-suspended in fresh media before plating.

2.4 Cryopreservation

For cryopreservation cells where collected as described in section 2.3. After centrifugation, cells were resuspended in complete cell culture media supplemented with 10% volume/volume (v/v) dimethyl sulphoxide (DMSO) at an approximate density of 1-2 x 10^6 cells/mL and aliquoted into cryo-preservation tubes. Tubes were cooled at a constant -1ºC / minute to -80ºC using isopropanol, for long term storage in the vapour phase of liquid nitrogen.

To resuscitate cells from cryo-preservation, vials were thawed at 37ºC. The cell suspension was carefully transferred to a universal container. 10 mL of complete cell culture was added drop by drop and the cell suspension subjected to centrifugation at 500g to pellet the cells and discard the DMSO. The cell pellet was resuspended in 1mL of complete culture media and cells were transferred to a T-150 flask containing 25 mL of pre-warmed cell culture media and then incubated at 37ºC / 5% CO_2 / 95%.

2.5 Primary cardiomyocyte isolation

Adult rabbit left ventricular cardiomyocytes were used as the primary cell model due to lack of availability of rat primary cardiomyocytes. Twenty weeks of age adult rabbit left ventricular cardiomyocytes were isolated by cardiac retrograde aortic reperfusion. The full heart was digested with collagenase type I (400 U/ml) at 37ºC. The left ventricle was chopped into small pieces and placed into a sterile tube containing Kreb's solution (20 mM NaCl, 20 mM HEPES, 5.4 mM KCl, 0.52 mM NaH_2PO_4, 3.5 mM MgCl2, 20 mM taurine, 10 mM creatine, glucose and bovine serum albumin (BSA) at 37°C, pH 7.4, at 37ºC). Cells were isolated by cycles of: inverting the tube constantly for ten minutes to enable the tissue to sink to the bottom

of the tube and then the supernatant was transferred into a fresh sterile tube, until the supernatant turned clear. Cells were then allowed to pellet by gravity over a period of approximately 30 minutes and the supernatant discarded carefully with a sterile pasteur pipette. Cells were resuspended in Kreb's solution and washed 4 consecutive times with Kreb's supplemented with $CaCl_2$ at 100 μM, 400 μM, 700 μM and 1 mM, respectively. Between washes cells were allowed to pellet by gravity and the Kreb's discarded carefully. Following the final wash cells were gently resuspended in M199 medium supplemented with 5 mM taurine, 5 mM carnitine, 5 mM creatine and 1 IU penicillin, 100 μg/mL streptomycin pre-heated at 37ºC. Cells were then plated in 6 well plates at a density of $3x10^4$ cells/well in supplemented M199 medium and used immediately for experiments.

2.6 Hypertrophy model

A hypertrophy model was established in H9c2 cells and adult rabbit left ventricular primary cardiomyocytes.

H9c2 cells were seeded at a density of $3x10^4$ cells per well and maintained at 37ºC in a humidified chamber 24 hours prior to initiation of experiments. Cells were then changed to serum free media for 1 hours, followed by stimulation with 100 nM AngII and cells incubated for 96 hours at 37°C in order to induce hypertrophy. After 96 hours of incubation, cells were washed twice with PBS and fixed with 2% (w/v) paraformaldehyde (PFA) for 15 minutes on ice. PFA was removed with 2 washes of PBS and 2% (v/v) crystal violet added overnight to stain the cells. Cell size was measured with ImageProPlus 4.1 software (Media Cybernetics, USA). Alternatively, cells were stimulated with 1 μM arg-vasopressin in a similar manner to that described above. Cell size was assessed by measurement of cell length. For each condition 100 cells were measured (ten fields of view in each condition) and each experiment was repeated 3 times.

To investigate the role of Ang1-7 and Ang1-9 in hypertrophy H9c2 or adult rabbit primary left ventricular cardiomyocytes were incubated with either Ang1-7 or Ang1-9 30 min before addition of AngII or arg-vasopressin. Alternatively, agonists for Mas (MBP7 10 μM and 100 μM) and AT2R (CGP42112A 10 nM, 100 nM and 1

μM) at the indicated concentrations were added 30 minutes before adding AngII. To assess receptor use the following antagonists were used at the indicated concentrations: ATR1 (Losartan 1 μM, 10 μM), ATR2 (PD123,319 100 nM, 500 nM, 1 μM) Mas receptor (A779 10 μM), B_2 bradykinin receptor (HOE 140 1 μM) and the ACE inhibitor (Captopril, 1 mM). Antagonists were added 15 minutes before addition of Ang1-7 or Ang1-9.

Left ventricular rabbit cardiomyocytes were seeded as above following extraction and then stimulated with AngII (500 nM) or arg-vasopressin (1 μM), followed by incubation for 24 hours at 37°C. In primary cardiomyocytes length and midpoint width was measured to calculate volume of the cell. Cell volume was determined via a modified calculation as first described by Satoh *et al.* (Satoh *et al.*, 1996). Using confocal microscopy, Satoh et al. reconstructed the volume of spherical latex beads. Based on this data they were able to calculate a correction factor for spherical aberration of 0.68. Assuming cardiomyocytes as an ellipsoid and applying the correction factor, volume was calculated as:

$$\text{Volume} = (\text{Width} \times \text{Length} \times \pi) \times 0.68$$

Ang1-7 (500 nM) and Ang1-9 (500 nM) were added 30 minutes before the addition of the prohypertrophic peptide (AngII or arg-vasopressin). Losartan (1 μM), PD123,319 (500 nM) and Captopril (10 μM) were added 15 minutes before Ang1-7 or Ang1-9.

For pertussis toxin (PTX), cells were seeded in 6 well plates at $3x10^4$ cells per well and incubated for 8 hours. Media was exchanged for culture media (DMEM, 10% (v/v) FCS, 2 mM L-glutamine, 1 IU penicillin, 100 μg/mL streptomycin, 1% (v/v) sodium pyruvate) supplemented with PTX (25 ng/ml) and cells incubated overnight. Media was exchanged for serum free media supplemented with PTX (25 ng/ml), and Ang1-7, Ang1-9 and AngII stimulation was performed as described above.

2.7 Protein extraction

Protein was extracted from unstimulated and AngII and/or Ang1-7 or Ang1-9 stimulated H9c2 cardiomyocytes seeded in 6 well plates by washing twice with PBS and adding 300 µl of lysis buffer containing 50 mM tris(hydroxymethyl) aminomethane (Tris)-HCl, 150 mM NaCl, 2 mM EDTA, 2 mM ethylene glycol tetraacetic acid (EGTA), 0.2% (v/v) Triton-X, 0.3% (v/v) nonyl phenoxylpolyethoxylethanol (NP-40), 100 mM phenylmethanesulfonyl fluoride, 1 M sodium fluoride (NaF) and 1 x proteinase and phosphatase cocktail (Sigma, Dorset, UK) to each well. Each well was scraped, the lysed cells collected in a microcentrifuge tube and then incubated on ice for 1 hour, with agitation every 15 minutes. Samples were subjected to centrifugation at 12,000 g to pellet the cell debris. Protein containing supernatant was transferred to a new microcentrifuge tube and the pellet discarded.

2.7.1 Determination of protein concentration

Protein concentration in samples was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, Illinois, USA), a colorimetric detection and quantification protocol, following manufacturer's instructions. Briefly, a standard curve was generated using the following BSA dilutions: 2000 µg/mL, 1500 µg/mL, 1000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 25 µg/mL. 200 µL of working reagent was added to 25 µL of sample or standard in duplicate in a 96 well plate, and incubated for 30 minutes at 37ºC in the dark. The plate was then analysed on a Wallac Victor^2 plate reader (Wallac, Turku, Finland) with absorbance at 570 nm. Results were then calculated according to the linear equation based on the standard curve generated.

2.8 Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblotting was used to detect specific proteins. First, samples were prepared at the appropriate concentration with 5x loading buffer containing: 10%

weight/volume (w/v) SDS, 30% (v/v) glycerol, 10% (v/v) Tris-HCl pH 6.8, 0.01% (w/v) bromophenol blue and 2% (v/v) β-mercaptoethanol. Samples were heated at 95ºC for 5 minutes to denature the protein, mildly cooled and loaded into the well.

The polyacrylamide (PA) gel consisted of a 4% stacking gel containing 13.3% (v/v) N,N'-methylene-bis-acrylamide (polyacrylamide 30%), 25% (v/v) Tris pH 6.8 (3.75 mM), 0.1% (v/v) SDS, 1% (v/v) ammonium persulphate (APS) and 0.1% (v/v) of N,N,N',N'-Tetramethylethylenediamine (TEMED). Depending on the molecular mass of the protein to be detected different percentage resolving gels were used: To detect the Ang1-7 and Ang1-9 fusion protein (32 kDa) (section5.1) following adenoviral gene transfer a 12% resolving gel which contained 40% (v/v) of PA (30%), 25% (v/v) of Tris pH 8.8 (11.25 mM), 0.1% (v/v) SDS, 1% (v/v) APS and 0.1% (v/v) TEMED was used.

Following sample loading, gels were electrophoresed at 100 V through the stacking gel, then switched to 200 V for electrophoresis through the resolving gel in running buffer (0.025 M Tris-HCl, 0.2 M glycine, 0.001 M SDS) for approximately 3 hours. Protein was then transferred to Hybond-P polyvinylidene difluoride membrane (Amersham Bioscience UK Limited, Buckingham, UK), to enable antibody binding and detection. Protein transfer was performed using an electric current which promotes protein migration from the gel to the membrane. Protein transfer was performed overnight at 80 mV in transfer buffer [0.025 M Tris, 0.2 M glycine, 20% (v/v) methanol, 0.01M SDS] at 4ºC. To confirm the samples had transferred correctly, the gel was stained with Coomassie brilliant blue stain [0.5% (w/v) Coomassie Brilliant Blue R-250 (Biorad, Hemel Hempstead, UK), 50% (v/v) methanol and 10% (v/v) glacial acetic acid) for 30 minutes and then destained with destaining solution containing 40% (v/v) methanol and 10% (v/v) glacial acetic acid for another 30 minutes.

2.9 Western immunoblotting

Once proteins were transferred to the membrane, antibody detection was performed. The membranes were first blocked in TBS-T [150 mM NaCl, 50 mM Tris, 0.1% (v/v) Tween-20] + 10% (w/v) fat-free milk powder (blocking buffer) for 8 hours at

4ºC. Membranes were incubated with the primary antibody diluted in blocking buffer at the recommended dilution (Table 2) overnight at 4ºC with shaking.

Following overnight incubation the membrane was washed twice in blocking solution for five minutes each at room temperature, followed by incubation with an 1:2000 dilution of the appropriate secondary antibody, rabbit anti-mouse or swine anti-rabbit horseradish peroxidase (HRP) (Neomarkers, Fremont, CA, USA) for 1 hour at room temperature with shaking. The membrane was then washed six times for 15 minutes each at room temperature with shaking, four times with blocking solution and two times with tris buffered saline-tween (TBS-T). Proteins were visualized using Enhanced Chemiluminescent (ECL) Detection System (Amersham Biosciences UK Limited, Buckingham, UK) following the manufacturer's instructions. Films were exposed for various lengths of time, ranging from 10 seconds to overnight.

Antibody				Dilution	Stock Concentration
Name	**Specie**	**Clone #**	**Company**		
Anti-Mouse IgG2b	Rabbit polyclonal	Ab9171	Abcam	1:250	4 µg/mL
Anti-PKC	Mouse monoclonal	MC5	Abcam	1:100	2.70 mg/mL
FITC-labelled	Rabbit anti-mouse polyclonal	F0232	Dako	1:40	400 mg/L

Table 2. Primary antibodies and conditions used in western blot protein detection.

2.10 Immunocytochemistry

H9c2 cardiomyocytes were plated on to silane-coated coverslips at $3x10^5$ cells per coverslip. 24 hours later cells were stimulated for different times and then fixed with 4% (w/v) paraformaldehyde for 15 minutes on ice. PFA was removed following two washes in PBS and the cells permeabilised with 0.1% (v/v) Triton-X-100 for 30 minutes at room temperature. Cells were washed 3 times in PBS, and incubated for 1 hour with the primary antibody, mouse monoclonal anti-PKC (diluted 1:100 in PBS and 20% (v/v) rabbit serum; 22 µg/mL). The primary antibody was removed by washing the cells 3 times with PBS, for 5 minutes. Next, cells were incubated with secondary fluorescein isothiocyanate (FITC)-labelled antibody (Dako, Ely, UK) (rabbit anti-mouse antibody diluted 1:40 in PBS and 20% (v/v) rabbit serum) for 30 minutes in the dark. Next, cells were washed 3 times for 5 minutes each with PBS to remove secondary antibody and then mounted onto a glass slide using Vectashield (Vector laboratories, Peterborough, UK) with propidium iodide as nuclear counterstain.

2.11 Phalloidin staining

Phalloidin (Sigma, Dorset, UK) is a fungal toxin able to bind to the F-actin subunit of actin filaments in cells. Phalloidin was used to stain actin filaments and image changes in their organization in H9c2 cardiomyocytes stimulated with AngII and/or Ang1-9 or Ang1-7. H9c2 cardiomyocytes were seeded at $3x10^4$ cells per well on coverslips. Hypertrophy was stimulated as described previously (section 2.6). After 4 days cells were washed twice with PBS, fixed with 4% (w/v) PFA for 20 minutes at room temperature, followed by two washes in PBS before permeabilization with 0.1% (v/v) Triton X-100 for 20 minutes. Cells were washed in PBS and stained with phalloidin solution [5 µg/ml (v/v) Phalloidin-FITC, 1% (w/v) BSA in PBS] for 1 hour at room temperature protected from light. Unbound phalloidin was removed by washing in PBS and coverslips mounted onto a glass slide using Vectashield containing 4',6-diamidino-2-phenylindole (DAPI).

2.12 RNA extractions

To extract RNA from H9c2 cardiomyocytes, cells were plated at $3x10^5$ cells per well in 6 well plates. RNA was extracted using the RNeasy mini kit (Qiagen, Crawley, West Sussex, UK). Briefly, cells were lysed and homogenized with RLT buffer which contains denaturing guanidine isothiocyanate. RLT buffer immediately inactivates RNases and ensures the extraction of the intact RNA. 70% (v/v) ethanol was then added to facilitate binding to the spin column and sample was loaded to an RNeasy spin column and subjected to centrifugation at 8,000 g for 15 seconds in order to bind the RNA onto the membrane. Once bound, the RNA sample was washed 3 times (700 µL of RW1 buffer x 1 and 500 µL of RPE buffer x 2) and subjected to centrifugation at 8,000 g for 15 seconds to eliminate contaminates. To elute purified RNA 30 µL of dH_2O was added to the column and spun at 8000g for 1 minute. Samples were stored at -80ºC.

2.13 DNase treatment

In order to remove the contaminating DNA from RNA extractions Turbo DNA-*free* kit (Applied Biosystems, Warrington, UK) was used. Following the manufacturer's instructions a 50 µL reaction was generated consisting of 40 µL RNA, 0.5 µL 10x buffer and 1 µL DNase. The reaction was incubated for 30 minutes at 37ºC in a water bath. DNase activity was suspended by adding DNase inactivation reagent (0.1% volume) and followed by incubation for 2 minutes at room temperature. To pellet the DNase inactivation reagent the reaction was subjected to centrifugation at 10,000 g for 1.5 minutes and the RNA transferred to a fresh Eppendorf.

2.14 Quantification of nucleic acids

Nucleic acids (DNA and RNA) were quantified using a Nanodrop Spectophotometer (Thermo Scientific, Loughborough, UK). This apparatus measures the pulse light produced by a xenon flash lamp that passes through the sample, using a linear charged-coupled device (CCD) array. Calculation of the amount of nucleic acid is

based on a modified Beer-Lambert equation with ng/µL as units. The equation is as follows:

$$\mathbf{c = (A * \varepsilon)/b}$$

c = the nucleic acid concentration in ng/µL

A = the absorbance in arbitrary units (AU)

ε = the wavelength-dependent extinction coefficient in ng / µL

b= the pathlength in cm

The extinction coefficients used for nucleic acids are:

• Double-stranded DNA: 50 ng/µL

• Single-stranded DNA: 33 ng/µL

• RNA: 40 ng/µL

The purity of the sample was assessed by calculating the A_{260}:A_{280} ratio. A ratio of ≥ 1.8 was considered pure.

2.15 Complementary deoxyribonucleic acid (cDNA) synthesis

mRNA extracted from cells was reverse transcribed into complementary DNA (cDNA). The Reverse Transcription Kit (Applied Biosystems, Warrington, UK) was used following the manufacturer's instructions to reverse transcribe 1 µg of mRNA in a 20 µL reaction. The reaction contained: 5.5 mM $MgCl_2$, 2 mM total deoxyribonucleotide triphosphates (dNTPs) mix (0.5 mM each), 8 U random hexamers, 25 U Multiscribe, and 1µg of RNA. Cycling conditions were as follow: 25ºC for 10 minutes for preannealing, 48ºC for 30 minutes to enable reverse transcription and 95ºC for 5 minutes for reverse transcription inactivation. CDNA was stored at -20ºC.

2.16 Polymerase chain reaction (PCR)

PCR is a technique used to amplify at a large scale a specific DNA sequence generating millions of copies. This method utilises differential thermal cycling conditions, where double stranded DNA is heated to separate the DNA strands through breaking the hydrogen bonds, then cooled to facilitate primer annealing followed by re-heating to elongate the DNA by the action of a thermostable DNA polymerase. This is followed by a final step of re-annealing of the new DNA strands. These steps are repeated to exponentially increase the amount of the DNA template. PCR was used to detect expression of Mas and AT2R in H9c2 cardiomyocytes. Primers were designed based on the Genebank sequences (Table 3). PCR reactions were as follows: 10 ng of cDNA in a 25 µL reaction containing 200 µM of each dNTP, 2 mM $MgCl_2$, 200 nM forward and reverse primers and 1 U *Taq*® DNA polymerase (Promega, Southampton, UK) in 1x PCR reaction buffer. Samples were submitted to 35 thermal cycles of denaturing at 95ºC for 1 minute, annealing at primer-specific temperatures (for details see table 3) for 1 minute and extension at 72ºC for 1 minute. PCR products were analysed following electrophoresis in an agarose gel (section 2.22.2.1).

PCR Primers		Sequence	Melting Point
Mas receptor	**Forward**	5'-CCATCAGTGTGGAGAGATGCCT-3'	68ºC
	Reverse	5'-CAGTAGTACCAGTGGTAATAGT-3'	62ºC
AT2 receptor	**Forward**	5'-CCCTGGCAAGCATCTTATGTAG-3'	66ºC
	Reverse	5'-GTCTTAATGGGCACTGGTTCAG-3'	66ºC
BNP	**Forward**	5'-CAGAACAATCCACGATGCAG-3'	57ºC
	Reverse	5'-CCTTTACCGAGTCTCTGTCG-3'	59ºC

Table 3. Primers used for gene expression detection.

Primers sequences used in PCR to detect gene expression of Mas, AT2R and BNP (brain natriuretic peptide).

2.17 Quantitative real-time polymerase chain reaction (QPCR)

QPCR is a laboratory technique used to amplify and quantify a target DNA sequence. This technique follows the same DNA amplification principle of standard PCR but with the production of a fluorescence signal that is proportional to the amount of product generated in each cycle. The fluorescence signal can be measured directly to give either an absolute number of copies or a relative amount when it is normalized to control DNA. There are two methods of quantification: using dyes that bind to double stranded DNA (dsDNA) or using fluorescent oligonucleotide probes. To quantify the expression of BNP the SYBR Green Detection System (Applied Biosystems, Warrington, UK) was used in which SYBR Green binds dsDNA. Reactions consisted of 1 µg cDNA, the SYBR Green PCR core reagents kit (6.25 µl v/v) and 400 nM of each specific primer (Table 3). QPCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) using the following conditions: 50 cycles at 95ºC for 10 minutes to denature DNA, 95ºC for 15 seconds to denature, 60ºC for 1 minute for annealing and elongation. This was followed by 95ºC for 15 seconds to dissociate, 60ºC for 15 seconds and 95ºC for 15 seconds.

BNP quantification was calculated relative to the expression of a control housekeeping gene 18S (Applied Biosystems, Warrington, UK). All samples were analysed in duplicate using TaqMan data analysis software.

2.18 Analysing calcium transients in single cells

Ca^{2+} stored in the endoplasmic reticulum is mobilized to the cytoplasm through a receptor mediated event, such as activation of a GPCR. To study calcium transient activation a single cell calcium assay was performed. Fura-2 Calcium Indicator (Invitrogen, Paisley, UK) is a fluorescent reagent that binds to intracellular calcium, changing its emission wavelength proportionally to the amount of Ca^{2+} bound enabling quantification. Based on this, intracellular Ca^{2+} can be estimated independently of dye concentration or cell thickness. H9c2 cardiomyocytes were

seeded on coverslips at $1x10^5$ cells per well, for 70% confluence the following day. Cells were loaded 1.5 µM Fura-2 in standard cell culture media, and incubated for 30 minutes at 37ºC. Coverslips were placed into a microscope chamber in a Nikon Diaphot inverted microscope equipped with a Nikon ×40 oil immersion Fluor objective lens (NA = 1.3) containing microscope buffer (130 mM NaCl, 5 mM KCl, 20 mM HEPES, 10 mM Glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) connected to a perfusion system and the microscope chamber was illuminated with an ultrahigh point intensity 75-W xenon arc lamp (Optosource, Cairn Research, Faversham, Kent, UK). A monochromator (Optoscan, Cairn Research) was used to alternate the excitation wavelength between 340/380 nm and control the excitation band pass (10 nm for 340 nm and 8 nm for 380 nm). Sequential images (2 x 2 binding) were taken every 60 ms, with exposure to excitation light for 100 ms / image. AngII, Ang1-7, Ang1-9 and ionomycin agonists (Sigma, Dorset, UK) were diluted to a concentration of 1 µM in microscope buffer, while vasopressin was diluted to 10 µM. Cells were perfused with agonists after 60 basal images were recorded and further 140 images recorded following stimulation. Agonist was then removed, 200 images recorded and cells then perfused with the positive control agonist (ionomycin 1 µM or vasopressin 10 µM). Cell images were obtained using a Nikon Diaphot inverted microscope coupled to a Nikon (Tokyo, Japan) 40x oil immersion Fluor objective lens. To monitor Fura-2 fluorescence emission at 510 nm, a high-resolution interline-transfer cooled digital camera (Cool Snap-HQ, Roper Scientific/Photometrics, Tucson, AZ, USA) was used. MetaFluor imaging software (Universal Imaging Corp., Downing, PA) was used for control of the monochromator, charge-coupled device camera, and for processing of the data.

2.19 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Assay

CellTiter 96® Non-radioactive Cell Proliferation Assay (Promega, Southampton, UK) was used to determine viable cell number in the hypertrophy assay. This assay is based on the conversion of a tetrazolium salt to a formazan product by viable cells. H9c2 cardiomyocytes were plated at a seeding density of $3x10^4$ cells per well and the hypertrophy assay protocol followed (section 2.6). After the 96 hours incubation

period, dye solution containing tetrazolium was added to the culture medium of each well in a 15:100 ratio and incubated for 4 hours at 37°C in 5% CO_2 / 95% air. After incubation 1 mL of Solubilization Solution/Stop Mix was added to each well to solubilize the formazan product. Absorbance was recorded at 570 nm using a Wallac Victor^2 plate reader (Wallac. Turku, Finland).

2.20 Animal husbandry and *in vivo* experimentation

To study the role of Ang1-9 the SHRSP was used (Figure 2-1). SHRSP are maintained "in-house" by brother-sister mating. Routine in lab microsatellite screening is performed to confirm homozygosity of all loci within a random group of the strain. All animals are housed under controlled environmental conditions, maintained with a 12 hours light/dark cycles at ambient temperature. Rats were fed standard rat chow (rat and mouse No.1 maintenance diet, Special Diet Services) and water provided *ad libitum*. Male rats were used in all experiments. Work with experimental animals was in accordance with the Animals Scientific Procedures Act 1986 under the project license 60/2874 held by Professor A.F. Dominiczak. At 11 weeks of age an exploratory transthoracic echocardiography was performed followed by implantation of the radio-telemetry probe to monitor blood pressure. 10 days after telemetry probe implantation (13 weeks of age), osmotic minipumps were implanted subcutaneously to secrete peptides/pharmacological reagents for 4 weeks. Two weeks following implantation of the minipump (15 weeks of age) a second echocardiogram was performed. At 17 weeks of age the third echocardiogram was performed before SHRSP were sacrificed and organs processed for analysis.

2.20.1 Radiotelemetry

Hemodynamics of SHRSP were monitored through the study from one week prior to implantation of the osmotic minipumps using the Radio-Telemetry Monitoring of Blood Pressure and Heart Rate Dataquest IV Telemetry System (Data Science International, Minneapolis, USA). Radiotelemetry provides 24 hours monitoring and data records of systolic and diastolic blood pressure, mean arterial pressure, heart rate and motor activity, as previously described (Davidson *et al.*, 1995). Briefly, before the transmitter (radio frequency transducer model TA11PA) was implanted,

calibration was performed to verify accuracy to within 3 mmHg. 11 week old SHRSP were anesthetised with 5% isofluorane and the flexible catheter of the transmitter was inserted in the abdominal aorta below the renal arteries, orientated against flow and sutured to the abdominal wall. Rats were housed in individual cages following the procedure. Each cage was placed on a receiver panel connected to a computer for data acquisition. Rats were unrestrained and free to move within their cages. Data was sampled every 5 minutes for 10 seconds. Following the procedure a recovery period of 7 days for blood pressure and heart rate return to normal was allowed (Davidson *et al.*, 1995) before interventions were initiated.

2.20.2 Osmotic minipump studies

Osmotic minipump secretion (Alzet, CA, USA) was used to assess the systemic effects of Ang1-9 in the SHRSP in the presence and absence of the AT2R blocker PD123,319 for 4 weeks. Control animals were implanted with a minipump secreting water. The osmotic minipump operates through an osmotic pressure difference between a compartment of the pump and the tissue where it is set (Figure 2-2). The compartment has a high osmolality causing water to enter, compressing the peptide reservoir and ejecting at a controlled and predetermined rate the agent inside. The secretion rate depends on the model and varies between 0.11 and 10 µL/h.

Osmotic minipumps secreted 100 ng/kg^{-1}/min^{-1} Ang1-9 or PD123,319 for 4 weeks following implantation. In order to determine the stock concentration of Ang1-9 required prior to filling the minipumps the following equation was used:

$$\mathbf{C = K/Q}$$

C = concentration of solution (µg/µL)
K = peptide delivered per day (µg/kg)
Q = release rate of the pump (µL/h)

The flow rate of the osmotic minipump used (Azlet # 2004) was 0.25 µL/h so the concentration required was:

C = (144 µg/kg) / (0.25 µL/h) = 5.76 µg/µL.

The maximum reservoir of the 2004 model is 200 µL, so to achieve a concentration of 5.76µg/µl, the amount of peptide that should be dissolved in 200 µL is:

$$(5.76\ \mu g/\mu L) \times 200\ \mu L = 1.2\ \mu g$$

Minipumps were filled the day before implantation and left overnight in isotonic solution at 37ºC to allow equilibration to be reached. Minipumps were subcutaneously implanted under anaesthesia 10 days after the surgical placement of the telemetry radio transducer. For double pump implantation, minipumps were implanted back to back in the same site.

2.20.3 Echocardiography (ECHO)

SHRSP were mildly anesthetized with 1.25% to 1.5% isofluorane in 1.5 L/min O_2 before image acquisition and were positioned in left lateral decubitus on a heated pad. An Acuson Sequoia c512 ultrasound system was used to acquire non-invasive 2-D guided M-mode images at a depth of 20 mm and at the tip of the papillary muscles. Left ventricle anterior and posterior wall thickness as well as chamber diameter were measured in a short axis view using the leading edge-to-lead edge convention during both systole and diastole. The velocity of blood passing through the mitral valve was measured with colour Doppler and pulse wave. The sample volume was placed at the tip of the mitral leaflets and adjusted to the maximal velocity position. The sample volume was set at 2.5 mm. This method was also used to qualitatively examine the valve for evidence of mitral regurgitation. All ECHO data was measured over at least three consecutive cardiac cycles, except for Doppler spectra which were recorded over 5 to 10 cardiac cycles at a sweep speed of 150 mm/s.

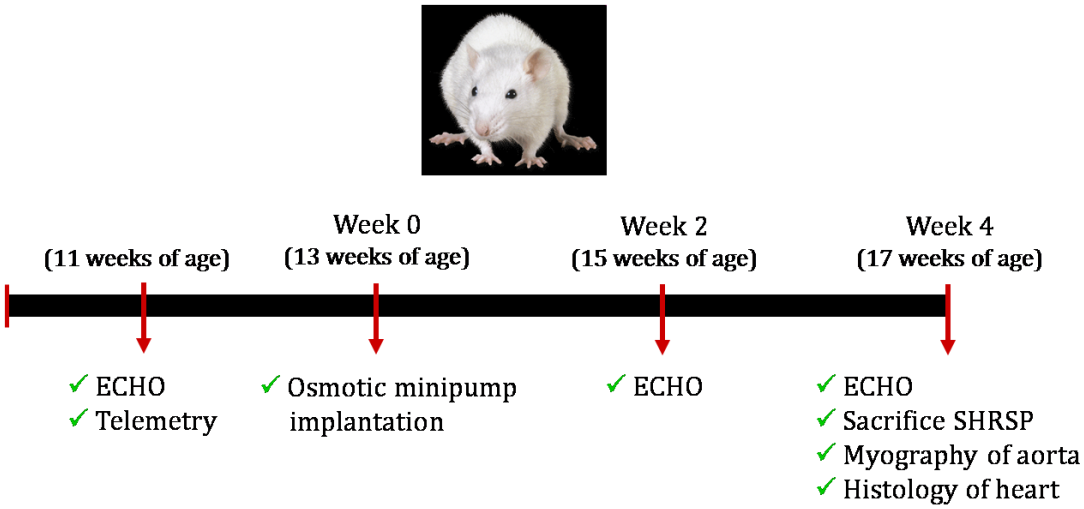


Figure 2-1. Experimental protocol diagram for SHRSP.

At 13 weeks of age SHRSP were infused with Ang1-9 peptide for 4 weeks. During this time blood pressure was monitored with a radiotelemetry probe implanted at the 11th week of age. Cardiac function was monitored with echocardiogram performed 3 times: pretreatment (11 weeks of age), during treatment (15 weeks of age) and at the end of the study (17 weeks of age). After SHRSP were sacrificed organs were collected for myography and histology analysis.

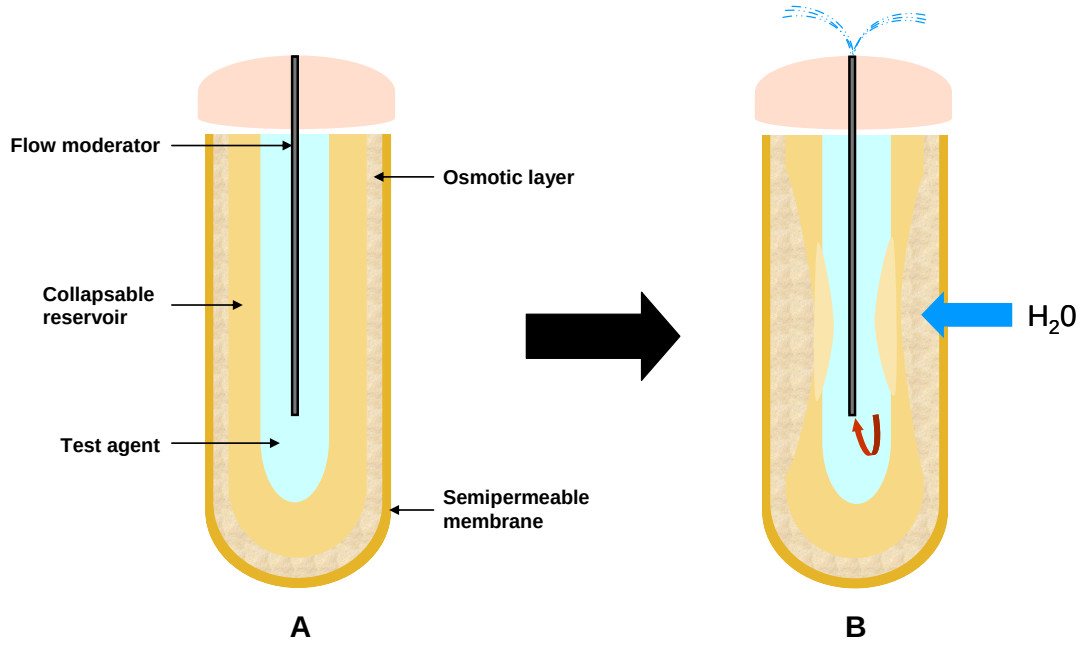


Figure 2-2. Schematic of osmotic minipump.

Osmotic minipumps function based on the difference in osmolarity between compartments. These pumps have a semipermeable membrane which allows water to enter the osmotic layer driven by its high osmolarity. (A) This osmotic layer surrounds the cylindrical collapsible reservoir containing the reagent for secretion. (B) When the pump is in an aqueous location the osmotic layer absorbs water collapsing the reservoir due to the hydrostatic pressure generated, resulting in secretion of the stored reagent [modified from Theeuwes *et al.* (Theeuwes and Yum, 1976)].

2.20.3.1 Formulae used in ECHO assessments

• **Ejection fraction** was defined as follows:

$$\mathbf{EF = [(LVEDV – LVESV) / LVESD \times 100]}$$

LVEDV = left ventricular end diastolic volume
LVESV = left ventricular end systolic volume

• **Fractional shortening** was derived from:

$$\mathbf{FS = [(LVEDD – LVESD) / LVEDD \times 100]}$$

LVEDD = left ventricular end diastolic diameter
LVESD = left ventricular end systolic diameter

• **Cardiac output** was derived from:

$$\mathbf{CO = [(ESV – EDV) \times HR]}$$

ESV = end systolic volume
EDV = end diastolic volume
HR = heart rate

• **Stroke volume** was calculated as follows:

$$\mathbf{SV = ESV - EDV}$$

ESV = end systolic volume
EDV = end diastolic volume

• **Left ventricular mass index** was calculates as follows:

$$\mathbf{LVMI = 0.8\ (1.04\ (LVAWd + LVIDd + LVPWd)^3 - (LVIDd)^3) + 0.6)}$$

LVAWd = left ventricular anterior wall in diastole
LVIDd = left ventricular internal diameter in diastole
LVPWd = left ventricular posterior wall in diastole

• **Relative wall thickness** was calculated as follows:

$$\mathbf{RWT = (AWTd + PWTd) / EDD}$$

AWTd = anterior wall thickness in diastole

PWTd = posterior wall thickness in diastole

EDD = end diastolic diameter

• **Change in interventricular septal wall thickness** was measured using the formula:

$$\mathbf{ISWT = [(AWTs – AWTd) / AWTs \times 100]}$$

AWTs = anterior wall thickness in systole

AWTd = anterior wall thickness in diastole

2.20.4 Tissue collection

SHRSP were sacrificed after 4 weeks of implantation of the minipump. Rats were deeply anesthetized with isofluorane and the thoracic cavity opened to expose heart and lungs. Thoracic aorta was excised from its insertion into the diaphragm and the aorta, lungs and heart isolated. Carotid arteries, kidneys, liver, spleen, muscle and brain were collected and either snap frozen in liquid nitrogen, embedded in optimal cutting temperature compound (OCT) (VWR, Lutterworth, UK) or fixed in 10% (v/v) formalin overnight for embedding in paraffin for histological analysis. A fresh section of the thoracic aorta was retained to perform large vessel pressure myography.

2.20.5 Large vessel pressure myography

Pressure myography is used to measure the physiological function of large vessels. In this technique, small segments of the vessel are mounted between two wire cannulae and a suitable transmural tension applied thereby enabling a study of the effects on myogenic response to certain vasoactive compounds.

Pressure myography was used to assess the thoracic aortic endothelial function of SHRSP in each group. To prepare thoracic aortas, vessels were cleaned from connective tissue (Figure 2-3 A and B), 2 mm rings cut and then mounted in a large vessel myograph baths containing Kreb's buffer (0.25 M NaCl; 0.001 M KCl; 2 mM $MgSO_4$; 50 mM $NaHCO_3$; 2 mM KH_2PO_4; 1 mM glucose; 2.5 mM $CaCl_2$) maintained at 37ºC, oxygenated with 95% O_2, 5% CO_2 (Figure 2-3 C and D). The vessel was pre-treated with potassium-buffered Kreb's (0.6 M KCl; 0.13 M $MgSO_4$; 0.25 M $NaHCO_3$; 11 mM KH_2PO_4; 0.1 M glucose; 0.025 M $CaCl_2$) followed by stimulation with PE and carbachol (1µM). Drugs were washed from the aortic rings and then rested for 30 minutes before cumulative concentration-response curves to PE (for contraction) and carbachol (for relaxation) (1 nM to 10 µM) were constructed. Aortic rings were then washed to remove drugs, rested for 30 minutes before treating the vessel with L-NAME (100 mM). After 20 minutes incubation period a concentration-response curve to PE (1 nM to 10 µM) was constructed.

2.20.6 Histology

Slides were coated with aminoalkylsilanes by placing blank slides in 2% (v/v) 3-aminopropyltriethoxysaline (APES) and acetone solution for 30 seconds. Slides were washed twice with acetone for 10 seconds followed by 2 washes with dH_2O for 5 minutes each. Slides were then dried at 37ºC overnight. Five µm sections were cut and mounted onto slides. Slides were baked for 3 hours at 65ºC and then at 40ºC overnight. Paraffin was removed from the tissue sample by 2 x 7 minute washes in Histoclear (Fisher Scientific, Leicestershire, UK). Samples were then rehydrated by passing through an ethanol concentration gradient (100%, 95%, and 75%) for 7 minutes each, followed by a final wash in dH_2O for 7 minutes.

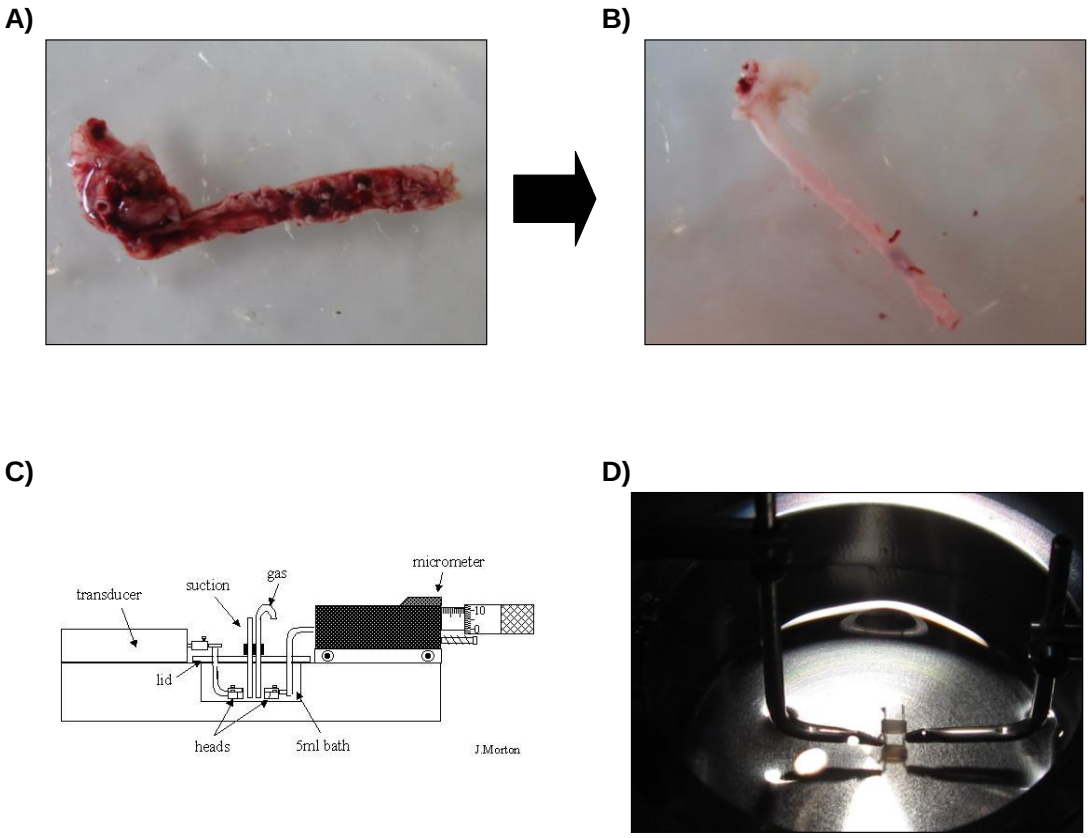


Figure 2-3. Pressure myograph.

(A) Thoracic aortas from each group were cleaned from connective tissue and (B) 2 mm aortic rings were cut to perform large vessel myography. (C and D) Aortic rings were placed in the heads of the bath to enable transmural tension on the vessel to be applied and controlled by the micrometer. The bath was filled with Kreb's buffer at 95% O_2/5% CO_2 pumped through the gas tube. Vessel contraction was recorded by the transducer and data collected.

2.20.6.1 Haematoxylin and eosin

Deparaffinised sections were stained with haematoxylin for 2 minutes, washed in running tap water for 10 minutes, and excess haematoxylin removed by dipping slides in 95% ethanol 10 times. Nuclei were counterstained in eosin for 2 minutes followed by a further 5 minutes wash in running tap water. Slides were dehydrated through increasing concentrations of ethanol (75%, 95% and 100%) for 7 minutes each. Sections were then cleared by 2 washes in histoclear (7 minutes each) and mounted in DPX (non-aqueous xylene based mounting media) (Sigma, Dorset, UK) for analysing. The nuclei stained blue while the cytoplasm stained pink.

2.20.6.2 Masson's trichrome staining

The Masson's Trichrome kit (Sigma, Dorset, UK) was used as per the manufacturer's instructions. Briefly, deparaffinised sections were incubated in Bouin's solution overnight at room temperature and the following day slides were washed under running water. Next, Weigert's Iron Haematoxylin solution was prepared by mixing equal parts of solution A (1% (v/v) haematoxylin in ethanol) and solution B [1.2% (w/v) ferric chloride and 1% (v/v) hydrochloric acid] and slides incubated for 5 minutes, followed by a 5 minutes wash under running dH_2O. Sections were incubated in Biebrich Scarlet-Acid Fuchsin (0.9% (v/v) Biebrich Scarlet, 0.1% (v/v) acid fuchsin, 0.1% (v/v) acetic acid) for 5 minutes, and then washed for 5 minutes in dH_2O. Slides were then placed in working phosphotungstic/phosphomolybdic acid solution (1 volume 10% (v/v) phosphotungstic acid, 1 volume 10% (v/v) phosphomolydic acid, 2 volumes of dH_2O) for 5 minutes and then in Aniline Blue solution (2.4% (v/v) aniline blue, 2% (v/v) acetic acid) for a further 5 minutes. Sections were then washed for 2 minutes in 1% (v/v) acetic acid followed by dehydration and mounted in DPX as described in haematoxylin and eosin section. The procedure stains cell nuclei black, the cytoplasm and muscle fibres appear red and collagen stains blue. Quantification of collagen stain was measured by transforming pixel values to optical density units using the Area of Interest selection tool and Macro within ImageProPlus software.

2.20.6.3 Picrosirius red staining

After deparaffinisation and rehydratation as described above, sections were incubated under dark conditions in 0.1% (v/v) picrosirius red solution (300 mg sirius red F3B, 300 ml saturated picric acid) for 90 minutes at room temperature. Sections were then washed twice for 5 minutes in 0.01 N HCl, followed by 2 washes of 5 minutes each with dH_2O. Sections were then dehydrated, cleared and mounted as described in haematoxylin and eosin section. Picrosirius red stains collagen bright red. Quantification of collagen stain was measured by transforming pixel values to optical density units using the Area of Interest selection tool and Macro within ImageProPlus software.

2.21 Plasmid cloning

2.21.1 Annealing of oligonucleotides

Oligonucleotides were resuspended in dH_2O to a concentration of 100 µM. To anneal the oligonucleotides 1 µL of each was added to 98 µL of dH_2O to a final reaction volume of 100 µL (final concentration of oligonucleotide 1 µM). Reactions were heated at 98ºC for 10 minutes and then cooled to 50ºC over 1 hour. Samples were subjected to electrophoresis in a 16% PA gel to confirm annealing (Section 2.22.2.2).

2.21.2 Plasmid preparation

A sample of glycerol stock of transformed bacteria containing the plasmid (see section 2.22.6) was streaked onto agar and incubated overnight at 37ºC to allow the bacteria to grow. Single bacterial colonies were picked to be amplified in 10 mL of Luria-Broth (LB) for 6 hours at 37ºC with shaking and then transferred to 500 mL of LB for overnight culture at 37ºC with shaking.

Plasmid DNA extraction

Plasmid DNA was isolated using either the QIAGEN® plasmid purification Maxi kit or the QIAprep® Miniprep kit (Qiagen, Crawley, West Sussex, UK).

● Small scale preparation of plasmid DNA (Miniprep)

This protocol is based on alkaline lysis (NaOH/SDS) of bacterial cells and the binding of DNA to a silica membrane in the presence of a high salt buffer followed by elution in low salt buffer. Briefly, individual plaques of competent bacteria transformed with the plasmid were collected and amplified in 5 mL LB overnight. Bacteria were pelleted and resuspended in 250 µL of P1 buffer (RNase A), lysed by addition of 250 µL of buffer P2 (NaOH/SDS) (which breaks down components of the bacterial cell wall and denatures proteins and chromosomal and plasmid DNA). The lysis buffer was neutralized by addition of 350 µL of N3 buffer (guanidinium chloride and acetic acid), which adjusts the reaction conditions to high salt binding and causes SDS, cellular debris and chromosomal DNA to precipitate. Precipitated samples were subjected to centrifugation at 18,000 g for 10 minutes at 4ºC and the supernatant transferred to the column containing a silica membrane to bind the plasmid DNA, followed by centrifugation for 1 minute at 4ºC. The eluted DNA was washed with 500 µL buffer PB (guanidinium chloride and propan-2-ol) to remove endonucleases and salts were removed by a further wash in 750 µL of buffer PE. DNA was eluted by incubation of the membrane with 50 µL of TE (Tris-EDTA) buffer and centrifugation at 18,000 g for 1 minute.

● Large scale preparation of plasmid DNA (Maxiprep)

This technique is based on the same principles of alkaline lysis as the Miniprep protocol, but utilises DNA binding via an anion-charge membrane under low salt and appropriate pH conditions. Briefly, a single colony of transformed competent bacteria was grown in 10 mL of LB for 6 hours at 37ºC with shaking at mid-log phase. The culture was then transferred to a new culture of 500 mL LB for amplification overnight at 37ºC with shaking. Bacteria were harvested by centrifugation at 6,000 g for 15 minutes at 4ºC. To lyse the bacteria the resulting pellet was resuspend in 10 mL P1 buffer (containing RNase A) and 10 mL of P2 buffer (NaOH-SDS), followed by incubation for 5 minutes in order to allow the SDS to disrupt the cell membrane and the NaOH to denature chromosomal DNA and proteins. The lysis step was then neutralized by the addition of P3 buffer (acidic potassium acetate) for 20 minutes incubation on ice. This incubation step triggers precipitation of potassium dodecyl sulphate, which traps denatured proteins and DNA. Samples were then subjected to centrifugation at 20,000 g for 30 minutes at

4ºC. The supernatant containing the plasmid DNA was transferred to the column allowing the solution to flow through the resin by gravity to permit binding of plasmid DNA to the resin. To completely remove residual RNA and cellular debris, the column was washed twice with 3 mL of buffer QC and plasmid DNA eluted via washing the column with 15 mL of buffer QF. Plasmid DNA was desalted and concentrated by precipitating with 10.5 mL isopropanol and subjected to centrifugation at 15,000 g for 30 minutes at 4ºC. DNA pellet was washed with 5 mL of 70% (v/v) ethanol, then subjected to a further step of centrifugation at 15,000 g for 10 minutes. Finally, the DNA pelleted was left to air dry for 30 minutes before resuspension in 200 µL 1x TE (pH 8.0).

2.22 Nucleic acid analysis and manipulations

2.22.1 Restriction endonuclease digestion

Restriction endonuclease digestion is a procedure to cut double stranded DNA by the action of a restriction endonuclease. These enzymes were isolated from bacteria for their ability to digest foreign double-stranded DNA at specific nucleotide recognition sequences in both strands (restriction sites). These restriction sites occur by chance in the DNA and are mainly palindromic, allowing the enzyme to cut the same sequence in both strands. Usually the restriction endonucleases leave an over-hanging end on each strand, termed the "sticky" end, facilitating ligation of nucleotide sequences with complementary "sticky" ends to generate recombinant DNA molecules. This technique was used to confirm plasmid DNA integrity and to prepare plasmid vectors for cloning. To verify plasmid DNA integrity 0.5-1.0 µg of plasmid DNA was digested in a 10 µL reaction containing 10 U of the specific restriction endonuclease and 1x of the appropriate reaction buffer. Reactions were incubated at 37ºC for 1 to 2 hours. To prepare plasmid vectors for cloning 50 – 100 µg of plasmid DNA was digested with 50 U of the restriction endonuclease and 1x reaction buffer in a 100 µl reaction at 37ºC overnight. Complete reactions were analysed via agarose gel electrophoresis (section 2.22.2.1).

2.22.2 Gel electrophoresis

Gel electrophoresis is used to separate nucleic acids or proteins using a crosslinked polymer set in an electrical current. Because DNA molecules and proteins carry a negative charge, when applying the electrical current, samples migrate towards the anode. When migration is performed through the polymer, the pores of the gel allow the separation of the nucleic acids or proteins depending on their size. The two main types of gel are agarose and acrylamide. They differentiate in the porosity of the polymer; acrylamide has smaller pores, so it is used for separating smaller fragments.

2.22.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis is typically used for analyzing PCR fragments or restriction endonuclease digest reactions. Products were electrophoresed through a 1% (w/v) agarose gel in 1x Tris/Borate/EDTA (TBE) (10 mM Tris, 10 mM boric acid, 10 mM EDTA, pH 8.3) and ethidium bromide (10 ng/mL). Typically, samples and DNA size markers (100 bp or 1 kb, depending on the size of the fragment of interest) were loaded in 6x loading gel (0.02% (w/v) bromophenol blue, 0.02% (v/v) xylene cyanole and 2.5% (v/v) glycerol) into pre-cast wells and current was applied. Gels were electrophoresed at a constant voltage of 90 to 100 V with TBE as a running buffer. Bands were visualised and photographed on a molecular imager ChemiDoc™ XRS+ Imaging System (Bio-Rad Laboratories, Hemel Hempstead, UK).

2.22.2.2 Polyacrylamide gel electrophoresis

In polyacrylamide gels the pores of the polymer are smaller, making it better suited to resolve small DNA fragments (up to a maximum of 1000 bp). PAGE was used to analyse single stranded oligonucleotides annealing efficiency. Oligonucleotides were analysed in 16% PA gels. Either 400 ng of annealed oligonucleotides or 200 ng of single stranded oligonucleotides were loaded in 6x loading gel (section 2.22.2.1). Gel was subjected to electrophoresis at a constant voltage of 100 V.

2.22.3 Gel extraction of DNA

This technique was design to extract PCR products or excised DNA fragments following restriction endonuclease digestion to eliminate excess nucleotides, primers or enzyme reaction reagents. The Wizard® SV Gel and PCR Clean-Up System (Promega, Southampton, UK) was used to extract plasmid DNA following the manufacturer's instructions. Briefly, following separation of plasmid DNA or PCR products by agarose gel electrophoresis, the gel portion containing the DNA segment of interest was cut with a clean scalpel blade and then transferred to a microcentrifuge tube containing membrane binding solution (4.5 M guanidine isothiocyanate, 0.5 M potassium acetate, pH 5) at a ratio of 10 µL of binding solution per 10 µg DNA. DNA was mixed well and incubated at 65ºC for 10 minutes (or until the gel was fully dissolved). The solution was then transferred to a minicolumn, incubated for 1 minute and then subjected to centrifugation at 16,000 g for 1 minute at 4ºC. The minicolumn was washed twice with Washing Solution (10 mM potassium acetate pH 5, 16.7 µM EDTA pH 8, 80% (v/v) ethanol) with 700 µL and 500 µL, respectively. After each wash samples were subjected to centrifugation at 16,000 g for 1 minute and then 5 minutes at 4ºC. Gel-purified DNA was eluted with 50 µL of TE and centrifugation at 16,000 g for 1 minute. DNA was stored at -20ºC.

2.22.4 Dephosphorylation of plasmid DNA ends

To avoid recircularization of digested plasmid DNA in ligation reaction the 5' phosphates from digested plasmid DNA were removed. Briefly, 5 U Shrimp Alkaline Phosphatase (SAP) (Promega, Southampton, UK) was added per 2.5 µg of plasmid DNA and the samples incubated for 15 minutes at 37ºC followed by inactivation of the SAP by 15 minutes incubation at 65ºC.

2.22.5 Ligation of oligonucleotides into digested plasmid DNA

To ligate annealed oligonucleotides the dephosphorylated plasmid DNA T4 DNA ligase (Promega, Southampton, UK) was used. Ligation reactions were performed at

ratios of 5:1, 3:1, 1:1, 1:3 and 1:5 molar ratio of vector:insert were performed to maximize the chance of obtaining the correct colonies. To calculate ratios the following formula was used:

$$\textbf{ng of insert} = \frac{\textbf{(ng of vector) x (kb size of insert)}}{\textbf{kb size of vector}} \textbf{ x (molar ratio)}$$

for example:

ng of vector = 50 ng

kb size of insert = for Ang1-7 0.048 kb

for Ang1-9 0.054 kb

kb size of the insert = 1 kb

1 U of T4 DNA ligase in a final reaction volume of 10 µL was added and samples incubated at 4ºC for 3 hours, before proceeding to transform mixture into competent bacteria to select ligated colonies (section 2.22.6).

2.22.6 Transformation of competent bacteria

DNA is a hydrophilic molecule and naturally repelled by cell membranes. In order for DNA to cross the cell membrane, bacteria have to be made "competent" by producing small holes in the bacteria cell membrane by suspending the cells in ice cold 50 mM $CaCl_2$. We transformed ligation reactions into competent *E.coli* JM109 (Promega, Southampton, UK). Briefly, 50 µL of competent bacteria were incubated with 5 µL ligation reaction for 30 minutes on ice allowing the DNA to bind to the membrane. Samples were then incubated for 1 minute at 42ºC in a water bath, heat-shocked and immediately put back on ice for 2 minutes. 450 µL SOC media (20g/L bactotryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose) was added and samples placed in an orbital shaker at 180 rpm / 37ºC for 1 hour. 250 µL bacteria was then pipetted onto a LB agar plate (10 g/L bactotryptone, 5 g/L bactoyeast extract, 5 g/L NaCl, 15 g/L agar, pH7.5) with 100 µg/mL of ampicillin, and sample was spread over the agar surface and incubated at 37ºC overnight.

Ratio	Ang1-7	Ang1-9
1:5	10.2 ng	13.5 ng
1:3	7.2 ng	8.1 ng
1:1	2.4 ng	2.7 ng
3:1	0.8 ng	0.9 ng
5:1	0.48 ng	0.54 ng

Table 4. Amount of Ang1-7 and Ang1-9 oligonucleotides duplexes used for plasmid ligation.

2.22.7 DNA sequencing

The dideoxy sequencing technique was used. This procedure is based on the use of dideoxynuclotides labelled with different fluorophores which have a missing hydroxy group at the 3' position, making it impossible for another nucleotide to bind, resulting in a series of different size DNA fragments which are visualized through the last nucleotide incorporated as a fluorescent labelled dNTP. The DNA sequencer utilises a laser beam focused in a constant position on the electrophoresis gel, to excite the fluorescence caused by the different fluorophores and according to the different wavelength that each fluorophore emits the sequencer translates the information to the 4 different nucleotides and generates a DNA sequence.

In order to confirm oligonucleotide sequence plasmid DNA was extracted from amplified single colonies of competent bacteria, see section 2.21.2. 300 ng DNA was used as a template for sequencing using a specific forward primer: 5'-TGGATGAATATAAACTCT-3'; and individual reverse primers (Table 5). Each sequencing reaction contained 1.6 nM primer (forward or reverse), 0.5 µL v.3.1 Ready reaction mix (Applied Biosystems, MA, USA), 3.5 µL v3.1 sequencing buffer (Applied Biosystems, MA, USA) in a 20 µL reaction. The cycle conditions were 25 cycles of 96ºC for 50 seconds to denature, 50ºC for 20 seconds to anneal and 60ºC for 3 minutes to extend the DNA sequenced fragment. The sequencing reaction was cleaned with CleanSEQ (Agencourt Bioscience Corporation, MA, USA) following the manufacturer's instructions. Results were then analysed using an ABI 3730 automated sequencer using SeqScape v2.0 analysis software.

2.22.8 Generation of glycerol stocks

For archiving of plasmid colonies glycerol stocks were generated. 800 µL of the bacterial culture containing the plasmid DNA was transferred to a 1.5 µL Eppendorf and 200 µL of sterile glycerol added. Glycerol stocks were stored at -80ºC.

2.23 Production of recombinant adenoviruses RAdAng1-7 and RAdAng1-9

Production of recombinant adenoviruses (RAd) was based on cotransfection of two plasmids via the method first described by McGrory *et al.* (McGrory *et al.*, 1988). Very briefly, two plasmids (an adenovirus genome vector and a shuttle vector containing a transgene expression cassette) are cotransfected into the 293 cell line [a human embryonic kidney cell line transformed with the entire left-hand end of the adenovirus serotype 5 genome containing the E1 gene, essential for virus replication (Graham *et al.*, 1977)] recombination between the 2 plasmids removes the early region 1 of the adenovirus and inserts the expression cassette encoding the transgene This produces replication deficient adenoviral vectors expressing the transgene that can be propagated in the 293 cell line.

2.23.1 Co-transfection of genome vector and shuttle vector for generation of recombinant adenovirus

Recombinant adenoviruses were generated in low passage 293 cells by recombination of pVQ-CMV-K-NpA shuttle vector (with the appropriate expression cassette in it) and the pacAd59.2-100 genome vector. Transfection of plasmid DNA was mediated by Ca^{2+}/phosphate-mediated gene transfer. This technique is commonly used to transfer DNA into the cell, via the DNA forming a complex with Ca^{2+} phosphate facilitating its association with the cell membrane, allowing the uptake of this precipitate by the cell and passive transport to the nucleus. Low passage 293 cells were plated at $2x10^6$ cells per 25 cm^2 tissue culture flask in order to reach 70-80% confluence next day. Cells were washed twice in PBS, and media replaced with 1.5 mL DMEM supplemented with 10% (v/v) FCS, 25 mM HEPES pH 7.9. Next, drop-wise addition of a solution containing 320 µL media (DMEM supplemented with 25 mM HEPES pH 7.1) 16 µL of 1 M $CaCl_2$ and 21 µg plasmid DNA (14 µg pacAd5 9.2-100 plasmid and 7 µg of pVQ-CMV-K-NpA-Ang1-7 or pVQ-CMV-K-NpA-Ang1-9) was performed with rocking of the flask. Flasks were incubated at 37ºC for 16 hours before washing the cells gently with PBS and

changing the media to 7 mL standard cell culture media. Cell media was replaced every 2 to 3 days until cytopathic effect was apparent.

2.23.2 Generation of crude adenovirus stock

Once the cytopathic effect was evident cells were harvested to extract recombinant adenoviral vectors. Adenovirus were recovered by lysing the cells using Arklone P (trichlorotrifluoroethane) (ICI Ltd, Cheshire,UK) or 3 freeze/thawing cycles in dry ice and a 37ºC water bath. Briefly, cells were collected in 50 mL tubes and subjected to centrifugation at 250 g for 15 minutes at room temperature. The pellet was resuspended in 1 mL PBS and an equal volume of Arklone P added. The tube was inverted for 10 seconds followed by shaking for 5 seconds. This step was repeated for 1 minute. Samples were subjected to centrifugation at 750 g for 15 minutes to form 3 layers. The upper layer consists of an aqueous solution, containing the virus, while the middle and lower layers contain cellular debris and solvent, respectively. The top layer containing the adenovirus was transferred to a fresh tube and stored at -80ºC until purification via CsCl density gradient centrifugation.

2.23.3 Plaque purification by end-point dilution of recombinant adenovirus

Crude stocks of recombinant adenovirus may be the result of several recombination events during the plasmid cotransfection in 293 cells. In order to generate stocks of single recombinant adenoviral vectors we purified the crude stock via serial dilutions in 293 cells. 293 cells were plated in a 96 well plate (10 wells in a row, 8 rows) at 50 to 60% confluence. Serial dilutions of crude adenovirus stocks were prepared at dilutions ranging from 10^{-2} to 10^{-11} in standard cell culture media. The media in each well of a row was replaced with 100 µL of sequential adenoviral dilutions (10 wells per row), leaving the bottom row for control cells (untransfected). The plate was then incubated in a humidified chamber at 37ºC in 5% CO_2 overnight. Media in each well was then changed for 200 µL of fresh media and every 2-3 days onwards until the 8^{th} day. Cells and media of 3 wells with a visible cytopathic effect at the highest dilution were collected for storage at -80ºC. For 1 well recombinant adenovirus was extracted by addition of an equal volume of Arklone P and a second round of plaque

purification performed. By the 8th day of the second plate, plaques of the highest dilution were consider plaque pure and were extracted by Arklone P (described above) and stored at -80ºC as recombinant adenovirus stock.

2.23.4 Generation of seed stocks of recombinant adenovirus

To generate seed stocks of recombinant adenoviral vectors, 50 µL of plaque pure virus was added to a 1x T-150 flask of 293 cells. Transduced cells were incubated for 3 to 5 days for the cytopathic effect to be complete. Cells were collected and subjected to centrifugation to pellet. The cell pellet was resuspended with Arklone P as previously described (section 2.23.2). Virus was aliquoted and stored at -80ºC.

2.23.5 Generation of high–titer stocks of recombinant adenovirus

To generate high-titer stocks of recombinant adenoviral vectors 293 cells were subcultured in 20-30 150 cm^2 tissue culture flasks. When cells were approximately 80-90% confluent they were infected with 50 µL of seed stock of recombinant adenovirus. Media was changed every 3 days until the cytopathic effect initiated, and then cells were fed with 10 mL of fresh media and incubated until all cells were detached from the flask. Once detached, cells were collected immediately to avoid prolonged incubation periods of detached cells which cause loss of virus titer. Cells were pelleted at 250 g for 10 minutes at room temperature and the pellet resuspended in 8 mL PBS, before extraction with Arklone P as described above, mixing it well for 1 minute.

2.23.6 CsCl density gradient purification

Virus extraction with solvents such as Arklone P leaves contaminating cellular debris in the preparation which may be cytotoxic when using the virus *in vitro and in vivo*. For this reason a CsCl density gradient was used to separate these cellular proteins and produce pure virus stocks. This method is also a simple and efficient method to concentrate adenovirus stocks. First 14 mL cellulose-nitrate ultra-clear

centrifuge tubes (Beckman Coulter Ltd, Buckinghamshire, UK) were sterilized with 70% (v/v) ethanol followed by rinsing in sterile dH_2O. In these tubes a 3 layer gradient was formed by pipetting a lower layer of 2 mL CsCl density 1.45 g/cm^3, followed by careful layering of 3 mL of CsCl density 1.32 g/cm^3 and finally 2 mL of 40% (v/v) glycerol. Next the crude adenovirus preparation was added drop-wise to the top layer and the tube topped up with PBS. Next tubes were loaded into a Sorvall Discovery 90 rotor bucket, placed in the rotor (RPS4OT-859) and subjected to ultracentrifugation at 90,000 g for 1.5 hours at room temperature with maximum acceleration and free deceleration. Following centrifugation a discrete white band is formed between the CsCl 1.45 and the CsCl 1.32 density layers containing the virus. To collect the band the tube was gently pierced with a 22 G needle and syringe just underneath the virus band, and the virus carefully collected into the syringe. The virus was then transferred to a Slide-A-Lyser dialysis cassette with a molecular weight cut off of 10,000 kDa (Perbio Science UK Ltd, Northumberland, UK) and dialysed for 2 hours against 2 L of 0.01 M Tris pH 8 / 0.001 M EDTA (dialysis buffer). After 2 hours the buffer was changed to 2 L of fresh dialysis buffer supplemented with 10% (v/v) glycerol and left overnight. Finally, the virus was removed from the dialysis cassette, aliquoted into 50 µL volumes and stored at -80ºC.

2.23.7 Titration of recombinant adenoviral stocks

Titration of recombinant adenoviral vectors was performed via two methods, determining the number of physical virus particles or by calculating the plaque forming unit (biological titer) per mL (pfu/mL).

2.23.7.1 Recombinant adenovirus particle titres

Viral particles titers were calculated by measuring the protein content of the virus stock by MicroBCA assay kit (Pierce, Rockford, IL, USA). Following manufacturer's instruction BSA standards ranging from 0.5 µg/mL to 200 µg/mL were prepared and 150 µL loaded in duplicate into wells of a clear 96 well plate. Next 1, 3 and 5 µL of virus stock in duplicate were added to the 96 well plate and the volume adjusted to 150 µL using PBS. Next, 150 µL of BCA working reagent was

added to all samples and standards and the plate incubated at 37ºC for 2 hours. Absorbance of each well at 570 nm was measured in a Wallac Victor^2 plate reader (Wallac, Turku, Finland). To calculate the protein content the blank absorbance was subtracted from the gross readings for each sample and standard. The value of the unknowns was then exported to the plotted standard curve to generate the virus particle titre according to the following formula described by Von Seggern *et al.* (Von Seggern *et al.*, 1998):

$$1\ \mu g\ protein = 1 \times 10^9\ viral\ particles$$

2.23.7.2 Titration of recombinant adenovirus by end-point dilution assay

This form of titration quantifies only the virus particles that are capable of forming a plaque. This technique is based on end-point dilution, through serial dilutions of the virus in 293 cells was described in section 2.23.3. On the 8th day of cell infection, pfu/ml was calculated according to the following equation (Nicklin, 1999):

The **proportionate distance** (PD) =

$$\frac{\%\ positive\ wells\ above\ 50\% - 50}{(\%\ positive\ wells\ above\ 50\%) - (\%\ positive\ below\ 50\%)}$$

and **log ID_{50}** (infectivity dose) =

log dilution above 50% + (proportionate distance x dilution factor)

For example; for a plate with 6 positive well at 10^{-10} dilution and 1 positive well at 10^{-11} dilution the calculation would be as follows:

$$PD = \frac{60\% - 50\%}{60\% - 10\%} = 0.2$$

$$\log ID_{50} = -10 + (0.2 \times -1) = -10.2$$

$$TCID_{50}\ (tissue\ culture\ infectivity\ dose) = \frac{1}{10^{-10.2}}$$

$$TCID_{50}/100\mu l = 10^{10.2}$$

X dilution factor (= 10)

$TCID_{50}/ml = 10^{11.2} = 1.584 \times 10^{11}$ $TCID_{50}/mL$

1 $TCID_{50} \approx 0.7$ pfu

Final titer = $(1.584 \times 10^{11}) \times 0.7 =$ **1.11×10^{11} pfu/mL**

2.24 *In vitro* infections

2.24.1 Optimization of virus infection dose with β-galactosidase expression

Different cell lines vary in the concentration of virus required for efficient transduction. In order to optimise adenoviral gene delivery to H9c2 cardiomyocytes a recombinant adenovirus containing a *lacZ* gene which expresses β-galactosidase (RAd35) (Wilkinson *et al.*, 1998) was used. This assay uses X-gal as a substrate for β-galactosidase (β-gal), converting it into a blue insoluble product, visualizing cells producing β-gal as a result of virus transduction.

H9c2 cardiomyocytes were seeded in 24 well plates at a density of $1x10^4$ cells per well and incubated overnight in a humidified chamber at 37ºC 5% CO_2. Cells were transduced with 10, 50, 100, 300, 500 and 1000 pfu/cell of RAd35 and incubated for 24 hours at 37ºC. Cells were then washed twice with PBS and fixed with 2% (w/v) PFA (Sigma, Dorset, UK) on ice for 15 minutes, the PFA removed by 2 washes in PBS, X-Gal stain (77mM Na_2HPO_4, 23 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN_6)$, 3 mM $K_4Fe(CN_6)$, 20 mg X-Gal) (Melford Laboratories Ltd, Ipswich, UK) added. Cells were incubated for 24 hours at 37ºC to facilitate staining. Cells were then visualized using light microscopy.

To assess RAdAng1-7 and RAdAng1-9 in the hypertrophy assay H9c2 cardiomyocytes were seeded in a 6 well plates at a density of $3x10^4$ cells per well and incubated overnight in a humidified chamber at 37ºC 5% CO_2. Cells were transduced with 500 and 1000 pfu/cell of RAdAng1-7 or RAdAng1-9 and incubated for 24 hours at 37ºC. Cells were then changed to serum free media followed by stimulation with 100 nM AngII and cell incubated for 96 hours at 37°C. After 4 days

of incubation cells were fixed with 2% (w/v) PFA, stained with 2% (v/v) crystal violet and cell size measures using Image ProPlus software as described previously. To asses receptor use A779 (10 μM) and PD123,319 (500 nM) were added at the same time as addition of RAdAng1-7 or RAdAng1-9.

To investigate RAdAng1-7 or RAdAng1-9 in adult rabbit primary left ventricular cardiomyocyte, these cells were seeded at $3x10^4$ cells per well and transduced with 500 and 1000 pfu/cell of RAdAng1-7 or RAdAng1-9 1 hour before stimulation with AngII, followed by incubation for 24 hours at 37°C. Length and midpoint width was measured and volume calculated as described.

2.25 Statistical analysis

Experiments were performed in triplicate on 3 different occasions. Data are shown as mean ± standard error of the mean (ME ± SE) and representative of three independent experiments. Paired Student's t test and one way ANOVA with Bonferroni's correction for multiple comparisons were applied and statistical difference was considered with p values <0.05.

In vivo experiments were performed with 6 animals per group. Comparison between groups for the myography data was performed by repeated measures ANOVA (analysis of variance) as described previously (Davidson *et al.*, 1995). Data from radio-telemetry was analysed by two way ANOVA with Bonferrroni's correction.

C H A P T E R 3

Assessment of the effects of AngII, Ang1-7 and Ang1-9 on cardiomyocyte hypertrophy

3.1 Introduction

The renin angiotensin system receptors AT1R, AT2R and Mas are all classified as GPCRs, however their mechanisms of action vary widely. Although, AngII is an agonist for the AT1R and AT2R, it is through the AT1R that AngII classically signals (Aplin *et al.*, 2009). AngII binds to the AT1R and induces hypertrophy through the $G_{\alpha q/11}$-subunit in vascular smooth muscle cells and by inducing transactivation of the epidermal growth factor receptor as well as phosphorylation of Rho-kinase (Ohtsu *et al.*, 2008). AngII also activates PLC beta 3 and the Erk1/2 pathway through $G_{\alpha q/11}$-subunit to induce cardiomyocyte hypertrophy (Lorenz et al., 2009b, Bai et al., 2004, Lorenz et al., 2009a). The function of the AT2R is less well understood. Although it is thought to act through the $G_{\alpha i}$-subunit there is conflicting evidence. Mukoyama *et al.* showed that the AT2R retained a 5-amino acid motif which impaired the coupling of a G-protein (Mukoyama *et al.*, 1993). However in 2000, Hansen *et al.* demonstrated activation of the $G_{\alpha i}$-subunit by stimulation of AT2R with AngII, AngIII or Ang1-7 (Hansen *et al.*, 2000). Very few studies have linked the Mas receptor to G-protein subunit activation, although there is evidence that $G_{\alpha q/11}$-subunit activation by Mas can lead to induction of AT1R expression (Canals *et al.*, 2006).

As well as the specificity in G-protein subunits in enabling coupling to specific cell signalling pathways homo and heterodimerization of GPCR has been shown to regulate activation of signalling pathways, adding further complexity to our understanding of cell signalling mechanisms (Milligan, 2007, Milligan, 2008, Schulte and Levy, 2007). Many studies have shown an interaction between the B2 bradykinin receptor and Mas receptor, where by Ang1-7 potentiates bradykinin-induced vasodilatation through the Mas receptor (Peiro et al., 2007, Ueda et al., 2001). Furthermore, Castro *et al.*, have shown an interaction between the AT1R, AT2R and Mas in murine hearts (Castro *et al.*, 2005).

Many cell signalling pathways converge on activation of sets of transcription factors. Transcription factors regulate the expression of certain genes and the primary response in many pathways is an increase in genes such as *fos*, *c-jun*, *myc* and *junB* within the first hour of stimulation. These genes are termed the immediate-early

genes and their expression is transitory, returning to normal levels after a couple of hours, independently of the presence of the stimuli (Kim and Iwao, 2000, Diez *et al.*, 2001, Rajabi *et al.*, 2007). Sadoshima *et al.* demonstrated the expression of these immediate-early genes in cultured cardiac myocytes stimulated with AngII (Sadoshima and Izumo, 1993). Because of their early expression these genes are believed to be expressed as the cardiomyocyte adapts to the stimuli. After induction of the early genes AngII induces myocyte gene reprogramming to a foetal phenotype increasing the expression of ANP, BNP, β-MHC, skeletal α-actin and TGF-β among others (Chien *et al.*, 1991). Concurrently the expression of genes related to intracellular Ca^{2+} metabolism such as phospholamban and Ca^{2+}-ATPase are decreased (Chien *et al.*, 1991).

Several studies have shown that AngII induces cardiac hypertrophy through the AT1R (Lamas and Pfeffer, 1991, Kaneko *et al.*, 1996) AngII promotes cardiac hypertrophy by activating several pathways, including activation of PKC and alterations in Ca^{2+} handling, to promote cardiomyocyte hypertrophy (Liao *et al.*, 1997, Qin *et al.*, 2001, Heineke and Molkentin, 2006, Liu *et al.*, 2008, Chintalgattu and Katwa, 2009). AngII also promotes cardiac hypertrophy and fibrosis by phosphorylation of ERKs (Liao *et al.*, 1997, Min *et al.*, 2005, Aplin *et al.*, 2007, Olson *et al.*, 2008). Activation of JNK and p38 phosphorylation by AngII has also been shown to increase the expression of hypertrophy markers (Kim and Iwao, 2000, Force et al., 1996). However, AngII also directly induces *c-fos, jun B, Egr-1, c-myb and c-myc* (Aaronson and Horvath, 2002, Kandalam and Clark, 2009, Mehta and Griendling, 2007).

AngII is also important in extracellular remodelling. It has been shown that phosphorylation of c-Src tyrosine kinase leads to extracellular matrix remodelling and activation of cytoskeletal proteins to allow cardiomyocyte remodelling (Kodama *et al.*, 1998, Taniyama *et al.*, 2003, Mehta and Griendling, 2007). AngII also regulates extracellular matrix remodelling by increasing the synthesis of collagen, fibronectin, promoting the accumulation of proteoglycans and increasing expression of TGF-β (Ju and Dixon, 1996, Huang et al., 2008, Tiede et al., 2003, Brilla et al., 1993, Schroder et al., 2006).

In cardiac hypertrophy the role of the AT2R is controversial. Blockade of AT2R in AngII-infused rats increased left ventricular growth (Bartunek *et al.*, 1999). AT2R inhibition also increased protein synthesis and augmented PKC translocation to the membrane (Bartunek *et al.*, 1999). Furthermore, lentivirus-mediated overexpression of the AT2R in SHRSP diminished left ventricular wall thickness and collagen without affecting blood pressure (Metcalfe *et al.*, 2004). Aortic banding in transgenic mice overexpressing the AT2R reduced left ventricular myocyte diameter as well as decreasing collagen levels (Yan *et al.*, 2008). Although the typical hypertrophy markers were not regulated, the expression of phospholamban (PLB), as well as the ratio between SERCA2 and iNOS were higher, indicating a role for the AT2R in hypertrophy regulation (Yan *et al.*, 2008). However, there have been studies where the AT2R seems to have a prohypertrophic effect. D'Amore *et al.* showed that adenoviral mediated overexpression of AT2R in cardiomyocytes resulted in hypertrophy (D'Amore *et al.*, 2005). Furthermore, this AT2R-mediated pro-hypertrophic effect seemed to be independent of any agonist-mediated stimulation of the AT2R. In accordance to this study, Yan *et al.* showed that transgenic mice overexpressing AT2R had altered left ventricular function and increased cardiac myocyte diameter and collagen levels which led to dilated cardiomyopathy (Yan *et al.*, 2003b). At the molecular level an increase in phosphorylated PKC- α and β was also demonstrated (Yan *et al.*, 2003b). Overall the role of AT2R signalling in cardiac hypertrophy is unclear.

Conversely the AT2R has also been demonstrated to mediate an anti-fibrotic role. AT2R blockade in rat heart and kidney increases fibrosis (Morrissey and Klahr, 1999). In cardiac fibroblasts overexpressing the AT2R, blocking its activation via the antagonist PD123,319 increased the production of collagenous protein, fibronectin, collagen type I and overall protein synthesis (Ohkubo *et al.*, 1997). In AT2R knockout mice an increase in interstitial and perivascular fibrosis has been observed (Wu *et al.*, 2002, Yan *et al.*, 2003b)(Ichihara *et al.*, 2001) . These results have also been reported in AT2R overexpressing transgenic mice, where AngII-induced perivascular fibrosis was abolished (Kurisu *et al.*, 2003).

Ang1-7 is one of the novel peptides of the renin-angiotensin system. ACE2 is responsible for its generation by cleaving AngII. It has been reported that Ang1-7

blocks the classical effects of AngII, such as proliferation in vascular smooth muscle cells, cardiomyocyte hypertrophy, and fibroblast proliferation (Santos et al., 2006, Mercure et al., 2008, Grobe et al., 2007b, Grobe et al., 2006, De Mello, 2009), among others by interacting with the Mas receptor (Santos *et al.*, 2003, Pinheiro *et al.*, 2009). Although described as one of the peptides formed by ACE2 (by cleaving AngI) (Donoghue *et al.*, 2000) and being a substrate to form Ang1-7 (by ACE cleavage) (Schluter and Wenzel, 2008), no direct effect of Ang1-9 has been described. Ang1-9 has, however, been described as a competitive inhibitor of ACE, as Ang1-9 is a substrate for this hormone (Snyder and Wintroub, 1986). Furthermore, Erdos *et al.* demonstrated that Ang1-9 had a potentiating effect on bradykinin-induced intracellular Ca^{2+} concentration and NO synthesis in cardiomyocytes and endothelial cells (Erdos *et al.*, 2002).

Here, the role of AngII, Ang1-7 and Ang1-9 were investigated in two *in vitro* models of cardiomyocyte hypertrophy using the rat H9c2 neonatal cardiomyocyte cell line and adult rabbit primary cardiomyocytes.

3.2 Results

3.2.1 AngII mediated induction of hypertrophy in rat neonatal H9c2 and adult rabbit left ventricular cardiomyocytes

It has been reported that when cardiomyocytes are stimulated with AngII an increase in hypertrophy markers, sarcomeric rearrangement and an increase in cell size can be observed (Chien *et al.*, 1991, Carreno *et al.*, 2006, Rajabi *et al.*, 2007). To study the role of angiotensin peptides in hypertrophy, we first established an AngII-induced hypertrophy model in H9c2 cardiomyocytes and adult rabbit left ventricular cardiomyocytes.

In order to determine the optimal dose of AngII for hypertrophy induction in H9c2 and rabbit primary cardiomyocytes, both cell types were stimulated with a range of AngII concentrations. The range of doses found in the literature for H9c2 cells were from 100 nM to 1 µM (Li et al., 2005d, Guo et al., 2009, Kodama et al., 1998, Nakagami et al., 2003). Therefore H9c2 cardiomyocytes were incubated with 100 nM, 300 nM, 500 nM and 1 µM AngII. Following 96 hours of incubation with AngII H9c2 cells were fixed and stained with 2% Crystal violet and cell size measured blindly (section 2.6). The same AngII dose range was used in rabbit primary cardiomyocytes, however cell width and length were measured and volume calculated following 24 hour incubation.

For AngII stimulated H9c2 cardiomyocytes there was a trend for an increase in cell size at all doses of AngII (Figure 3-1 A), however, only at 100nM and 500nM was a significant increase in cell size compared to unstimulated cells observed (unstimulated = 196.8 ± 8.3 µm; AngII 100 nM = 262.3 ± 10.8 µm; AngII 500 nM = 235.3 ± 7.9 µm; $p < 0.05$, $n = 50$ cells) (Figure 3-1 B). 100 nM of AngII was therefore selected for use in the H9c2 cardiomyocyte hypertrophy model.

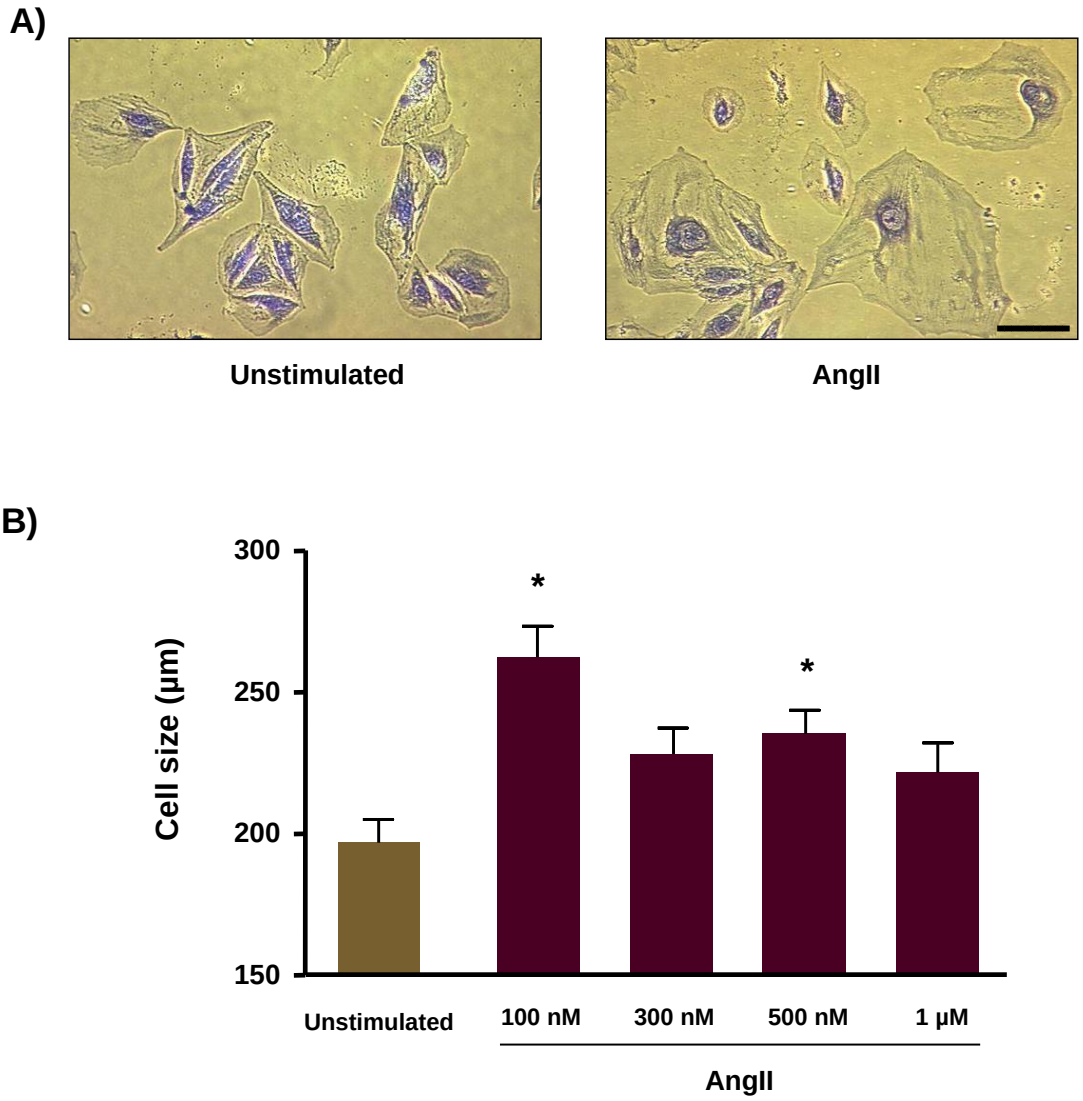
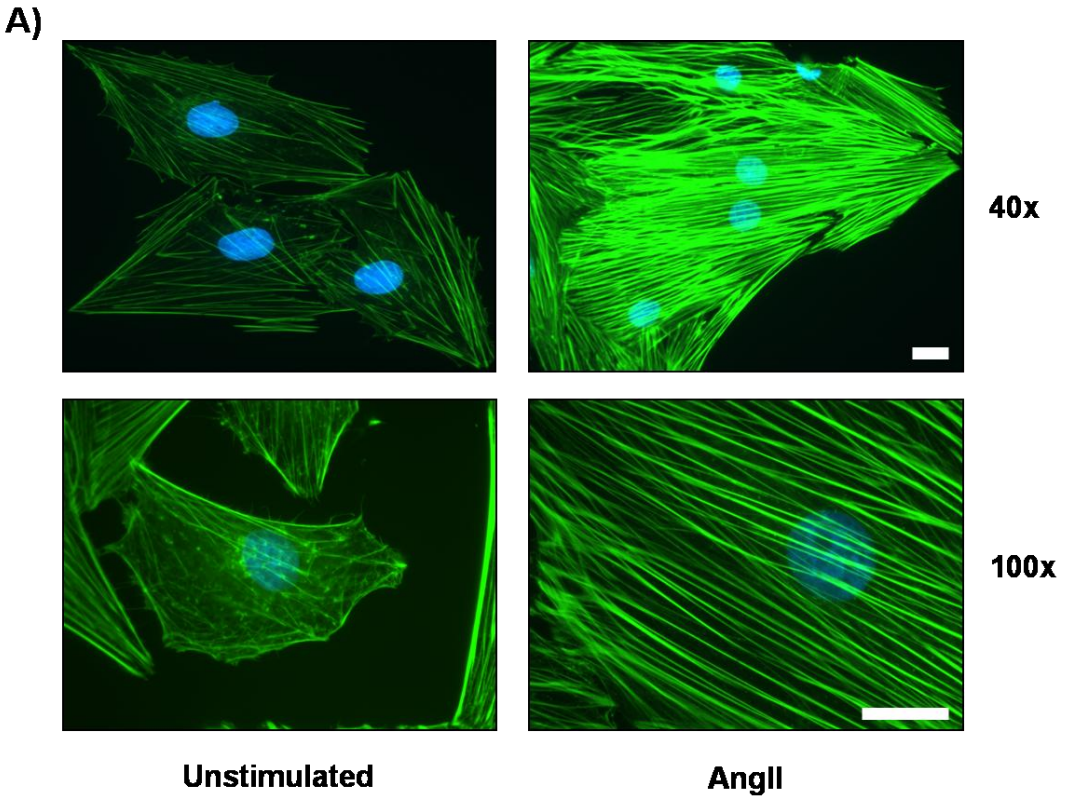


Figure 3-1. AngII-induced hypertrophy in H9c2 cardiomyocytes.

(A) Representative photomicrographs of unstimulated and AngII-stimulated H9c2 rat neonatal cardiomyocytes (Scale bar = 100µm. Magnification:10x). (B) H9c2 cardiomyocytes were incubated for 24 hours in serum free media before addition of AngII at 100, 300, 500 or 1000 nM AngII for 96 hours. Cells were then fixed, stained with crystal violet and cell size measured using ImageProPlus. * $p<0.05$ vs. control unstimulated cells.

Reorganization of α-actin microfilaments in cardiac myocytes occurs during hypertrophy (Samuel *et al.*, 1984) and has been established as a hypertrophic phenotype. Furthermore, in cultured cardiomyocytes AngII has been shown to induce reorganization of sarcomeres (Thomas *et al.*, 2002). In order to investigate any reorganization in cardiomyocytes sarcomeres induced by AngII, α-actin filaments of H9c2 cardiomyocytes were stained with a F-phalloidin-FITC conjugate following stimulation with AngII. Unstimulated cardiomyocytes displayed an irregular pattern of sacromere units throughout the cytoplasm of the cell (Figure 3-2). The increased organization of the sarcomeres was clearly evident in AngII-stimulated H9c2 cardiomyocytes (Figure 3-2 A). Next, BNP gene expression was analysed in AngII-stimulated H9c2 cardiomyocytes using Q-RT-PCR. Physiological BNP gene expression was significantly upregulated when cardiomyocytes were stimulated with 100 nM AngII compared to unstimulated H9c2 cells (Figure 3-2 B).

In adult rabbit left ventricular primary cardiomyocytes cell volume was calculated using width and length measurements (section 2.6). Upon quantifying measurements of width and length separately a significant difference in cell size was only apparent for cell width, suggesting that AngII may induce concentric hypertrophy in these cells (Figure 3-3 A, B and C). At 100nM AngII did not cause a significant increase in cell volume. However, at 300 nM, 500 nM and 1 µM a significant increase in cell width was induced (31.7 ± 7 nm vs. 37.8 ± 8.3 nm; $p<0.01$), leading to an increase in volume (unstimulated = 68.4 ± 1.8 μm^3; AngII 100 nM = 72.4 ± 1.9 μm^3; AngII 300 nM = 75.9 ± 2.1 μm^3; AngII 500 nM = 78.7 ± 2.1 μm^3; AngII 1 µM = 78.3 ± 2 μm^3) (Figure 3-3 D). Since 500 nM AngII induced the greatest difference in cell volume compared to unstimulated cells this dose was selected for use in further experiments in rabbit primary cardiomyocytes.



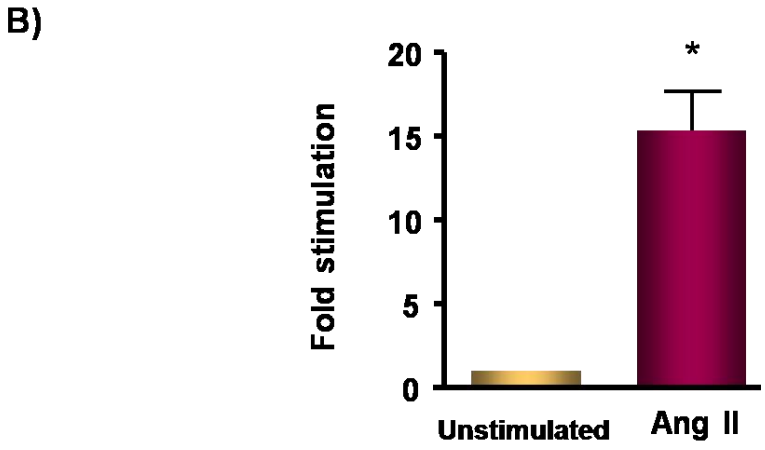


Figure 3-2. Characterization of AngII-induced hypertrophy in H9c2 cardiomyocytes.
H9c2 cardiomyocytes were incubated for 96 hours with 100 nM AngII to induce hypertrophy. To evaluate α-actin filament reorganization (A) cardiomyocytes were fixed and stained with FITC-labelled phalloidin (Magnification: top panel 40x, bottom panel 100x; scale bar = 100 µm); to evaluate BNP expression (B) the mRNA from H9c2 cardiomyocytes +/- AngII-stimulation was extracted and cDNA generated subjected to QRT-PCR for BNP. Results were normalized to expression of 18S RNA (* $p<0.001$).

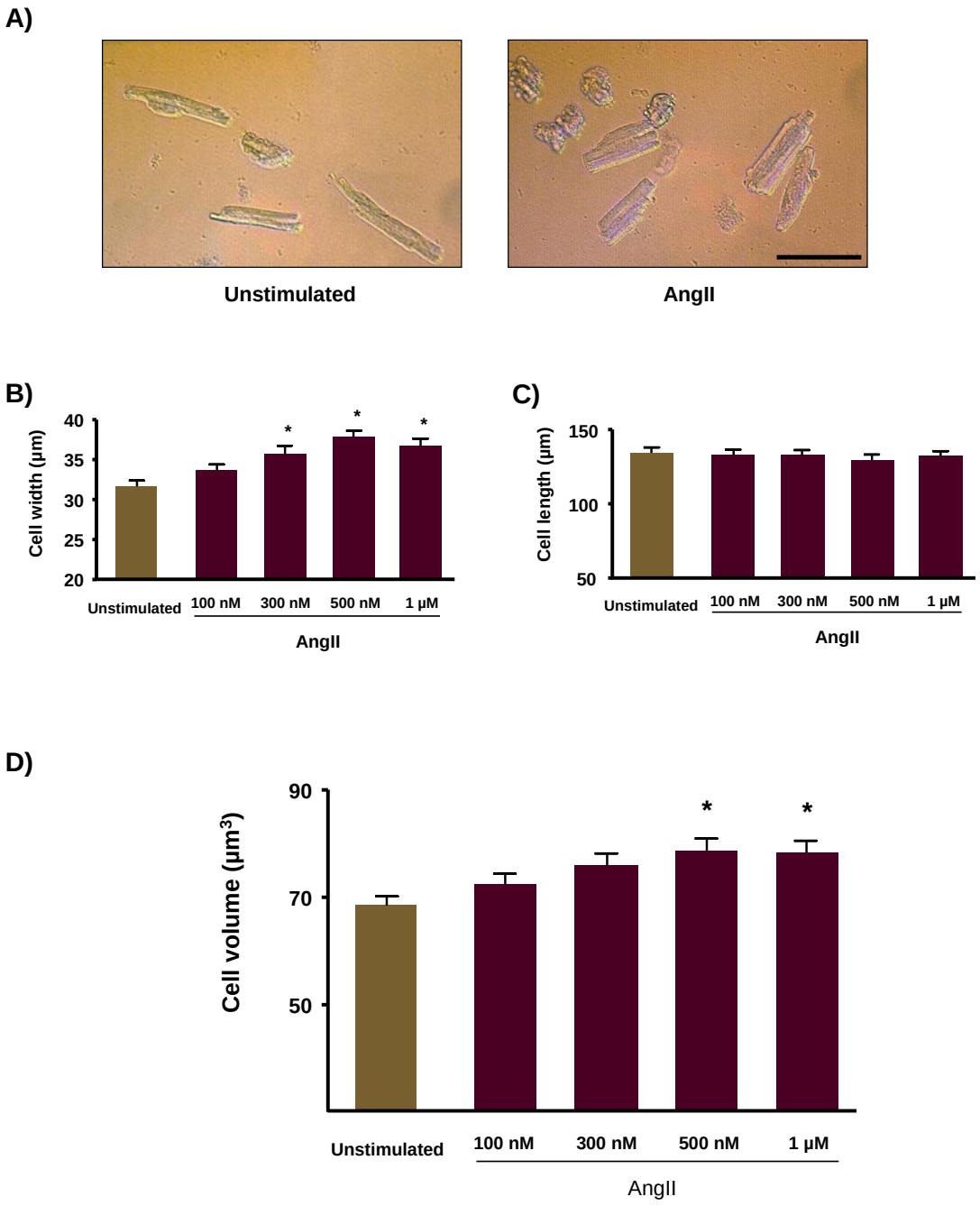


Figure 3-3. AngII-induced hypertrophy in left ventricular adult rabbit primary cardiomyocytes.

(A) Representative photomicrographs of unstimulated and AngII-stimulated left ventricular adult rabbit primary cardiomyocytes (Scale bar = 100 µm. Magnification: 20x). Left ventricular primary cardiomyocytes were extracted from adult rabbits via collagenase digestion, plated and immediately stimulated with 100 nM, 300 nM, 500 nM and 1 µM AngII for 24 hours. (B) Cell width and (C) length was measured using ImageProPlus. (D) Cell volume was calculated using cardiomyocyte width and length. The increase of volume in primary cardiomyocytes was due to increase of width in the cell. * $p<0.01$ vs. control unstimulated cells.

3.2.2 The effect of Ang1-7 and Ang1-9 on AngII-induced Hypertrophy

First any basal effects of Ang1-7 or Ang1-9 on cardiomyocyte phenotype were assessed. H9c2 cardiomyocytes were incubated with Ang1-7 or Ang1-9 at the dose used for blocking hypertrophy (100 nM) in the absence of AngII. Cardiomyocytes did not show any difference in cell size compared to unstimulated cells (unstimulated = 176.3 ± 6.1 μm; Ang1-7 100 nM = 182.4 ± 5.4 μm; Ang1-9 100 nM = 172.8 ± 4.9 μm) (Figure 3-4 A).

Next, the effect of Ang1-7 and Ang1-9 on AngII-induced hypertrophy was analysed. Either 10 nM, 100 nM or 1 μM Ang1-7 or Ang1-9 were added 30 minutes before addition of AngII. In H9c2 cardiomyocytes, all three doses of Ang1-7 blocked Ang-II induced hypertrophy and there was no significant difference when compared to unstimulated cardiomyocytes (Figure 3-4 B and C). With Ang1-9 similar results were observed however, only at concentrations of 100 nM and 1 μM was Ang1-9 able to completely block AngII-induced hypertrophy (Figure 3-4 B and C) (unstimulated = 186.4 ± 5.4 μm, AngII = 232.8 ± 9.9 μm, Ang1-7 = 203.8 ± 7.4 μm; Ang1-9 = 195.9 ± 7.1 μm; $p<0.05$). Furthermore, Ang1-7 and Ang1-9 also blocked AngII-induced BNP gene expression in H9c2 cardiomyocytes (Figure 3-5 A). Next the effects of Ang1-7 and Ang1-9 on sarcomeric α-actin re-organization in AngII-stimulated H9c2 cardiomyocytes were analysed. H9c2 cardiomyocytes were incubated with Ang1-7 and Ang1-9 before AngII stimulation. AngII-stimulated H9c2 cardiomyocytes showed an increased organization of actin filaments as expected, conversely, Ang1-7 and Ang1-9 stimulated H9c2 cardiomyocytes showed the same irregular sarcomeric pattern as unstimulated cells (Figure 3-5 B).

To assess effects of Ang1-7 or Ang1-9 on cardiomyocyte viability a MTT assay was performed. H9c2 cardiomyocytes were incubated with Ang1-7, Ang1-9 and AngII as described previously and MTT assay was performed to assess cell viability 96 hours after AngII-stimulation. Results showed no significant difference between unstimulated and stimulated cells, suggesting that Ang1-7, Ang1-9 or AngII did not have any effect on cardiomyocyte viability (Figure 3-6).

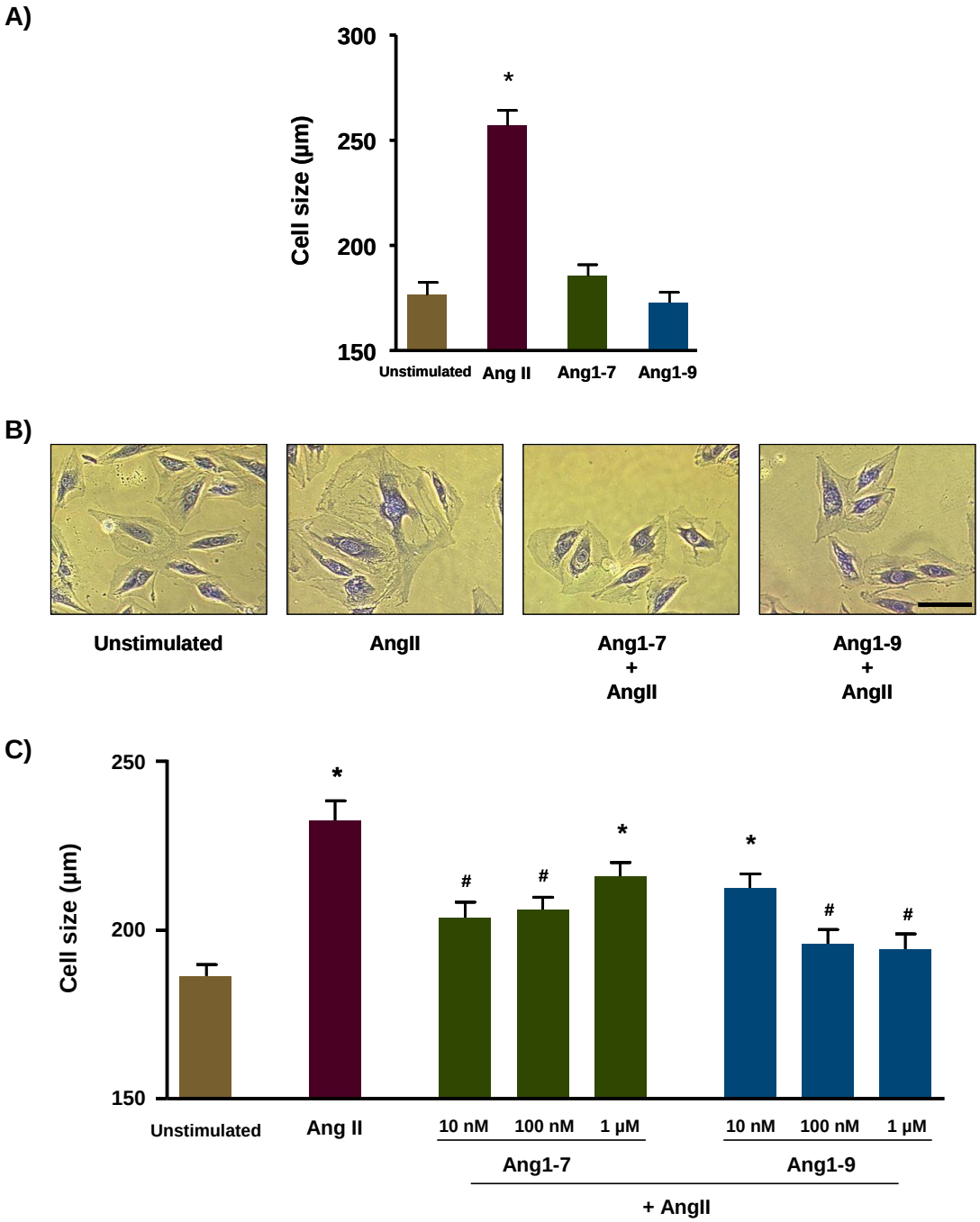


Figure 3-4. Effect of Ang1-7 and Ang1-9 on unstimulated and AngII-induced hypertrophy in H9c2 cardiomyocytes.

(A) H9c2 cardiomyocytes were incubated with 100 nM Ang1-7 or Ang1-9 alone for 96 hours. Cells were fixed, stained and measured using ImageProPlus; * $p<0.001$ vs. unstimulated cells. Next, H9c2 cardiomyocytes were incubated with Ang1-7 or Ang1-9 at 10 nM, 10 nM or 1 µM 30 minutes before stimulation with 100 nM AngII. Cells were then incubated for a further 96 hours before fixing, staining and cell size measured. (A) Representative photomicrographs of unstimulated and AngII-stimulated H9c2 cardiomyocytes +/- Ang1-7 or Ang1-9 (Scale bar = 100 µm. Magnification: 10x). (B) Cell size was measured using ImageProPlus (* $p<0.001$ vs. unstimulated cells, # $p<0.05$ vs. AngII-stimulated cells).

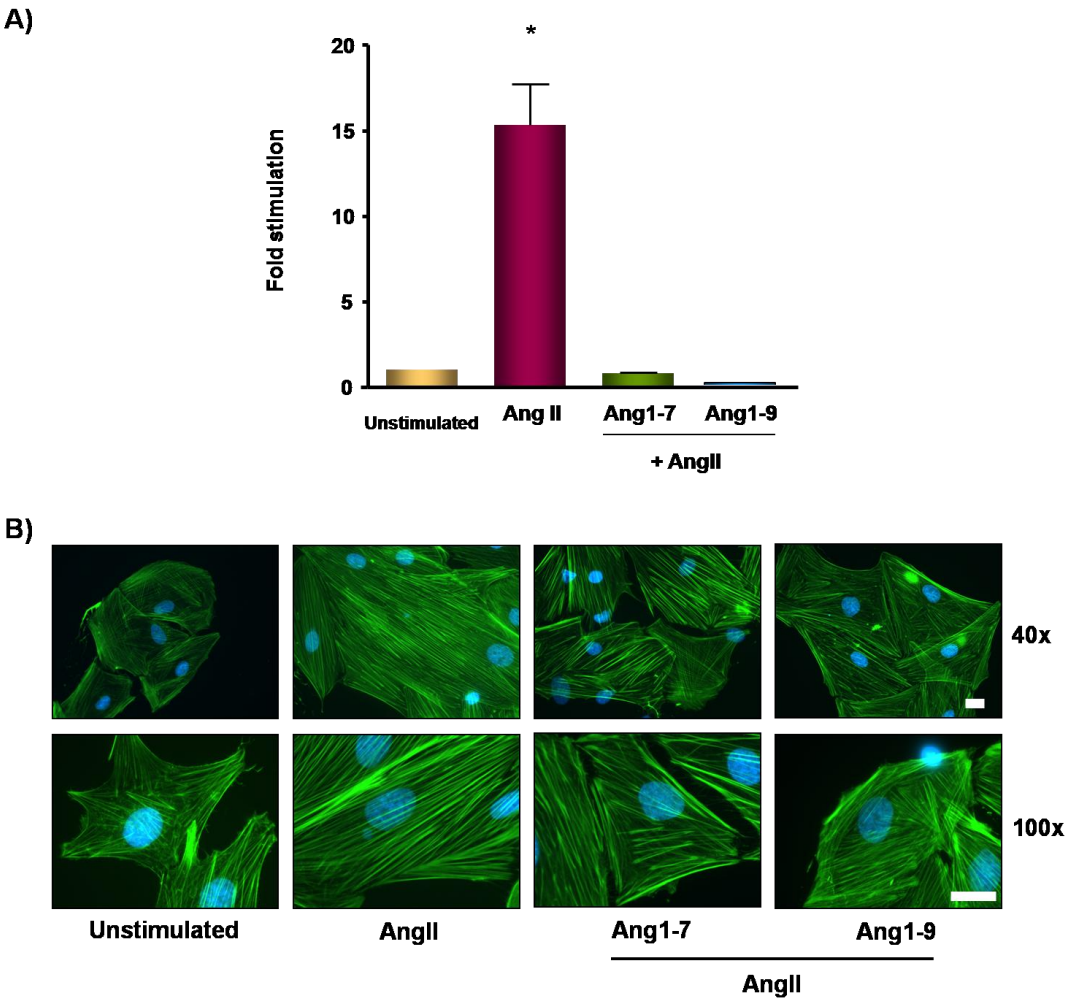


Figure 3-5. Characterization of the effects of Ang1-7 and Ang1-9 on AngII-induced hypertrophy in H9c2 cardiomyocytes.

Ang1-7 or Ang1-9 were added to H9c2 cardiomyocytes 30 minutes before addition of AngII and incubated for 96 hours. (A) BNP gene expression was quantified by TaqMan following mRNA extraction and cDNA generation from unstimulated and AngII-stimulated H9c2 cardiomyocytes +/- Ang1-7 or Ang1-9. Results were normalized to expression of 18S RNA. (B) At 96 hours α-actin filament reorganization was evaluated by fixing the cardiomyocytes and staining with FITC-labelled phalloidin (Magnification: top panel 40x, bottom panel 100x; scale bar = 100 µm). (* $p<0.001$ vs. unstimulated cells).

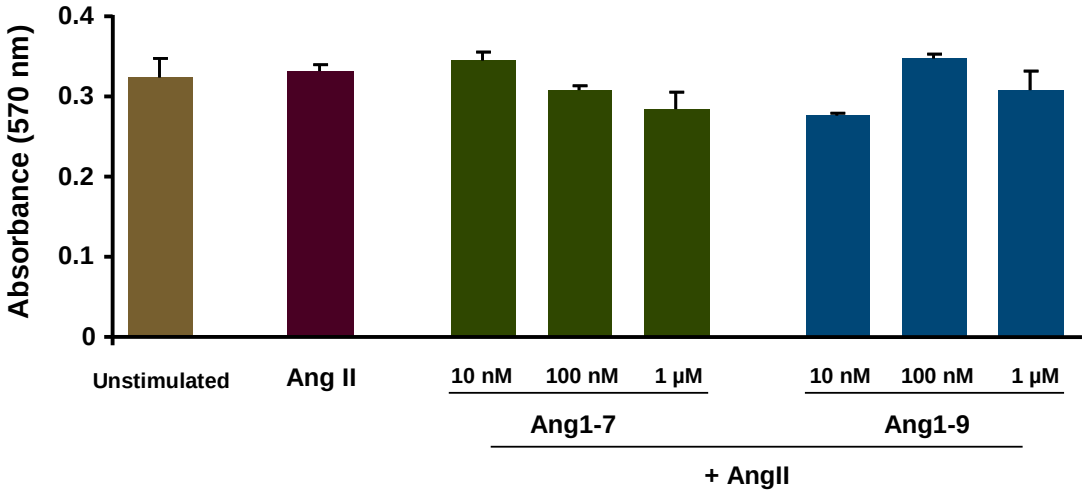


Figure 3-6. The effect of AngII, Ang1-7 and Ang1-9 on H9c2 cardiomyocyte viability.

H9c2 cardiomyocytes were incubated with 10 nM, 100 nM or 1 µM of Ang1-7 or Ang1-9 30 minutes prior to stimulation with AngII. After incubation for 96 hours, cardiomyocyte viability was assessed using the MTT assay, before absorbance was recorded at 570 nm on a Wallac Victor2 spectrophotometer.

The effect of Ang1-7 and Ang1-9 was also studied in rabbit primary cardiomyocytes (Figure 3-7 A). Primary cardiomyocytes were incubated with 100 nM, 500 nM and 1 µM Ang1-7 or Ang1-9 30 minutes before addition of AngII, and 24 hours later cell length and width were measured. Similar to H9c2 cardiomyocytes significant differences in cell size were observed, this was only apparent for width (Figure 3-7 B and C). All 3 doses of either Ang1-7 or Ang1-9 blocked AngII-induced hypertrophy produced by AngII (unstimulated = 67.1 ± 2.9 μm^3; AngII = 85.7 ± 4 μm^3; Ang1-7 100 nM = 61.4 ± 2.2 μm^3; Ang1-7 500 nM = 66.6 ± 4.3 μm^3; Ang1-7 1 µM = 64.7 ± 3.2 μm^3; Ang1-9 100 nM = 65.5 ± 2.5 μm^3; Ang1-9 500 nM = 59.6 ± 3.5 μm^3; Ang1-9 1 µM = 64.2 ± 3 μm^3; $p < 0.001$) (Figure 3-7 D).

Several studies have shown that higher concentration of Ang1-7 cause opposite effects to what was described with lower concentrations (De Mello, 2009, Garcia and Garvin, 1994, Haulica et al., 2003). To investigate if Ang1-7 or Ang1-9 had different effects at supra-physiological concentration, both peptided were added at 10 and 100µM. These concentrations were based on receptors pharmacology and a literature search. When H9c2 cardiomyocytes were stimulated with higher concentrations of Ang1-7 or Ang1-9 (10 µM and 100 µM), 100 µM Ang1-7 induced a significant increase in cell size compared to unstimulated cells (unstimulated = 187.1 ± 5 µm; AngII = 247.2 ± 6.6 µm; Ang1-7 10 µM = 219.3 ± 6.2 µm; Ang1-7 100 µM = 235 ± 6.7 µm; Ang1-9 10 µM = 194.2 ± 6.6 µm; Ang1-9 100 µM = 178.8 ± 4.9 µm; $p < 0.01$; n = 100 cells per group) (Figure 3-8). However when Ang1-9 was added to cardiomyocytes at these same concentrations, cell length was no different from unstimulated cells.

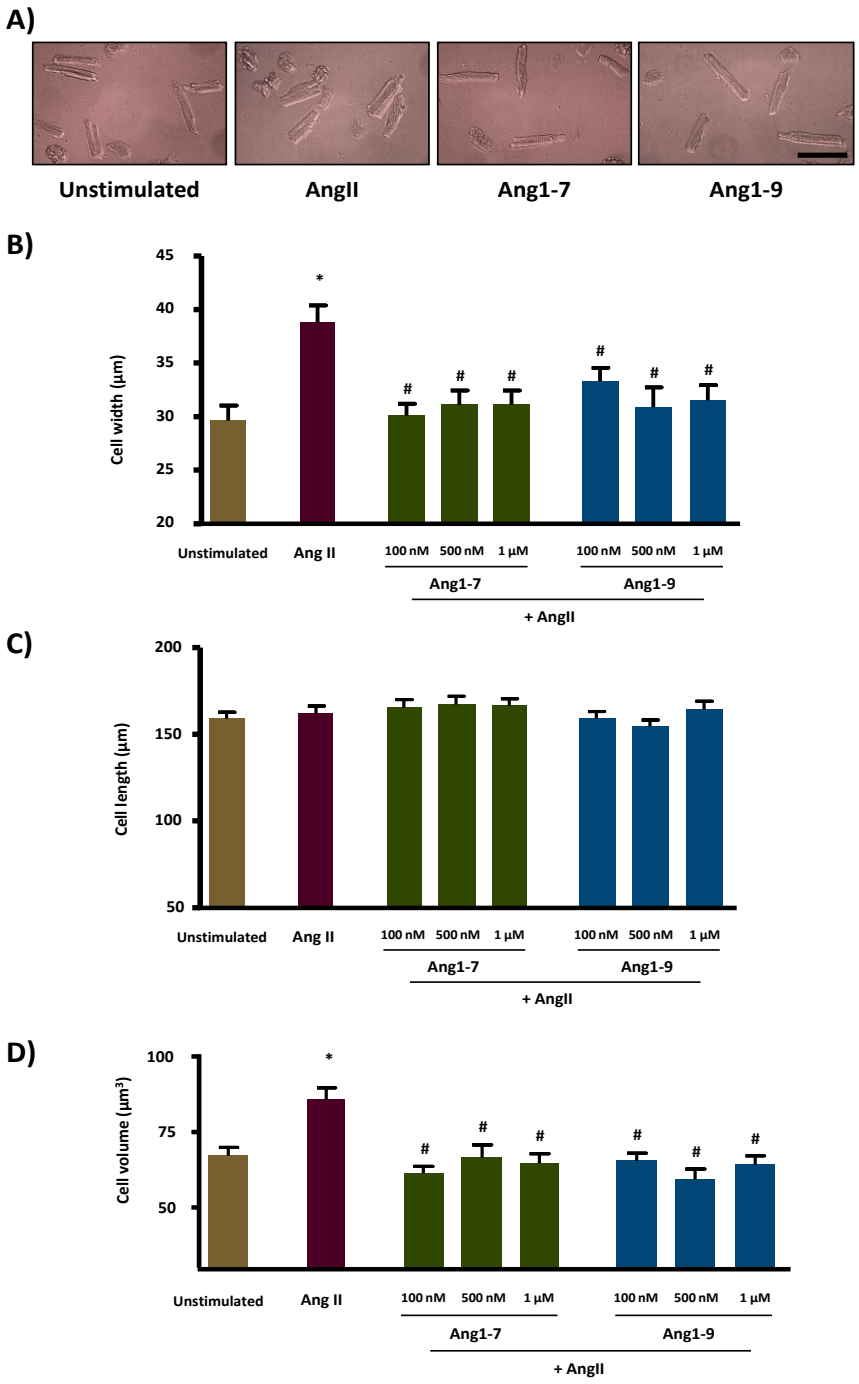


Figure 3-7. Role of Ang1-7 and Ang1-9 in AngII-induced hypertrophy in left ventricular adult rabbit primary cardiomyocytes.

(A) Representative photomicrographs of unstimulated and AngII-stimulated left ventricular adult rabbit primary cardiomyocytes +/- Ang1-7 or Ang1-9 (Scale bar = 100 µm. Magnification: 20x). Rabbit left ventricular primary cardiomyocytes were incubated with Ang1-7 or Ang1-9 at 100 nM, 500 nM or 1 µM 30 minutes before stimulation with 500 nM AngII. Cells were then incubated for a further 24 hours before measurement. (B) Cell width and (C) length was measured using ImageProPlus. (D) Cell volume was calculated using cardiomyocyte width and length. * $p<0.001$ vs. control unstimulated cells; # $p<0.05$ vs. AngII-stimulated cells.

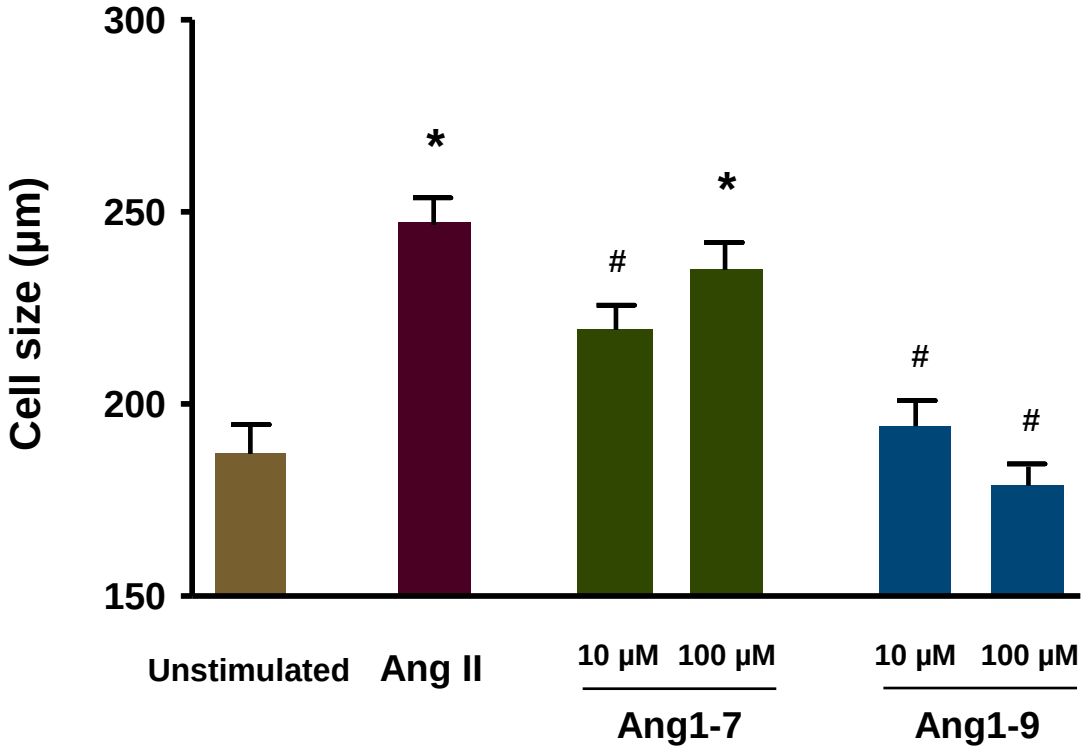


Figure 3-8. Supra-physiological effects of Ang1-7 or Ang1-9 on H9c2 cardiomyocytes. H9c2 cardiomyocytes were incubated with 10 µM or 100 µM Ang1-7 or Ang1-9 in the presence of 100 nM AngII. * $p<0.001$ vs. control unstimulated cells; # $p<0.01$ vs. AngII-stimulated cells.

3.2.3 Inhibition of Angiotensin Converting Enzyme

Ang1-9 has been described to generate Ang1-7 through its cleavage by ACE (Snyder and Wintroub, 1986). To test whether conversion to Ang1-7 was necessary for the effects of Ang1-9 in cardiomyocytes, hypertrophy was assessed in the presence of the ACE inhibitor captopril to block any conversion of Ang1-9 to Ang1-7. Captopril (1 mM) was added to Ang1-9 + AngII stimulated cells. Ang1-9 still blocked AngII-induced hypertrophy, even in the presence of Captopril (unstimulated = 135.7 ± 5.3 μm; AngII = 169.4 ± 8 μm; Ang1-9 = 133.9 ± 6.9 μm; Ang1-9 + captopril = 137.9 ± 7.4 μm; $p<0.01$) (Figure 3-9 A). Similar findings were observed in adult rabbit left ventricular primary cardiomyocytes (unstimulated = 71 ± 9.8 μm^3; AngII = 114.2 ± 14.1 μm^3; Ang1-9 = 86.8 ± 1.9 μm^3; Ang1-9 + captopril = 84.7 ± 13 μm^3; $p<0.001$) (Figure 3-9 A).

3.2.4 Analysis of the role of the Mas Receptor in cardiomyocyte hypertrophy

The orphan G-protein coupled receptor Mas has been described to interact with Ang1-7 (Santos *et al.*, 2003) and through this receptor, Ang1-7, antagonizes the effects of AngII (Pinheiro et al., 2009, Santos et al., 2006). To confirm Mas expression RT-PCR was performed on cDNA generated from H9c2 cardiomyocytes +/- addition of AngII (as described in section 2.16). Mas expression was detected in H9c2 cardiomyocytes and expression was not regulated by AngII stimulation. (Figure 3-10 A). Mas protein detection by western immunoblotting on H9c2 cardiomyocytes was also performed, however, the lack of specificity of the available antibodies for Mas resulted in no detection of the receptor. To assess if Ang1-7 or Ang1-9 were signalling their anti-hypertrophic effects via Mas we used the pharmacological antagonist, A779. In the H9c2 cardiomyocyte model A779 had no effect on unstimulated or AngII-stimulated cell size (unstimulated = 191.1 ± 6.6 μm; unstimulated + A779 = 208.4 ± 7.3 μm; AngII = 288.6 ± 12.3 μm; AngII + A779 = 273.6 ± 10.9 μm; $p < 0.001$) (Figure 3-10 B).

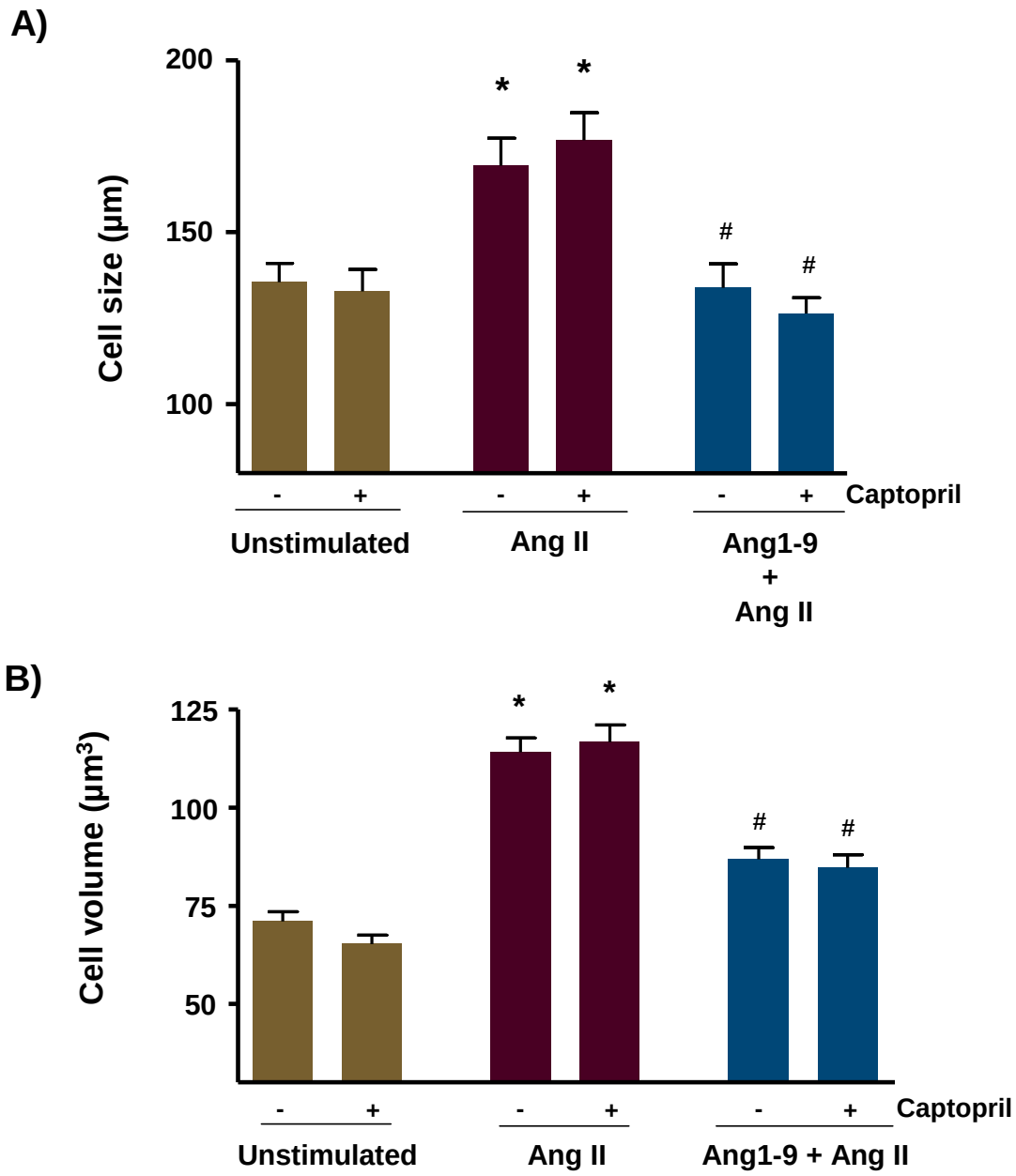


Figure 3-9. Ang1-9 mediated inhibition of hypertrophy in the presence of ACE inhibition.

(A) H9c2 cardiomyocytes were incubated with 1 mM captopril 15 minutes before addition of Ang1-9 (100 nM) and AngII. (B) Freshly isolated rabbit primary cardiomyocytes were plated, incubated with 1 mM captopril 15 minutes before addition of 500 nM Ang1-9. Cells were then incubated for a further 30 minutes period before stimulation with 500 nM AngII. Cell size was measured 24hours later using ImageProPlus. *p<0.001 vs. unstimulated cells; # p<0.001 vs. AngII stimulated cells.

Preincubation of AngII stimulated cardiomyocytes with A779 15 minutes prior to Ang1-7 addition blocked the antihypertrophic effect of Ang1-7 (Ang1-7 = 218.3 ± 9.8 μm; Ang1-7 + A779 10 μM = 275.5 ± 14.0 μm; Ang1-7 + A779 100 μM = 304.9 ± 13.0 μm; $p<0.001$). However, A779 did not effect on Ang1-9 (Ang1-9 = 242.4 ± 10.7 μm; Ang1-9 + A779 10 μM = 223.5 ± 8.4 μm; Ang1-9 + A779 100 μM = 213.4 ± 8.9 μm; $p<0.01$) (Figure 3-10 B).

In similarity with the data observed in H9c2 cardiomyocytes, in adult left ventricular rabbit primary cardiomyocytes A779 was also able to block the anti-hypertrophic effect of Ang1-7 without affecting the actions of Ang1-9 (Figure 3-10 C). These results provided further evidence that Ang1-9 has independent effects from those expected if it merely generated Ang1-7.

MBP7 is a phage-display derived peptide identified by Bikkavilli *et al.*, as a novel partial Mas agonist (Bikkavilli *et al.*, 2006). Using 2 different concentrations of MBP7 (10 and 100 μM) previous data have shown differential effects on Mas signalling, defining it as a partial agonist. To further study the role of Mas, AngII-stimulated H9c2 cardiomyocytes were incubated with 10 μM and 100 μM MBP in the presence or absence of Ang1-7 or Ang1-9. At 10 μM and 100 μM MBP7 had no effect on unstimulated cell size but did however block AngII-induced hypertrophy at both doses. Importantly, a significant difference between MBP7 doses was observed, suggesting an agonist effect at low doses and an antagonist effect at high dose, supporting its definition as a classical partial agonist. Furthermore, upon co-incubation with AngII and Ang1-7 at 10 μM MBP7 partially reversed the anti-hypertrophic effect of Ang1-7 suggesting that Ang1-7 was a more efficacious ligand for the Mas receptor, but that MBP7 might have higher affinity. This was supported by the finding that at 100 μM MBP7 completely blocked the anti-hypertrophic effects of Ang1-7. However, MBP7 had no effect on the anti-hypertrophic effects of Ang1-9 at either dose. These results demonstrate that at low concentrations MBP7 acts as a partial agonist of Mas, but acts with a lower efficiency but higher affinity than Ang1-7. Conversely, at higher concentrations (100 μM) MBP7 acts as an antagonist of Mas, blocking the engagement of Ang1-7 (Figure 3-11).

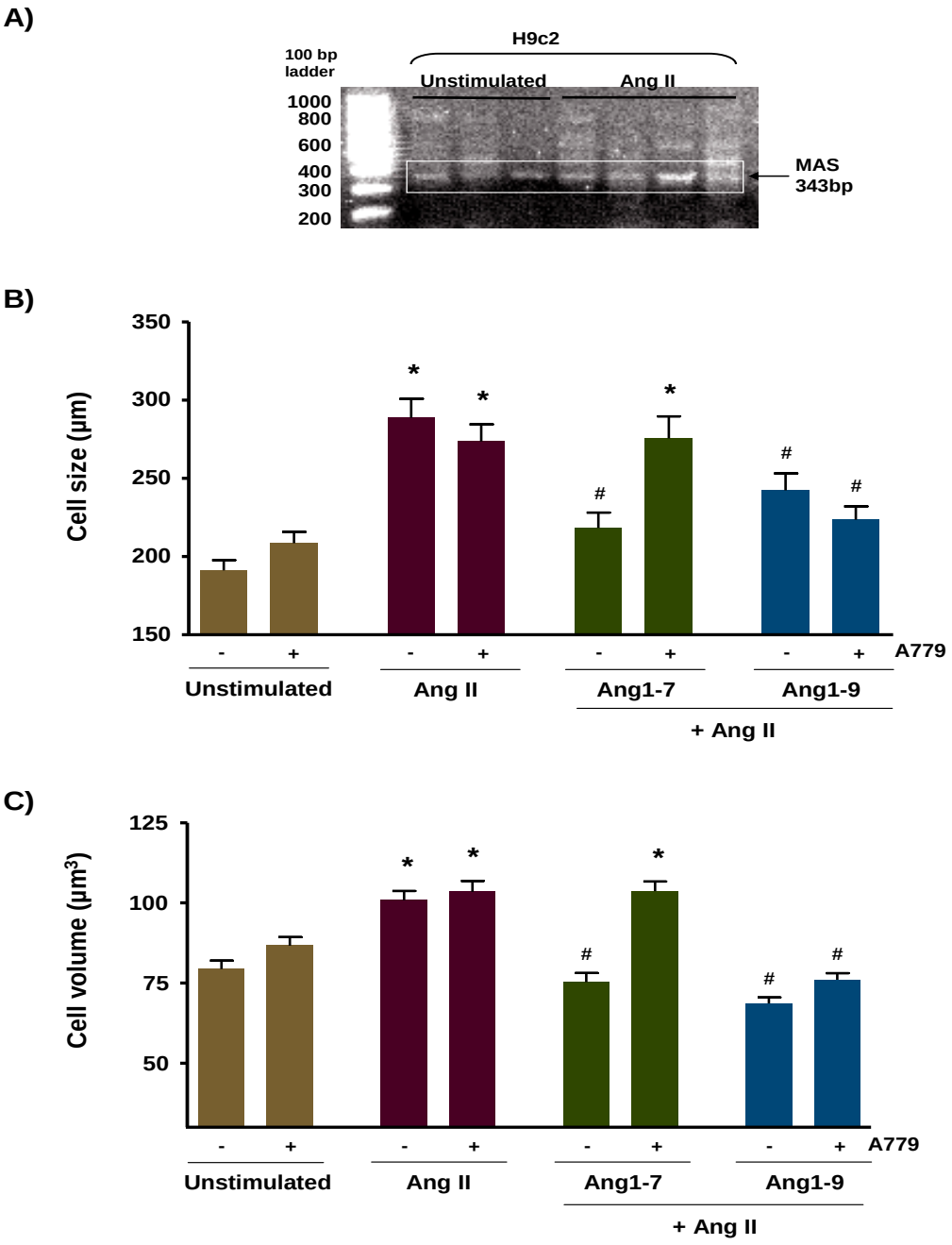


Figure 3-10. The role of the Mas receptor in Ang1-7 and Ang1-9 signalling cardiomyocytes.

(A) mRNA of unstimulated or AngII-stimulated (100 nM) H9c2 cardiomyocytes was extracted, cDNA generated and PCR performed to detect Mas. PCR product = 343bp. (B) Cells were preincubated with the Mas antagonist A779 (10 µM) 15 minutes before addition of Ang1-7 or Ang1-9. Cells were then incubated for 30 minutes before stimulation with 100 nM AngII and then incubated until 96 hours. * $p<0.001$ vs. unstimulated cells, # $p<0.01$ vs. AngII stimulated cells. (C) Isolated rabbit primary cardiomyocytes were plated, A779 (10 µM) added, 15 minutes before addition of Ang1-7 or Ang1-9 (500 nM), and 500 nM AngII. Cell size was measured at 24 h using ImageProPlus. * $p<0.001$ vs. unstimulated cells; # $p<0.001$ vs. AngII stimulated cells.

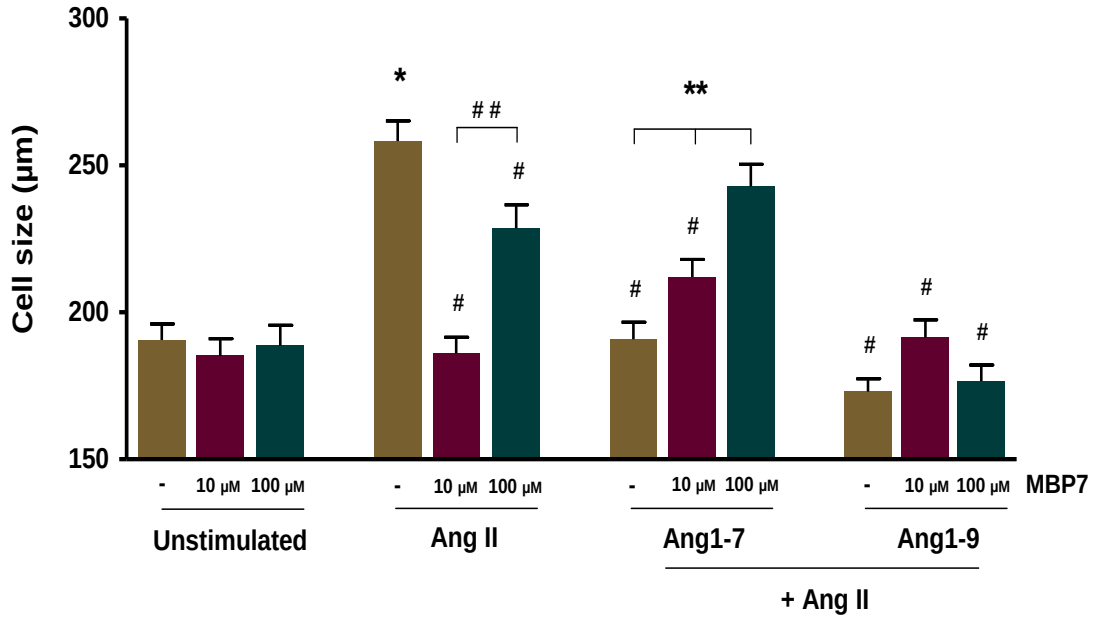


Figure 3-11. The effect of the partial Mas agonist MBP7 in H9c2 cardiomyocyte hypertrophy.

H9c2 cardiomyocytes were plated and incubated for 24 hours. MBP7 (10 and 100 µM) was added and cells incubated for 15 minutes before adding Ang1-7 or Ang1-9 (100 nM). Following a further 30 minutes incubation cells were stimulated with AngII (100 nM). After 96 hours incubation, cells were fixed, stained with crystal violet and cell size was measured using ImageProPlus. * $p< 0.001$ vs. unstimulated cells; # $p< 0.001$ vs. AngII stimulated cells; ** $p< 0.01$ vs. Ang1-7 stimulated cells; ## $p<0.001$ vs. AngII + MBP 10 µM stimulated cells.

3.2.5 Assessment of the role of the AT1R in cardiomyocyte hypertrophy

The AT1R is the AngII receptor associated with most of the classical actions of AngII, including vasoconstriction, proliferation, inflammation, coagulation, extracellular matrix remodelling and hypertrophy. To test any association of Ang1-9 with the AT1R or crosstalk between Mas and the AT1R, hypertrophy was induced via a different agonist. Arg-vasopressin triggers cardiomyocyte hypertrophy via the arginine-vasopressin receptor 1A (Hiroyama *et al.*, 2007, Sharma *et al.*, 2007).

H9c2 and rabbit primary cardiomyocytes were stimulated with different concentrations of arg-vasopressin (10 nM, 100 nM and 1µM) to establish a role in inducing hypertrophy. In H9c2 cardiomyocytes all three concentrations of arg-vasopressin induced hypertrophy (unstimulated = 199 ± 3.9 µm; arg-vasopressin 10 nM = 236 ± 5.3 µm; arg-vasopressin 100 nM = 258.67 ±7.6 µm; arg-vasopressin 1µM = 253.9 ± 5.3 µm, $p < 0.01$) (Figure 3-12 A).

Additionally, in rabbit primary cardiomyocytes all 3 doses of vasopressin also induced significant increases in cell volume (unstimulated = 75.7 ± 2.3 μm^3; arg-vasopressin 10 nM = 96.5 ± 2 μm^3; arg-vasopressin 100 nM = 92.4 ± 3.6 μm^3; arg-vasopressin 1 µM = 92.8 ± 3.1 μm^3; $p < 0.01$) (Figure 3-12 B, C, D). This increase in cardiomyocyte volume was also due to the increase in the width of the cells, in similarity to AngII-induced hypertrophy (Figure 3-12 B and C); therefore 1 µM was selected for further experiments.

Next, the AT1R antagonist losartan was utilised at either 1 or 10 µM in the arg-vasopressin-induced hypertrophy model. In H9c2 cardiomyocytes preincubation with losartan blocked AngII-induced hypertrophy (Figure 3-13 A) however, it did not affect the pro-hypertrophic effect of vasopressin (unstimulated = 179.2 ± 5.4 µm;

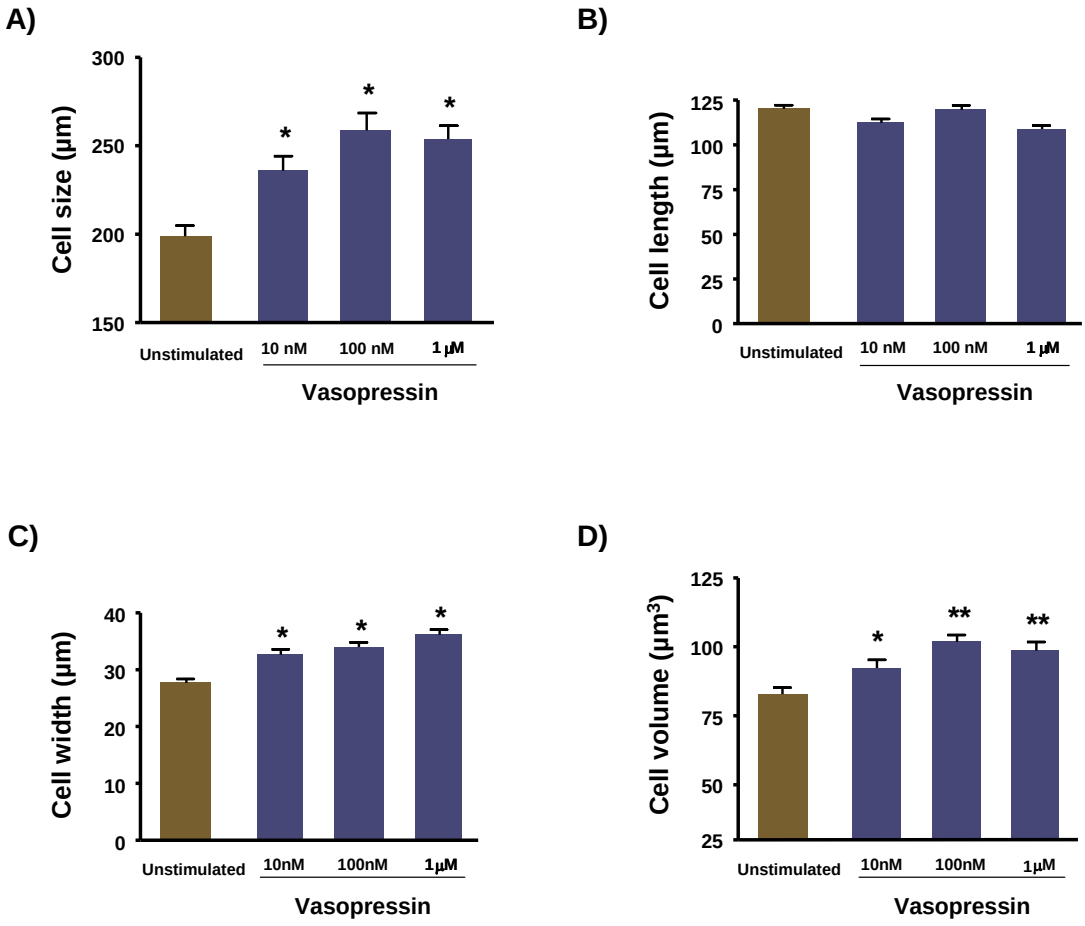


Figure 3-12. Vasopressin-induced hypertrophy in cardiomyocytes.

(A) H9c2 cardiomyocytes were plated and incubated for 24 hours in serum free media before addition of different concentrations of arg-vasopressin (10 nM, 100 nM and 1 µM) for 96 hours to induce hypertrophy. Cells were then fixed, stained with crystal violet and cell size measured using ImageProPlus. * $p<0.01$ vs. control unstimulated cells. Furthermore, freshly isolated primary ventricular cardiomyocytes were plated and immediately stimulated with 10, 100 and 1 µM of arg-vasopressin and then incubated for 24 hours. (D) Cell volume was measured using ImageProPlus. *$p<0.01$ vs. control unstimulated cells. The increase in volume of primary cardiomyocytes was due to an increase in the width of the cells (C), as the length of the primary cardiomyocytes (B) did not differ between groups.

AngII = 253.4 ± 8.4μm; AngII + losartan 10 μM = 201 ± 6.1 μm; arg-vasopressin = 227.4 ± 9.8 μm; arg-vasopressin + losartan 10 μM = 247.8 ± 7.2 μm; $p<0.001$) (Figure 3-13 A). Next, arg-vasopressin-stimulated H9c2 cardiomyocytes were incubated with losartan, and Ang1-7 or Ang1-9. Blocking AT1R with 1 or 10 μM losartan had no effect on the anti-hypertrophic effects of Ang1-7 or Ang1-9 (Ang1-7 = 190.4 ± 4.8 μm; Ang1-7 + losartan 10 μM = 181.9 ± 6.1 μm; Ang1-9 = 189.7 ± 5.3 μm; Ang1-9 + losartan 10 μM = 173.4 ± 4.6 μm; $p< 0.001$) (Figure 3-13 A). This suggested that Ang1-7 or Ang1-9 did not antagonize AngII signalling by competition for the AT1R. In rabbit primary cardiomyocytes blocking AT1R with 10 μM losartan confirmed the findings as both Ang1-7 and Ang1-9 blocked the pro-hypertrophic effects of arg-vasopressin in the presence of losartan (Figure 3-13 B).

3.2.6 Assessment of the role of the AT2R in cardiomyocyte hypertrophy

Although the AT2R was described over 2 decades ago, the mechanisms of how it acts are still controversial. AT2R expression has been described only in foetal, neonatal and in diseased rodent cardiomyocytes (Mehta and Griendling, 2007)(Bastien *et al.*, 1996). However, in both normal adult and failing human hearts the predominant AngII receptor was found to be the AT2R accounting for 70% of AngII binding sites (Regitz-Zagrosek *et al.*, 1995), suggesting a difference in AngII receptor expression between species. First, the expression of AT2R was confirmed in cDNA generated from H9c2 cardiomyocytes +/- AngII via RT-PCR (Figure 3-14 A).

Next, the effects of the commercial AT2R agonist (CGP42112A) (Ohkubo *et al.*, 1997) were assessed (Figure 3-14 B). The addition of CGP42112A (10 nM, 100 nM and 1 μM) efficiently blocked AngII-induced hypertrophy (30% increase in cell size induced by AngII was normalized by addition of CGP42112A; $p < 0.001$).

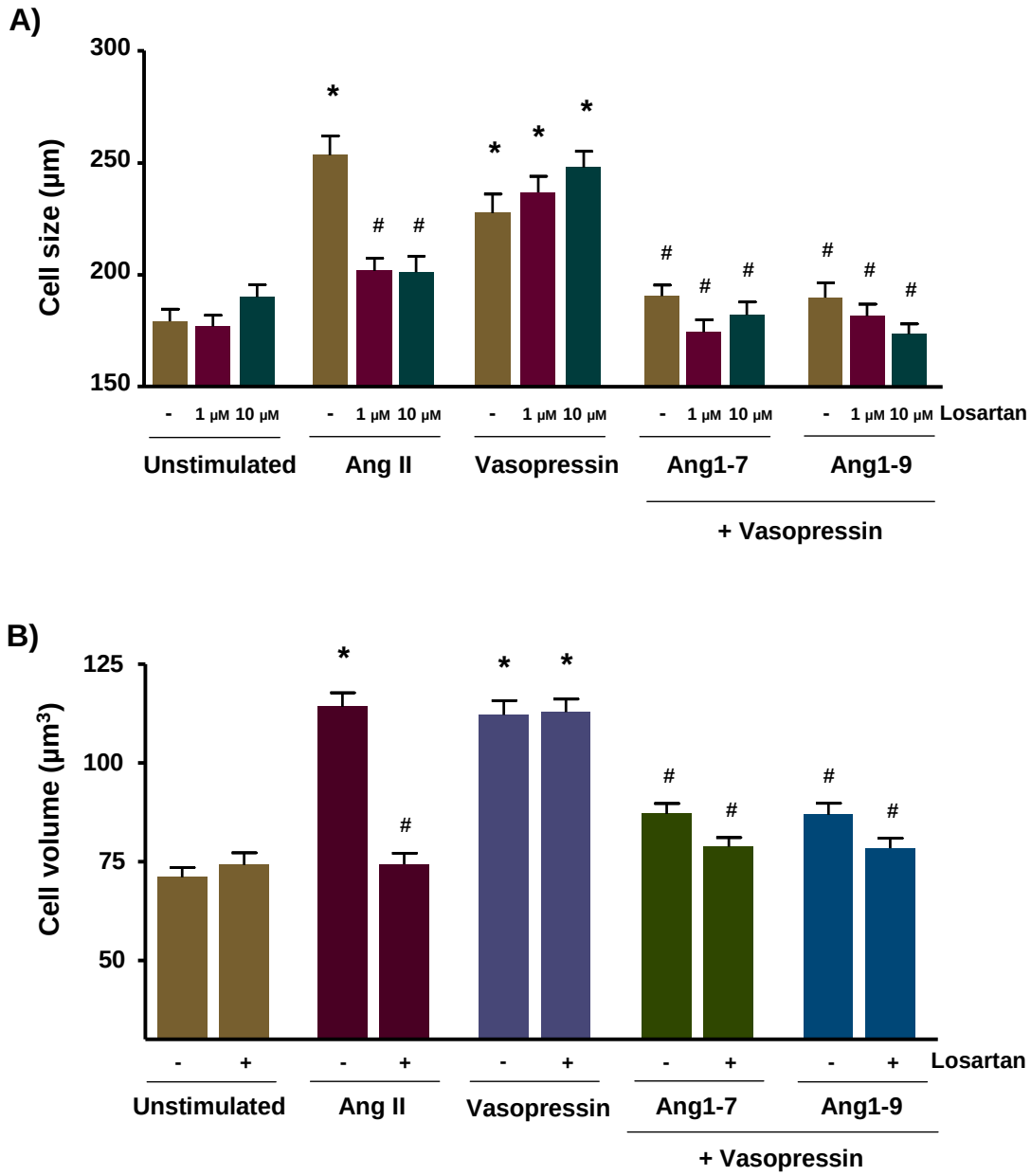


Figure 3-13. The role of the AT1R in Ang1-7 and Ang1-9 signalling in cardiomyocytes. (A) H9c2 cardiomyocytes were stimulated with arg-vasopressin (1 µM) to induce hypertrophy. Losartan (1 µM and 10 µM) blocked AngII-induced hypertrophy but not the action of arg-vasopressin. Cells were preincubated with losartan before addition of Ang1-7 or Ang1-9. After incubation for 30 minutes cells were stimulated with arg-vasopressin and incubated for 96 hours. (B) Isolated primary cardiomyocytes were plated and incubated with losartan (10 µM) 15 minutes before the addition of Ang1-7 or Ang1-9 (500 nM). Cells were incubated for 30 minutes before the addition of AngII (500 nM) or arg-vasopressin (1 µM) to induce hypertrophy and cells were incubated for a further 24 hours. * $p<0.001$ vs. control unstimulated cells; # $p<0.001$ vs. arg-vasopressin stimulated cells.

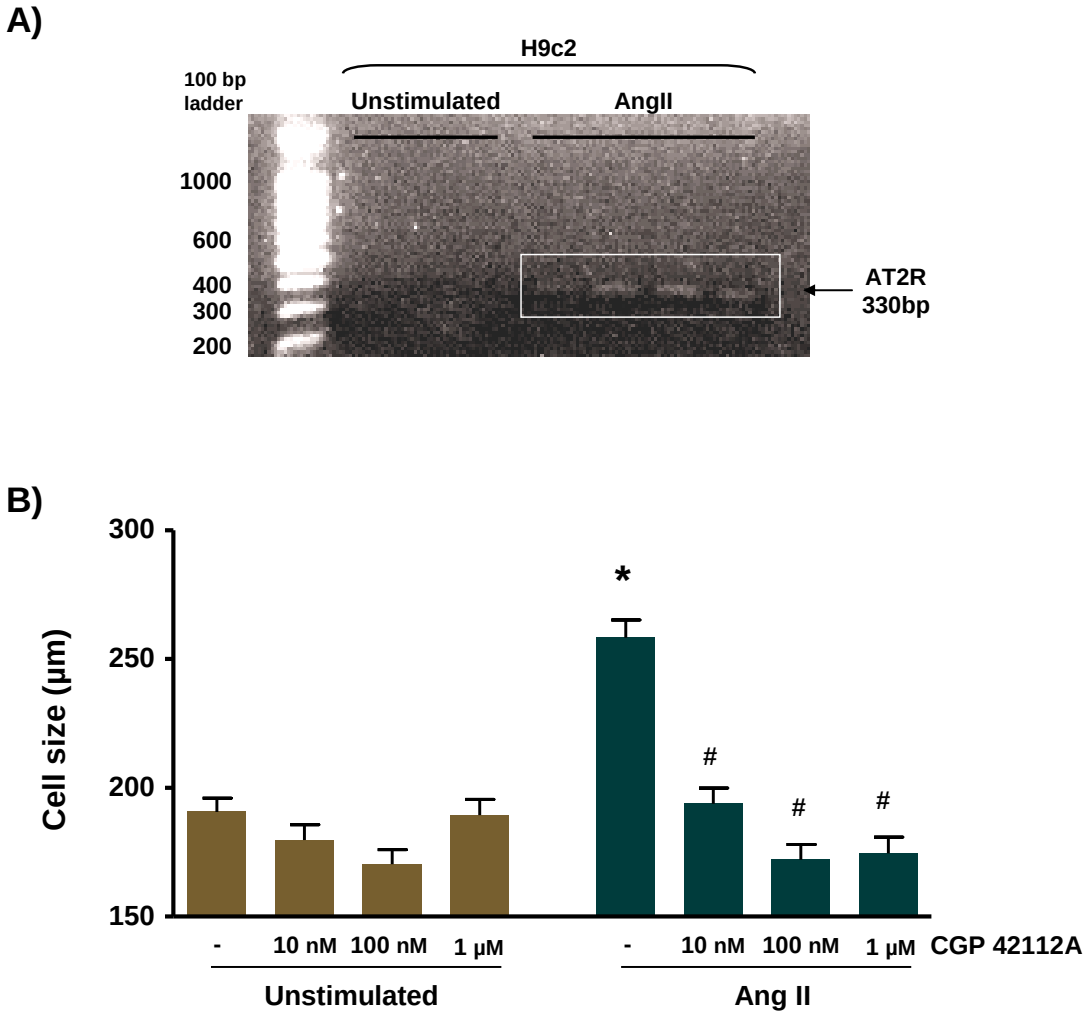


Figure 3-14. AT2R expression and activation in H9c2 cardiomyocytes.

(A) H9c2 cardiomyocytes were plated and stimulated with AngII (100 nM) for 24 hours. MRNA from unstimulated and AngII-stimulated H9c2 cardiomyocytes was extracted and cDNA generated. PCR was performed to detect AT2R. PCR product 330 bp (100 bp ladder). (B) H9c2 cardiomyocytes were incubated for 24 hours in serum free media before addition of the specific AT2R agonist CGP42112A at 10 nM, 100 nM or 1 µM for 30 minutes followed by addition of 100 nM AngII. Cells were incubated for a further 96 hours, fixed, stained with crystal violet and cell size measured using ImageProPlus. * $p<0.001$ vs. unstimulated cells; # $p<0.001$ vs. AngII stimulated cells.

To assess the role of the AT2R in the effects of Ang1-7 and Ang1-9 the AT2R antagonist PD123,319 was utilised. In H9c2 cardiomyocytes preincubation with Ang1-7 or Ang1-9 blocked AngII-induced hypertrophy as shown previously (Figure 3-15 A). Addition of PD123,319 at 100, 500 or 1000 nM alone did not affect AngII-induced hypertrophy. Additionally, the anti-hypertrophic effect of Ang1-7 was not abolished in the presence of PD123,319 (Ang1-7 = 221.1 ± 6.4 µm; Ang1-7 + PD123,319 500 nM = 216.2 ± 7.0 µm; $p < 0.001$). However, when PD123,319 was added to Ang1-9-stimulated cardiomyocytes, the anti-hypertrophic effect of Ang1-9 was blocked in a dose dependent manner (Ang1-9 = 216.3 ± 6.1µm; Ang1-9 + PD123,319 500 nM = 290.8 ± 8.3 µm; $p<0.001$). These findings were replicated in rabbit primary cardiomyocytes (Figure 3-15 B) suggesting that Ang1-9 signals through the AT2R.

3.2.7 Assessment of the role of the B2 bradykinin receptor in cardiomyocyte hypertrophy

The bradykinin B_2 receptor has been reported to heterodimerize with the AT2R and Mas receptor, leading to inhibition of fibrosis and promotion of vasodilatation (Soares de Moura et al., 2004, Peiro et al., 2007, Kurisu et al., 2003). In addition Erdos *et al.* showed that Ang1-9 potentiated bradykinin B_2 receptor activity, by enhancing the release of arachidonic acid in human atria and ventricles and an increase in intracellular Ca^{2+} concentration and NO synthesis in endothelial cells (Erdos *et al.*, 2002). In order to investigate if the bradykinin receptor was participating in Ang1-7 or Ang1-9 signalling the B_2 blocker HOE 140 was utilized. H9c2 cardiomyocytes were incubated with either A779 (10 µM) or PD123,319 (500 nM) and HOE 140 (1 µM) for 15 minutes before addition of Ang1-7 or Ang1-9. HOE 140 was unable to effect AngII-induced hypertrophy indicating there was no cross-talk between the B_2 receptor and the AT1R (unstimulated = 174.7 ± 5.5 µm; unstimulated + HOE 140= 161.6 ± 4.4 µm; AngII = 246.5 ± 9.8 µm; AngII + HOE 140= 248.3 ± 7.5 µm; $p < 0.001$) (Figure 3-16). However, blockade of the B2 receptor in cardiomyocytes stimulated with Ang1-7, had a reduced effect on the anti-hypertrophic effect of Ang1-7 (Figure 3-16).

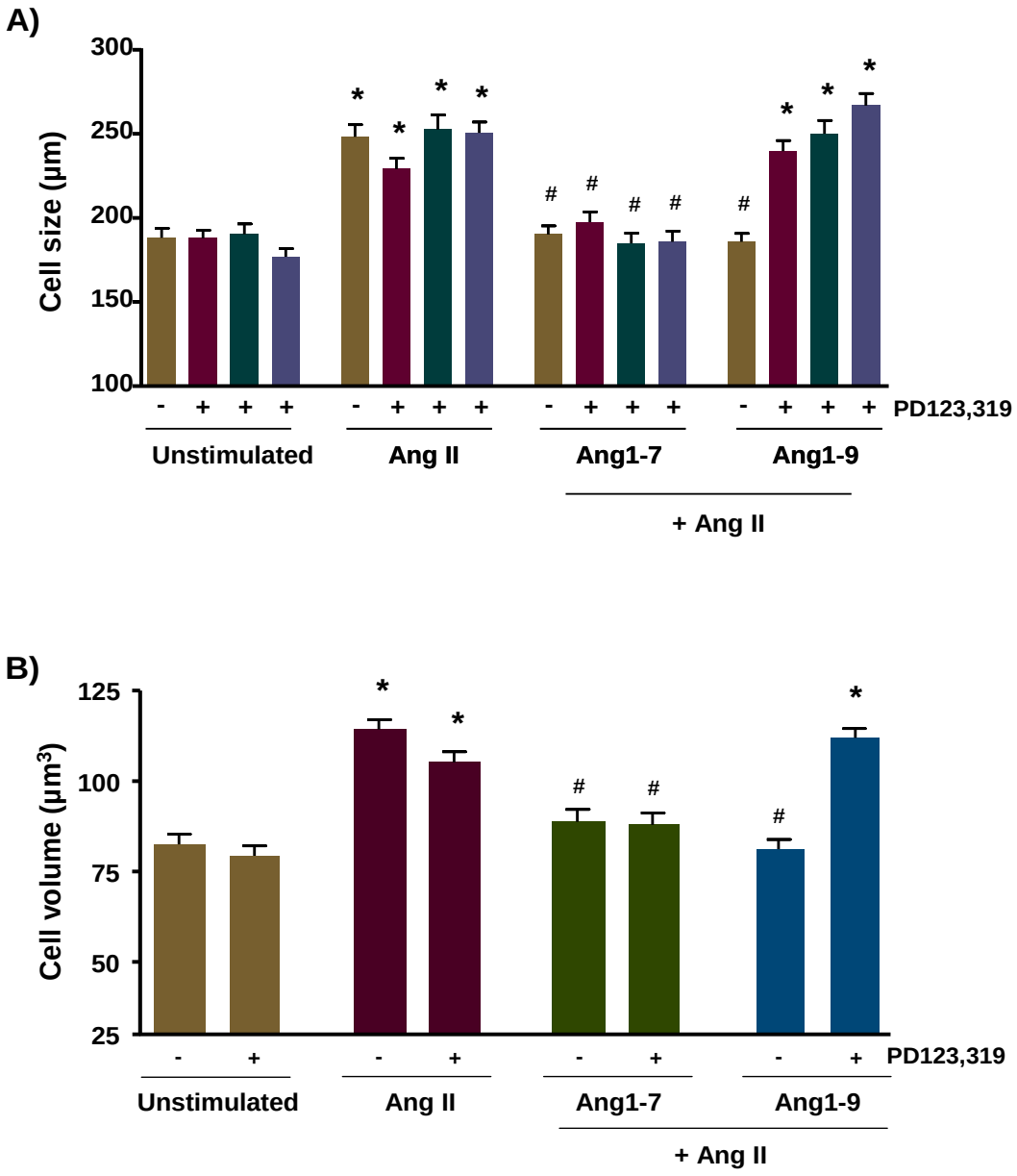


Figure 3-15. The role of the AT2R in Ang1-7 and Ang1-9 signalling in cardiomyocytes. (A) H9c2 cardiomyocytes were incubated for 24 hours in serum free media before addition of the AT2R antagonist PD123,319 (100 nM, 500 nM or 1 µM) followed by incubation for 15 minutes before addition of Ang1-7 (100 nM) or Ang1-9 (100 nM). Cells were incubated for a further 30 minutes and then stimulated with AngII (100 nM). After 96 hours incubation, cells were fixed, stained with crystal violet and cell size measured using ImageProPlus. * $p<0.001$ vs. unstimulated cells, #$p<0.001$ vs. AngII stimulated cells. (00 nM PD123,319; 500 nM PD123,319; 1 µM PD123,319). (B) Freshly isolated rabbit primary cardiomyocytes were plated and stimulated with PD123,319 (500 nM) for 15 minutes prior to addition of Ang1-7 or Ang1-9. AngII (500 nM) was added 30 minutes later, and cells were incubated for 24 hours. Cell volume was calculated from length and width measurements using ImageProPlus. * $p<0.01$ vs. unstimulated cells; # $p<0.01$ vs. AngII stimulated cells.

Although there was no significant difference between AngII-stimulated cells and Ang1-7 + HOE 140 stimulated cells, there was a significant increase in cell size observed between cardiomyocytes incubated with HOE 140 and Ang1-7 compared to cardiomyocytes incubated with Ang1-7 alone (Ang1-7 = 167.8 ± 4.2 µm; Ang1-7 + A779 = 226.4 ± 7 µm; Ang1-7 + HOE = 209.1 ± 6.2 µm) (Figure 3-16). Furthermore, HOE 140 had no effect on the anti-hypertrophic effect of Ang1-9 (Ang1-9 = 174.7 ± 4.5 µm; Ang1-9 + PD123,319 = 228.2 ± 8.0 µm; Ang1-9 + HOE 140 = 185.5 ± 5.5 µm) (Figure 3-16).

3.2.8 Cell Signalling

3.2.8.1 Protein Kinase C Translocation

PKC is activated and translocates rapidly from the cytosol to the cell membrane following exposure of cardiomyocytes to AngII (Jalili *et al.*, 1999a)(Li *et al.*, 2002). PKC membrane translocation was assessed in unstimulated and AngII-stimulated H9c2 cardiomyocytes at 1, 5 and 10 minutes. A marked mobilization of PKC from the cytosol to the membrane when H9c2 cardiomyocytes were stimulated with AngII was observed which was apparent following 1 minute of AngII stimulation (Figure 3-17). After 5 minutes PKC was still localized to the cell membrane but by 10 minutes the stain had returned to the same levels of unstimulated cells (Figure 3-17). This loss of fluorescence at 10 minutes led to the assessment of PKC translocation in H9c2 cells incubated with the Ang1-7 or Ang1-9 at 1 and 5 minutes. Co-addition of Ang1-7 or Ang1-9 to AngII stimulated cells produced a similar relocalization pattern of PKC staining to that observed with AngII alone.

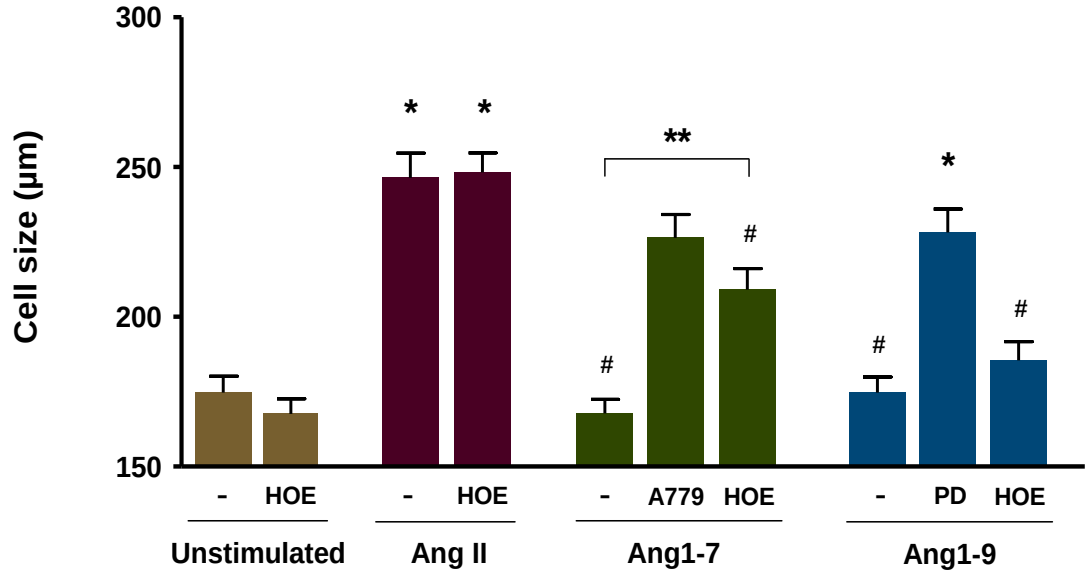


Figure 3-16. The role of the B2 bradykinin receptor in AngII-induced hypertrophy in H9c2 cardiomyocytes.

H9c2 cardiomyocytes were incubated for 24 hours in serum free media before adding HOE 140 (1 µM), A779 (10 µM) or PD123,319 (500 nM). Cells were incubated for 15 minutes before addition Ang1-7 or Ang1-9 (100 nM) followed by AngII 30 minutes later. Following 96 hours incubation, cells were fixed, stained with crystal violet and cell size measured with ImageProPlus. * $p<0.001$ vs. unstimulated cells; # $p<0.001$ vs. AngII stimulated cells; ** $p<0.001$ vs. Ang1-7 stimulated cells. (PD = PD123,319; HOE = HOE140).

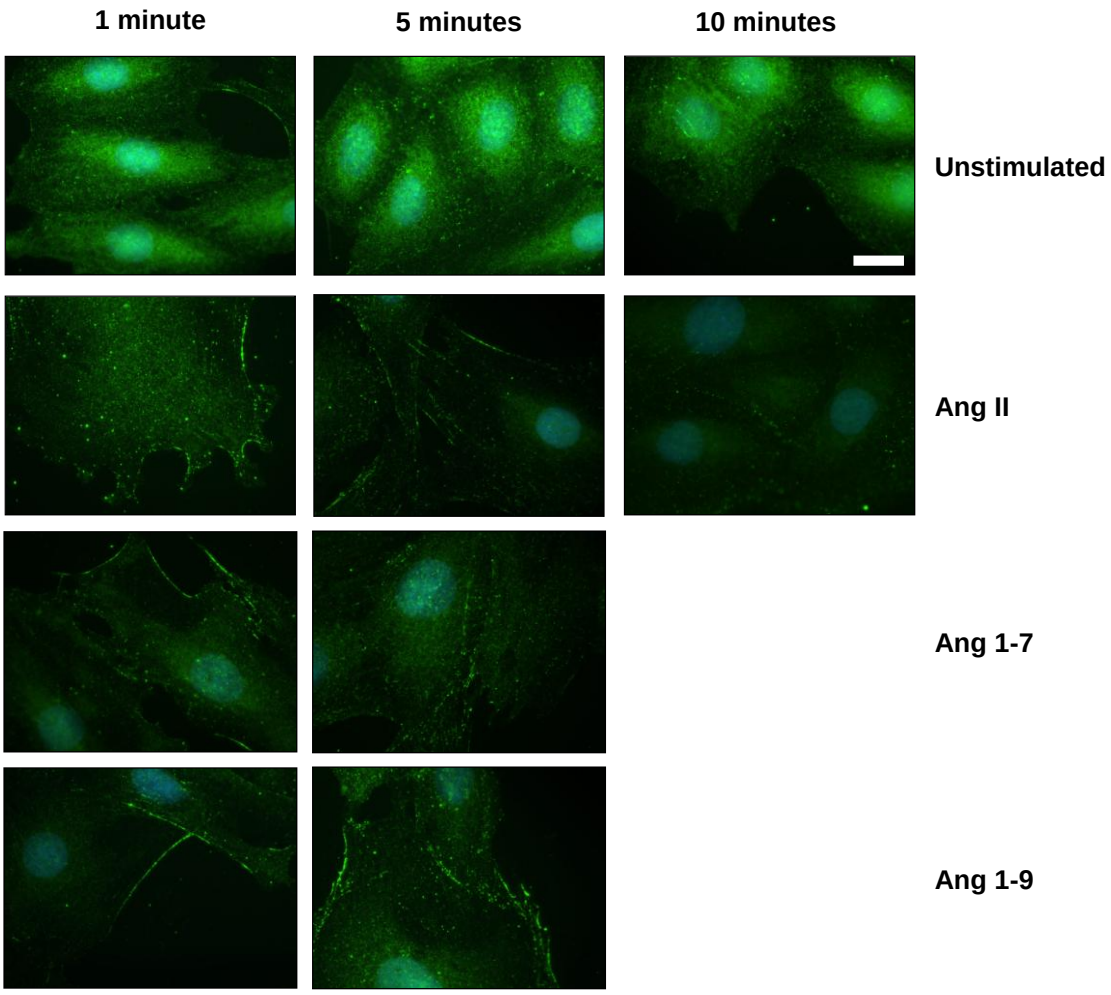


Figure 3-17. PKC translocation in the H9c2 hypertrophy model.

H9c2 cardiomyocytes were plated and incubated for 24 hours. Cells were then incubated with AngII for 1, 5 and 10 minutes and PKC translocation assessed in unstimulated and AngII-stimulated H9c2 cardiomyocytes. Next, H9c2 cardiomyocytes were preincubated with Ang1-7 or Ang1-9 (100 nM) and PKC translocation assessed after 1 and 5 minutes of AngII stimulation. Scale bar = 100µm; Magnification: 100x.

3.2.8.2 Assessment of receptor activation via the measurement of calcium transients in individual cells

To explore the functionality of the AT1R, AT2R and Mas receptor more fully Ca^{2+} transients were assessed in single cells following Fura-2 loading (section 2-18). H9c2 cardiomyocytes loaded with Fura-2 were exposed to AngII, Ang1-9 or Ang1-7 (1 µM) and Ca^{2+} mobilization recorded as an indicator of receptor activation. Cytosolic Ca^{2+} increased when H9c2 cardiomyocytes were exposed to AngII (Figure 3-18 A). Although mobilization of Ca^{2+} induced by AngII has been previously described (Samain *et al.*, 2002), our findings showed that only one third of the cardiomyocytes actually responded to AngII in this setting (Figure 3-18 B). In order to evaluate that engagement to a GPCR induced Ca^{2+} transients in these cells we assessed arg-vasopressin-induced Ca^{2+} mobilization as positive control. Arg-vasopressin also induced an increase in cytosolic Ca^{2+} in H9c2 cardiomyocytes.

When cardiomyocytes were exposed to Ang1-9 or Ang1-7, no increase in Ca^{2+} was registered (Figure 3-19 A and B). Moreover, exposure of H9c2 cardiomyocytes to the commercial AT2R agonist CGP42112A also failed to produce a Ca^{2+} transient, similar to that observed when perfusing with Ang1-9 (Figure 3-19 C). However, when H9c2 cardiomyocytes were re-exposed to ionomycin as a positive control an increase in intracellular Ca^{2+} was observed indicating the assay was functioning.

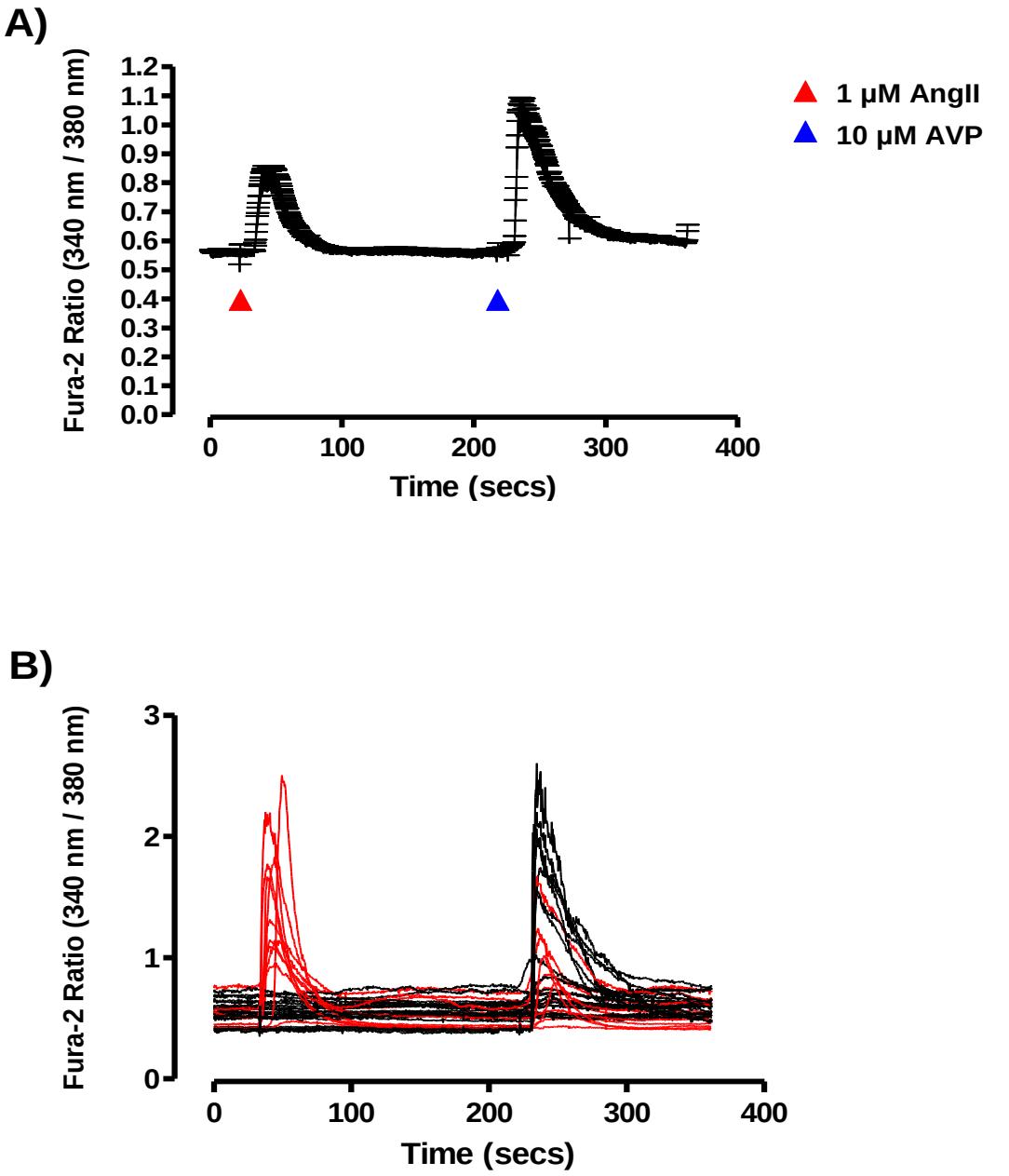


Figure 3-18. Assessment of calcium transients in H9c2 cardiomyocytes.

H9c2 cardiomyocytes were plated and incubated for 24 hours. Cells were loaded with the Ca^{2+} indicator Fura-2 (1.5 µM) and incubated for a further 30 minutes at 37ºC. Cells were placed into a microscope chamber which was then perfused with AngII (1 µM), following acquisition of 60 images the change in cytoplasmic Ca^{2+} was recorded for a further 140 images. The agonist was then removed and a further 200 images collected followed by perfusion with (arg-vasopressin 10 µM). (A) Traces of grouped cells perfused with AngII. (B) Traces of individual cardiomyocytes showing the actual number of cells that responded to AngII stimulation. Results are expressed as fluorescence ratio at 340 / 380 nM. Red traces represent cardiomyocytes that responded to AngII stimulation; black traces represent cardiomyocytes not responding to AngII stimulation (AVP = arg-vasopressin).

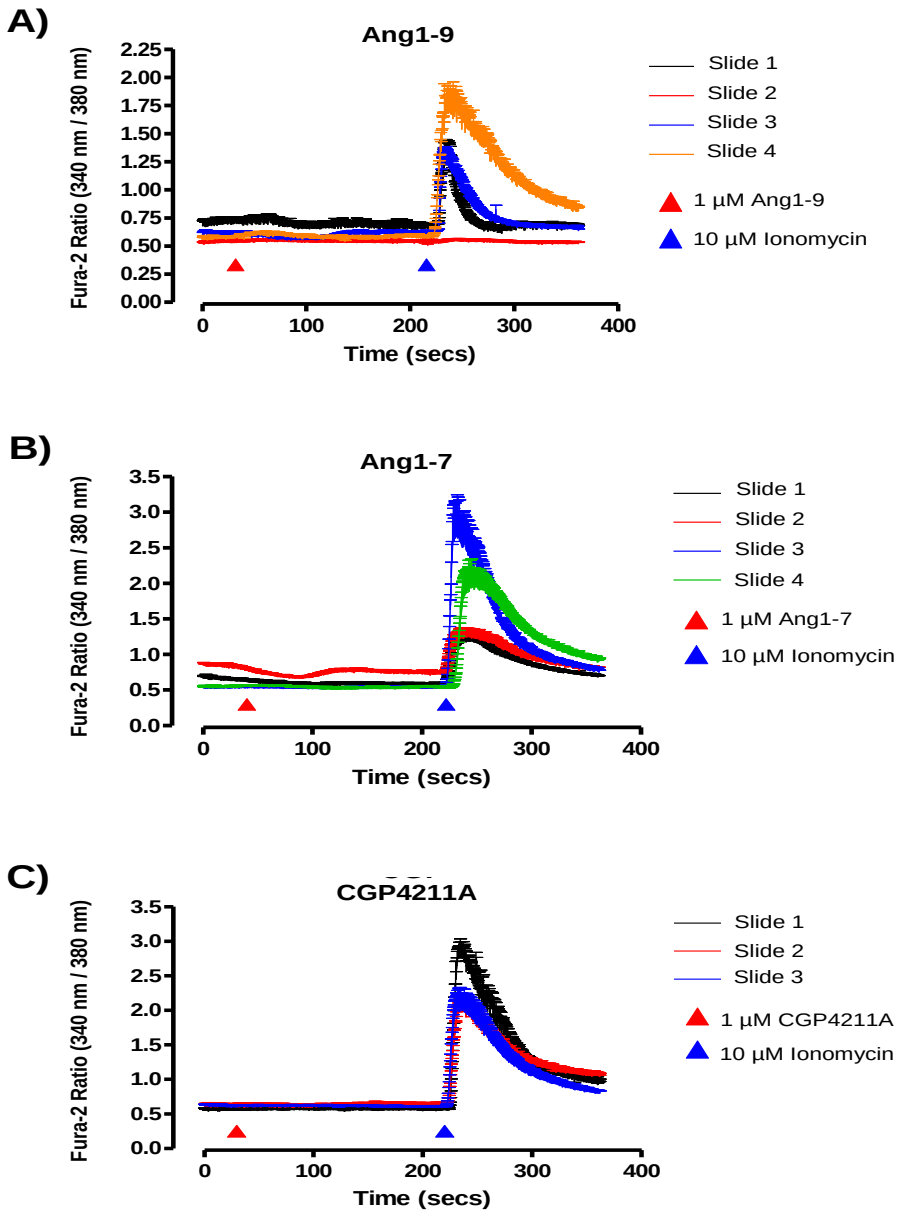


Figure 3-19. Assessment of the effects of Ang1-7, Ang1-9 and CGP42112A on calcium signalling.

H9c2 cardiomyocytes were loaded with the Ca^{2+} indicator Fura-2 (1.5 µM) and incubated for 30 minutes at 37ºC. Cells were placed into a microscope chamber and perfused with Ang1-7, Ang1-9 or CGP42112A (1 µM) followed by acquisition of 60 images before any change in cytoplasmic Ca^{2+} was recorded for a further 140 images. Once agonist was removed, a further 200 images were collected before the positive control agonist (ionomycin 1 µM) was added and a further 140 images collected. (A) Traces of changes in cytoplasmic Ca^{2+} in Ang1-9 stimulated cardiomyocytes grouped in slides; (B) Traces of changes in cytoplasmic Ca^{2+} in Ang1-7 stimulated cardiomyocytes grouped in slides; (C) Traces of cardiomyocytes stimulated with CGP42112A grouped in slides. Results are expressed as fluorescence at a ratio of 340 / 380 nm.

3.2.9 Assessment of the role of individual G-Protein Coupled Receptor Subunits

PTX has been described to inactivate $G_{\alpha i}$ proteins (Teschemacher and Seward, 2000, Huang et al., 2009, Lee et al., 2009, Lane et al., 2007). To investigate whether $G_{\alpha i}$ mediates the antihypertrophic effects of Ang1-7 and Ang1-9 on AngII-stimulated H9c2 cardiomyocytes, cell size was assessed in the presence of PTX. In the presence and absence of PTX, AngII was able to induce hypertrophy (unstimulated = 189.7 ± 5.5 µm; unstimulated + PTX = 189.3 ± 5.6 µm; AngII = 241.5 ± 6.2 µm; AngII + PTX = 239.8 ± 4.9 µm; $p < 0.001$) (Figure 3-20). However, the anti-hypertrophic effect of both Ang1-7 and Ang1-9 were abolished by PTX (Ang1-7 = 201.0 ± 5.0 µm; Ang1-7 + PTX = 235.0 ± 4.8 µm; Ang1-9 = 184.5 ± 4.0 µm; Ang1-9 + PTX = 222.0 ± 4.7 µm) (Figure 3-20). These preliminary results suggest that when Mas and AT2R are bound to Ang1-7 and Ang1-9 respectively, they may initiate cell signalling cascades through activation of the $G_{\alpha i}$-subunit.

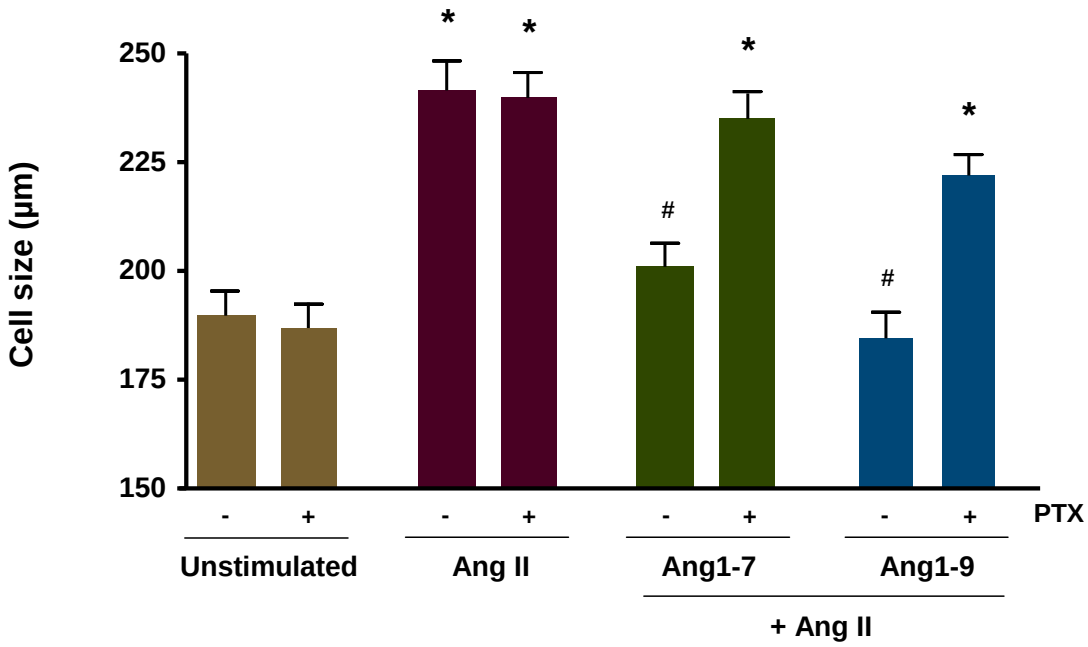


Figure 3-20. $G_{\alpha i}$ mediates the anti-hypertrophic effect of Ang1-7 and Ang1-9 in H9c2 cardiomyocytes.

H9c2 cardiomyocytes were incubated for 8 hours before exchanging the media for serum free media supplemented with PTX (25 ng/ml), followed by addition of Ang1-7 or Ang1-9 (100 nM). Cells were incubated for 30 minutes before addition of AngII (100 nM). Cells were then incubated for a further 96 hours before fixing, staining with crystal violet and cell size measured with ImageProPlus. * $p<0.001$ vs. control; # $p<0.001$ vs. AngII.

3.3 Discussion

In this chapter, a novel role for the angiotensin peptide Ang1-9 has been established. The data show that Ang1-9 is an antagonist of *in vitro* cardiomyocyte hypertrophy. This peptide metabolite of AngII has not been shown to mediate any specific receptor interactions, although it was thought to contribute to reduced AngII (through being generated from AngI via the actions of ACE2) and also to act as a substrate for Ang1-7 generation via ACE metabolism. These data indicate that Ang1-9 appears to have direct effects, antagonizing pro-hypertrophic signals from different stimuli in cardiomyocytes from different species, representing both neonatal and adult lineages. Additionally, Ang1-9 utilizes the AT2R to mediate its signals, segregating its actions from those of Ang1-7, which engages the Mas receptor.

The difference between neonatal and adult cardiomyocytes is well established. Neonatal cardiomyocytes have the ability to divide, while adult cardiomyocytes have lost this ability. This makes neonatal cells easier to culture and they are the most widely used. However, adult cardiomyocytes are more reflective of the adult heart *in vivo*. Adult cardiomyocytes are larger and the transverse tubule system is fully developed (Schaub *et al.*, 1997). Adult and neonatal cardiomyocytes not only differ in morphology and mitotic ability but also gene expression is different, for example, the AT2R is expressed at lower levels in normal adult rodent cardiomyocytes (Lijnen and Petrov, 1999, Schaub et al., 1997). Therefore it is important to assess hypertrophy in different developmental stages of cardiomyocytes and in different species to reinforce the importance and relevance of the results.

The classical definition of the RAS as a systemically acting hormone system is increasingly being re-defined by tissue-specific actions following the description of local generation of RAS components in individual organs. The heart has been a major focus for studying the local RAS since the discovery of ACE2 (Crackower *et al.*, 2002). In the heart, Ang1-7 has been described to antagonize the pathophysiological signalling of Ang-II. Furthermore, Ang1-7 blocks hypertrophy and interstitial fibrosis in heart of rats infused with AngII (Grobe *et al.*, 2007b, Mercure *et al.*, 2008). Furthermore, Grobe *et al.* showed that Ang1-7 not only

blocked AngII-induced hypertrophy but also hypertrophy induced by other stimuli such as corticosteroids (Grobe *et al.*, 2006). In addition, Ang1-7 antagonizes AngII signalling in other tissues. For example, in blood vessels intraarterial infusion of Ang1-7 into the human forearm antagonized AngII-induced vasoconstriction (Ueda *et al.*, 2000), while in rat aortas Ang1-7 prevented superoxide formation induced by AngII (Polizio *et al.*, 2007). In kidney proximal tubular cells Ang1-7 inhibited AngII-induced activation of p30, ERK1/2 and JNK (Su *et al.*, 2006). In the brain Ang1-7 has been shown to block the increased release of norepinephrine from the hypothalamus of rats following stimulation with AngII (Gironacci *et al.*, 2004). All these studies highlight the importance of Ang1-7 in the counter-regulatory arm of the RAS. Although certain studies have proposed that Ang1-7 may interact with the AT1R and AT2R (Silva et al., 2007, Walters et al., 2005) it is now established that Ang1-7 utilises the proto-oncogene G-protein coupled receptor Mas (Santos *et al.*, 2003, Santos *et al.*, 2006, Sampaio *et al.*, 2007b, Dias-Peixoto *et al.*, 2008).

Hypertrophy in cardiomyocytes has been defined as an increase in cell size and in protein synthesis, reorganization of sarcomeres and expression of hypertrophy markers (Carreno et al., 2006, Frey and Olson, 2003, Dorn et al., 2003). The most established markers are ANP, BNP, β-MHC and skeletal α-actin (Dorn *et al.*, 2003). However the difference in expression of ANP, β-MHC and skeletal α-actin can depend on the species and the developmental stage of the cardiomyocyte thus BNP has been defined as the most reliable acute hypertrophy marker (Schaub *et al.*, 1997, Stuck *et al.*, 2008). In accordance with this, we were able to induce increases in cell size following AngII stimulation in both neonatal rat cardiomyocyte line (H9c2) and in adult primary rabbit ventricular cardiomyocytes. AngII also induced an increase expression of BNP as well as inducing reorganization of α-actin filaments in H9c2 cardiomyocytes. Importantly, in adult rabbit primary cardiomyocytes the increase in cell volume induced by AngII was due to an increase in cell width. This has been reported to be related to the addition of sarcomeres in parallel in the cardiomyocyte, characteristic of concentric hypertrophic growth (Dorn *et al.*, 2003). Assessment of sarcomere staining using phalloidin in primary adult cardiomyocytes would help address this. The addition of both Ang1-7 and Ang1-9 was able to block the increase in cell size, sarcomere reorganization and increase in BNP gene expression, induced by AngII stimulation, resulting in an effective block of hypertrophy at least *in vitro*.

The first interesting finding which segregated the actions of Ang1-7 from Ang1-9 was the observation of a bi-phasic mechanism of Ang1-7 action (Figure 3-8). At concentrations up to 1 μM Ang1-7 antagonized the pro-hypertrophic effects of AngII, however at higher concentrations (100 μM) Ang1-7 induced hypertrophy even in the absence of AngII (Figure 3-8). In contrast, Ang1-9 was not pro-hypertrophic at any concentration tested and retained the ability to antagonize AngII signalling. Since a bi-phasic effect was also observed with the partial Mas agonist MBP7 (Figure 3-11), Mas signalling effects may underline the biphasic effects of Ang1-7. Previous data have demonstrated that MBP7 promotes internalization of the Mas receptor with no increase in [H^3] inositol phosphate in CHO cells expressing the Mas receptor (Bikkavilli *et al.*, 2006). However, MBP7 at 100 μM significantly increased the accumulation of [H^3] inositol phosphate, suggesting MBP7 acted as a partial agonist. A bi-phasic mechanism of action for Ang1-7 has been reported previously in several studies (Garcia and Garvin, 1994, Haulica *et al.*, 2003, De Mello, 2009). In rat aorta Ang1-7 concentrations above 10 μM stimulated vasoconstriction while lower concentrations produced vasodilatation (Haulica *et al.*, 2003). In kidney proximal tubules 1 pM Ang1-7 stimulated fluid absorption, however at 10 nM fluid absorption decreased (Garcia and Garvin, 1994). In cardiomyopathy 10 nM Ang1-7, hyperpolarized cardiomyocytes and increased conduction velocity through the heart, while 100 nM triggered the opposite effect (De Mello *et al.*, 2007). The data here provide further evidence for a bi-phasic role for Mas signalling via Ang1-7 and highlight differences between Ang1-7 and Ang1-9 function.

In contrast to Ang1-7 very little is known about Ang1-9. Ang1-9 was originally reported to be generated in the heart through metabolism of AngI via ACE2 and further metabolised to Ang1-7 through ACE cleavage (Donoghue *et al.*, 2000). Jackman *et al.* demonstrated that cathepsin A could stimulate Ang1-7 and Ang1-9 production in human heart tissue (Jackman *et al.*, 2002) but experiments to dissect differential effects of Ang1-7 or Ang1-9 were not performed. In the study performed here inhibition of ACE to prevent Ang1-9 conversion to Ang1-7 did not block its effects. It is important to mention that when attempting to asses the efficacy of captopril we were unable to mesure ACE activity *in vitro*. However, blockade of the Mas receptor with the antagonist A779, did not abolish the antihypertrophic effects

of Ang1-9, supporting the theory of an independent role for Ang1-9 in cardiomyocyte hypertrophy and furthermore highlighting that Ang1-9 signalled via a different receptor.

Through the antagonism of the AT1R with losartan, it was demonstrated that Ang1-9 does not act as a competitive inhibitor of AngII signalling via the AT1R. Furthermore, Ang1-9 and Ang1-7 blocked arg-vasopressin-induced hypertrophy, demonstrating that they could both inhibit hypertrophy induced by alternative agonists.

The results here demonstrate the use of the AT2R by Ang1-9 in mediating its effects. The role of the AT2R in the RAS is controversial. AngII is reported to signal via both the AT1R and the AT2R (Bartunek *et al.*, 1999, Bai *et al.*, 2004)(Oudot et al., 2005, Aranguiz-Urroz et al., 2009) however the AT1R is the main mediator of the classical effects of AngII and the AT2R is considered a counter-regulatory signalling mechanism by which AngII could negatively feedback on signals mediated via the AT1R. This was due to the discovery that engagement of the AT2R by AngII led to bradykinin and NO release (Abadir *et al.*, 2006).

It is important to mention that even though A779 and PD123,319 are the most established antagonist for Mas and the AT2R, there is no certainty that these peptides are complete selective to the receptors (Brechler et al., 1993). More studies to evaluate their pharmacology are required.

There is evidence of heterodimerization between the bradykinin B2 receptor and the AT1R, as well as the AT2R and Mas (Soares de Moura *et al.*, 2004, AbdAlla *et al.*, 2005, Canals *et al.*, 2006). It has also been reported that captopril, besides inhibiting ACE, potentiates the antihypertrophic effect of bradykinin (Carreno *et al.*, 2006). Jackman *et al.* showed that Ang1-9 and Ang1-7 potentiated the B2 receptor by enhancing the release of arachidonic acid and NO generation in CHO cells expressing human ACE and B_2 receptor and also in endothelial cells (Jackman *et al.*, 2002). However, Ang1-9 is also further transformed to Ang1-7 via ACE mediated-cleavage, and a study to differentiate a direct role to segregate Ang1-7 from Ang1-9 was not performed. Here, no interaction between bradykinin B2 receptor and the

AT2R was observed as blocking the B_2 receptor by HOE 140 did not affect Ang1-9. However, the antihypertrophic effect of Ang1-7, although not abolished completely, was affected by antagonism of the B_2 receptor, suggesting a potential interaction between Mas and B_2 receptors. Furthermore, antagonism of the B_2 receptor supports the direct antihypertrophic effect of Ang1-9, rather than potentiation of bradykinin and/or inhibition of ACE.

Intracellular Ca^{2+} mobilization has been previously demonstrated for agonist binding to a G-protein coupled receptor (Thomas *et al.*, 1996, Samain *et al.*, 2002, Emkey and Rankl, 2009). The results here suggest that such a Ca^{2+} transient is not induced by Ang1-7 or Ang1-9 acutely, supporting the idea that Mas and the AT2R are not classical GPCR. In agreement with the data here presented, Dias-Peixoto *et al.* was unable to induce changes in intracellular Ca^{2+} in cardiomyocytes when stimulated with Ang1-7 (Dias-Peixoto *et al.*, 2008). It is important to mention that even though grouped results of AngII perfusion of H9c2 cardiomyocytes stimulated a Ca^{2+} transient, however when analysing traces for single cells, only one third of cells responded. This suggests that there may be low levels of receptor expression in H9c2 cardiomyocytes preventing observation of acute effects. This agrees with the requirement for 96 hour incubation to induce hypertrophy with AngII. Further experiments in primary cells in which hypertrophy occurs in a 24 hour time period are important.

G-proteins can couple to several different receptors and depending on the G-protein subunit, different pathways can be activated (Fredholm *et al.*, 2007, Rang, 2007, Milligan, 2008). It is well established that AngII induces hypertrophy through $G_{\alpha q/11}$ in vascular smooth muscle cells and cardiomyocytes (Bai *et al.*, 2004, Ohtsu *et al.*, 2008, Lorenz *et al.*, 2009b). However AT2R signalling is still not well defined and the findings are conflicting. In COS-7 cells over-expressing recombinant AT2R some studies have showed activation of the $G_{\alpha i}$-subunit following AT2R stimulation (Hansen *et al.*, 2000), while others have failed to show AT2R association with G-proteins (Mukoyama *et al.*, 1993). The Mas receptor is also less clearly understood but evidence does indicate $G_{\alpha q/11}$-subunit activation (Canals *et al.*, 2006). Here, blocking the $G_{\alpha i}$-subunit with PTX completely blocked the anti-hypertrophic effects of Ang1-7 and Ang1-9 suggesting that they may signal via the $G_{\alpha i}$-subunit.

Stimulation of cardiomyocytes with AngII has been demonstrated to induce translocation of PKC from the cytoplasm to the cell membrane (Li *et al.*, 2002). Detection of PKC in H9c2 cardiomyocytes stimulated with AngII triggered a rapid movement of PKC as within 1 minute of stimulation PKC was detected in the cell membrane where it was retained for at least 5 minutes. When Ang1-7 and Ang1-9 were added to AngII-stimulated cardiomyocytes PKC translocation to the membrane was also observed within a similar timeframe and with a similar distribution. The antibody used detects several isoforms of PKC (α, β and γ), therefore, does not allow identification of any difference in specific PKC isoforms following differential stimuli. Different PKC isoforms are activated following exposure of cardiomyocytes to different hypertrophic stimuli leading to activation of different pathways such as intracellular Ca^{2+} handling and ANP production (Church *et al.*, 1994, Takeishi *et al.*, 2000). It is therefore important that further experiments are performed in the future to distinguish such differences. Furthermore, Ang1-7 and Ang1-9 induced PKC translocation was detected in the presence of AngII and not the peptides alone; therefore no data regarding the basal effects of the peptides was generated.

The findings reported here have implications for our understanding of the integrated functioning of the RAS. Although Ang1-9 is a previously reported angiotensin metabolite, this is the first demonstration of a direct biological effect of this peptide which can be separated from the actions of either AngII or Ang1-7 via the use of pharmacological reagents. Our findings are replicated in two different cardiomyocyte types representing different species and both adult and neonatal lineages. In summary, the data presented here demonstrates that Ang1-9 is able to antagonize AngII signalling in cardiomyocytes, selectively via the AT2R.

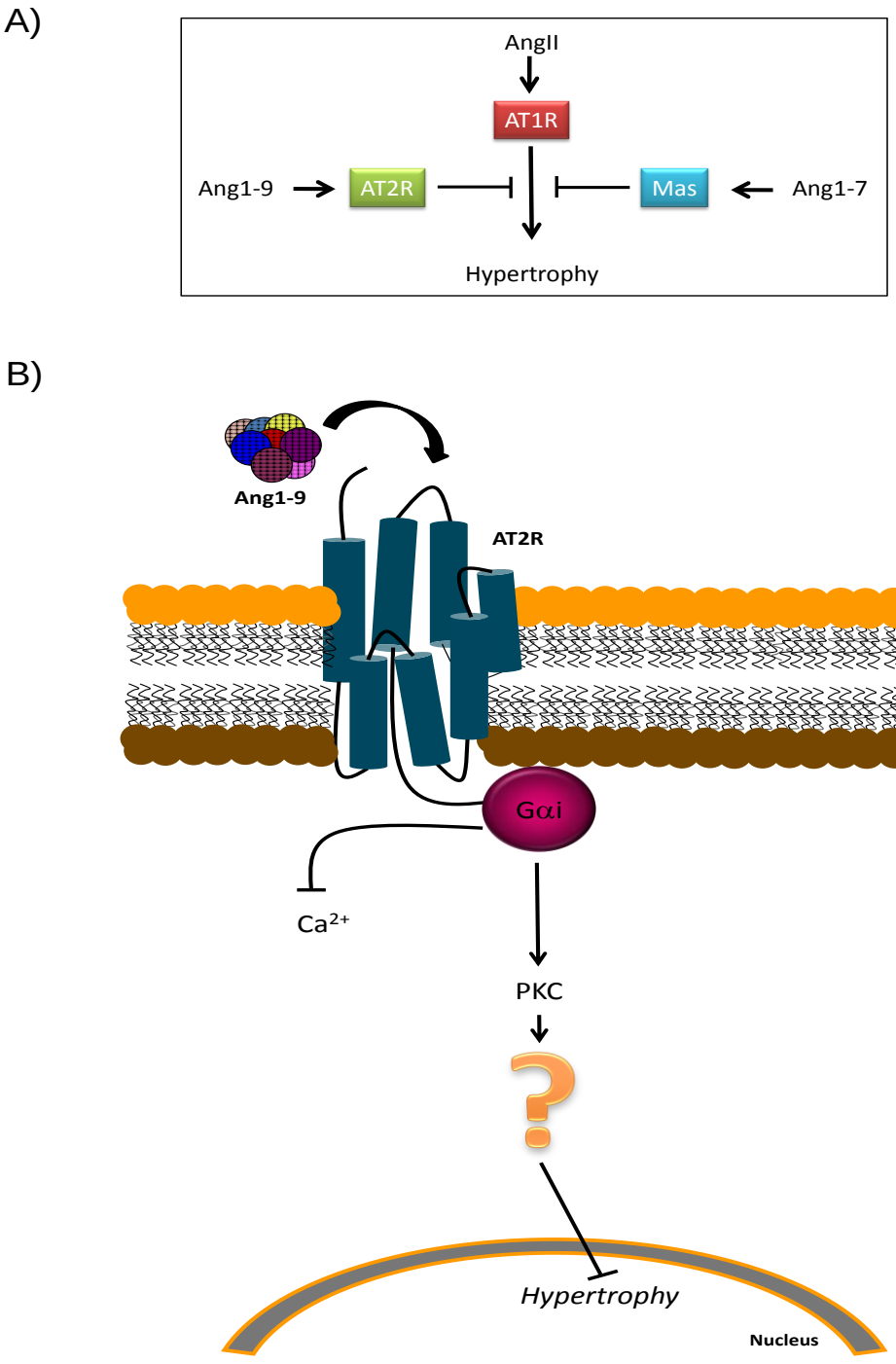


Figure 3-21. Diagram of findings.

Figure representing the findings of this chapter. A) AngII induces cardiomyocyte hypertrophy signalling through the AT1R. Both Ang1-9 and Ang1-7 block AngII-induce hypertrophy by coupling to the AT2R and Mas receptor respectively. B) Through coupling to the AT2R, Ang1-9 is able to block hypertrophy. Once bound to the AT2R it is the $G_{\alpha i}$-subunit that mediates translocation of PKC to the cell membrane and block hypertrophy. However, there is still a gap between PKC translocation and blocking hypertrophy to be elucidated.

C H A P T E R 4

Assessment of the effects of angiotensin 1-9 in the stroke prone spontaneously hypertensive rat

4.1 Introduction

The SHRSP is an experimental model of hypertension developed over 30 years ago by Okamoto *et al.* and frequently used for the study of CVD (Okamoto and Aoki, 1963). This rat strain was developed by inbreeding the SHR strain with selected offspring of parents that developed stroke (Okamoto and Aoki, 1963). The main characteristics of this strain include lower body weight compared to SHR, early development of high blood pressure (10 weeks of age) and stroke. High blood pressure in the SHRSP is maintained by later development of high peripheral vascular resistance due to neurogenic vasoconstriction to maintain normal cardiac output (Tanase *et al.*, 1982). Stroke is most frequently due to cerebral haemorrhage or infarction in the anteriomedial and occipital cortex, and the basal ganglia around 100 to 150 days of age (Tanase *et al.*, 1982). SHRSP portrays a phenotype similar to human essential hypertension as it presents end-organ damage such as left ventricular hypertrophy, stroke and renal failure and is sexually dimorphic (Badyal, 2003, Tanase et al., 1982).

The role of the RAS as an important regulator of blood pressure and its involvement in the pathophysiology of hypertension is very clear. *In vivo* animal models have demonstrated the participation of the different components of the RAS in the control of blood pressure. For example, in animals infused with AngII elevated blood pressure has been shown (Frank *et al.*, 2007, Guzik *et al.*, 2007)(Kawada *et al.*, 2002). *In vivo* delivery of AngII induces cardiac hypertrophy (Majalahti *et al.*, 2007, Sriramula *et al.*, 2008) and fibrosis (Brilla *et al.*, 1993, Chintalgattu and Katwa, 2009). In transgenic mice that over-express the AT1R it has been shown that this receptor mediates AngII-induced hypertension, cardiac hypertrophy and fibrosis (Majalahti *et al.*, 2007)(Billet *et al.*, 2008). Although there is clear evidence for AT2R-mediated signalling in hypertension (Metcalfe et al., 2004, Kaschina et al., 2008, Bartunek et al., 1999), the exact role of it is controversial. *In vivo* over-expression of AT2R using vectors or transgenic mice attenuates cardiac hypertrophy, fibrosis and promotes vasodilatation (Kurisu et al., 2003, Metcalfe et al., 2004, Yayama and Okamoto, 2008, Yan et al., 2008). Stimulation of AT2R after myocardial infarction has been demonstrated to improve ventricular function

(Kaschina *et al.*, 2008), while blockade or deletion of the AT2R gene promotes cardiac hypertrophy and high blood pressure (Ichiki *et al.*, 1995, Bartunek *et al.*, 1999). Conversely, it has also been shown that over-expression of AT2R promotes dilated cardiomyopathy (Yan *et al.*, 2003b) and cardiac fibrosis (Masson *et al.*, 2009).

The role of the more recently discovered components of RAS is not well defined. Over-expression of ACE2 has given controversial results. Some studies show a reduction in blood pressure, cardiac hypertrophy and fibrosis (Huentelman *et al.*, 2005, Diez-Freire *et al.*, 2006, Grobe *et al.*, 2007a) as well as improved cardiac and endothelial function (Der Sarkissian *et al.*, 2008, Rentzsch *et al.*, 2008). Alternatively, ACE2 over-expressing mice demonstrated abnormalities in heart conduction as well as fibrosis and poor cardiac function (Donoghue *et al.*, 2003), whereas overexpression via gene delivery results in severe cardiac fibrosis and heart failure (Masson *et al.*, 2009). The role of Ang1-7 has also been studied *in vivo*. Benter *et al.* showed that Ang1-7 had a protective role against hypertension and cardiac damage in the SHR (Benter *et al.*, 2006). Ang1-7 has also shown antihypertrophic and antifibrotic effects in hypertensive animal models (Grobe *et al.*, 2007b, Mercure *et al.*, 2008). Vasodilatation and anti-inflammatory effects have also been described as actions of Ang1-7 *in vivo* (Lee et al., 2009)(Sampaio *et al.*, 2003). Pharmacological blockade and deletion of the Ang1-7 receptor Mas has provided evidence that Ang1-7's *in vivo* effects are mediated by the Mas receptor (Soares de Moura *et al.*, 2004, Santos *et al.*, 2006, Dias-Peixoto *et al.*, 2008).

Osmotic minipumps have been a useful tool to investigate the physiological and pathophysiological role of specific agents in a systemic environment. A major advantage of osmotic minipumps is the subcutaneous delivery of a reagent at a constant dose over a defined period of time which would not be affected by any other manipulations, procedures or homeostatic adjustments (Struyker-Boudier and Smits, 1978). Osmotic minipumps have been used widely to study the systemic effects of angiotensin peptides hormones. AngII has been delivered through osmotic minipumps in several studies (Metsarinne *et al.*, 1996, Castoldi *et al.*, 2007)(Saito *et al.*, 2004). Metsarinne *et al.* described AngII-mediated regulation of ACE expression in lung, heart and kidney as subcutaneous delivery for 3 days to normal rats

decreased ACE activity and expression in lung, but did not affect ACE expression in heart and kidney (Metsarinne *et al.*, 1996). In the kidney, Saito *et al.* demonstrated that AngII was an important regulator of renal damage (Saito *et al.*, 2004). AngII delivered by osmotic minipumps to rats for 7 days increased kidney expression of TGF-β1 and deposition of collagen I and IV. The involvement of AngII in vascular remodeling has been studied using osmotic minipumps, leading to increased expression of tissue inhibitors of metalloproteinases-2 (TIMP-2) in aortic smooth muscle cells (Castoldi *et al.*, 2007). In these studies AngII infusion also triggered increased blood pressure and cardiac hypertrophy. Osmotic minipumps have also been useful to study pharmacological reagents. Delivery of AT1R and AT2R antagonists has been used to study the regulation of the local RAS (Zhou et al., 2006, Ishiyama et al., 2004). Blockade of the AT1R using candersatan in rats resulted in increased expression of angiotensinogen, ACE, and AT2R in the microvasculature of the brain (Zhou *et al.*, 2006) and also decreased cardiac hypertrophy with improved contractility (Ishiyama *et al.*, 2004). Furthermore, osmotic minipump-mediated delivery of Ang1-7 has also been used to prove its effects in different tissues (Lu et al., 2008, Mendes et al., 2005). Analysis of expression of different components of the RAS in heart and kidney when Ang1-7 was subcutaneously delivered showed a decrease in AngII levels and increased ACE 2 expression in the heart, while in kidney AngII was not affected but Ang1-7 and AngI levels were increased (Mendes *et al.*, 2005).

Other features of hypertension include endothelial dysfunction and altered cardiac function parameters such as cardiac performance are cardiac output, stroke volume and ejection fraction. Cardiac output (CO) refers to the amount of blood ejected from the heart per minute, and is a product of stroke volume (SV) and heart rate (HR). In SHRSP CO is approximately 250ml/min (Badyal, 2003). SV is the difference between the end diastolic volume and the end systolic volume and represents the mechanical function of the heart. SV can be affected by changes in preload, afterload and contractility of the cardiac muscle. Afterload represents the force the ventricular muscle needs to contract in order to eject the blood, while preload refers to the amount of blood in the ventricle at the end of the diastole. Ejection fraction is the main parameter to evaluate ventricular function as it represents the fraction of blood in the ventricle that is ejected in every ventricular contraction. This parameter is

usually 60% and is measured as the ratio between SV and end diastolic volume (Fauci, 2008, Guyton, 2007, Andreoli, 2001). According to Frank-Starling Law, if the preload increases, the ventricular muscle increases contractile tension in order to pump blood efficiently and maintain CO and tissue perfusion. (Fauci, 2008, Andreoli, 2001). With the advent of radiotelemetry and ECHO, assessment of blood pressure and cardiac function *in vivo* became non-invasive and less stressful for the animal and hence more accurate. Here, Ang1-9 was infused via osmotic minipumps into SHRSP to assess its effects on blood pressure, cardiac and endothelial function.

4.2 Results

4.2.1 Effects of Ang1-9 infusion on blood pressure

Analysis of blood pressure was performed by radio-telemetry. The transmitter was implanted in the peritoneum of the rats 10 days before osmotic minipump implantation (Figure 2-1) and blood pressure was measured constantly over the 4 weeks of the study. The average of one week blood pressure readings was used to analyse the data. All three groups started the study with similar values of mean arterial blood pressure (control = 155.8 ± 1.6 mmHg; Ang1-9 = 160.3 ± 5.9 mmHg; Ang1-9 + PD123,319 = 164.5 ± 4.2 mmHg). Following minipump implantation, mean arterial blood pressure changed and an approximate increase of 10 mmHg in Ang1-9 infused rats and 20 mmHg in SHRSP co-infused with Ang1-9 and PD123,319 was observed compared to controls, although this difference did not reach statistical significance (control = 149.0 ± 1.0 mmHg; Ang1-9 = 158.8 ± 8.0 mmHg; Ang1-9 + PD123,319 = 162.9 ± 5.9 mmHg) (Figure 4-1 A). This tendency was sustained until the 4 weeks end point of the study (control = 152.7 ± 1.0 mmHg; Ang1-9 = 164.3 ± 9.8 mmHg; Ang1-9 + PD123,319 = 172.0 ± 8.4 mmHg). Similar results were observed in systolic and diastolic blood pressure readings (Figure 4-1 B and C).

4.2.2 Effects of Ang1-9 on cardiac function

In order to evaluate cardiac function ECHO was performed during the study. The first ECHO was performed at the 11^{th} week of age prior to implantation of the minipump. The second ECHO was performed 2 weeks after osmotic minipump implantation (15 weeks of age) and on the third day before animal sacrifice (17 weeks of age). Left ventricular motion mode (M-mode) images captured at the papillary muscle level were used to calculate wall thickness and left ventricular internal diameter. Left ventricular end diastolic measurements, endocardial fractional shortening and ejection fraction were used to assess left ventricular systolic function. Wall thickness measurements included those for the posterior wall (LVPW) and anterior wall (LVAW). Internal left ventricular diameter (ILVD) and wall thickness

were measured in diastole and systole to allow accurate assessment of left ventricle mass index (LVMI) and left ventricular systolic function (Figure 4-2).

To calculate LVMI from M-mode imaging the cube function formula (ASE-cube formula) with Deveroux correction was used (see section 2.19.3.1) (Devereux *et al.*, 1986) and then normalized to tibia length. ASE-cube formula is used based on the assumption that the left ventricle's shape is a prolate ellipsoid of regular configuration and a ratio of long- to short-axis lengths of 2:l (Devereux *et al.*, 1986). Left ventricle mass index (normalized through tibia length) was not different between the control and Ang1-9 groups (control = 1.8 ± 0.1; Ang1-9 = 1.9 ± 0.1) (Table 6; Figure 4-3). However, in animals co-infused with Ang1-9 and PD123,319 a significant increase in LVMI was observed when compared to control or Ang1-9 infused animals (Ang1-9 + PD123,319 = 2.8 ± 0.1; $p < 0.001$) (Table 6; Figure 4-3).

Extrapolation of the data from M-mode ECHO to calculate cardiac output showed a significant difference in cardiac output between groups (Figure 4-4). SHRSP infused with Ang1-9 had a significant increase in cardiac output when compared to control rats (control = 188.5 ± 14.5 mL/min; Ang1-9 = 229.4 ± 1 mL/min; $p < 0.05$). Furthermore, the Ang1-9-induced increase in cardiac output was abolished when rats were coinfused with PD123,319 (Ang1-9 + PD123,319 = 194.3 ± 7 mL/min). Interestingly, the change in cardiac output was not induced by increases in stroke volume or heart rate as either of these were significantly different between any of the groups. No changes in ejection fraction or fractional shortening were observed between groups (Table 6).

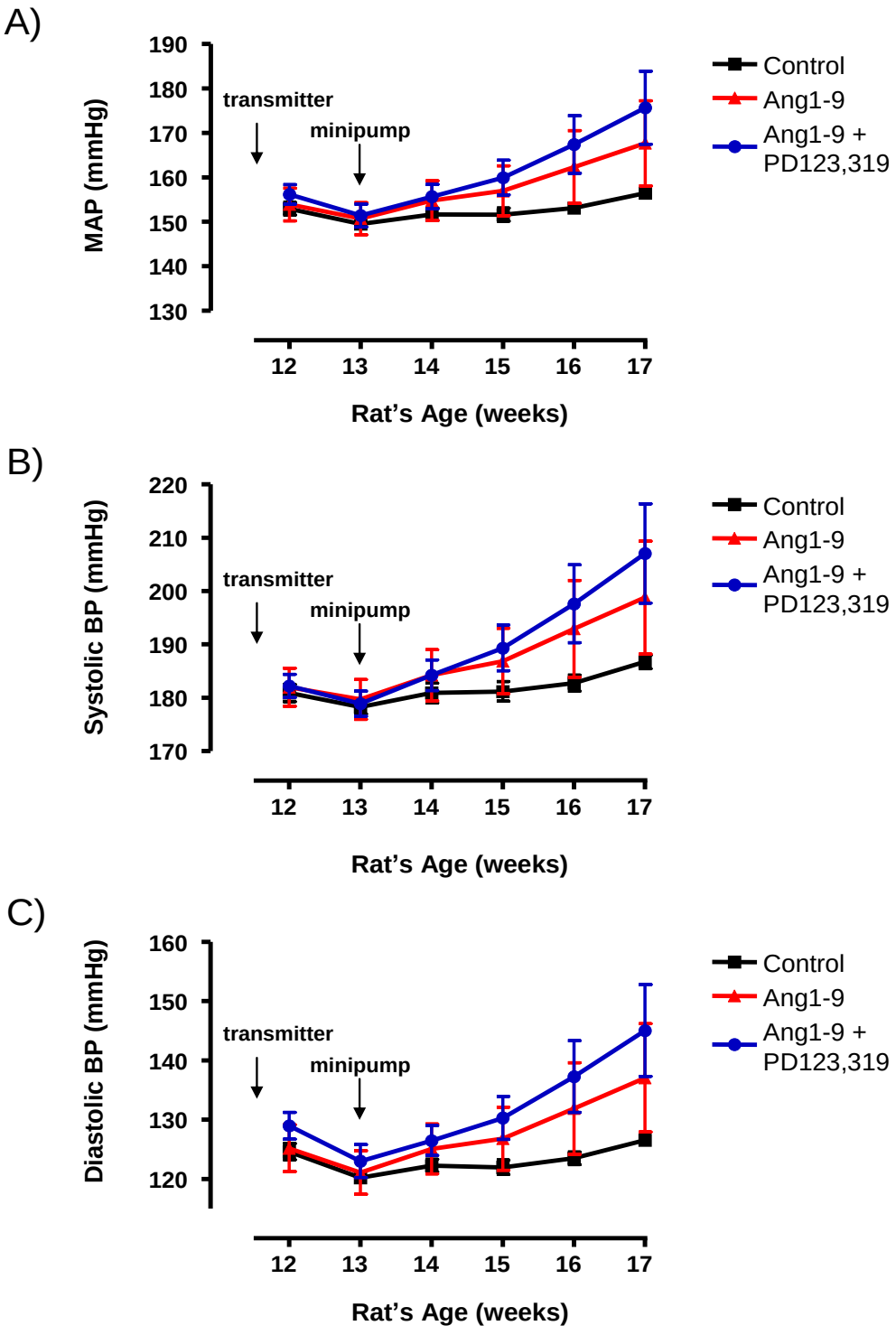


Figure 4-1. Effects of Ang1-9 infusion on blood pressure in SHRSP.

Blood pressure was monitored via radiotelemetry from 11 weeks of age until the end of the experimental protocol. Infusion of either water (control), Ang1-9 or Ang1-9 + PD123,319 initiated at week 13 and continued for 4 weeks. (A) Mean arterial pressure (MAP); (B) Systolic blood pressure (BP); (C) Diastolic blood pressure (BP). Rat's age in weeks. Two way ANOVA analysis was performed and no significant difference between groups was found. n = 6 rats per group.

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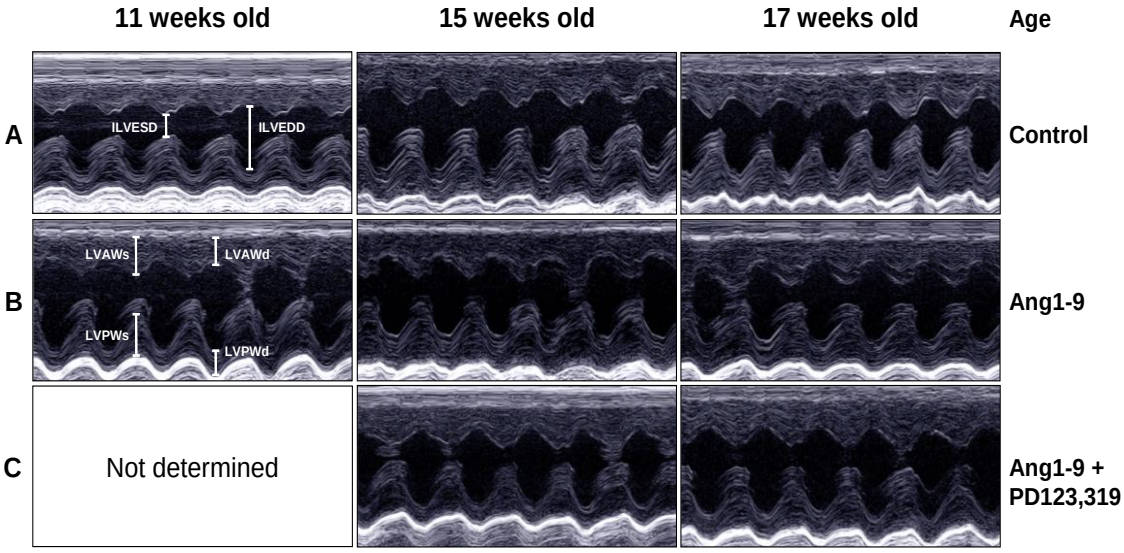


Figure 4-2. M-mode echocardiography.

Representative traces for the M-mode echocardiography performed in SHRSP at 11, 15 and 17 weeks of age. (A) Control group, (B) Ang1-9 infused, (C) Ang1-9 + PD123,319 infused. ILVESD = internal left ventricular end systolic diameter; ILVEDD = internal left ventricular end diastolic diameter; LVAWd = left ventricle anterior wall in diastole; LVPWd = left ventricle posterior wall in diastole; LVAWs = left ventricle anterior wall in systole; LVPWs = left ventricle posterior wall in systole; PD = PD123,319.

	Control			Ang1-9			Ang1-9 + PD123,319			Statistics		
Age	11	15	17	11	15	17	11	15	17	11	15	17
LVMI	1.48 ± 0.03	1.60 ±0.03	1.80 ± 0.10	1.50 ± 0.01	1.67 ± 0.03	1.88 ± 0.07	N/D	2.80 ± 0.08**#	2.78 ± 0.09**#	NS	P<0.001	P<0.001
RWT	0.41 ± 0.01	0.42 ± 0.01	0.44 ± 0.02	0.42 ± 0.02	0.45 ± 0.02	0.44 ± 0.02	N/D	0.41 ± 0.003	0.41 ± 0.002	NS	NS	NS
HR	458.0 ± 8.1	441.8 ± 6.5	436.9 ± 15.8	475.0 ± 7.4	447.3 ± 8.5	426.9 ± 6.5	N/D	433.9 ± 10.3	435.3 ± 9.0	NS	NS	NS
SV	0.34 ± 0.03	0.40 ± 0.03	0.43 ± 0.04	0.36 ± 0.02	0.40 ± 0.03	0.45 ± 0.04	N/D	0.44 ± 0.02	0.44 ± 0.01	NS	NS	NS
CO	156.2 ± 13.6	179.5 ± 14.7	188.5 ± 14.5	172.1 ± 9.1	180.9 ± 10.5	229.4 ± 0.9*##	N/D	191.2 ± 7.3	194.3 ± 7.0	NS	NS	P<0.05
EF%	81.0 ± 1.9	84.1 ± 1.0	82.3 ± 2.1	86.9 ± 1.4	84.6 ± 1.6	82.9 ± 1.8	N/D	82.1 ± 2.3	79.0 ± 1.5	NS	NS	NS
FS%	44.9 ± 1.9	45.9 ± 2.3	45.9 ± 2.2	51.7 ± 1.9	48.9 ± 2.0	47.2 ± 2.1	N/D	46.2 ± 2.3	42.5 ± 1.1	NS	NS	NS

Table 5. Cardiac Function measurements.

The table illustrates the Transthoracic ECHO findings for the different parameters to evaluate cardiac function. Left ventricular motion mode images, taken at the level of the papillary muscles, were used to measure left ventricle posterior and anterior wall thickness and left ventricular internal diameters at end systole and end diastole. Based on these data left ventricular mass index (LVMI), relative wall thickness (RWT), heart rate (HR), stroke volume (SV), cardiac output (CO), ejection fraction (EF) and fractional shortening (FS) were calculated. Age of rats in weeks, n = 6 rats per group. ND = Not determined; NS = not significant. ** = p<0.001 compared to control; * p< 0.05 compared to control; # p<0.001 compared to Ang1-9; ## p<0.05 compared to Ang1-9 + PD123,319.

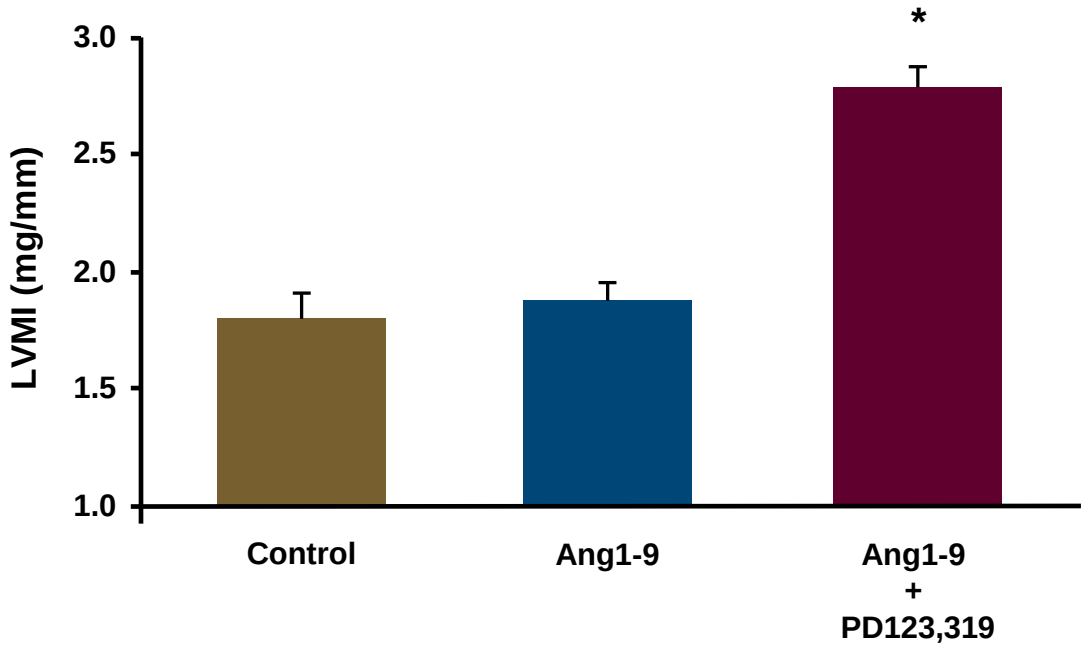


Figure 4-3. Quantification of left ventricular mass index.

ECHO performed on 17 weeks old rats following 4 weeks infusion via osmotic minipump. Left ventricular mass (LVM) is the difference between the epicardium delimited volume and the left ventricular chamber volume multiplied by an estimate of myocardial density (1.04). LVM was normalized to tibia length to adjust physiologic variation between animals producing a value for left ventricular mass index (LVMI). LVMI showed a significant difference between the Ang1-9 + PD123,319 group and control and Ang1-9 groups. * $p < 0.001$; $n = 6$ rats per group.

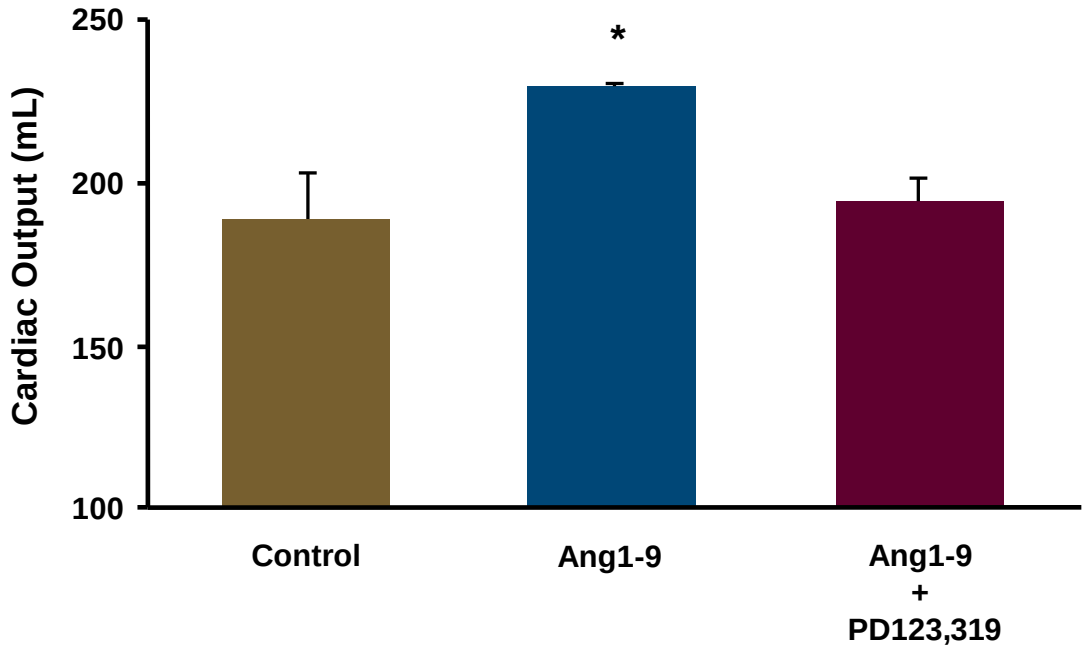


Figure 4-4. Assessment of cardiac output.

Based on ECHO data, cardiac output was calculated at sacrifice in control, Ang1-9 and Ang1-9 + PD123,319 infused rats. A significant increase was observed in cardiac output in SHRSP infused with Ang1-9 for 4 weeks. Co-infusion of PD123,319 abolished the increase in cardiac output observed in Ang1-9 infused animals. * $p < 0.05$; $n = 6$ rats per group.

4.2.3 Effects of Ang1-9 on fibrosis

Signalling via the AT2R has been reported to mediate reduced cardiac fibrosis in different models (Falcon et al., 2004, Morrissey and Klahr, 1999, Wu et al., 2002, Yan et al., 2003b). To assess the effects of Ang1-9 on cardiac fibrosis, hearts of SHRSP from each group were stained with Masson's trichrome and picrosirius red staining. Masson's trichrome selectively stains muscle, collagen fibers, and erythrocytes, with nuclei dyed black, muscle fibers and erythrocytes dyed red and collagen fibers blue. Picrosirius red stains collagen I and III in a brilliant red colour. There was no obvious evidence of irregularity in cardiac structure between groups when assessing heart sections with haematoxylin and eosin stain (Figure 4-5). Histological analysis of hearts of control SHRSP with Masson's trichrome revealed a marked increase in collagen fibers in the perivascular region (Figure 4-6 A). This observation is in agreement to that reported, where perivascular fibrosis is observed at 8 weeks of age (Sawamura *et al.*, 1990). In contrast, the extent of perivascular fibrosis was attenuated in SHRSP infused with Ang1-9 for 4 weeks. Interestingly, following co-infusion of PD123,319 and Ang1-9, fibrosis levels were similar to those observed in control animals, however, fibrosis was apparent with a different distribution. Whereas, in control animals only perivascular fibrosis was observed, in Ang1-9 + PD123,319 infused animals the deposits of collagen were interstitial, with negligible collagen deposits observed in perivascular regions (Figure 4-6 A). Quantification of cardiac fibrosis indicated that Ang1-9 infusion significantly blocked the endogenous fibrosis evident in the SHRSP and this appeared to be mediated through the AT2R since PD123,319 abolished the anti-fibrotic effect of Ang1-9 (Figure 4-6 B). In similarity to what was observed with Masson's trichrome, picrosirius red staining of control SHRSP hearts revealed perivascular and interstitial deposits of collagen type I and III (Figure 4-7 A). In Ang1-9 infused animals the collagen deposition was reduced, an effect reversed by co-infusion of PD123,319. Quantification of collagen fibers in the heart sections also showed a significant difference between control and Ang1-9 infused rats (Figure 4-7 B). Furthermore, PD123,319 significantly attenuated the effects of Ang1-9 on fibrosis (Figure 4-7 A and B). These findings suggest Ang1-9 has an anti-fibrotic effect on the heart *in vivo* and that these actions are mediated by the AT2R.

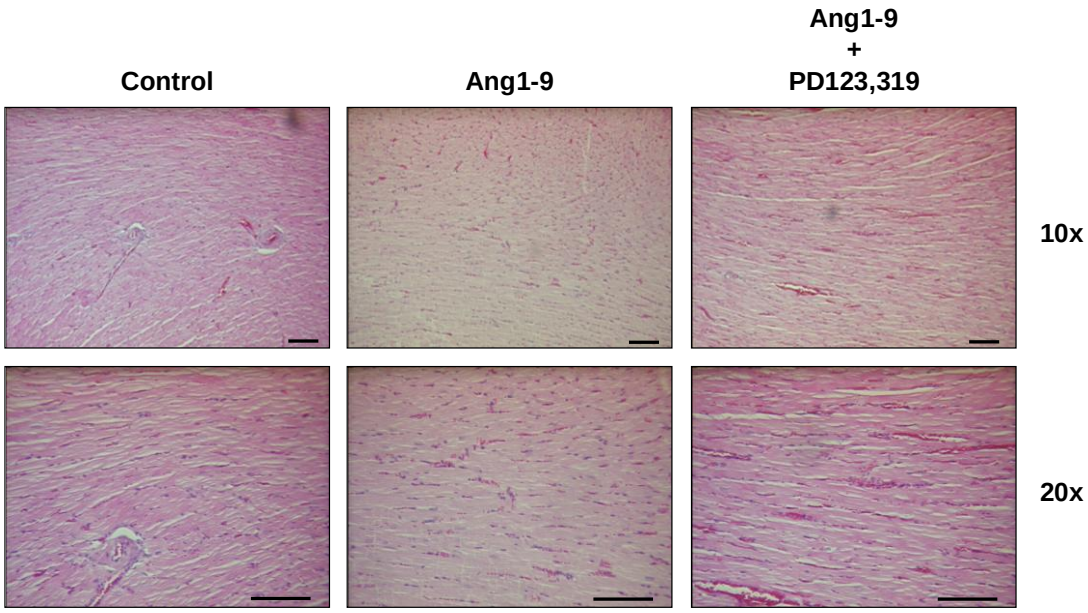


Figure 4-5. Histological analysis of cardiac structure.

Cardiac structure of SHRSP infused for 4 weeks with water (control), Ang1-9 or Ang1-9 + PD123,319 was assessed on histological sections with haematoxylin and eosin. No apparent difference was found between groups in haematoxylin and eosin stain. Scale bar = 10 µm, top panel magnification 10x, bottom panel magnification 20x.

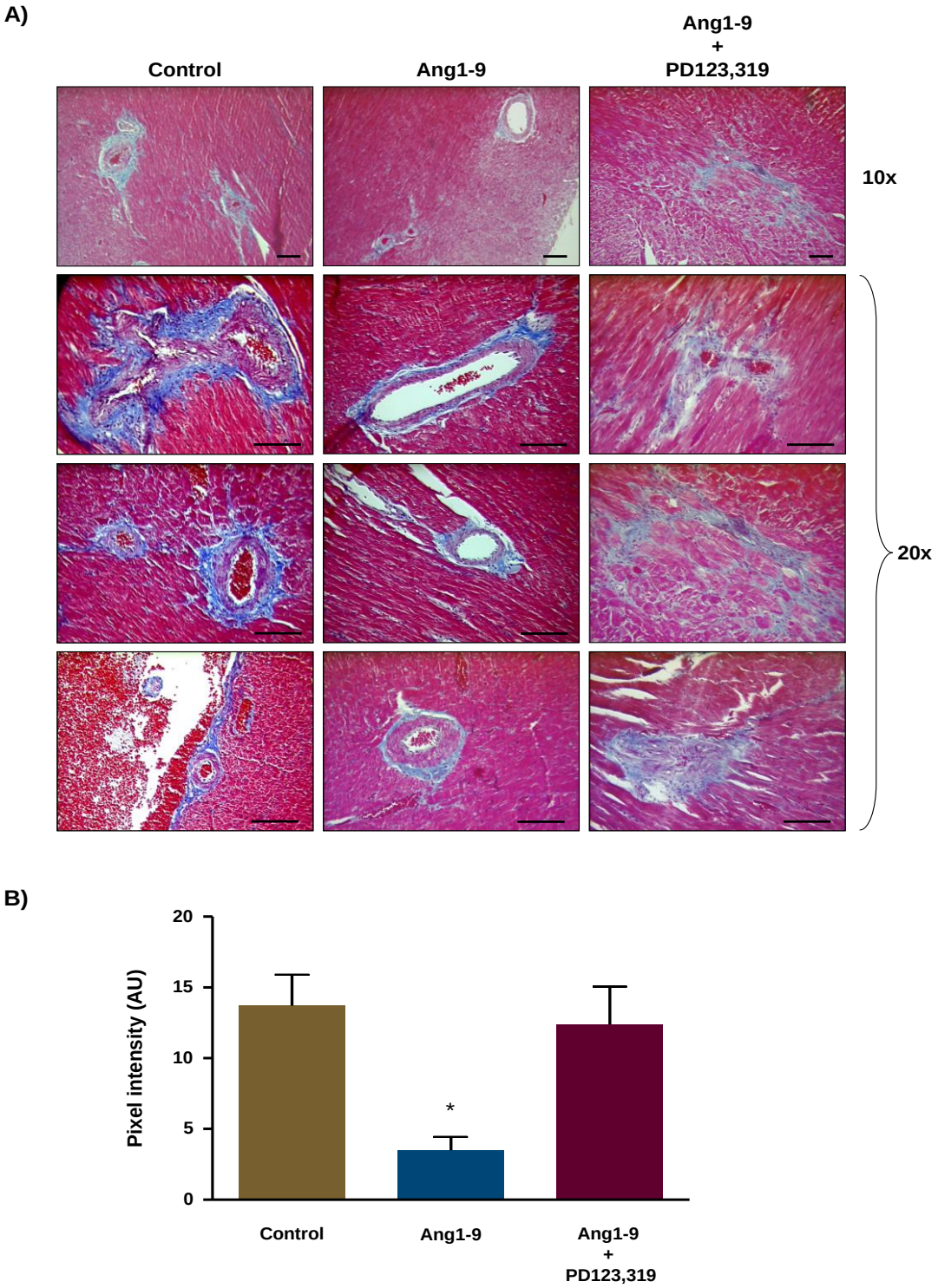


Figure 4-6. Effects of Ang1-9 on cardiac fibrosis assessed with Masson's trichrome staining.

Hearts from SHRSP infused with water (control), Ang1-9 or Ang1-9 + PD123,319 for 4 weeks were analysed histologically with Masson's trichrome staining. Masson's trichrome revealed perivascular fibrosis in the control group, which was reduced by infusion of Ang1-9. However, the anti-fibrotic effect of Ang1-9 was reversed by co-infusion with PD123,319. Scale bar = 10 μm, top panel magnification 10x. 3 bottom panels magnification 20x. * $p<0.01$ compared to control. AU = arbitrary units

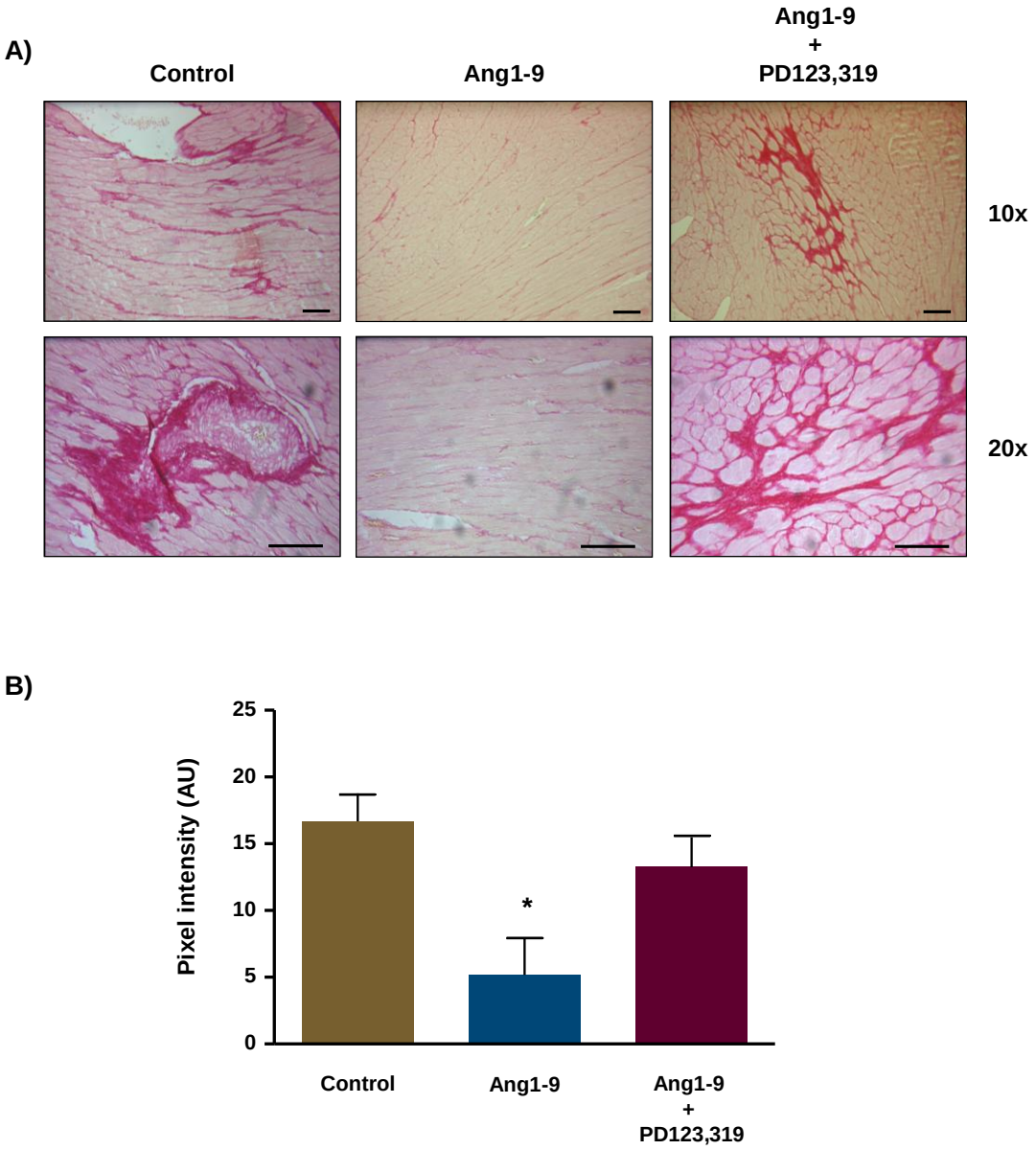


Figure 4-7. Effects of Ang1-9 on cardiac fibrosis assessed with picrosirius red staining.

Histological sections from SHRSP hearts infused with water (control), Ang1-9 or Ang1-9 + PD123,319 for 4 weeks were analysed for collagen type I and III content with picrosirius red staining. Heart sections from control animals showed increased perivascular and interstitial deposition of collagen. Ang1-9 infusion blocked collagen deposition, whereas co-infusion of PD123,319 with Ang1-9 reversed the antifibrotic effect of Ang1-9. Scale bar = 10 µm, top panel magnification 10x, bottom panel magnification 20x. * $p < 0.05$ compared to control. AU = arbitrary units.

4.2.4 Effects of Ang1-9 on endothelial function

Immediately following sacrifice, aortas were isolated to perform large vessel pressure myography to evaluate the direct effect of Ang1-9 on endothelial function. The principle of myography is based on the bioavailability of NO in vessel endothelium (Anderson *et al.*, 1995). NO is released by vascular endothelial cells producing vessel relaxation. The synthesis of NO is catalyzed by nitric oxide synthases (eNOS, nNOS and iNOS) (Palmer *et al.*, 1988). Inhibition of NOS with L-NAME triggers vessel contraction due to the loss of NO production (Ibarra *et al.*, 2006). Performing pressure myography in the absence and presence of L-NAME gives an indication of endothelial function as the difference between the two curves reflects the amount of NO produced. In control SHRSP inhibition of NOS with L-NAME did not modify basal arterial tension [induced by phenylephrine (PE)] as there was no significant difference between the contraction curves (Control PE = 64.1 ± 10.3%; Control PE + L-NAME = 74.0 ± 10.4%)(Figure 4-8 A). This indicates a lack of basal NO availability suggesting dysfunctional endothelium in the control SHRSP, which is consistent with observations in previous studies in the SHRSP (Kerr *et al.*, 1999, Hamilton *et al.*, 2002). However, when pressure myography was performed on aortas from Ang1-9 infused animals L-NAME significantly increased the contractile response of the aorta (Figure 4-8 B). An approximately 30% difference was observed between the curves (Ang1-9 PE = 68.3 ± 11.0%; Ang1-9 PE + L-NAME = 99.0 ± 12.0%; $p<0.01$) (Figure 4-8 B). These findings indicate that Ang1-9 improves NO bioavailability, suggesting Ang1-9 is able to improve endothelial function. In contrast, animals co-infused with Ang1-9 and the AT2R antagonist PD123,319 showed no significant difference between basal contraction and contraction in the presence of L-NAME (Ang1-9 + PD123,319 PE = 62.6 ± 11.0%; Ang1-9 + PD123,319 PE + L-NAME = 72.5 ± 17.7%) (Figure 4-8 C), suggesting PD123,319 reversed the effects of Ang1-9 on endothelial function.

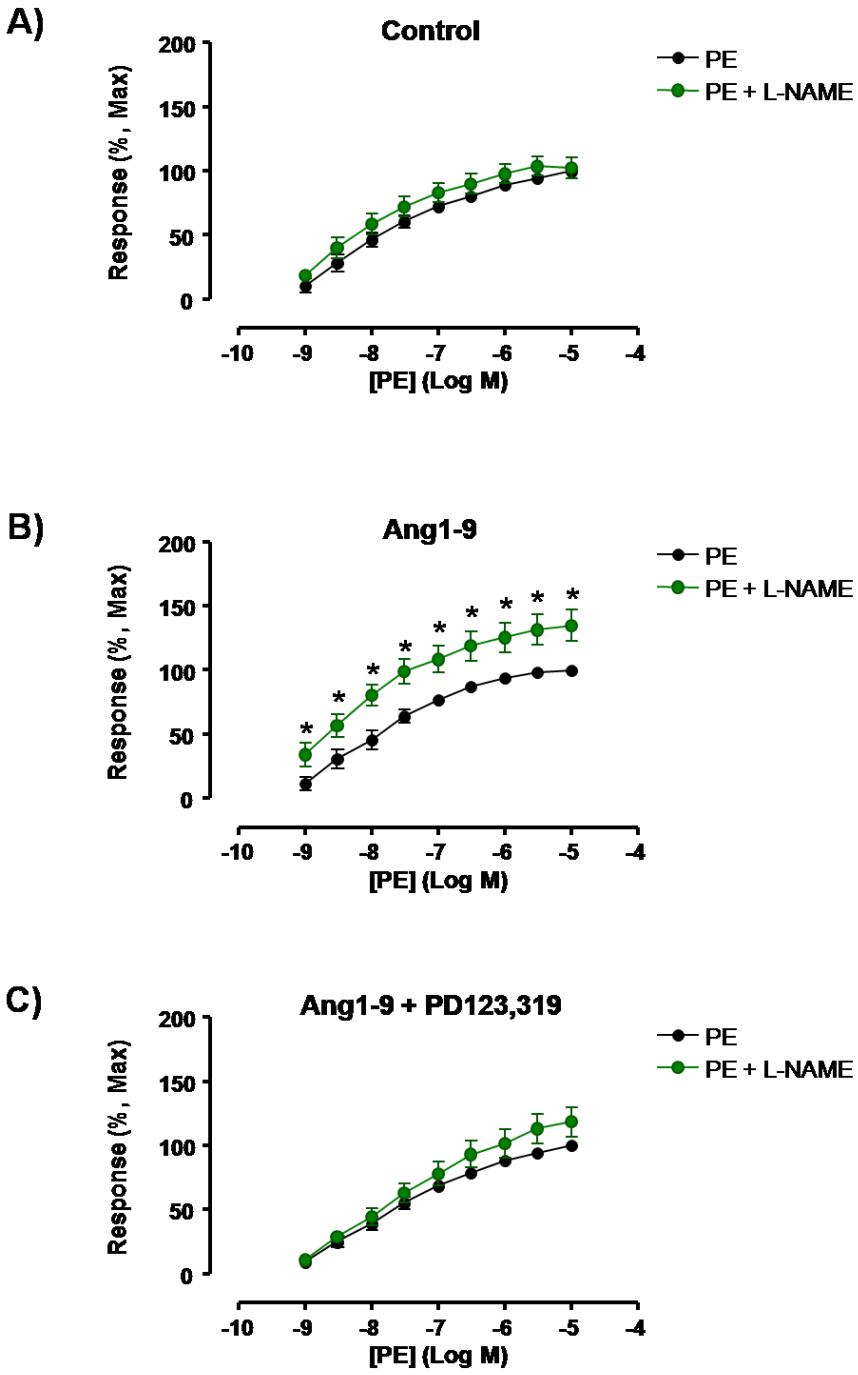


Figure 4-8. Effects of Ang1-9 infusion on endothelial function.

Large vessel myography was performed in freshly isolated aortic rings of SHRSP infused with (A) water (control), (B) Ang1-9 or (C) Ang1-9 + PD123,319 for 4 weeks. Basal contractile response was induced by phenylephrine (PE). To inhibit endogenous production of NO, vessels were incubated with L-NAME and contraction induced with PE. Endothelial function was evaluated as NO bioavailability assessed as the difference between contractile response curves. * $p < 0.01$; $n = 6$ animals per group.

4.3 Discussion

Here, SHRSP were used to study the effects of Ang1-9 on blood pressure, cardiac function, hypertrophy and fibrosis and endothelial function. SHRSP were infused for 4 weeks with water (control), Ang1-9 or Ang1-9 + PD123,319 via osmotic minipumps. Ang1-9 infusion had no effect on blood pressure. However, Ang1-9 increased cardiac output, blocked cardiac fibrosis assessed histologically and improved endothelial function assessed by *ex-vivo* vessel myography. Importantly, PD123,319 was able to reverse all the effects of Ang1-9, suggesting Ang1-9 actions were mediated by the AT2R. Interestingly, PD123,319 also had effects on cardiac hypertrophy, increasing left ventricular mass index when compared to control or Ang1-9-infused animals. #

Human hypertension is a, complex multifactorial, polygenic disease. To study this entity many animal models have been developed. SHRSP is one of the most used animal models to study hypertension. As already mentioned, this model develops end-organ damage such as left ventricular hypertrophy, stroke and renal failure resembling a human hypertensive phenotype (Tanase *et al.*, 1982, Badyal, 2003). However there are better rodent models to study heart failure such as transverse aortic constriction in mice. Cardiac overload in this mice cause dilatated cardiomyopathy and heart failure and has been extensively used as a reproducible model for cardiac hypertrophy. As future work, assessment of Ang1-9 effects on this model would be fundamental.

The RAS is the main regulator of systemic blood pressure and furthermore, in animal models it has been shown that systemic delivery of AngII induces an increase in blood pressure, cardiac remodelling (increasing left ventricular mass and cardiomyocyte size and induction of fibrosis) (Chintalgattu and Katwa, 2009, Frank et al., 2007, Guzik et al., 2007). Novel components of the local tissue-specific RAS have been shown to mediate beneficial effects on blood pressure and cardiac remodelling when delivered in animal models via minipump or overexpression. ACE2 and Ang1-7 have both been shown to reduce cardiac hypertrophy and fibrosis, as well as lower blood pressure but by different mechanisms (Benter *et al.*, 2006, Diez-Freire *et al.*, 2006). However, it has also been described that delivery of ACE2

can promote the development of cardiac fibrosis and HF, while delivery of Ang1-7 does not affect blood pressure (Grobe *et al.*, 2007b, Masson *et al.*, 2009) indicating the difficulty in defining the direct roles of these proteins. Even though results are conflicting these local novel components of the RAS are clearly importantly involved in the pathophysiology of hypertension and end organ damage. Based on this we assessed the role of Ang1-9 in a hypertensive rat model, the SHRSP, with associated end organ damage. Ang1-9 has been described to have an indirect effect on cardiac tissue via potentiating bradykinin in endothelial cells (Jackman *et al.*, 2002) or by forming Ang1-7 through ACE cleavage (Donoghue *et al.*, 2000). Until now there is no evidence of direct actions of this peptide. In the present study Ang1-9 was delivered into SHRSP for 4 weeks and assessed its effects in a hypertensive *in vivo* model by analysing blood pressure, cardiac function and remodelling, and endothelial function.

Even though a cardinal sign of hypertension is high blood pressure, in the present study Ang1-9 had no effect on blood pressure. However, it has been shown that lentiviral over-expression of AT2R in the myocardium of the SHRSP after completion of embryonic development had no effect on blood pressure when compared to control SHR (Metcalfe *et al.*, 2004). In addition in an AngII chronic infusion rat model to induce hypertension, Ang1-7 had no effects on blood pressure, however, Ang1-7 was able to attenuate AngII-induced cardiomyocyte hypertrophy and interstitial fibrosis (Grobe *et al.*, 2007b). Furthermore, Mercure *et al.* showed that under a hypertensive challenge transgenic mice over-expressing Ang1-7 in the heart, had reduced ventricular hypertrophy and fibrosis when compare to control animal, but had no effect on hypertension development (Mercure *et al.*, 2008). Hence, there is evidence for target end-organ effects of angiotensin peptides and for AT2R function in the absence of systemic effects on blood pressure. However it is important to mention that in both treated groups (Ang1-9 infused and Ang1-9 + Pd123,319 coinfused rats) there is an increased variability in blood pressure (observed in standard error bars). It has been described that arterial blood pressure has a beat-to-beat fluctuation. This fluctuation has been explained as the dynamic response of the cardiovascular system to control perturbations, and the renin-angiotensin system together with the autonomic nervous system have been considered to be the main players on this short-term control (Akselrod et al., 1981).

In this study further analysis of these fluctuations between groups should be carried out using power spectrum analysis to assess the short-term cardiovascular control systems and address if there is any difference between groups.

In order to evaluate cardiac function transthoracic ECHO was performed in the SHRSP. From the parameters studied only CO was significantly higher in the Ang1-9 group, an effect that was blocked when the AT2R antagonist PD123,319 was co-infused with Ang1-9. Despite CO being derived from SV and HR via the formula CO = SV x HR, here neither HR nor SV were significantly different between groups. However, according to Darcy's Law, CO is influenced in an inverse manner by vascular resistance (Fauci, 2008, Andreoli, 2001) and therefore even with no changes in SV or HR the increase in CO in SHRSP infused with Ang1-9 could be related to the decreased vascular resistance resulting in increased CO to maintain tissue perfusion. This hypothesis is supported by the large vessel myography in which Ang1-9 improves endothelial function. Since, NO is a vasodilatator agent that can decrease vascular resistance, Ang1-9 may reduce vascular resistance by improving endothelial function leading to increased NO bioavailability. Interestingly, when Ang1-9 infused rats were co-infused with the AT2R antagonist PD123,319, both CO and vasorelaxation / NO bioavailability decreased to basal values equivalent to control SHRSP. This further supports a role for the AT2R in mediating Ang1-9 signalling. Furthermore, and supporting these findings, AT2R stimulation has been described to increase synthesis of NO in cardiac and endothelial cells and promote vasodilatation (Abadir et al., 2006, Ritter et al., 2003).

An interesting finding is the lack of effect of Ang1-9 on cardiac hypertrophy *in vivo*. This obviously does not correlate with the *in vitro* data where Ang1-9 showed clear antihypertrophic effects. However, the *in vitro* and *in vivo* results do support the notion that Ang1-9 may signal through the AT2R *in vitro* and *in vivo*. The AT2R has been described as a non-classical GPCR (Schluter and Wenzel, 2008, Funke-Kaiser *et al.*, 2009). With this knowledge the different *in vivo* and *in vitro* actions of Ang1-9 maybe due to the AT2R having different roles in different conditions. In addition left ventricule weigth normalized with tibia length as well as cross sectional staining with wheat germ agglutinin to measure cardiomyocyte area histologically would support any changes in cardiac hypertrophy. However echocardiography

measurements of cardiac hypertrophy have been previously validated (Graham et al., 2004). As most of the *in vitro* data is performed in neonatal rat cardiomyocytes, younger rats might be needed to extrapolate results from the *in vitro* to the *in vivo* stimulation. Furthermore, the *in vitro* data is the result of the preventive effects of Ang1-9, whereas *in vivo* Ang1-9 was infused to reverse cardiac hypertrophy and hypertension in an in-bred rat strain which has been in-bred for many generations for the phenotype and is extremely well compensated. However, Ang1-9 was able to reverse interstitial and perivascular fibrosis, an effect that was blocked by co-infusion with PD123,319. In addition to this data, other studies have shown that AT2R activation can mediate an antifibrotic effect *in vivo*, without having an antihypertrophic effect supporting the findings presented here (Yan *et al.*, 2003b, D'Amore *et al.*, 2005). Studies have implicated the AT2R as a therapeutic target in cardiac remodeling, though transgenic data is controversial. Two groups have developed cardiac-selective AT2R over-expressing mouse lines (Sugino *et al.*, 2001, Yan *et al.*, 2003b). The first line shows no differences in blood pressure, baseline or pressure overload-induced cardiac hypertrophy compared to wild type mice, however there was a reduction in cardiac fibrosis (Sugino *et al.*, 2001). The second study reported an increase in basal hypertrophy, a decrease in pressure overload-induced hypertrophy, an increase in cardiac fibrosis and reduction in blood pressure (Yan *et al.*, 2003b). Conversely, in the two knockout strains described to date one demonstrates decreases in cardiac hypertrophy and fibrosis without any changes in blood pressure (Ichihara *et al.*, 2001), while the other demonstrates increases in blood pressure, although cardiac parameters have not been reported (Ichiki *et al.*, 1995). Although some of the differences in these studies maybe due to technical differences in the methods used for phenotyping, the true function of the AT2R remains elusive. Activation of AT2R with the oral agonist C21 blocked phosphorylation of p38 and p44/42 in infarcted cardiac tissue increasing cell survival, as well as blocking the inflammatory response. In the data here we did not observe competition between AngII and Ang1-9, suggesting it may be a preferential ligand for the AT2R. This is also supported by the fact that the AT2R agonist CGP4211A produces similar effects to those seen with Ang1-9. Recently the development of a new AT2R agonist, compound 21 (C21) has indicated that the AT2R may be a therapeutic target in myocardial infarction (Kaschina *et al.*, 2008). Our data shows that co-infusion of Ang1-9 + PD123,319 significantly increased

LVMI. Furthermore, even though the AT2R antagonist was able to reverse the anti-fibrotic effect of Ang1-9, the collagen deposition observed in the Ang1-9 + PD123,319 co-infused rat hearts was interstitial instead of perivascular as observed in control SHRSP. This data suggest that PD123,319 may have an intrinsic effect on basal AT2R signalling. In addition, based on the evidence of beneficial effects of the AT2R on cardiac remodeling described earlier, blocking the AT2R with PD123,319 might cause blockade of the receptor effects resulting in an increase in LVMI.

In animal models the local RAS components have been shown to have a role in cardiac remodeling (Huentelman *et al.*, 2005, Grobe *et al.*, 2006, Grobe *et al.*, 2007a, Mercure *et al.*, 2008). Chronic infusion of Ang1-7 to rats blocked AngII-induced interstitial cardiac fibrosis and cardiomyocyte hypertrophy (Grobe *et al.*, 2007b). Similarly, in deoxycorticosterone acetate-salt rats Ang1-7 prevented interstitial and perivascular collagen deposition in the heart, resulting in prevention of cardiac fibrosis (Grobe *et al.*, 2006). Lentiviral over-expression of ACE2 in rat hearts also blocked AngII-induced hypertrophy and fibrosis (Diez-Freire et al., 2006, Huentelman et al., 2005). In relevance to this, our data shows reduced perivascular and interstitial fibrosis in animals infused with Ang1-9 compared to control SHRSP. This data suggests that the anti-fibrotic effect of Ang1-9 can act as a counter-regulatory mechanism to AngII signalling, supporting a potential therapeutic role for Ang1-9. Since, this anti-fibrotic property of Ang1-9 is reversed when PD123,319 is co-infused in the SHRSP these results support the hypothesis that Ang1-9 signals through the AT2R.

The data here shows an effect of Ang1-9 on interstitial and perivascular fibrosis. In addition Ang1-9 improved endothelial function leading to decreased perivascular resistance and increased cardiac output. Furthermore, PD123,319 blocked all the observed effects of Ang1-9 supporting the assertion that the AT2R mediates Ang1-9 signalling.

C H A P T E R 5

Development and validation of adenoviral vectors for over-expression of Ang1-7 and Ang1-9

5.1 Introduction

The development of gene transfer is a powerful approach for research in the cardiovascular system. The ability to over-express specific genes in different cardiovascular tissues has permitted the assessment of their mechanisms of action in cardiovascular physiology and pathophysiology, increasing understanding of CVD and highlighting novel pathological targets for the development of treatments.

To characterise the effects and molecular pathways of the RAS *in vivo* has been a challenge. Although classical systemic actions have been widely investigated, studying new tissue-specific components of the RAS *in vivo* is difficult, limiting the ability to distinguish systemic from local effects. A system which enabled delivery of peptides in a tissue-specific manner would be useful to study the mechanisms of these peptides action in specific cardiovascular disease phenotypes, such as cardiac hypertrophy or heart failure. This is important as recent studies have highlighted the importance of separate tissue specific actions of the RAS in kidney (Crowley *et al.*, 2005) and central nervous system (CNS) (Lee-Kirsch *et al.*, 1999). Generation of gene transfer tools able to overexpress RAS components and peptides in specific tissues would facilitate research into the tissue-specific actions of the RAS.

Due to the involvement of the RAS in hypertension this system has been a target for gene transfer strategies to treat hypertension. Inhibition of different components of the RAS using short oligonucleotides or full length antisense DNA was one of the first approaches for RAS gene transfer. Tomita *et al.* cloned antisense oligonucleotides for angiotensinogen into the hemagglutinating virus of Japan for delivery into the SHR, resulting in reduced blood pressure (Tomita *et al.*, 1995). Gene transfer of antisense mRNA for the AT1R delivered to SHR also reduced blood pressure (Phillips *et al.*, 1997). Furthermore, intracardiac delivery of retroviral vectors expressing full-length antisense DNA for the AT1R in SHR neonates prevented development of hypertension and changes in Ca^{2+} regulation in the kidney (Gelband *et al.*, 1999). Inhibition of ACE by antisense oligonucleotides has also been studied by the use of retroviral vectors and increased AngII-induced

intracellular Ca^{2+} transients into pulmonary artery endothelial cells and decreased blood pressure when delivered in SHR (Wang *et al.*, 1999, Wang *et al.*, 2000).

In order to design technology to enable the study of the tissue-specific roles of the RAS peptides Methot *et al.* developed a fusion protein able to direct the production of RAS peptides in transgenic mice (Methot *et al.*, 1997). This protein is able to over-express these peptides in specific tissues or cells due to the unique design of its conformation consisting of a signal peptide from human prorenin linked to a portion of the mouse heavy chain constant region immunoglobulin (Ig) G. This is followed by a portion of the human prorenin prosegment containing a cleavage site for furin protease and finally the biologically active RAS peptide (Figure 5-1 A) (Methot *et al.*, 1997).

The individual expression cassette components function in several ways to facilitate secretion of the biologically active RAS peptide from the cell. First the renin signal peptide ensures the entrance of the fusion protein to the endoplasmic reticulum (Methot *et al.*, 2001). This is possible due to the binding of the renin signal peptide to the signal recognition particle (SRP) during synthesis in the ribosome (Walter *et al.*, 1981). The SRP then delivers the protein along with the ribosome to the SRP receptor on the endoplasmic reticulum (Walter *et al.*, 1981). The protein passes through the endoplasmic reticulum membrane via the membrane protein translocator complex and into the secretory pathway (Leslie, 2005, Kanner et al., 2003). The heavy chain immunoglobulins bind to the binding immunoglobulin protein (BiP) in the secretory pathway while they await arrival of the light chain to form disulfide bridges (Methot *et al.*, 2001). The IgG in the fusion protein has the characteristic that it is secreted as a monomer and so does not bind to the BiP or form di-sulphide bridges, therefore facilitating efficient transgene expression and mature peptide production. Furthermore, the IgG maintains a stable conformation enabling the expression of proteins that would otherwise be poorly folded and therefore degraded, such as small peptides. The prosegment of human prorenin is a molecular spacer that is necessary to expose the protease processing site for the cleavage of the peptide (Methot *et al.*, 2001).

The cleavage site is the key element for successful secretion of the peptide from the cell. Proteases are enzymes that destroy proteins by hydrolysis of the bonds between amino acids. Furin is a serine protease present in the Golgi apparatus bound to the membrane of almost all mammalian cells. Methot *et al.* designed a peptide cleavage site for furin modifying the prorenin prosegment cleavage site from PMKR to RVRTKR. After cleavage, the encoded peptide is released into the secretory pathway of the cell (Methot *et al.*, 2001). Although amino acids in the amino-terminal side of the cleavage are essential for furin, the amino acids in the carboxy-terminal are also important. Reudelhuber *et al.* analysed the array of sequences for successful furin cleavage and concluded that although a broad spectrum of sequences can be used not all amino acid combinations are compatible limiting the biologically active peptides that can be expressed by the fusion protein (Figure 5-1 B) (Reudelhuber *et al.*, 1998). The first amino acid of most angiotensin peptides is aspartic acid (D) which is compatible with furin cleavage (Methot *et al.*, 2001).

The angiotensin peptide fusion protein approach has been used to generate several transgenic models. Van Kats *et al.* developed the first transgenic mouse model over-expressing AngII specifically in the heart using this fusion protein (van Kats *et al.*, 2001). This group showed that the increase of AngII production in the mice hearts lead to left ventricular hypertrophy and cardiac fibrosis, confirming that the secreted AngII was functional. A transgenic rat with targeted over-expression of Ang1-7 in the testis resulted in increased plasma levels of Ang1-7 (Santos *et al.*, 2004, Botelho-Santos *et al.*, 2007). Furthermore, Santos *et al.* showed that in these transgenic rats Ang1-7 over-expression protected against isoproterenol-induced cardiac hypertrophy as well as having a protective effect on postischemic cardiac function (Santos *et al.*, 2004). Furthermore, in the same rat model a decrease in vascular resistance in lung, spleen, kidney, brain and testis has been described, suggesting Ang1-7 as an important regulator of regional hemodynamics and highlighting the potential of this transgenic approach to study actions of angiotensin peptides (Botelho-Santos *et al.*, 2007).

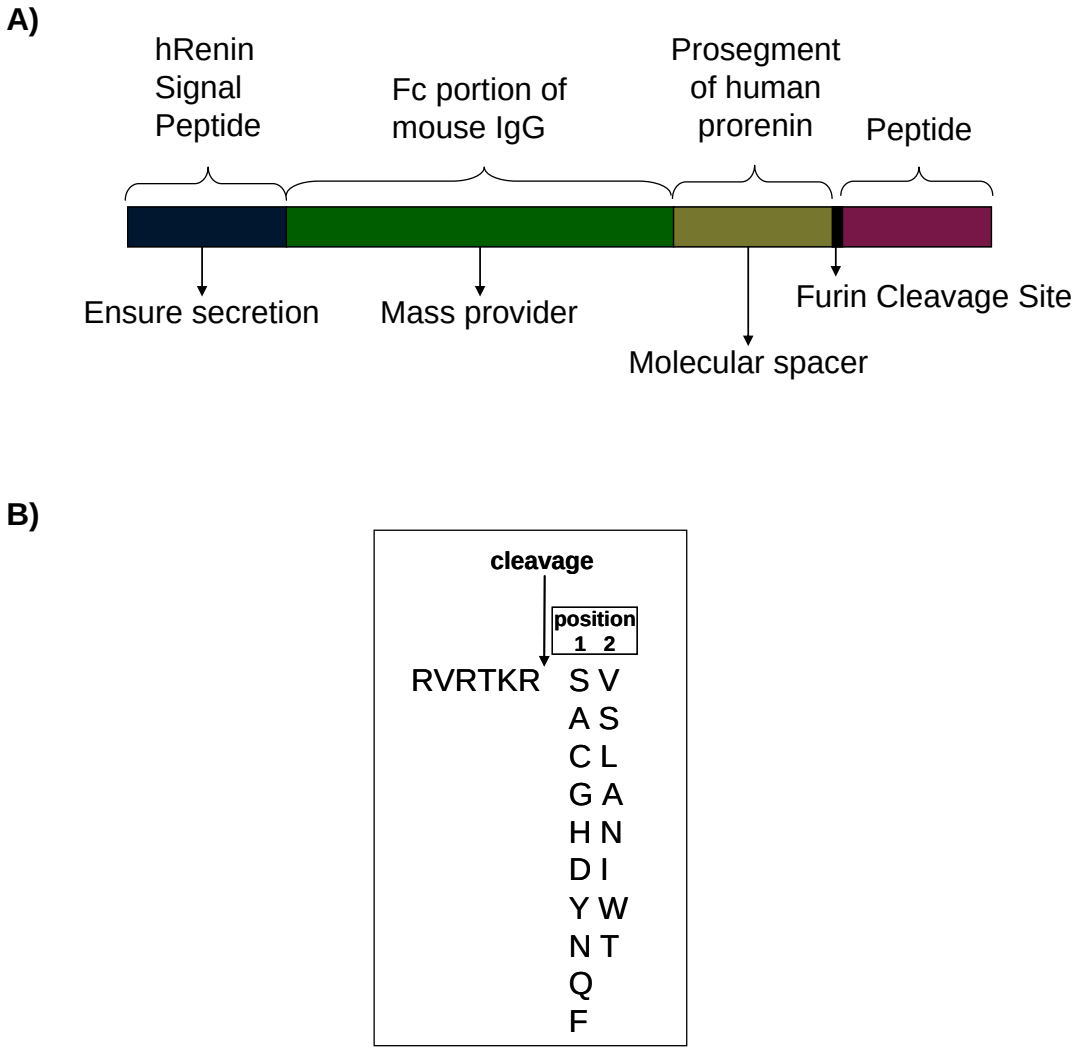


Figure 5-1. Schematic of expression cassette used to express RAS peptides in transgenic models.

(A) Explanatory diagram of fusion protein used to over-express RAS peptides. The expression cassette consists of a renin signal peptide to ensure secretion, a mouse heavy chain IgG to provide mass for efficient production of the protein, a furin protease cleavage domain (to release the peptide for secretion), and each peptide (figure modified from Methot *et al.* 2001). (B) Compatible amino acids on the carboxy-terminal side of the furin cleavage. For example the first amino acid of AngII and Ang1-7 is aspartic acid (D) or for ANP it is serine (S), making these peptides potential candidates for expression.

Other studies have used the fusion protein to over-express Ang1-7 selectively in other organs to study its effects (Burgelova et al., 2009, Ferreira et al., 2010, Ferreira et al., 2006, Gomes et al., 2010, Mercure et al., 2008). In a transgenic rat model over-expressing Ang1-7 selectively in the heart from the α-MHC promoter, Ang1-7 was protective against AngII and isoproterenol induced hypertrophy and fibrosis, by blocking activation of p38 and c-Src and deposition of interstitial collagen (Mercure *et al.*, 2008, Ferreira *et al.*, 2010). Recently using the same transgenic model Ang1-7 was described to drive production of nNOS in ventricular myocytes, leading to activation of cGMP, counteracting AngII-mediated stimulation of the calcineurin/NFAT hypertrophic pathway (Gomes *et al.*, 2010). Furthermore, selective over-expression of Ang1-7 in the rat kidney using this approach indicated that Ang1-7 controlled urinary volume by increasing urine osmolality and decreasing free water clearance, as well as having a protective role against renal hypertension (Ferreira *et al.*, 2006, Burgelova *et al.*, 2009).

Gene transfer via vector-mediated delivery is an alternative potential approach to drive angiotensin peptide production. Gene transfer vectors, in general, have been classified as non-viral and viral. Non-viral vectors have the advantage that they are safe, effective, and have the potential for repeated applications but they have a poor transfection efficiency and require high concentrations to achieve a therapeutic level (Bobek et al., 2006, Gardlik et al., 2005, Melo et al., 2004, Melo et al., 2005). Viruses are the vectors of choice for gene transfer in cardiovascular tissue as they provide high efficiency gene transfer compared to non-viral vectors. Although vectors based on viruses still have some safety issues, they are the most efficient way to transfer genetic material into most target cardiovascular cells. Viral vectors efficiently transduce genetic material into a wide range of cells and are the most used vectors for manipulating gene expression in cardiovascular disease. Viral vectors have been developed based on adenovirus (Ad), adeno-associated virus (AAV), lentivirus, retrovirus, herpes simplex virus and alphavirus. Although certain viral vectors show promise for CVD applications, e.g. AAV1 and 6 which are in gene therapy clinical trials for heart failure for over-expression of SERCA2a (Pleger et al., 2007, Rengo et al., 2009, Hajjar et al., 2008), Ad vectors have been the most widely

investigated for acute over-expression of genes in cardiovascular tissues for both therapeutic approaches and for probing molecular mechanisms of disease.

Ads are a family of 51 serotypes divided into 6 subspecies (A-F), mainly classified according to their ability to haemagglutinate erythrocytes. Adenoviral vectors are the most widely investigated as gene therapy vectors including in clinical trials, and of them species C, particularly serotype 5 (Ad5) (Chien, 1999). This virus is the most used viral vector and the most widely used for clinical trials.

Ads are icosahedral non-enveloped, double stranded DNA viruses of approximately 70 nm in diameter, with a 36 kB genome encoding for 50 polypeptides. The capsid of the virus has 3 principal protein components: the hexon, the penton base and the fiber. These structures are responsible for the immune response and for the cell tropism of the virus (Kim *et al.*, 2002) (Figure 5-2).

The adenoviral genome is organized into 2 sets of expressed regions; the early regions (E1, E2, E3 and E4) expressed before DNA replication and the late regions (L1, L2, L3, L4 and L5) expressed after DNA replication initiates. The E1 region encodes for E1a and E1b proteins and is essential for viral replication as it induces expression of multiple transcripts that promote DNA transcription. For example, E1a forces the cell to enter S phase so the virus can replicate and E1b expression inhibits the p53 pathway in the cell in order to prevent cell apoptosis triggered by the forced entry to S phase (Debbas and White, 1993, Cardoso *et al.*, 2008). The transcripts needed for DNA replication are encoded by the E2 region (Caravokyri and Leppard, 1996) and E3 encodes products that block the transport of the major histocompatibility complex to the cell surface inhibiting the host immune response (Flomenberg *et al.*, 1992). E4 is responsible for encoding the products needed for inhibiting host mRNA trafficking to the Golgi apparatus and facilitating viral mRNA shuttling to the Golgi complex to ensure viral production. The late region encodes for the fiber, penton base and hexon needed for packaging mature virions (Schaack, 2005).

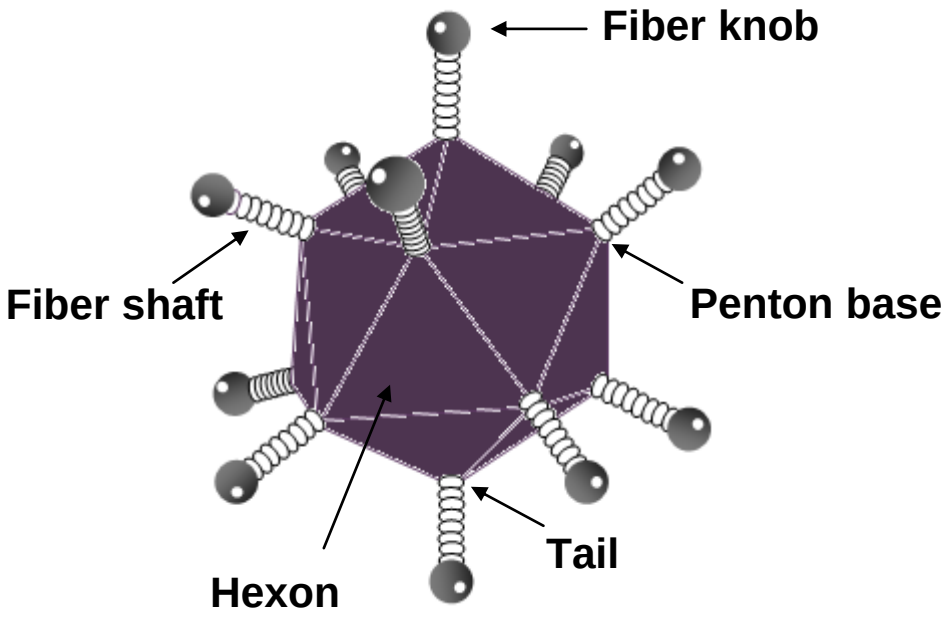


Figure 5-2. Diagram of an adenovirus.

Representation of the icosahedral structure of the adenovirus. The viral capsid has 3 principal proteins: the trimeric hexon (240 copies), the pentameric base (12 copies) and the trimeric fiber that consists of tail, shaft and knob domain (12 copies).

First generation replication-deficient Ads are deleted of the E1a and E1b genes which are essential for viral replication, thus rendering the virus replication defective and providing space for insertion of the expression cassette. Ad vectors are produced in a permissive cell line such as the 293 cell line, a human embryonic kidney cell line transformed with the left hand end of the Ad genome, including the E1 region, to provide the missing E1a and E1b genes *in trans* (Graham *et al.*, 1977). Generation of first generation Ad is either by a two plasmid or one plasmid transfection method. For the two plasmid transfection method, the first plasmid encodes the expression cassette flanked by sequences of the E1 region, and the second plasmid encodes the adenoviral genome, with a large insertion in the E1 region to avoid self packaging. Co-transfection into 293 cells and recombination between the plasmids generates a 1st generation replication deficient recombinant Ad expressing the transgene. One of the problems encountered in the production of these Ads is the spontaneous generation of replication-competent Ad caused by the recombination between the plasmid containing the Ad genome and the E1 region of 293 cells. The second method generates an intact E1-deleted Ad genome containing the expression cassette which is then transfected into 293 cells to package virus and is termed AdEASY (He *et al.*, 1998). Even though the region for initiating viral DNA replication is deleted in these viruses there is evidence of leaky expression of viral genes which is thought to occur via transactivation by endogenous transcription factors, inducing host inflammatory and immune responses which leads to inactivation of transgene expression and death of virally transduced cells (Yang *et al.*, 1994).

First generation adenoviral gene transfer vectors have the advantage of infecting dividing and non-dividing cells. Ads are relatively easy to manipulate, grow efficiently in tissue culture and can encode ≤8kB expression cassettes, they are stable, and high titer production is readily achievable. Disadvantages of Ad vectors include the innate and humoral immune response they induce preventing repeat administrations and approximately 70% of the general population carry antibodies and memory T-cells to the common serotypes (Muruve, 2004). In the cardiovascular setting Lemarchand *et al.* first described Ad gene delivery to endothelial cells and transgene expression in more than 90% of cells *in vitro*, but *in situ* in blood vessels just 2-5% of cells were transduced, indicating that gene delivery may not be as

efficient into intact blood vessels (Lemarchand *et al.*, 1993, Merrick *et al.*, 1996, Setlow, 2003). The transient expression from 1st generation Ads can be advantageous in some clinical scenarios such as gene therapy for vein graft failure, where only short term expression of the transgene is needed, to avoid acute remodeling of the graft that leads to its failure (Baker, 2002, Baker *et al.*, 2006). Recently 3rd generation Ads (with all viral genes deleted) have been demonstrated to produce up to 2.5 years expression after a single delivery, indicating the inflammatory response can be overcome (Brunetti-Pierri *et al.*, 2005, Toietta *et al.*, 2005, Brunetti-Pierri *et al.*, 2006). In summary, adenoviral vectors provide an efficient tool to deliver genes *in vitro* and *in vivo* for transient over-expression of genes to study their role in physiology and pathophysiology.

In order to develop tools to study the effects of Ang1-7 and Ang1-9 specifically in cardiac myocytes *in vitro* and *in vivo* a fusion protein expression cassette was engineered into Ad vectors to over-express angiotensin peptides.

5.2 Results

5.2.1 Production of recombinant adenovirus Ang1-7 (RAdAng1-7) and Ang1-9 (RAdAng1-9)

pBluescript-pre-fc-proAngII fusion protein over-expressing AngII contains an AngII fusion protein encoding sequence flanked by *Bgl*II and *Eco*RI restriction sites. To replace AngII with Ang1-7 or Ang1-9 encoding sequences (Figure 5-3), oligonucleotides encoding Ang1-7 or Ang1-9 flanked by *Bgl*II and *Eco*RI restriction sites were commercially synthesised (Table 5). Single stranded complementary oligonucleotides were annealed as described (section 2.21.1). To confirm successful annealing, both double stranded and single stranded oligonucleotides were subjected to electrophoresis which indicated slower migration for annealed double stranded oligonucleotides compared to the single stranded oligonucleotides confirming successful annealing (Figure 5-4 A and B). Next pBluescript-pre-fc-proAngII was digested with *Bgl*II and *Eco*RI to excise the AngII encoding oligonucleotides, which was confirmed by electrophoresis (Figure 5-4 C).

Ligation of the annealed oligonucleotides encoding Ang1-7 or Ang1-9 with pBluescript-pre-fc-pro was performed in competent *E.coli* JM109. Ligation reaction was used at 5:1, 3:1, 1:1, 1:3 and 1:5 molar ratio of vector:insert. Single colonies were picked and amplified to extract plasmid DNA and sequenced to confirm correct ligation of the peptide encoding sequence into the fusion protein (Figure 5-3).

Peptide	Sequence of Oligonucleotides Encoding Angiotensin Peptides
Ang1-7	
Forward	5′-GATCTCGCGTACGCACTAAACGCGACCGGGTGTACATACACCCCTGAG-3′ ***Bgl*II Furin Ang1-7 Stop *Eco*RI**
Reverse	3′-AGCGCATGCGTGATTTGCGCTGGCCCACATGTATGTGGGGACTCTTAA-5′ ***Bgl*II Furin Ang1-7 Stop *Eco*RI**
Ang1-9	
Forward	5′-GATCTCGCGTACGCACTAAACGCGACCGGGTGTACATACACCCCTTCCACTGAG-3′ ***Bgl*II Furin Ang1-9 Stop *Eco*RI**
Reverse	3′-AGCGCATGCGTGATTTGCGCTGGCCCACATGTATGTGGGGAAGGTGACTCTTAA-5′ ***Bgl*II Furin Ang1-9 Stop *Eco*RI**

Table 6. Ang1-7 and Ang1-9 oligonucleotides.

Oligonucleotides encoding Ang1-7 and Ang1-9 peptides used to produce RAdAng1-7 and RAdAng1-9. BglII and *Eco*RI restriction enzymes flank the sequence encoding the furin site, the peptide and a stop codon.

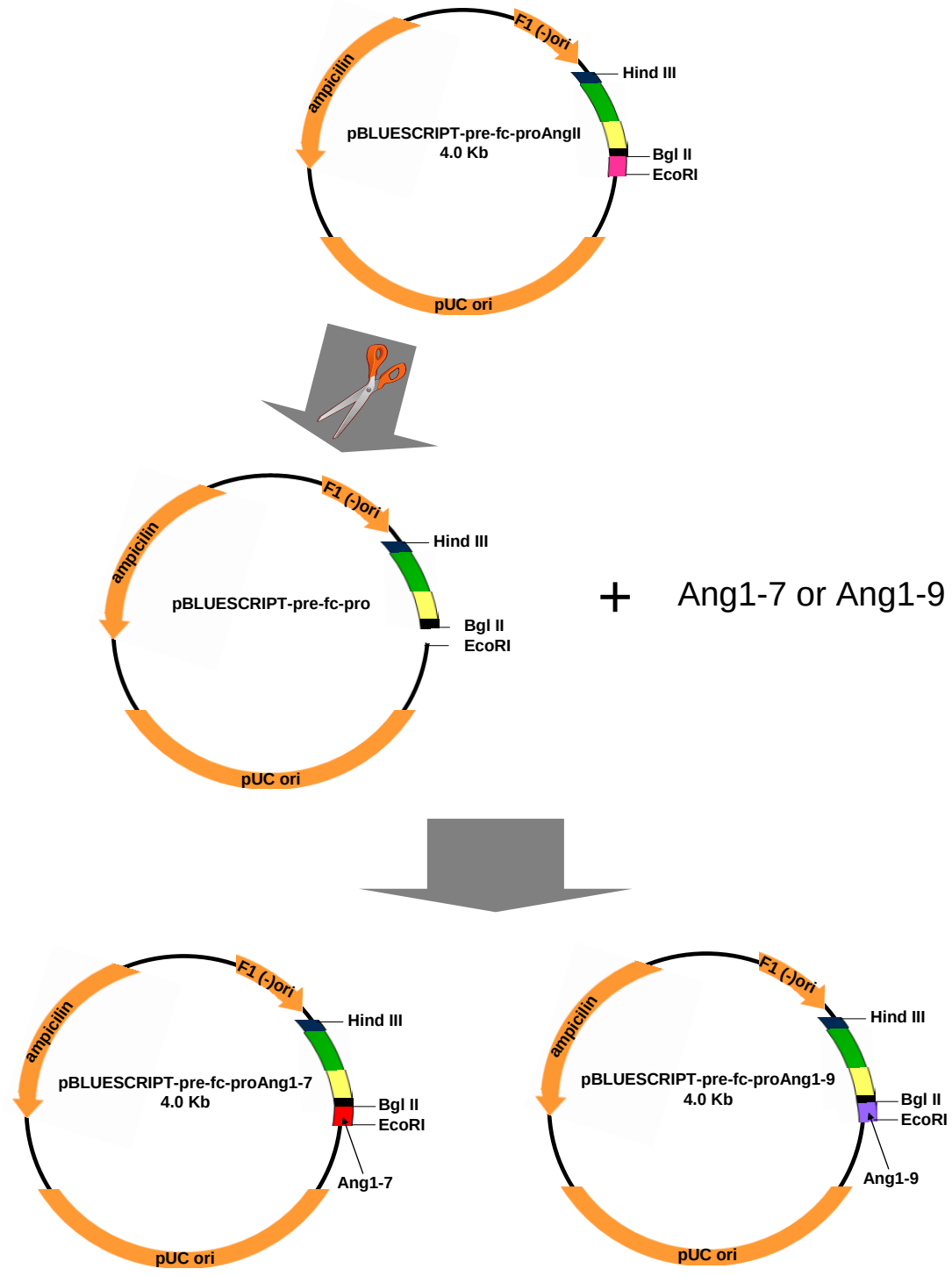


Figure 5-3. Cloning of Ang1-7 or Ang1-9 into pBLUESCRIPT-pre-fc-pro plasmid.

The sequence of events to clone Ang1-7 or Ang1-9 into the expression cassette. AngII was excised from pBLUESCRIPT-pre-fc-pro-AngII. pBLUESCRIPT-pre-fc-pro was then ligated with annealed Ang1-7 or Ang1-9 encoding oligonucleotides in *E.Coli* JM109, resulting in the generation of pBLUESCRIPT-pre-fc-pro-Ang1-7 and pBLUESCRIPT-pre-fc-pro-Ang1-9.

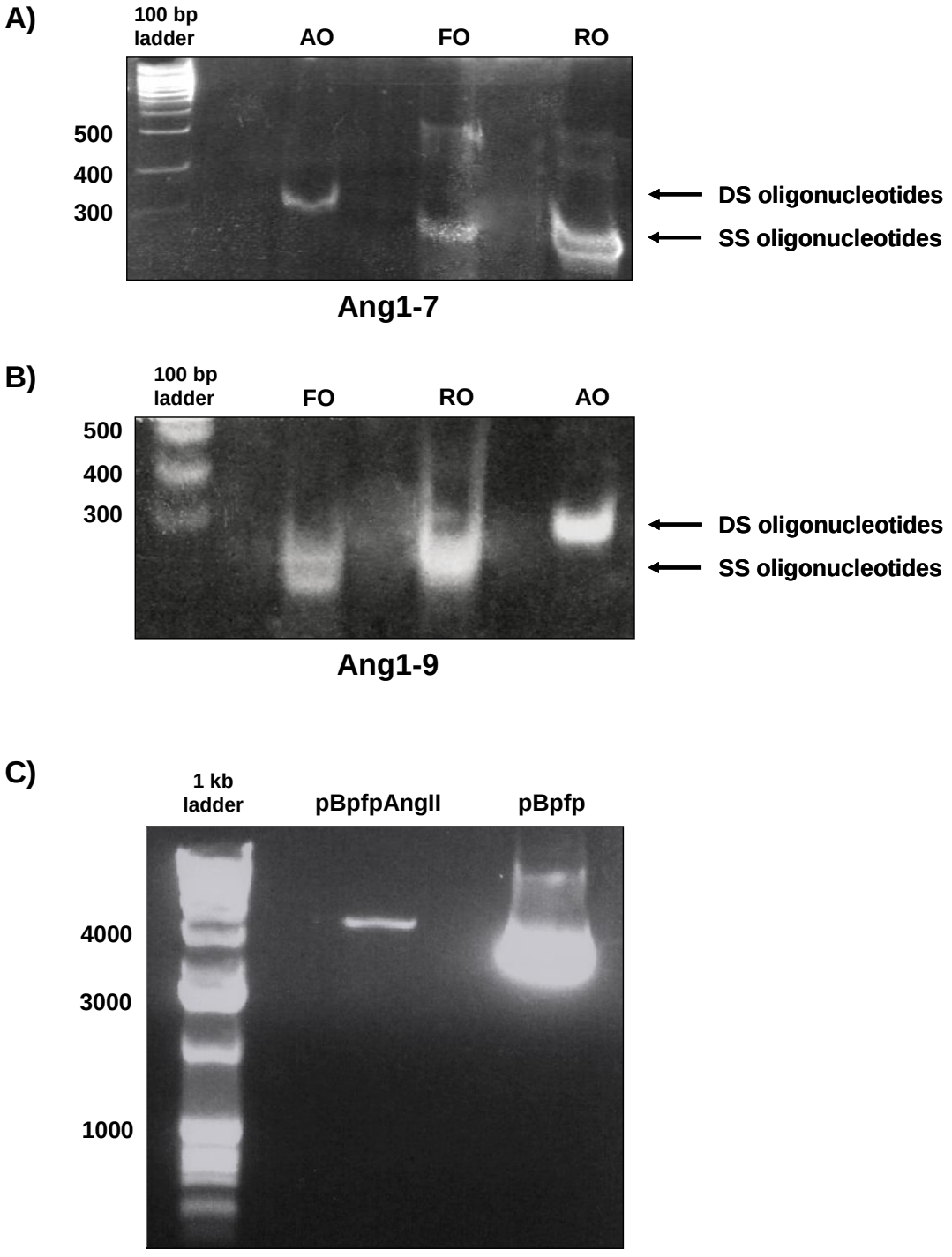


Figure 5-4. Cloning of Ang1-7 and Ang1-9 into pBluescript-pre-fc-pro.

Oligonucleotides encoding for (A) Ang1-7 or (B) Ang1-9 were annealed and subjected to electrophoresis in a 16% acrylamide gel (L: 100 bp ladder; FO: forward oligonucleotide; RO: reverse oligonucleotide; AO: annealed oligonucleotides). C) Digestion of 50 µg of pBLUESCRIPT-pre-fc-pro-AngII with 5 U of BglII and *Eco*RI (L: 1 kb ladder; pBpfp: pBLUESCRIPT-pre-fc-pro digested; pBpfpAngII: pBLUESCRIPT-pre-fc-pro-AngII native DNA; DS = double stranded; SS = single stranded).

5.2.1.1 Transfer of the expression cassette to an adenoviral shuttle vector

Next the fusion protein encoding Ang1-7 or Ang1-9 was excised from pBluescript-pre-fc-pro via *Hind*III and *Eco*RI digestion (Figure 5-5 A and B), and purified expression cassettes were cloned into *Hind*III/*Eco*RI digested pVQ-CMV-KNpA (the Ad shuttle vector) (Figure 5-6). Single colonies were picked, amplified and DNA extracted and successful ligation of the expression cassette into pVQ-CMV-KNpA was confirmed by digestion of the DNA with *Hind*III and *Eco*RI (Figure 5-7 [pVQ colonies]).

5.2.1.2 Generation of recombinant adenoviral vectors

To generate recombinant adenoviral vectors (RAd) a two plasmid transfection method was used (Figure 5-8 A) utilising pacAd5 9·2-100 (Anderson *et al.*, 2000), which expresses the adenovirus genome deleted of the E1 and E3 genes and each representative Ad shuttle vector, pVQ-CMV-KNpA Ang1-7 or Ang1-9. First, pVQ-CMV-KNpA-Ang1-7, pVQ-CMV-KNpA-Ang1-9 and pacAd5 9·2-100 were linearized with *Pac*I (Figure 5-8 B). Co-transfection of the linearized plasmid into 293 cells was mediated by Ca^{2+}-phosphate-mediated gene transfer. Recombination of plasmids and generation of recombinant adenovirus Ang1-7 or Ang1-9 (RAdAng1-7 or RAdAng1-9) was apparent within two weeks following co-transfection.

Crude adenoviral stocks were tested for expression of the fusion protein in Ad infected 293 cells lysate by western immunoblotting using a mouse anti-IgG antibody. Western immunoblotting indicated expression of fusion protein in its uncleaved (32 kDa) and cleaved (30 kDa) form, indirectly confirming expression, cleavage and secretion of Ang1-7 or Ang1-9 (Figure 5-9).

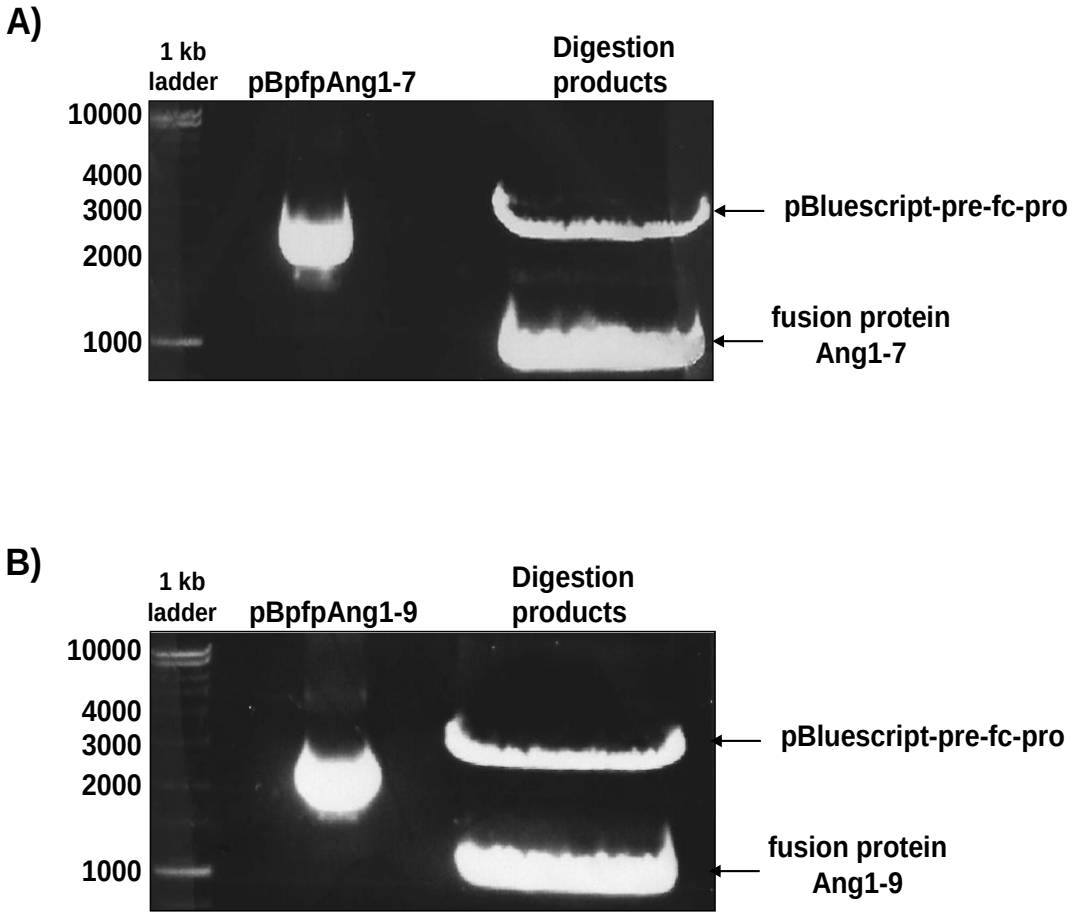


Figure 5-5. Digestion of pBLUESCRIPT-pre-fc-pro-Ang1-7 or pBLUESCRIPT-pre-fc-pro-Ang1-9 with *Hind*III and *Eco*RI.

50 µg of pBLUESCRIPT-pre-fc-pro-Ang1-7 or pBLUESCRIPT-pre-fc-pro-Ang1-9 was digested with 5 U of *Hind*III and *Eco*RI (L: 1 kb ladder) to confirm the correct insertion of the expression cassette.

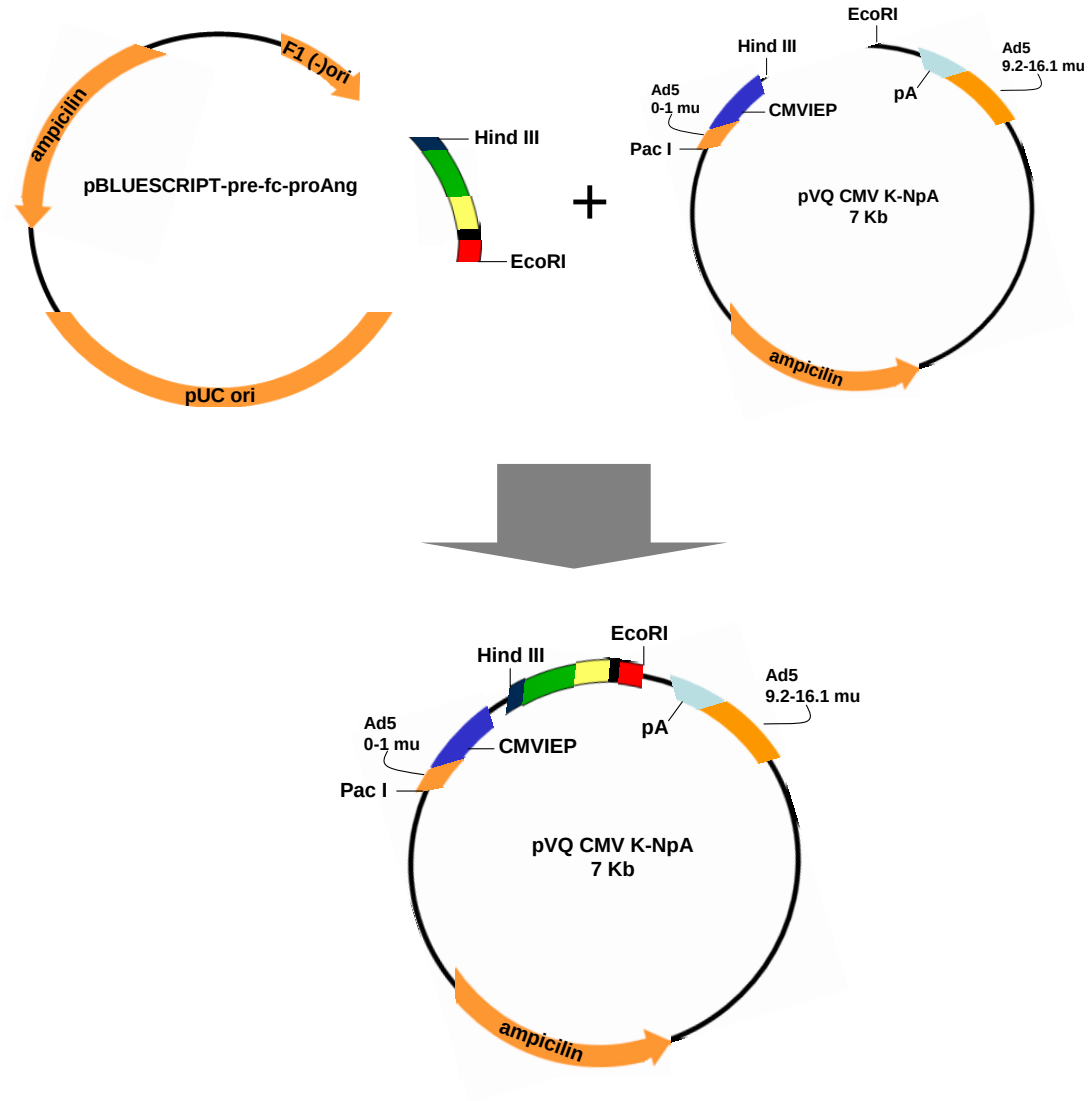


Figure 5-6. Cloning each expression cassette into the adenoviral shuttle vector pVQ CMV-NpA.

Schematic representing the transfer of the fusion protein from pBLUESCRIPT-pre-fc-pro to pVQ-CMV KNpA to generate pVQ-CMV-KNpA-Ang1-7 and pVQ-CMV-KNpA-Ang1-9 adenoviral shuttle vectors.

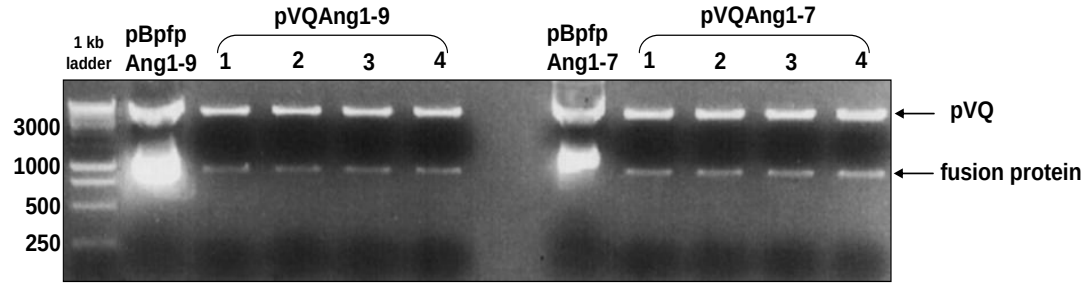


Figure 5-7. Confirmation of the correct cloning of the expression cassette into pVQ-CMV-KNpA plasmid.

10 µg of plasmid DNA extracted from single bacterial colonies was digested with 5 U of *Hind*III and *Eco*RI to confirm successful insertion of expression cassettes. p-Bluescript-pre-fc-pro-Ang1-7 and p-Bluescript-pre-fc-pro-Ang1-9 were also digested as positive controls. Lanes 1 – 4 individual screened colonies (L: 1 kb ladder; pBpfp: pBluescript-pre-fc-pro).

RAds were purified as described in the section 2.23.3. Protein titer was carried out via microBCA assay to quantify virus particle and end-point dilution in 293 cells to calculate plaque forming units. Mean titer calculated with microBCA assay were:

RAdAng1-7 = 2.6 x 10^{12} vp/ml
RAdAng1-9 = 2.9 x 10^{12} vp/ml

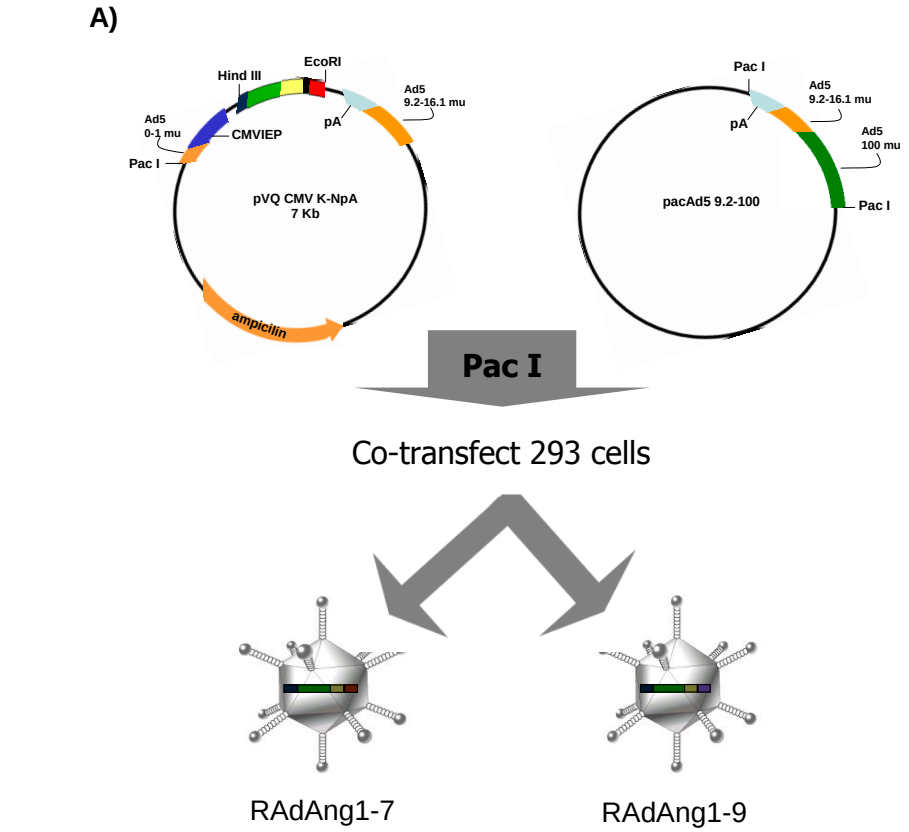
Plaque forming units was performed as described in the section 2.23.7.2. Pfu/cell titers were as followed:

RAdAng1-7 = 1.11 x 10^{11} pfu/cell

RAdAng1-9 = 1.11 x 10^{11} pfu/cell

The ratio between virus particle and plaque forming units titer for each RAd prep was 23.9 for RAdAng1-7 and 26.1 for RAdAng1-9, which is within expected limits.

To confirm that RAdAng1-7 and RAdAng1-9 were replicant incompetent, end point dilution assays in HeLa cells in parallel with 293 cell titers were performed. HeLa cells demonstrated no sign of viral replication within 8 days at any viral dilution, confirming the lack of contamination of the adenoviral preparations with replication-competent adenovirus.



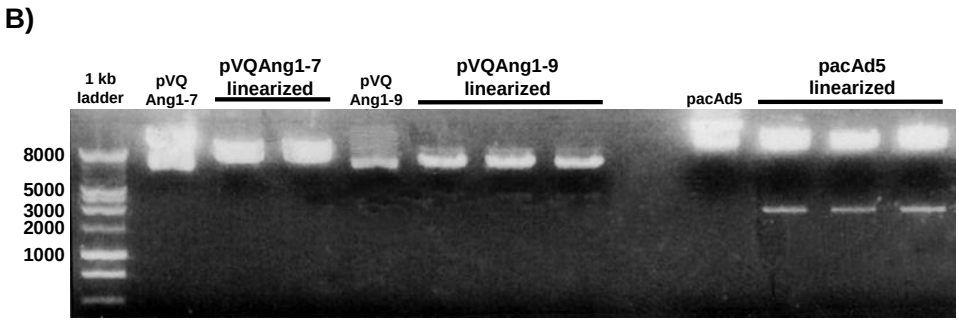


Figure 5-8. Method for generation of Adenovirus.

(A) Schematic of Ad generation. *Pac*I linearized pVQ-CMV-KNpA plasmids encoding each fusion protein producing Ang1-7 or Ang1-9 and pacAd5 9.2-100 were co-transfected into 293 cells to enable recombination to take place to generate crude stocks of replication-deficient adenoviral vectors over-expressing Ang1-7 or Ang1-9. (B) *Pac*I digestion of pVQAng1-7, pVQAng1-9 and pacAd5 9.2-100. 50 µg of pVQAng1-7 or pVQAng1-9 and 50 µg of pacAd5 9.2-100 were digested with 5 U of PacI to linearize the plasmids followed by electrophoresis. Lanes: 1-1 kb ladder; 2-pVQ-CMV-KNpA-Ang1-7; 3 and 4-pVQ-CMV-KNpA-Ang1-7 digested with *Pac*I; 5-pVQ-CMV-KNpA-Ang1-9; 6-8-pVQ-CMV-KNpA-Ang1-9 digested with *Pac*I; 10-pacAd5 9.2-100; 11-13-pacAd5 9.2-100 linearized with *Pac*I.

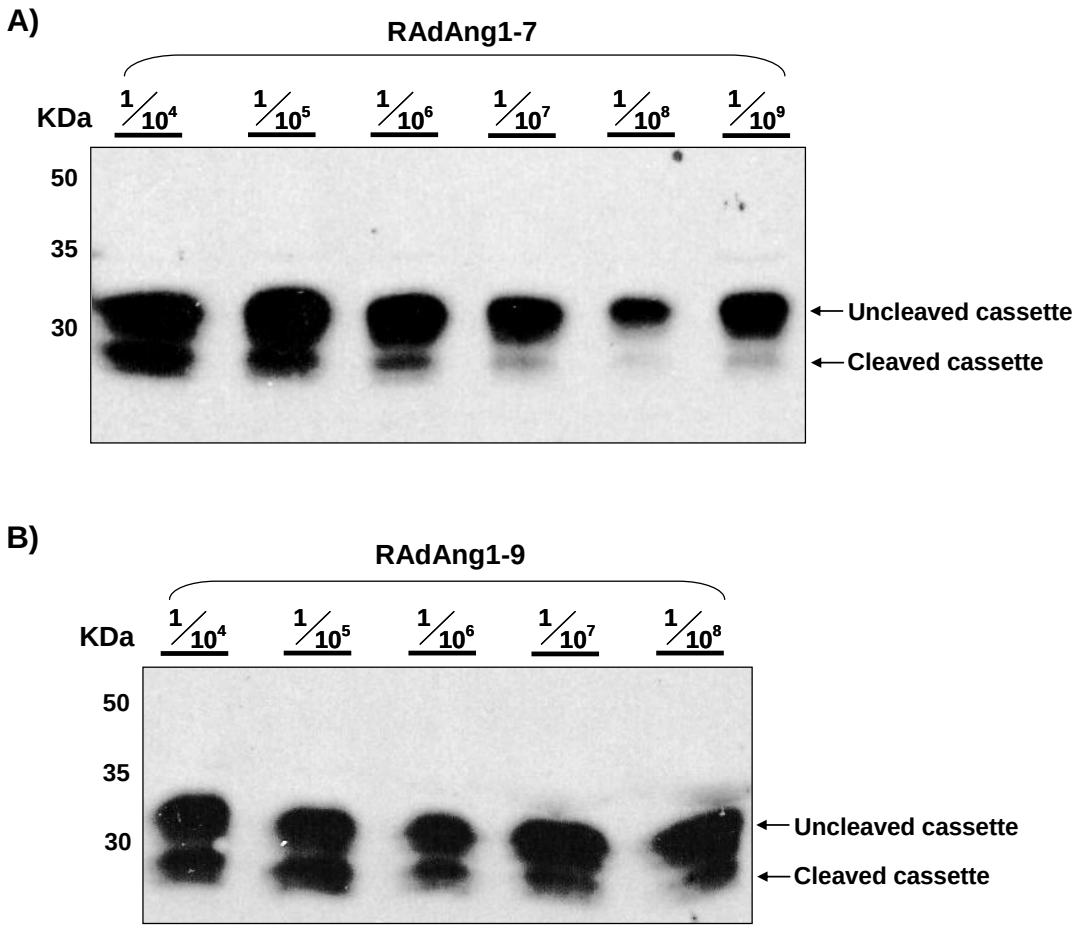


Figure 5-9. Detection of fusion protein expression in 293 cells.

293 cells transduced with RAdAng1-7 or RAdAng1-9 at different dilutions were lysed and subjected to electrophoresis and fusion protein expression was detected by western immunoblotting using an α-IgG2b antibody to detect the cleaved (30 kDa) and uncleaved (32 kDa) forms of the fusion protein.

5.2.2 Optimizing adenoviral transduction into H9c2 cardiomyocytes

To establish the dose at which RAds efficiently transduced H9c2 cardiomyocytes, cells were transduced with the reporter gene expressing RAd35. RAd35 expresses the *lacZ* gene which encodes β-galactosidase from the cytomegalovirus immediate early promoter (CMVIEP) (Wilkinson *et al.*, 1998). H9c2 cardiomyocytes were transduced with RAd35 at 50, 100, 300, 500 and 1000 pfu/cell and transgene expression assessed 24 hours later using X-gal staining (section 2.24.1). RAd35 transduction efficiency increased proportionally to the dose (Figure 5-10). At 500 and 1000 pfu/cell 70 to 90% of the cells expressed β-galactosidase. Furthermore, no sign of toxicity was observed at any concentration (Figure 5-10). Therefore, titers of 500 and 1000 pfu/cell were selected for characterization of the effects of RAdAng1-7 and RAdAng1-9 in H9c2 cardiomyocytes.

RAds efficiency transduction in left ventricular rabbit cardiomyocytes was previously established in the lab.

5.2.3 Fusion protein expression in H9c2 cardiomyocytes and HeLa cells

Western immunoblotting was used to detect fusion protein expression in H9c2 cardiomyocytes and HeLa cells following delivery of RAdAng1-7 and RAdAng1-9. HeLa cells were plated and transduced with recombinant RAdAng1-7, RAdAng1-9 or RAd60 (negative control) at 10 and 50 pfu/cell and incubated at 37 ºC for 24 hours to assess transgene expression. Similarly, H9c2 cardiomyocytes were plated and transduced with RAdAng1-7, RAdAng1-9 or RAd60 at 500 and 1000 pfu/cell to assay each RAd in the hypertrophy model. Cells were then lysed and western immunoblotting was performed as previously described (section 2.9) with an anti-mouse IgG2b antibody (Table 2) to detect the expression cassette. Detection of the secreted peptides directly in transduced cell media by western immunoblotting was not possible as the peptides were too small to resolve (~ 1 kDa). The western immunoblot showed 1 band of approximately 32 kDa, equivalent to the fusion

protein for both doses of RAdAng1-7 and RAdAng1-9 (Figure 5-11). This band corresponds to the uncleaved fusion protein, as the cleaved form is secreted from the cell to the media.

Transduction with RAd60 produced no signal following western immunoblotting, as expected (Figure 5-11).

5.2.4 Assessment of the effects of RAdAng1-7 and RAdAng1-9 in the cardiomyocyte hypertrophy

H9c2 cardiomyocytes were transduced with 500 and 1000 pfu/cell of RAdAng1-7, RAdAng1-9 or RAd60 (negative control). After 24 hour incubation, transduced H9c2 cardiomyocytes were stimulated with AngII (100 nM) and incubated for 96 hours. AngII induced cardiomyocyte hypertrophy as expected (Figure 5-12). Ad gene delivery *per se* did not have any effect as the size of the cardiomyocytes transduced with RAd60 was not different to AngII stimulated cells. However, at either concentration both RAdAng1-7 and RAdAng1-9 were able to block AngII-induced hypertrophy (AngII = 231.4 ± 79 μm; RAdAng1-7 500pfu = 176.9 ± 4.8 μm; RAdAng1-7 1000pfu = 189.6 ± 6.2 μm; RAdAng1-9 500pfu = 192.7 ± 5.8 μm; RAdAng1-9 1000pfu = 176.1 ± 4.2 μm; $p<0.05$) (Figure 5-12), indicating that each viral vector was functional and able to antagonize AngII-induced hypertrophy as was previously observed with exogenous peptide addition (see chapter 3). Next, RAdAng1-7 and RAdAng1-9 were assessed in the adult rabbit left ventricular primary cardiomyocyte hypertrophy model. Primary cardiomyocytes were transduced with 50, 100 and 300 pfu/cell of RAdAng1-7, RAdAng1-9 or RAd60, before stimulating with 500 nM AngII. Similarly to what was observed in H9c2 cardiomyocytes, RAd60 had no effect on AngII-induced hypertrophy (Figure 5-13). Importantly, RAdAng1-7 and RAdAng1-9 were able to block AngII-induced hypertrophy at all doses by normalizing cell width and hence volume (Figure 5-13).

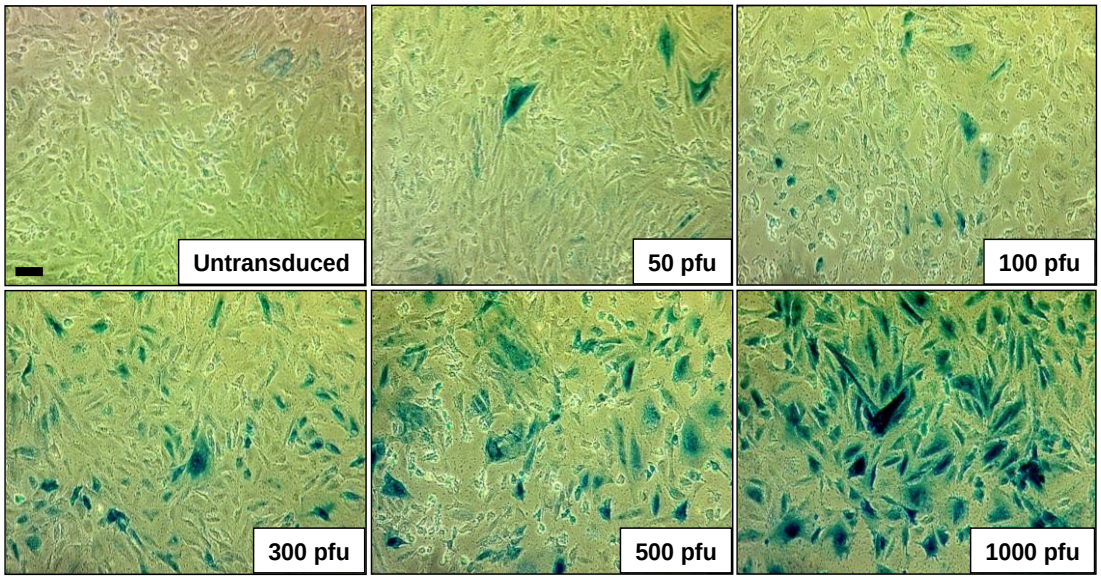


Figure 5-10. Transduction optimization in H9c2 cardiomyocytes.

H9c2 cardiomyocytes were transduced with RAd35 which encodes the *lac*Z gene at 50, 100, 300, 500 and 100 pfu/cell. After 48 hours incubation cells were fixed and β-galactosidase expression visualized using X-gal (Magnification = 10x; Scale bar = 10 µm).

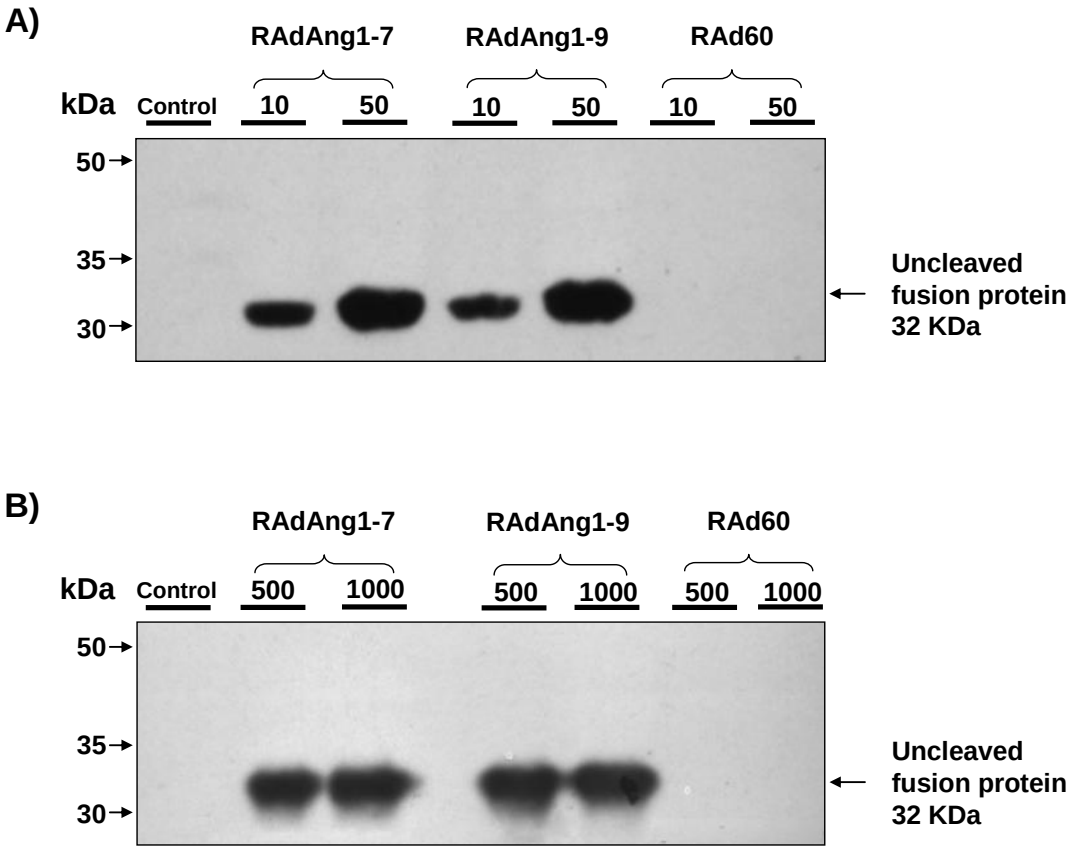


Figure 5-11. Fusion protein expression in transduced cells.

The uncleaved fusion protein (32 kDa) was detected by western immunoblotting in (A) HeLa cells transduced with 10 and 50 pfu/cell of RAdAng1-7, RAdAng1-9 or RAd60 or in (B) H9c2 cardiomyocytes transduced with 500 and 1000 pfu/cell. Control = unstimulated cells.

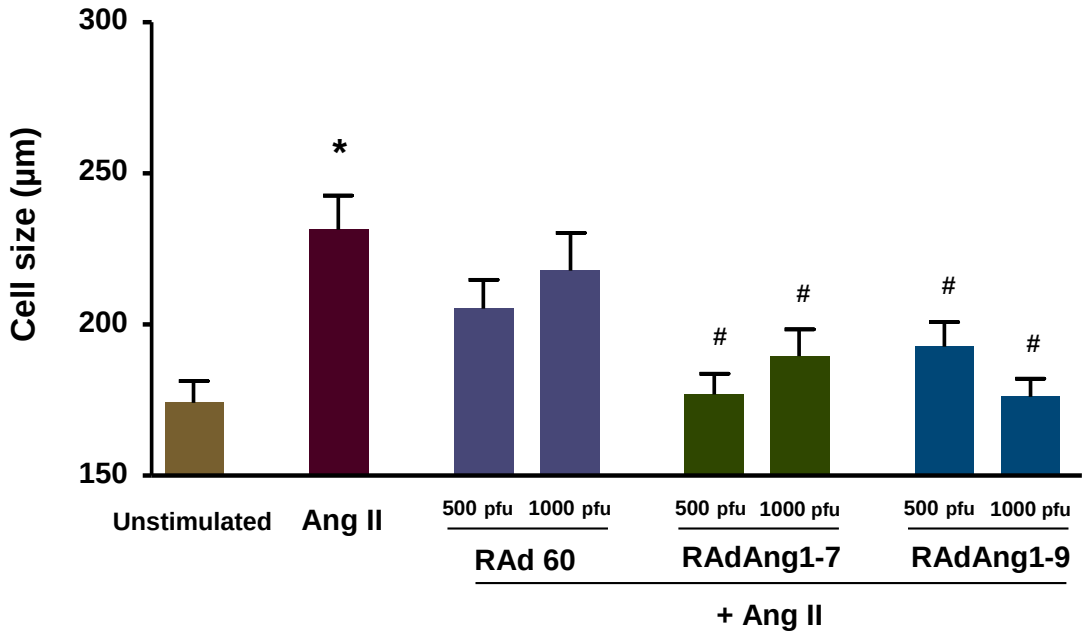


Figure 5-12. Assessment of the effects of RAdAng1-7 and RAdAng1-9 in AngII-induced hypertrophy in H9c2 cardiomyocytes.

H9c2 cardiomyocytes were transduced with RAdAng1-7, RAdAng1-9 or RAd60 at 500 and 1000 pfu/cell 24 hours before stimulation with AngII. Cells were fixed, stained with crystal violet and measured after 96 hours incubation with AngII. * $p<0.01$ vs. unstimulated cells; # $p<0.05$ vs. AngII stimulated cells.

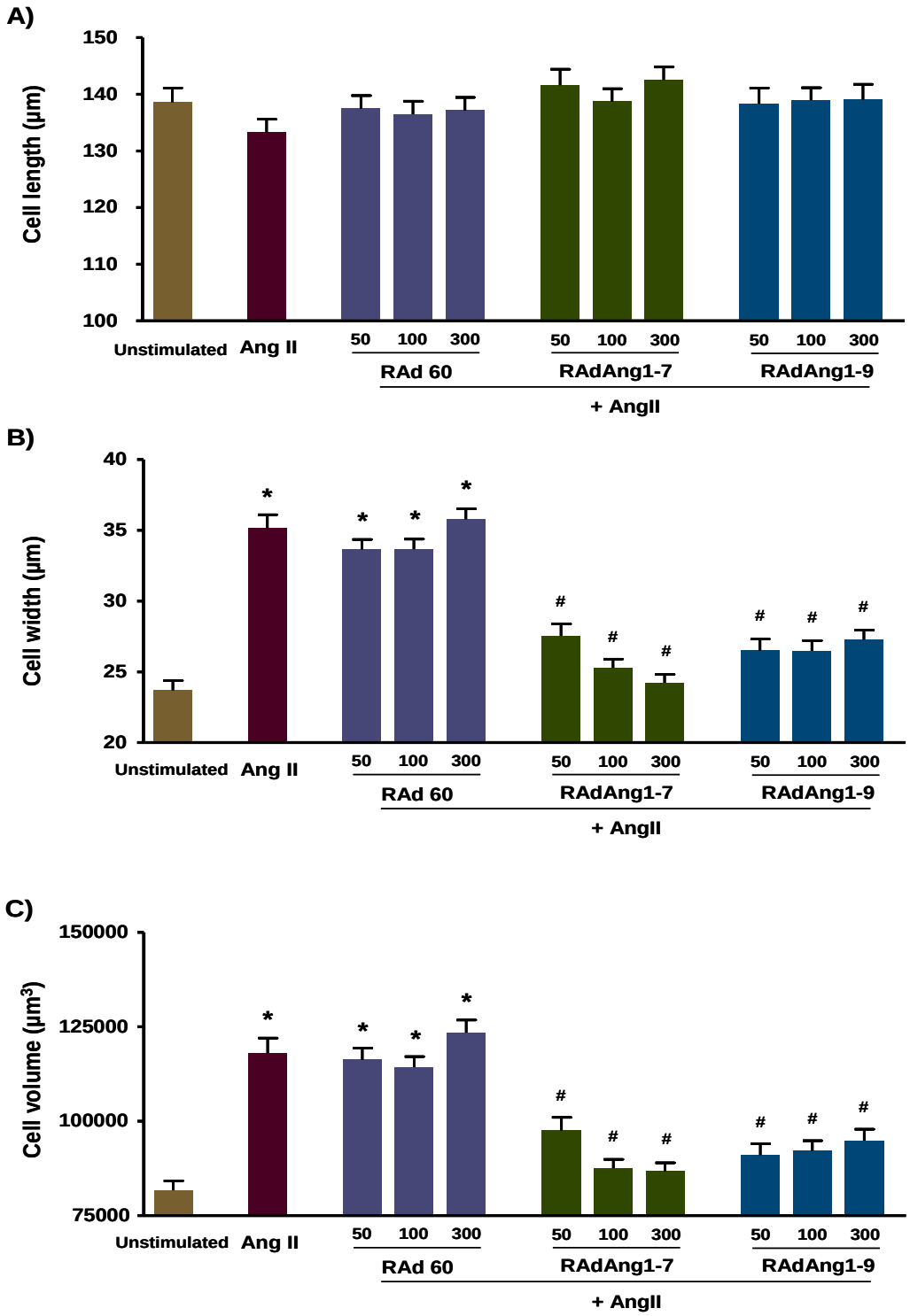


Figure 5-13. Effect of RAdAng1-7 and RAdAng1-9 in AngII-induced hypertrophy in primary cardiomyocytes.

Freshly isolated left ventricular adult rabbit primary cardiomyocytes were transduced with RAdAng1-7, RAdAng1-9 or RAd60 (50, 100 and 300 pfu/cell) 1 hour before stimulation with AngII (500 nM). After 24 hours incubation (A) cell length, (B) cell width and (C) cell volume of the cardiomyocytes was calculated. * $p<0.01$ vs. unstimulated cells; # $p<0.01$ vs. AngII stimulation.

5.2.5 Assessment of peptide secretion from RAdAng1-7 and RAdAng1-9 transduced cells

As secretion of Ang1-7 and Ang1-9 by each Ad vector was unable to be detected by standard western blotting due to the small molecular weight of the peptides, an indirect method was used to provide evidence that the peptides were secreted using a conditioned media assay. HeLa cells were transduced with 100 pfu/cell of either RAdAng1-7, RAdAng1-9 or RAd60 and incubated in serum free media for 48 hours at 37ºC. The media then was collected and added to AngII-stimulated H9c2 cardiomyocytes. (Figure 5-14 A).

Transfer of conditioned media from untransduced or RAd60 transduced HeLa cells to AngII-stimulated cells had no effect on AngII induced hypertrophy (RAd60 = 253.4 ± 12.7 µm). However, transfer of conditioned media from RAdAng1-7 or RAdAng1-9 transduced HeLa cells to AngII-stimulated cardiomyocytes blocked hypertrophy (RAdAng1-7 conditioned media = 162.3 ± 8.5 µm; RAdAng1-9 conditioned media = 176.2 ± 7 µm; $p<0.01$) (Figure 5-14 B). This provides indirect evidence that each RAd expressed the fusion protein and secreted the active peptide from the cell.

To demonstrate that the peptides secreted from each RAd function similarly to the exogenous peptides the effects of antagonists of both Mas and AT2R function were assessed. H9c2 cardiomyocytes transduced with 500 and 1000 pfu/cell of RAdAng1-7, RAdAng1-9 or RAd60 in the presence or absence of A779 (10 µM) or PD123,319 (500 nM). 24 hours later, transduced cardiomyocytes were stimulated with AngII (100 nM) and incubated for 96 hours. In similarity to the observations with exogenous peptides, addition of A779 abolished the anti-hypertrophic effect of RAdAng1-7, while RAdAng1-9 was able to block AngII-induced hypertrophy in the absence and presence of A779 (Figure 5-15 A). Addition of PD123,319 had no effect on the antihypertrophic effect of Ang1-7, but, completely eliminated the antihypertrophic effect of RAdAng1-9 (Figure 5-15 B).

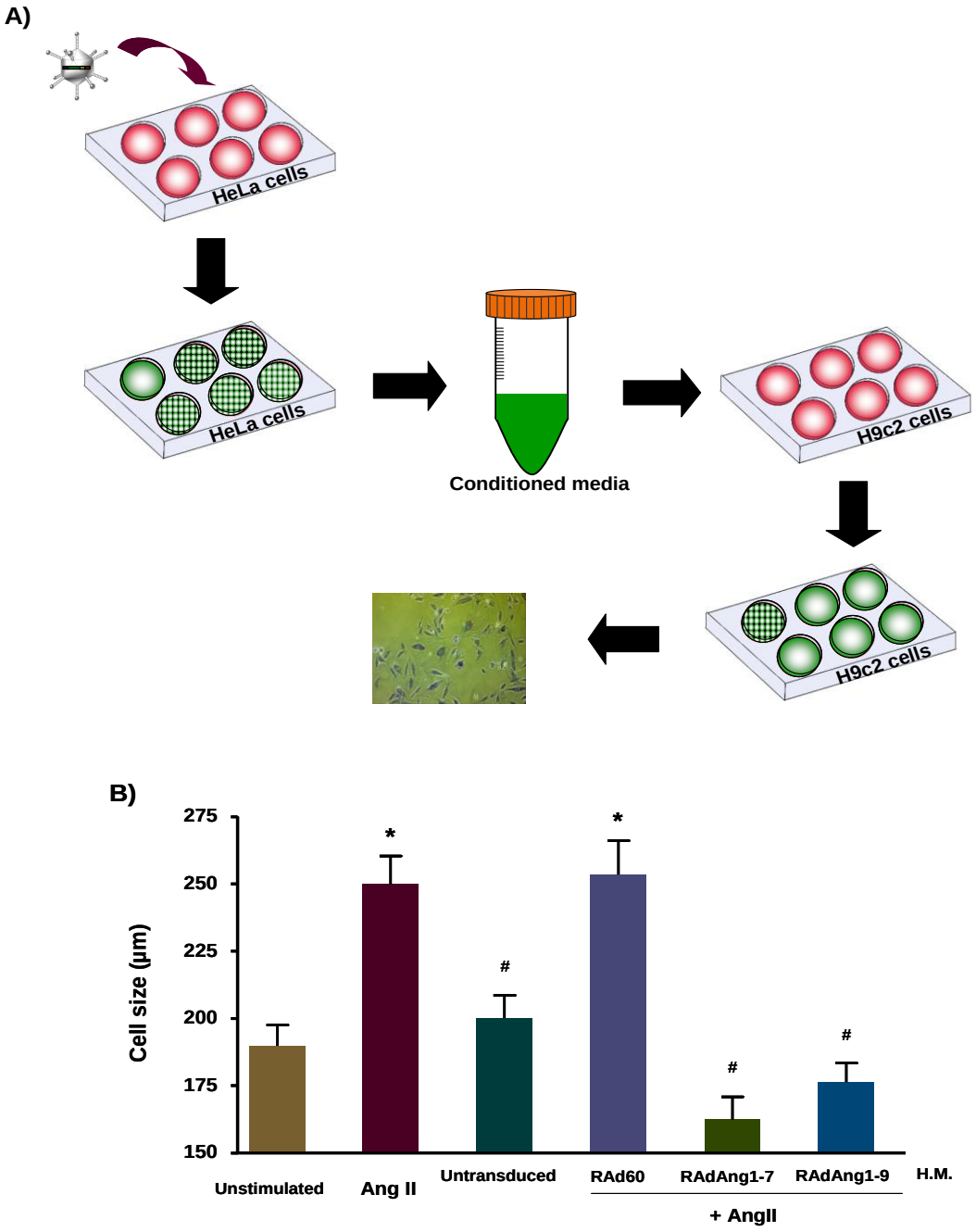


Figure 5-14. Conditioned media assay.

(A) HeLa cells were transduced with RAdAng1-7, RAdAng1-9 or RAd60 (R60) (negative control) (100 pfu/cell) and incubated for 48 hours. Culture media from HeLa transduced cells was transferred to H9c2 cardiomyocytes and incubated for 30 minutes before stimulating with AngII (100 nM). 96 hours later cells were fixed, stained with crystal violet and cell size measured. (B) Culture media from untransduced HeLa cells or cells transduced with RAd60 had no effect on AngII-induced hypertrophy. Conditioned media from RAdAng1-7 or RAdAng1-9 transduced HeLa cells blocked AngII-induced hypertrophy in H9c2 cardiomyocytes. * $p<0.01$ vs. unstimulated cells; # $p<0.01$ vs. AngII stimulated cells (H.M. = HeLa Media).

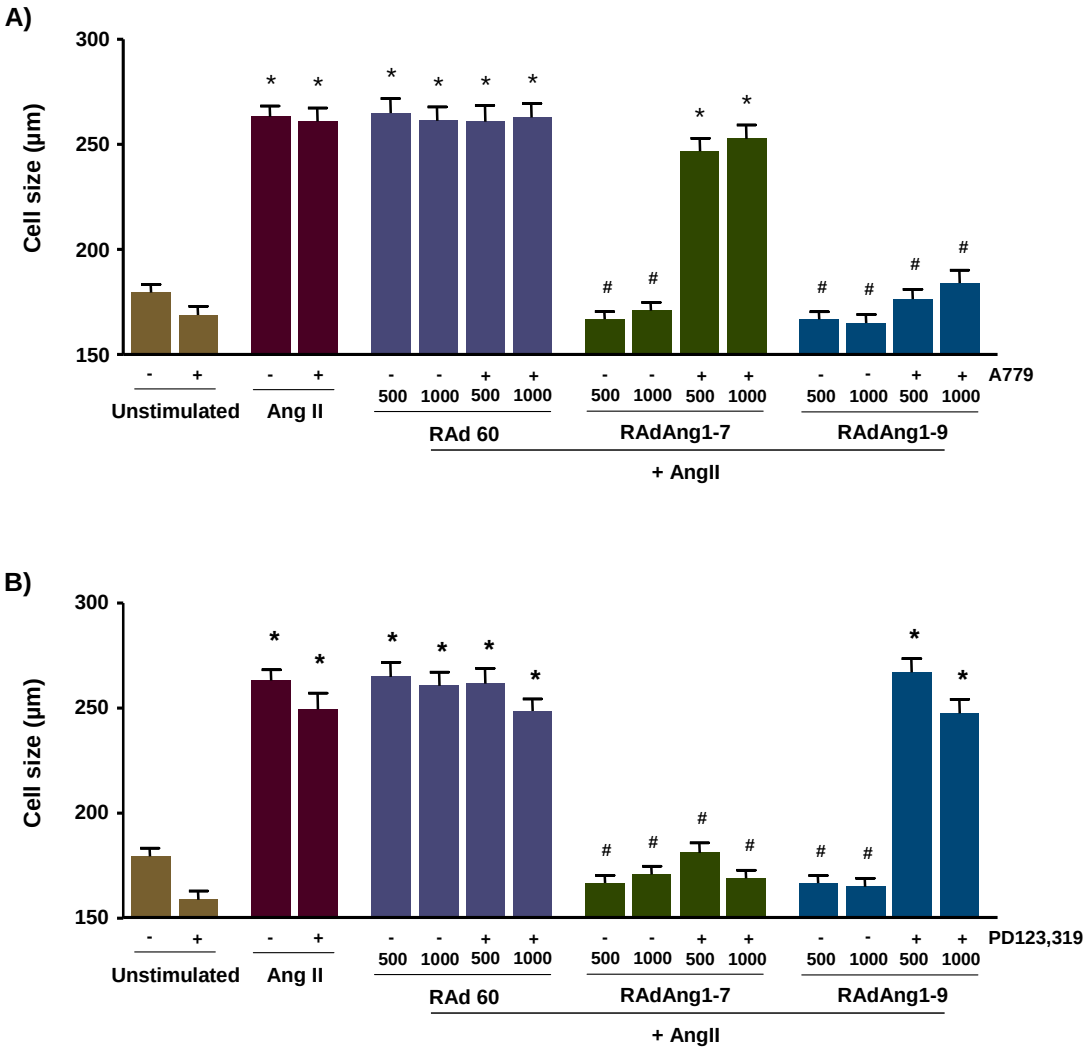


Figure 5-15. Effect of Mas and AT2R antagonism on AngII-stimulated H9c2 cardiomyocytes transduced with recombinant adenoviruses.

H9c2 cardiomyocytes were transduced with RAdAng1-7, RAdAng1-9 or RAd60 (negative control) in the presence or absence of (A) the Mas antagonist A779 (10 µM) or (B) the AT2R antagonist PD123,319 (500 nM) 24 hours before AngII stimulation. Cells were incubated for a further 96 hours before fixing, staining and measurement of cell size. * $p<0.01$ vs. unstimulated cells, # $p<0.001$ vs. AngII stimulated cells.

5.3 Discussion

In this chapter tools for the selective over-expression of Ang1-7 or Ang1-9 were generated. This will enable study of their function selectively in tissues and assessment of their utility as potential therapeutic gene targets. RAdAng1-7 and RAdAng1-9 were able to transduce neonatal rat and adult rabbit cardiomyocytes and secrete the RAS peptides. Furthermore, Ang1-7 and Ang1-9 secreted from transduced cardiomyocyte were able to block AngII induced hypertrophy in the same manner as exogenous peptides, via Mas and AT2R respectively, confirming over-expression of active peptides via RAdAng1-7 and RAdAng1-9 transduction.

In the last decade genetic approaches to study cardiac hypertrophy have been developed. Transgenic mice have become one of the most used tools to study single pathways leading to cardiac hypertrophy by knocking out a gene, for over-expression or over-expression of a mutant form of the gene. For example Ahmad *et al.*, demonstrated a diminished activation of Akt and RhoA by AngII signalling in transgenic mice over-expressing a mutant AT1R resulting in reduction of cardiac hypertrophy and blood pressure when compared to wild type animals (Ahmad *et al.*, 2009). Gusev *et al.* showed a direct effect of AngII on Ca^{2+} handling and cardiomyocyte contractility of transgenic mice over-expressing angiotensinogen (Gusev *et al.*, 2009), while by knocking out the Mas receptor, Pinheiro *et al.* showed an increase in fibrosis and microalbuminuria in transgenic mice (Pinheiro *et al.*, 2009). Although clearly transgenic models have revolutionised our understanding of the molecular basis of cardiac hypertrophy there are disadvantages. First, the cost of producing transgenic animals is very high and time consuming. Second, manipulation of genomic DNA is still random increasing the risks for epigenetic effects and the possibility of developmental abnormalities caused by embryonic over-expression. Also, transgenic mice represent the end-point of a disease which differs greatly to the initial stages. These effects may explain the discrepancies between transgenic studies and gene over-expression studies. For example ACE2 null mice have been reported to have increased blood pressure with no change in heart morphology (Gurley *et al.*, 2006) while over-expression of ACE2 driven by

AAV-mediated gene delivery in the SHRSP led to severe fibrosis in the heart (Masson *et al.*, 2009).

Cardiomyocytes are a difficult target for gene therapy as they differentiate into their mature terminally differentiated form within the first few weeks of life. Due to these characteristics specific vectors to transduce these cells would be valuable. Adenoviral vectors have the attributes to mediate gene delivery to adult cardiomyocyte *in vitro* and *in vivo* (Woo et al., 2007, Cutler et al., 2009, Porrello et al., 2009a, Maddaford et al., 2009, Heineke et al., 2007). Although there are more efficient vectors for long term gene delivery to cardiomyocytes, there are several advantages to using adenoviral vectors. Adenoviral vectors are capable of transducing quiesced cells, the tropism is efficient for both cardiomyocytes and other tissues such as kidney, brain, skeletal muscle and reproductive organs which are also important targets to study tissue-specific RAS effects (Paul et al., 2006, Hirsch et al., 1991, Tipnis et al., 2000, Roks et al., 1999, Elased et al., 2008, Leal et al., 2009, Pinheiro et al., 2009, Xia and Lazartigues, 2008). Although AAV vectors, particularly serotypes such as 1, 6 and 9 (Rengo et al., 2009, Palomeque et al., 2007, Yang et al., 2009) can produce long term expression in myocardium, high titers are required and the use of adenoviral vectors enables acute over-expression to further dissect the molecular mechanisms of action of Ang1-7 and Ang1-9.

The recombinant adenoviral vectors generated here efficiently transduced cardiomyocytes and expressed the RAS fusion protein. Ang1-7 and Ang1-9 were secreted from transduced cells and were able to block AngII-induced hypertrophy in cardiomyocytes from 2 different species, in similarity to the observations with the exogenous peptides. This confirmed that the Ad vectors secreted a functional peptide. Future work with these vectors is required *in vivo* to assess the phenotype induced following cardiac-selective delivery. Furthermore, RAdAng1-7 and RAdAng1-9 will be potentially useful to also investigate each peptides role in blood vessels, the kidney and in the brain.

C H A P T E R 6

General Discussion

Overall summary

The renin angiotensin system is a vital hormonal cascade that regulates blood pressure, volume and electrolytes by acting in the cardiovascular and renal system. Overactivity of the RAS contributes to the pathophysiology of hypertension and heart failure making the RAS a target for its treatment. Blocking various facets of the RAS including ACE via ACE inhibitors and the AT1R via AT1R blockers, as well as renin inhibitors are some of the most successful pharmacological treatments for hypertension and heart failure, supporting the fundamental importance of this cascade in the development of these diseases. The recent description of expression of all "classical" RAS components as well as novel components in specific tissues has altered the view of the RAS being simply a systemic acting system, to one with tissue-specific effects. Our understanding of the role of RAS in individual tissues is far from complete. Many studies have been developed to elucidate the tissue-specific actions of the novel components including ACE2 and Ang1-7 and although this has generated a large body of literature, findings still remain controversial.

The main focus of this thesis was the assessment of the novel RAS peptides Ang1-7 and Ang1-9 in cardiac hypertrophy. First, an *in vitro* hypertrophy model induced by AngII in rat neonatal H9c2 cardiomyocyte cell line and adult rabbit left ventricular primary cells was established. Confirmation of AngII-induced hypertrophy was performed assessing increases in cell size, in BNP gene expression and re-organization of α-actin sarcomeres. Next we evaluated the effects of Ang1-7 and Ang1-9 in the hypertrophy model. Both Ang1-7 and Ang1-9 blocked AngII-induced increases in cell size, as well as increased BNP expression and α-actin sarcomere re-organization. To confirm if Ang1-9 cleavage and Ang1-7 generation was necessary for its anti-hypertrophic effect, captopril (an ACE inhibitor) was used. Interestingly, Ang1-9 blocked AngII-induced hypertrophy in the presence of captopril. Furthermore, when blocking the Mas receptor, using A779, the effects of Ang1-9 were not affected, while A779 completely abolished the anti-hypertrophic effect of Ang1-7. Then, engagement of Ang1-9 with the AT1R and AT2R was assessed. To evaluate the AT1R, arg-vasopressin was used to induce hypertrophy. Blockade of the AT1R with losartan had no effect on either the effects of Ang1-7 or Ang1-9. Importantly, when blocking the AT2R with its antagonist PD123,319, the anti-

hypertrophic effects of Ang1-9 were completely abolished. To further investigate if the bradykinin receptor B_2 was participating in the Ang1-7 or Ang1-9 signalling the selective B2R inhibitor HOE 140 was used. Resulted showed interaction between the Mas receptor and the B_2 receptor but not with the AT2R. To start investigating the cell signalling pathways, PKC membrane translocation was assessed by immunofluorescence. AngII was able to induce rapid PKC translocation from the cytoplasm to the membrane , an effect also observed with Ang1-7 or Ang1-9. Next, the functionality of the AT1R, AT2R and Mas was evaluated by induction of Ca^{2+} transients with AngII, Ang1-9 or Ang1-7 respectively. Although AngII was able to induce Ca^{2+} mobilization, only one third of the cardiomyocytes responded. No increase in Ca^{2+} was observed when the AT2R was stimulated with Ang1-9 or the Mas receptor with Ang1-7. Finally, $G_{\alpha i}$-subunit activation by Ang1-7 or Ang1-9 through the Mas and the AT2R was investigated. The $G_{\alpha i}$-subunit antagonist, PTX, abolished the anti-hypertrophic effect of both Ang1-7 and Ang1-9.

Second, to develop tools to facilitate the research of Ang1-7 and Ang1-9, adenoviral vectors encoding a fusion protein expression cassette which drives over-expression and secretion of either Ang1-7 or Ang1-9 was engineered. Successful over-expression and secretion was confirmed when RAdAng1-7 and RAdAng1-9 blocked AngII-induced hypertrophy either by direct transduction of cardiomyocytes or by transfer of conditioned media from Ad transduced HeLa cells to AngII-stimulated cardiomyocytes. Moreover, the effects of RAdAng1-7 and RAdAng1-9 could be inhibited in the presence of 779 or PD123,310, respectively.

Finally, the role of Ang1-9 in an *in vivo* setting was assessed by delivering the peptide for a 4 weeks period into SHRSP using osmotic minipumps. Co-infusion of Ang1-9 and PD12,319 was also performed in order to evaluate the role of the AT2R in any observed effects of Ang1-9. Blood pressure, monitored via radiotelemetry, was not different between any of the groups, however, echocardiography, performed during the study, showed a significant increase in cardiac output in the Ang1-9 infused group compared to the control rats, an effect that was abolished by co-infusion of PD123,319. In addition, LVMI was significantly increased in rats co-infused with Ang1-9 and PD123,319. Histological assessment of the hearts showed reduced perivascular fibrosis in the Ang1-9 group, effect that was abolished with co-

infusion of PD123,319. Interestingly, fibrosis in the PD123,319 co-infused group was predominantly interstitial. Furthermore, assessment of NO bioavailability with pressure myography in aortic rings showed an increase in basal NO bioavailability in Ang1-9 infused SHRSP compared to control animals. Co-infusion of PD123,319 also abolished this effect. Overall, the data presented in this thesis supports the notion that Ang1-9 is an active RAS hormone, which is able to mediate beneficial effects in the cardiovascular system, including pathological effects of AngII, via the AT2R. These effects segregate its actions from those of Ang1-7, which signals via Mas.

Future work

Ang1-9 has been reported to act as a competitive inhibitor of ACE as well as to potentiate bradykinin B_2 receptor activity (Snyder and Wintroub, 1986, Erdos *et al.*, 2002). However, no direct functions have been ascribe to Ang1-9 to date. Two different models of cardiomyocyte hypertrophy were used to demonstrate the effects of Ang1-9, representing different species and adult and neonatal lineages. In both these models cardiomyocyte hypertrophy was induced by the addition of AngII, which was assessed by increased cell size as well as imaging of actin cytoskeleton using phalloidin in H9c2 cardiomyocytes. Depending upon whether sarcomeres are added in parallel or in series, cardiac hypertrophy can be classified as either concentric or eccentric (Carreno *et al.*, 2006). Here, width of the primary adult rabbit cardiomyocytes was increased significantly, presumably defining AngII as inducing a concentric hypertrophy phenotype. In retrospect, staining of the sarcomeres in rabbit cardiomyocytes with phalloidin to image the sarcomeres would have given an additional assessment of the type of hypertrophy induced by AngII. H9c2 cardiomyocytes are a widely used cell line when investigating cell signalling in cardiomyocytes (Kee and Kook, 2009, Villeneuve et al., 2009, Stuck et al., 2008) therefore selected for initial assessment of RAS peptides in cardiomyocyte hypertrophy. However, the fact they are a proliferative cell line, may not reflect the *in vivo* setting as well as primary cultures therefore future work in Ang1-9 cell signalling assessment should be performed in freshly isolated cultures of primary neonatal cardiomyocytes. Importantly all data was reproduced in freshly isolated primary cardiomyocyte from rabbits, providing confidence in the data. Although

cardiomyocyte hypertrophy was blocked *in vitro* by Ang1-9, in the *in vivo* study no difference was observed between control rats and rats infused with Ang1-9. Since Ang1-9 was also shown to have effects on cardiac fibrosis *in vivo* and the AT2R has also been recognized in several studies to reduce cardiac fibrosis (Falcon et al., 2004, Ohkubo et al., 1997, Oishi et al., 2003), primary fibroblast cultures would be essential in the future to study Ang1-9 signalling in response to proliferative agents such as AngII or TGF-β. Additionally, the *in vivo* data also support the notion that Ang1-9 signals through the AT2R to increase NO bioavailability in aortic rings. Therefore, future work to study the effects of Ang1-9 in endothelial cells would support the dissection of *in vitro* mechanisms of Ang1-9 actions.

A full understanding of the role of Ang1-9 would be important to understand its role within the RAS and its potential as a future therapy. The use of pharmacological antagonists was an important tool to demonstrate differences in the cellular receptors engaged by Ang1-7 and Ang1-9. Through this procedure the actions of Ang1-9 were established as antihypertrophic and anti-fibrotic as well as promotion of improved endothelial function, effects which were all mediated by the AT2R. Nevertheless, future experiments showing direct binding of Ang1-9 to the AT2R would support this data. A radio labelled peptide binding assay would be a logical step to probe Ang1-9 / AT2R engagement, by assessing endogenous binding in the presence and absence of receptor inhibitors. Several groups have shown the use of a recombinant adenoviral vector for over-expression of the AT2R (Li et al., 2005a, Warnecke et al., 2001, Porrello et al., 2009a). Transduction of non-AT2R expressing cell line with a recombinant adenovirus over-expressing AT2R and assessment of fluorescently labelled Ang1-9 binding would also help probe a direct Ang1-9 / AT2R association. In addition, analysis of Ca^{2+} transient and IP_3 activation by Ang1-9 in AT2R transduced cells may also support Ang1-9 binding to the AT2R. Although, there is still controversy as to whether the AT2R engages G-proteins and therefore other activation mechanisms could also be studied such as the interaction with β-arrestins, as has been well established as for the AT1R (Kim *et al.*, 2009).

The AT2R has been described to produce non-classical GPCR signals. In human myometrium and bovine cerebellar cortex AngII was unable to induce AT2R-mediated GTP gamma incorporation (Bottari *et al.*, 1991). However, Zhang et al. demonstrated AT2R interaction with the $G_{\alpha i}$-subunit (Zhang and Pratt, 1996).

Although our data shows that blockade of the $G_{\alpha i}$- subunit abolishes Ang1-9 effects, initial future work focusing on the G-protein subunits involved in Ang1-9 / AT2R signalling is needed. Selective inhibitors of the $G_{\alpha q}$ and $G_{\alpha s}$-subunits, YM-25489 (Canals *et al.*, 2006) and cholera toxin (Pei *et al.*, 2000), in AngII-stimulated cardiomyocytes, fibroblast and endothelial cells in the presence and absence of Ang1-9 would be an important tool to address this. Furthermore, short interfering RNA transfection to block expression of individual G-proteins will be helpful to define the role of each subunit in Ang1-9 signalling. To start differentiating downstream signalling induced by Ang1-9 through the AT2R, future work should be based on the knowledge of AngII cell signalling pathways. Blockade of PI3K, MAP kinase and PKC pathways with pharmacological inhibitors could identify potential differences between AngII and Ang1-9 signalling, in addition to identifying key pathways to focus on. In addition results in aortic rings showed a potential role of Ang1-9 in NO production. To further study this effect, the use of assays that measure NO production in Ang1-9 stimulated endothelial cells compared to AngII stimulated endothelial cells will be an important tool to investigate the ROS pathway. Finally, analysis of transcription factors and gene expression through reporter gene arrays, could lead to identification of novel targets regulated by Ang1-9. These could be further studied with immunofluorescence antibodies detecting specific transcription factors and evaluating nuclear translocation as western immunoblotting in Ang1-9 stimulated cell lysates with phospho-antibodies, for example.

Delivery of Ang1-9 with osmotic mini-pump implantation only enables the study of systemic effects. A system which enables delivery of peptides in a tissue-specific manner would be useful to study the mechanisms of Ang1-9 actions in specific cardiovascular disease phenotypes such as cardiac remodelling. This is important as recent studies have highlighted the importance of separating tissue-specific actions of the RAS in the kidney (Crowley *et al.*, 2005) and CNS (Lee-Kirsch *et al.*, 1999). Based on the fusion protein approach, generation of transgenic mice over-expressing Ang1-9 in the heart would also contribute to the understanding of Ang1-9's local activity and dissect them from its systemic actions. The development of these mice will also allow for future cross-breeding between them and other available transgenic mouse strains (e.g. AT1R and AT2R knockout (Sugaya et al., 1995, Hein et al., 1995 to further study the in vivo role of Ang1-9. Alternatively, tissue-specific effects of

Ang1-7 or Ang1-9 could be studied using direct delivery into the heart via intracardiac injection of the Ad vectors developed. Moreover adeno-associated viruses have been describe to be a better choice for cardiovascular gene transfer{Melo, 2002 #203, Woo et al., 2005). Cloning fusion protein into AAV 6 would also improve delivery of Ang1-9 and Ang1-7 to cardiovascular tissue. Furthermore, the use of RAdAng1-7 or RAdAng1-9 would avoid surgical implantation of the minipumps and all the associated side effects and risks. Since recombinant adenoviruses delivered intravenously target the liver, systemic delivery would enable the liver to express and secrete the peptides into the systemic circulation.

In the SHRSP experimental model no significant difference in blood pressure between any groups was observed, suggesting Ang1-9 had no effect on this. Previously, it has been shown that the AT2R has a role in cardiac remodelling decreasing left ventricular wall thickness and cardiac fibrosis in hypertrophy animal models without affecting blood pressure (Metcalfe et al., 2004, Falcon et al., 2004). Conversely, in the SHRSP study here Ang1-9 increased cardiac output and improved NO bioavailability, data that can be translated into decreased vascular resistance, suggesting Ang1-9 promotes vasodilatation. SHRSP represents an experimental model of chronic hypertension. Over time SHRSP compensate the increase of blood pressure in order to survive as well as develop other pathophysiological traits that contribute to the disease phenotype, making it an extremely well compensated disease model. A prevention rather than a reversal study may provide additional important information regarding the effects of Ang1-9. Furthermore, for future work the role of Ang1-9 in the systemic circulation could be studied in an acute hypertensive model, such as infusion of AngII via minipumps to trigger hypertension and cardiac remodelling acutely as has been described previously in normal rodents (Falcon *et al.*, 2004, Wang *et al.*, 2006a, Huang *et al.*, 2008).

Interestingly, although no effect of Ang1-9 on cardiac hypertrophy was observed, there was a significant increase in LVMI when Ang1-9 was co-infused with PD123,319, suggesting that PD123,319 may be having an intrinsic effect on basal AT2R activity in the SHRSP. Furthermore, although PD123,319 was able to abolish the anti-fibrotic effect of Ang1-9, rats co-infused with Ang1-9 and PD123,319

presented differential fibrosis staining patterns, supporting the hypothesis of an intrinsic effect of PD123,319. To further study this, a group of SHRSP infused with PD123,319 alone is being performed. Due to the time of the thesis submission this group was still ongoing and data was not available. Additionally, to complete the *in vivo* study a SHRSP group co-infused with Ang1-9 and A779 would be useful to rule out any potential effects mediated by Ang1-9 conversion to Ang1-7. Together future work to address the issues described above will enable a detailed dissection of the true role of Ang1-9 in the RAS.

Translational studies

Although animal models are useful tool to study actions of the novel RAS components, human biology can differ from this models, and assessment of the actions of peptides in clinical studies is the fundamental to determining the relevance of novel findings to human health and disease. Little is known about the local-tissue specific components of RAS in humans. Ang1-7 and Ang1-9 have been demonstrated to potentiate bradykinin in human pulmonary artery endothelial cells *in vitro* (Erdos *et al.*, 2002), but further study in humans is important. The study here presented sets the grounds for the development of clinical studies. Although the actions of the tissue-specific RAS are starting to be widely documented most of the studies are performed in animal models. However, using cultures of human cells and patient screening a few groups have addressed the effects of Ang1-7 in humans. In patients with essential hypertension, plasma levels of Ang1-7 were measured and compared to normal subjects (Luque *et al.*, 1996). The increase of Ang1-7 levels correlated inversely with the diastolic blood pressure of the patients with essential hypertension. In addition, Kono *et al.* showed a decrease in plasma levels of renin when Ang1-7 was infused to Bartter's syndrome patients (Kono *et al.*, 1986). Moreover, when Ang1-7 was infused into the brachial artery of heart failure patients, Ang1-7 was unable to induce vasodilatation compared to bradykinin infusion (Davie and McMurray, 1999). Based on these examples, detection of Ang1-9 in systemic circulation of normal subjects compared to hypertensive or groups of other patients, such as post-MI or heart failure by mass spectrometry or high performance liquid chromatography would be important to start elucidating Ang1-9 mechanisms in human pathologies. One group has recently reported the measurement of Ang1-9

using these techniques in human plasma (Campbell *et al.*, 2004). This study would provide information on the systemic actions of Ang1-9 in such diseases. Moreover, comparing levels of Ang1-9 at the different stages of disease development in patients will increase the understanding of the role of Ang1-9. Furthermore, quantification of other RAS components such as AngII and Ang1-7 in hypertensive patients infused with Ang1-9, for example via the forearm, as described for Ang1-7 (Davie and McMurray, 1999) would provide information on the effects and interactions of Ang1-9 in the RAS.

To determine the levels of Ang1-9 in specific tissues and organs immunodetection in human tissues would be a useful approach. Heart homogenates obtain from patients undergoing cardiac transplantation due to heart failure, idiopathic dilated cardiomyopathy or congenital disease, have been used to study Ang1-7 and Ang1-9 formation in human tissue (Zisman et al., 2003, Kokkonen et al., 1997). In both cases the production of these peptides was higher in diseased hearts compared to normal hearts (Kokkonen *et al.*, 1997, Zisman *et al.*, 2003). The use of myocardial biopsy of patients undergoing catheterism to obtain cardiac tissue would provide with human tissue to detect Ang1-9 in the heart using h*igh performance liquid chromatography*. Furthermore, the use of an Ang1-7 antibody has been useful to detect this peptide in human placenta by immunohistochemistry (Valdes *et al.*, 2006). However, the development of a human antibody to detect Ang1-9 is still needed, but this would allow visualizing the distribution of Ang1-9 in the human heart. Human heart homogenates would also provide the means to test Ang1-9 signalling in cardiac tissue. To address the effects of Ang1-9 on NO production in human blood vessels, analysis of contraction of human arteries rings obtained from coronary artery bypass grafting patients subjected to Ang1-9 treatment could be performed, as described for the study of Ang1-7, where Ang1-7 was able to block AngII-induce vasoconstriction (Roks *et al.*, 1999). In addition, the use of human endothelial cells has been helpful in the elucidation of Ang1-7 cell signalling pathways and has demonstrated that Ang1-7 blocked AngII-mediated induction of ERK 1/2 and NAD(P)H (Sampaio *et al.*, 2007a). This highlights that simple preliminary investigations in human cells *in vitro* would help dissect the translational relevance of the actions of Ang1-9.

In conclusion the data here presented demonstrates Ang1-9 as an independent and biological active peptide of the RAS. Importantly, we demonstrate that Ang1-9 acts as an antihypertrophic and anti-fibrotic peptide which also improved endothelial function, actions that are mediated by binding to the AT2R. These results give an initial view of the actions of Ang1-9 and its potential as a new therapeutic target in cardiovascular disease. Further work is required to determine its true role and relevance to human cardiovascular (patho)physiology.

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