Mechanisms involved in the pathophysiology of hypertension models Studies on transient receptor potential vanilloid 1 (TRPV1) and RAMP2

Liang, Lihuan

Awarding institution:
King's College London

Mechanisms involved in the pathophysiology of hypertension models: studies on transient receptor potential vanilloid 1 (TRPV1) and RAMP2

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King's College London

Lihuan Liang

Cardiovascular Division

King's College London

Franklin-Wilkins Building

Waterloo Capus

London SE1 9NH

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ABSTRACT

Hypertension has been described as an important risk factor for cardiovascular morbidity and mortality, despite current treatments. Therefore an increased understanding of the mechanisms in the development of hypertension has become essential. This project involves the study of the vascular component of murine models of hypertension. Firstly the protective role of calcitonin gene-related peptide (CGRP)-like peptide adrenomedullin (AM) was examined using transgenic mice for the AM receptor. Secondly transient receptor potential vanilloid 1 (TRPV1) was studied, activation of which is known to release the sensory neuropeptide CGRP.

The angiotensin II (ATII) induced hypertension murine model is considered to mimic essential hypertension. Here, hypertension was induced in 3-4 month old mice (mixed gender) over 14 days following implantation of ATII-containing osmotic mini-pumps. Blood pressure was monitored before mini-pump implantation and until day 14 by either tail cuff plethysmography or telemetry. Post-hoc study analysis of vascular hypertrophy and vascular dysfunction biomarkers (e.g. VCAM-1 and endothelin) was performed. Studies in AM receptor transgenic mice revealed no effect on hypertension, but significant protection against aortic vascular remodelling. This indicates that adrenomedullin, like CGRP, is a protective peptide in the vasculature. Surprizingly, the TRPV1 KO mice were protected against hypertension and aortic remodelling, suggesting a lack of critical involvement of CGRP.

To further understand the role of TRPV1, a second more chronic model was used. Obesity-induced hypertension was established in mice following high fat diet (HDF, 35% fat), from 3-15 weeks. Blood pressure and glucose tolerance were monitored during the last week of the study. Post-hoc study analyses revealed TRPV1 KO-HFD mice were protected against the hypertension.

This PhD project provides evidence that i) up-regulation of the functional AM1 receptor, protects against vascular hypertrophy and inflammation despite no change in blood pressure. ii) The AM1 receptor mechanisms involved in inhibition of vascular smooth muscle cell proliferation. iii) Deletion of TRPV1 protected mice from the hypertension and vascular hypertrophy induced by ATII or high fat diet. iv) Deletion of TRPV1 is also associated with improving glucose tolerance. Collectively these results provide evidence of two distinct mechanisms involved in cardiovascular regulation, of relevance to hypertension.

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LIST OF ABBREVIATIONS

12-HPETE	(S)-12-(hydroperoxy)eicosatetraenoic acid
15-HPETE	(S)-15-(hydroperoxy)eicosatetraenoic acid
2K1C	2 kidneys 1 clip model
20-HETE	20-hydroxyeicosatetraenoic acid
ACE	Angiotensin converting enzyme
ADH	Anti-diuretic hormone
Akt	Also know as Protein Kinase B
AM	Adrenomedullin
AM1	Adrenomedullin 1
AM2	Adrenomedullin 2
AMY	Amylin
$AM^{+/-}$	AM heterozygous knockout mice
APE	3-aminopropyletriethoxysilane
$ApoE^{-/-}$	Apolipoprotein E KO mice
ARBs	ATII receptor 1 blockers
AT1	Angiotensin II receptor 1
AT2	Angiotensin II receptor 2
ATII	Angiotensin II
ATP	Adenosine triphosphate
BCA	Bicinchoniinc acid
BMI	Body mass index
BP	Blood pressure
bp	Base pairs
C terminal	Carboxylic acid chain terminal
cAMP	Cyclic adenosine monophosphate

CB1	Cannabinoid1 receptor
CGRP	Calcitonin gene-related peptide
CGRPKO	Calcitonin gene-related peptide knockout
-$CONH_2$	Amidated C-terminal tyrosine
αCGRP	Alpha CGRP
βCGRP	Beta CGRP
CL	Calcitonin receptor-like receptor
CONH2	Amidated C-terminal tyrosine residue
CVDs	Cardiovascular diseases
DAB	3,3’ diaminobenzidine
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DRG	Dorsal root ganglion
eNOS	Endothelial NOS
ERK	Extracellular signal regulated kinases
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ETA	Endothelin-1 receptor A
ETB	Endothelin-1 receptor B
GPCR	G-protein-coupled receptor
H&E	Haematoxylin and eosin Staining
HFD	High (35%) fat diet
ICAM-1	Intracellular adhesion molecule 1
IL-1β	Interleukin 1β

IMD	Intermedin
i.p.	Intra-peritoneal injection
i.v.	Intra-venous injection
KO	Knockout mouse
2K1C	Two kidneys one clip model
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein 1
mRNA	Messenger ribonucleic acid
N terminal	Amino acid chain terminal
NADA	N-arachidonoyl dopamine
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NGF	Nerve growth factor
NICE	National Institute for Health and Clinical Excellence
NKA	Neurokinin A
NO	Nitric oxide
OLDA	N-oleoyldopamine
PAMP	Proadrenomedullin N-terminal 20 amino acid peptide
p38 MAPK	p38 Mitogen-activated protein kinase
PI3k	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol(4,5)-bisphosphate
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
RAMP	Receptor activity-modifying protein

RAMP1	Receptor activity-modifying protein 1
RAMP2	Receptor activity-modifying protein 2
RAMP3	Receptor activity-modifying protein 3
RAS	Renin-angiotensin system
RAAS	Renin-angiotensin-aldosterone system
RCP	Receptor component protein
ROS	Reactive Oxygen Species
RT-PCR	Reverse transcription-polymerase chain reaction
RTX	Resinferatonxin
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
SP	Substance P
TGFβ1	Transforming growth factor beta-1
TNFα	Tumour necrosis factor α
TRP	The transient receptor potential
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPML	Transient receptor potential muclolipin
TRPP	Transient receptor potential polycycstin
TRPV1	Transient receptor potential vanilloid type 1 receptor
TRPV1 KO	TRPV1 knockout
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells
WHO	World Health Organization

WT	Wildtype mouse
12-HPETE	(S)-12-(hydroperoxy)eicosatetraenoic acid
15-HPETE	(S)-15-(hydroperoxy)eicosatetraenoic acid

CHAPTER ONE: INTRODUCTION

1.1 Background

Blood pressure is the pressure exerted by circulating blood on the vessel walls. Hypertension, is a chronic medical condition, also called arterial hypertension, and is the state at which the blood pressure is increased from the normal range (Chobanian *et al*., 2003). This causes extra pressure on the heart, in order that blood can circulate normally. Hypertension has been described as the leading cause of morbidity and mortality all over the world. This is mainly due to the development of secondary hypertension-linked diseases, for instance heart failure and renal dysfunction (Kearney *et al*., 2005). Therefore, understanding the mechanisms underpinning the development of hypertension has become critical. This is especially important as one report has indicated that more than 25% of adults aged > 20 years old in the world were hypertensive in 2000, and this is predicted to increase to 29% by the year 2025 (Kearney *et al*., 2005).

This thesis contains a study of mechanisms involved in the development and maintenance of hypertension using two different murine models of hypertension; an angiotensin II (ATII)-induced hypertension model and an obesity-induced hypertension model. Firstly, this PhD project has involved the study of the CGRP-related peptide, adrenomedullin (AM). Secondly, this PhD project has concentrated on the role of the ion channel, transient receptor potential vanilloid 1, (TRPV1) which is believed to be primarily localized to the sensory nerves (Caterina *et al*., 1997). Activation of TRPV1 can lead to release of calcitonin gene related peptide (CGRP) a potent vasodilator neuropeptide (Brain *et al*., 1985). This introduction will begin by discussing the current knowledge about the CGRP family (mainly AM and CGRP) and then focusing on TRPV1. Then, I will discuss the pathophysiology of hypertension, and mechanisms involved in its development. Finally, I will explain the main hypertensive models and the models used in my PhD.

1.2 CGRP family

The calcitonin-gene related peptide superfamily (CGRP family, also known as the calcitonin family) includes CGRP, AM, amylin (AMY) and intermedin (IMD). CGRP,

formed from the tissue-specific splicing of the primary transcript of the calcitonin/CGRP gene, is a 37 amino acid neuropeptide. It was the first of the non-calcitonin peptides discovered in 1982 (Bell and McDermott 1996; Brain and Grant, 2004). AM, a 52 amino acid peptide, is ubiquitously expressed. It was first isolated in 1993 from human phaeochromocytoma cells (Kitamura *et al*., 1998). AMY, a 37-amino acid peptide, was first identified in 1986 as amyloid deposits from the pancreas from non-insulin-dependent diabetics (Westermark *et al*., 1987). The latest peptide to be discovered is IMD, a 47 amino acid peptide, also known as adrenomedullin 2 (AM2). It was discovered by two different groups at the same time (Roh *et al*., 2004; Takei *et al*., 2004a). CGRP, AM, IMD and AMY are grouped because they share similar structures (shown in Fig. 1.1), such as one 6-amino acid ring formed by the disulfide bond and an amidated C-terminal tyrosine residue (-CONH2), which is different from calcitonin which only has a 5-amino-acid ring. Both the disulfide bond and its amidated C-terminus are essential for the peptides' biological activity as they are essential for receptor binding and then cAMP generation (Eguchi *et al*., 1997). Removal of the first 15 amino acids for AM does not result in any loss of biological activity (Eguchi *et al*., 1997).

	16 21
hAM	H_2N-YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY - $CONH_2$
mAM	H_2N-YRQSMN—QGSRSNGCRFGTCTFQKLAHQTYQLTDKDKDGMAPRNKISPQGY-$CONH_2$
hCGRP	H_2N-ACDTATCVTHRLAGLLSRSGGVVKNFVPTNVGSKAF-$CONH_2$
mCGRP	H_2N-SCNTATCVTHRLAGLLSRGGVVKDNFVPTNVGSEAF-$CONH_2$
hAMY	H_2N-KCNTATCATQRLANFLVHSSNNFGAI LSSTNVGSNTY-$CONH_2$
mAMY	H_2N-KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY-$CONH_2$
hIMD	H_2N-VGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-$CONH_2$
mIMD	H_2N-VGCVLGTCQVQNLSHRLWQLVRPAGRRDSAPVDPSSPHSY-$CONH_2$

Figure 1.1 Sequence alignment for the calcitonin/intermedin family peptides from human and mouse. The two cysteine residues (in red), form a disulphide bond to create a ring and are shared by all family peptides. (h) Human; (m) mouse.

1.2.1 CGRP

CGRP has two different isoforms, alpha CGRP (αCGRP) and beta CGRP (βCGRP), where they differ from each other by only three amino acids in both humans (Morris *et al*. 1984, Van Rossum *et al*., 1997) and mice (Rezaeian *et al*., 2009,). αCGRP is the main isoform of CGRP in the body (Smillie *et al*., 2011).

CGRP is the most potent microvascular vasodilator identified to date (Brain and Grant., 2004). There is evidence to suggest the involvement of CGRP in controlling blood pressure. The circulating levels of CGRP are decreased in patients with uncomplicated essential hypertension (Portaluppi *et al*., 1992). On the other hand, the lack of CGRP may also contribute the development of hypertension (Portaluppi *et al*., 1992). Additionally, it is suggested that CGRP plays a role in certain pathological and physiological events such as vasodilatation, cardioprotection, and neurogenic inflammation (Qi and Hay, 2010). First of all, injection of picomole doses of CGRP can cause vasodilation of resistance vessels in human skin (Brain et al., 1985). Moreover, it is suggested that CGRP can cause coronary artery dilation which would delay the start of myocardial ischemia in patients with chronic angina (Uren *et al*., 1993). More recently an *in vitro* study in isolated perfused hearts from α-CGRP knockout (KO) and wild type (WT) mice demonstrated the cardioprotective role of CGRP against ischemia/reperfusion injury (Huang *et al*., 2008).

1.2.2 AM

AM, a 52 amino acid peptide, is modified by amidation from an immature 53-amino acid precursor (shown in Fig 1.2). A second peptide product is known to be generated from the AM gene. This is the proadrenomedullin N-terminal 20 amino acid peptide (PAMP). Even though AM and PAMP are generated by the same precursor molecule, the PAMP/AM ratio depends on the tissue or organ (Garcia *et al*., 2006). It has been demonstrated in the human forearm that PAMP is 60 times less potent than AM in regulating blood flow (Wilkinson *et al*., 2001). Therefore, it is believed that PAMP is less important in regulating both the blood pressure and blood flow. In addition to sharing its sequence homology with CGRP (Brain and Grant, 2004), AM also shares some cardiovascular activities with CGRP, such as vasodilatation but with 3-30 times less potency than CGRP (Brain and Grant, 2004). Perhaps, the most important function of

AM is during foetal development. The AM gene is believed to be essential for survival as $AM^{-/-}$ (the null mutation of AM gene) mice fail to survive beyond embryonic E14.5 (embryonic days 14.5). The reason for the fatalities was suggested to be linked with hydrops fetalis (a condition of accumulation of fluid or oedema in the fetus) and incomplete development of the heart and arterial vasculature (Caron and Smithies 2001).

AM is found in a range of tissues and cell types. Together with the potent vasodilatory activity of AM, AM also plays a protective role in the pathophysiology of cardiovascular diseases. Elevated plasma levels of AM are reported in a variety of cardiovascular disorders, including myocardial infarction, hypertension and atherosclerosis (Tsuruda *et al*., 2003; Nishikimi *et al*., 1995; Nagaya *et al*., 1999). In addition, secretion of AM was studied in isolated endothelial cells; vascular smooth muscle cells (VSMCs) and cardiomyocytes. It is suggested that production of AM is up-regulated by several factors, such as oxidative stress, angiotensin II (ATII), pro-inflammatory cytokines, hypoxia, hyperglycaemia and so on (Beltowski *et al*., 2004). AM can be produced as an immature glycine-extended form (comprising 85% of the total plasma AM) which is then converted to mature AM intracellularly by enzymatic amidation (Beltowski *et al*., 2004). Circulating AM is metabolised rapidly with an approximate half-life of 20 minutes. The lung is suggested to be the main site for peptide clearance of AM (Dupuis *et al*., 2005). More details about the cardiovascular function of AM will be discussed in section 1.6.2.

1.2.3 IMD and AMY

IMD (the AM2) is closely related to AM. There are two version of IMD. The first, a 53 amino acid version, is produced from a pre-prohormone (Yang *et al*., 2005). The second, a 47 amino acid version, is generated from processing the first one (Roh *et al*., 2004). However it is still difficult to confirm which version of these two is the major peptide. IMD is found in both peripheral tissues and the central nervous system (Bell and McDermott, 2008; Hong *et al*., 2012).

It is suggested that IMD interacts with all three receptor activity-modifying protein (RAMP)/ calcitonin receptor-like receptor (CL) complexes (details about the receptor complexes will be discussed in section 1.4) and displays a distinct profile from both AM

and CGRP but generally with less potency (Bell and McDermott, 2008; Jolly *et al*., 2009). Similar to the actions of AM and CGRP, IMD inhibits the progression of vascular hypertrophy and remodelling in terms of fibroblast and VSMC proliferation (Cai *et al*. 2010; Bell and McDermott, 2008). Moreover as a vasodilator, it is also thought to have cardiovascular protective benefits such as hypotensive actions (Fujisawa *et al*. 2007).

AMY, a 37-amino acid peptide, was first discovered in 1986 (Westermark *et al*., 1987). AMY shares some structural homology as well as biological activities with CGRP and AM, but it is suggested that its major physiological effect is to regulate glucose metabolism and energy balance (Trevaskis *et al*., 2010, Zhang *et al*., 2011, Roth 2013). For example, it was shown that mice lacking islet amylin exhibited increased insulin secretion and glucose tolerance (Gebre-Medhin *et al*., 1998).

1.3 Distribution of CGRP and AM

1.3.1 Distribution of CGRP

The distribution of CGRP is not closely linked to AM, as CGRP is widely expressed in both the central and peripheral nervous system and is classically known as a neuropeptide. In peripheral systems, it is primarily located within sensory neurons such as small unmyelinated C-fibres and myelinated Aδ-fibres (Brain and Grant, 2004). As mentioned before, a classic mechanism to release sensory CGRP is mediated by TRPV1 receptor activation on sensory C and Aδ fibers. CGRP is co-localized with other peptides in these fibres, such as tachykinins, especially substance P and neurokinin A (Brain and Grant 2004). In relation to the CGRP isoforms, αCGRP is primarily expressed in neurons of the central nervous and peripheral system (Schutz *et al*., 2004), while βCGRP was suggested to be primarily found in the gut, specifically, in the enteric nerves and pituitary gland (Petermann *et al*. 1987; Jonas *et al*. 1985). Recently both subtypes of CGRP were also reported in cultured human umbilical vein endothelial cells as determined by immunocytochemical analysis and mRNA expression (Luo *et al*., 2008).

CGRP is also widely expressed in the cardiovascular system and CGRP containing sensory neurons are embedded in most blood vessels. This CGRPergic network is

located at the junction of the adventia and the media and into the smooth muscle layer (Marshall 1992, Bell and McDermott 1996, Onuoha et al., 1998, Gulbenkian et al., 1993, Wimalawansa, 2001). Indeed a high density of CGRP immunoreactive fibres in the abdominal aorta and pial arterioles of the cat were identified (McCulloch *et al*., 1986) Such fibres have also been identified in the surrounding nervous and connective tissue of the myocardium and coronary vessels in the rat (Yoshizaki *et al*. 1987) and pig (Miyauchi *et al*. 1988). However, it is less densely invervated in the myocardium than the epicardium, endocardium or pericardium (Wimalawansa, 2001). In addition to the cardiovascular system, CGRP containing nerve fibres are also found in the gastrointestinal tract, lungs, thyroid gland (close to the C cells), human skin and splenic vein and sinusoids (Hagner *et al*., 2002a,b c). Moreover, non-neuronal sources of CGRP were also suggested recently to be present in human lymphocytes (Wang *et al*., 2002), keratinocytes (Hou *et al*., 2011) and endothelial progenitor cells (Fang *et al*., 2011).

1.3.2 Distribution of AM

Although AM is found circulating in humans (ranging from 1-10pM), levels are increased in some disease states as well as several physiological conditions (Brain and Grant, 2004). AM in plasma is specifically bound to factor H (Pio *et al*., 2001) making it difficult to determine the exact concentration in circulation. Circulating levels of AM were shown to be raised during pregnancy (Hata *et al*., 1997), in cardiovascular diseases such as hypertension (Hohno *et al*., 1996), and ischemic stoke (Hosomi *et al*., 2004). Such increased levels are suggested to be linked to the disease severity (Richards *et al*., 1996; Wang *et al*., 1995).

AM gene expression is widely expressed in most organs during both embryonic development as well as adulthood. Since AMs discovery it has been found in a range of tissues, such as the vasculature, heart, lung, kidney, adipose tissue, and macrophages. The expression of AM also can be stimulated by inflammatory cytokines such as TNFα and IL-1β (Brain and Grant, 2004). In addition, AM is also produced by fibroblasts, and cardiomyocytes (Sugo *et al*., 1994, Sugo *et al*., 1994a). It is primarily localised in the ventricles in the heart and aorta (Ichiki *et al*., 1994, Fukai *et al*., 2004, Yanagawa *et al*., 2007). Moreover, immunohistochemical studies demonstrated that the AM peptide is

present in all three layers of vessel wall, an observation confirmed by cell culture of endothelial cells, VSMCs and adventitial fibroblasts (Ishihara *et al*., 1997; Uemura *et al*., 2002; Jiang *et al.,* 2004). Immunoreactivity for AM was detected in SMCs of intima and media of human atherosclerotic lesions (Marutsuka *et al*., 2003). The AM expression in coronary artery plaques was increased in patients with unstable angina when compared to those with stable angina (Ishikawa *et al*., 2004). This finding was supported by VSMC culture studies showing that AM production and secretion were increased by factors like ATII, endothelin-1 and aldosterone (Uemura *et al*., 2002, Sugo *et al*., 1995) which are presumably pro-atherogenic.

1.4 The receptors

The active receptors for CGRP and AM are G-protein-coupled (Figure 1.3), composed of calcitonin-receptor like receptor (CL) and one of the three available RAMP isoforms (McLatchie *et al*., 1998). CL, consisting of 461 amino acids, was cloned in 1993 and belongs to the seven transmembrane domain G-protein-coupled receptor (GPCR) family (Njuki, *et al*., 1993). Depending on its co-expression with different RAMPs, CL together with RAMP1 forms a CGRP receptor, which can also be activated by AM and in some cases by IMD. In contrast, CL associates with RAMP2 to produce the functional AM1 receptor that is activated by AM and in some cases by IMD as well. CL links with RAMP3 to produce the AM2 receptor which also can be activated by CGRP and by IMD albeit at a lower potency than AM (Brain and Grant 2004; Bell and McDermott, 2008). The CGRP receptor can be antagonized by the CGRP peptide fragment $CGRP_{8–37}$ (Chiba *et al*., 1989) or by the non-peptide antagonist BIBN4096BS (Doods *et al*., 2000), while the AM1 receptor can be inhibted by $AM_{22–52,}$ a weak selective AM peptide receptor antagonist (Gardiner *et al*., 1999). Blocking activities induced by AM through the AM1 receptor can inhibit angiogenesis e.g. (reduce vessel number and impede tumour growth; Ishikawa *et al*., 2003). In terms of the binding of AM_{22-52} to the AM receptor, it was recently been revealed that Y52, G51 and I47 of AM_{22-52} are essential for the binding to the AM1 receptor (Watkins *et al*., 2013). Removing or substituting those amino acids will result in more than 100 times reduction in antagonist potency when compared to AM_{22-52} (Watkins *et al*., 2013).

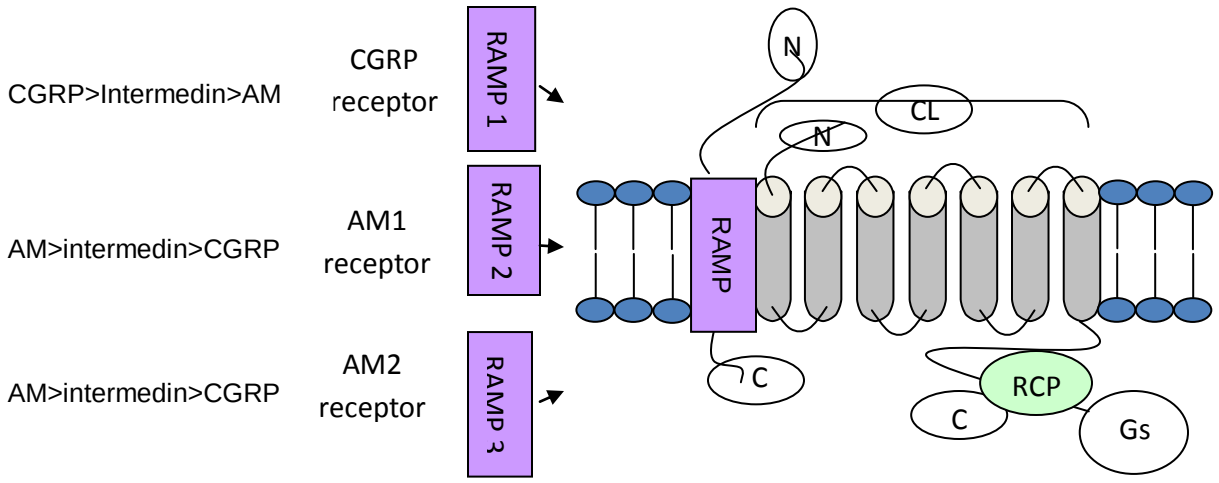


Figure 1.2: Model of CGRP/AM receptors. CL is a G-protein-coupled 7-transmembrane receptor. The RAMP is a single transmembrane domain protein. The interaction of RAMP1 with CL forms a CGRP receptor, RAMP2 with CL to produce an AM receptor, while RAMP3 with CL forms a CGRP/AM receptor.

CL belongs to the subfamily of GPCRs known as the 'B' family (Kolakowski, 1994) which also includes the calcitonin receptor. CL is composed of a long N-terminus extracellular domain, three extracellular loops, seven transmembrane domains, three intracellular loops as well as an intracellular C-terminus (as shown in figure 1.3). CL is poorly expressed on the surface of cells and it does not bind to any known endogenous ligand (Sexton *et al*., 2009). The N-terminus is believed to be essential for mediating the binding between CL and RAMPs to form a functional receptor (Barwell *et al*., 2011,). It was reported that the extreme N-terminus of CL (residues 23-60) is not only is responsible for the selectivity of the interaction with either AM or CGRP (Koller *et al*., 2002) but is also necessary for RAMP1 association as residue 41 in the N-terminus α-helix of CL was shown to be essential for CGRP binding (Barwell *et al*., 2010). Moreover, CL regions (from transmembrane domain 1 to 5) are responsible for transporting RAMPs to the plasma membrane. In addition, Kuwasako *et al*. also demonstrated that the human CL third extracellular loop is essential for AM signaling (Kuwasako *et al*., 2012).

Each of the three RAMP isoforms (RAMP1, RAMP2, and RAMP3) is about ~160 amino acids, long. They share some common structures including an extended extracellular N-terminus (approximately 100 amino acid residues), a single membrane spanning domain, and a short intercellular C-terminus (approximately 100 amino acid residues). However, they share no more than 30% sequence identity and their tissue distributions are different (Mclatchie *et al*., 1998; Sexton *et al*., 2001, Spielman and Parameswaran 2012). Studies have shown that the N-terminus of RAMP1 determines the expression of CGRP binding (Steiner *et al*., 2002). Similar conclusions have been drawn from studies on all RAMPs interactions with the CL, where the N-terminus of the RAMP is the chief determinant of receptor pharmacology (Mclatchie *et al*.,1998; Qi and Hay 2010). RAMP1 can be found in many tissues such as heart, uterus, brain, bladder, pancreas and skeletal muscle while RAMP2 is expressed widely in many organs, such as lung, heart, placenta, skeletal muscle, spleen, liver as well as pancreas. RAMP3 is also widely distributed in humans but expression is at low levels in the rat (Chakravarty *et al*., 2000; Nagae *et al*., 2000). The relatively highest expression in rats was found in lung, spleen, spinal cord (Chakravarty *et al*., 2000) and kidney (Nagae *et al*., 2000). RAMP expression has also been studied in a range of cardiovascular conditions, such as heart failure in rats where increased levels with RAMP2 were detected but not RAMP1 or 3 (Totsune *et al*., 2000). Similar observations were made during myocardial ischemia in rats (Qi *et al*., 2003) and spontaneously hypertensive stroke-prone rats (Wang *et al*., 2003). The expression and functions of RAMPs have been reviewed in details by others (Kuwasako *et al*., 2004; Udawela *et al*., 2004, Spielman and Parameswaran 2012). RAMP2 plays an essential role in blood vessel development as homozygous KO of RAMP2 but not RAMP1 or RAMP3 is lethal. It was suggested that the lethal effect was due to the lack of abiligty of AM to act on the AM1 receptor to maintain the blood vessel barrier (Kadmiel *et al*., 2012). This indicates the important function of RAMP2, which can not be compensated byother RAMPs. RAMP2 expression is not only observed in healthy conditions but also in some cardiovascular disease conditions. Nishikimi *et al*. have shown that increased gene expression of RAMP2 occurs in left ventricular hypertrophy and this is also observed even at a higher level in heart failure (Nishikimi *et al*., 2003).

CL co-expressed with RAMP2 produces the AM-selective AM1 receptor, which does not recognize CGRP and $CGRP_{(8-37)}$ to any great extent (Muff *et al*., 2003). In addition, Kuwasako *et al*. have suggested that on the N-terminus, the third intercellular loop and/or transmembrane 6 within CL are essential for selective cell surface relocation of RAMP2 (Kuwasako *et al*., 2009). As soon as AM binds to AM1 receptors, these receptors are soon internalized which leads to lysosomal degradation. Human RAMP2 is 26 amino acids longer than either RAMP1 or RAMP3, and human RAMP2 is unlike RAMP1 and 3 which possess 11 conserved amino acids in their N-terminus and transmembrane domains (McLatchie *et al*., 1998, Hay *et al*., 2006). Such distinct features of RAMP2 may account for its specific trafficking interaction with CL. Therefore, in the presence of RAMP2, the specificity of CL will change from RAMP1/CL to RAMP2/CL radically.

In addition to the CL and RAMP components, immunoprecipitation studies have identified the requirement of an intracellular peripheral membrane protein, termed receptor component protein (RCP) for facilitating signal transduction by CGRP and AM (Prado *et al*., 2001). RCP is an intracellular peripheral membrane protein that associates with CL (as Figure 1.3). Indeed, CL/RAMPs complex requires RCP for intracellular signaling cascade. Evans *et al*. (2000) demonstrated that knockdown of RCP expression will decrease CGRP-mediated cAMP production without affecting binding to the CGRP receptor (Evans *et al*., 2000). More recently the role of RCP in regulating the function of CL/RAMP1 complex has been investigated further. It has been demonstrated that RCP enables signal transduction of CL by interacting with the second intracellular cytoplasmic loops of CL (Egea and Dickerson 2012).

1.5 Cellular signaling of CGRP and AM

1.5.1 CGRP signaling

CGRP is a very potent vasodilator, approximately 3-30 times more potent than AM. Several mechanisms have been suggested by which CGRP produces vascular relaxation (Brain and Grant 2004). The first one is an endothelium-independent pathway: via activating CGRP receptors on the smooth muscle cells. This leads to increased cAMP and PKA activities, which causes vascular relaxation. The second

pathway is an endothelium dependent pathway, by which CGRP interacts with receptors on the endothelial cells to stimulate the cAMP and PKA pathway. It leads to the production of nitric oxide (NO) by stimulation of endothelial NO synthase (Brain and Grant 2004). Furthermore, apart from the vasodilator effect, CGRP was also reported to inhibit rat ATII stimulated VSMC proliferation in an *in vitro* study by inhibiting ERK1/2 activity (Qin *et al*., 2004).

1.5.2 AM signaling

As mentioned before, the actions of AM are mediated by the AM1 and AM2 receptors. Since the multifunctional AM was discovered, the molecular mechanisms of its action have been studied (Fig 1.4). AM dilates blood vessels via an endothelial-dependent and independent mechanism. Ishizaka *et al*., (1994) have shown that AM regulates vasodilatation by triggering cAMP accumulation and activating cyclic AMP-dependent protein kinase (protein kinase A, PKA) in smooth muscle cells (Ishizaka *et al*., 1994). In addition, Shimekake *et al*. suggested that the hypotensive effect of AM can be explained by two pathways: it increases the intracellular cAMP by acting on smooth muscle cells directly which leads to relaxation; and it increases intracellular Ca^{2+} level in bovine aortic endothelial cells, which leads to activation of NO synthase and NO release (Shimekake *et al*., 1995). Indeed, AM is more potent than CGRP at increasing the cAMP levels in rat vascular smooth muscle cells (Eguchi *et al*., 1994). The increased cAMP results in decreasing concentrations of Ca^{2+} and, in turn, induces SMC vasodilatation. Moreover, Nishimatsu *et al*. studied the NO-dependent relaxation in rat aorta and demonstrated that AM induces endothelial cell-dependent vasodilatation by activating Akt via a Ca^{2+}/calmodulin-dependent pathway, an established stimulant of eNOS (Nishimatsu *et al*., 2001). Furthermore, $AM^{+/-}$ mice demonstrated ischemia-induced renal damage to a greater extent than wildtype mice, whereas AM-overexpressing mice showed lesser damage after ischemia (Nishimatsu *et al*., 2002). This effect was again dependent on the level of NO production. Thus, these studies suggest that AM is likely to regulate vascular tone and blood pressure and to protect renal tissues from ischemia/reperfusion injury through its NO-releasing activity.

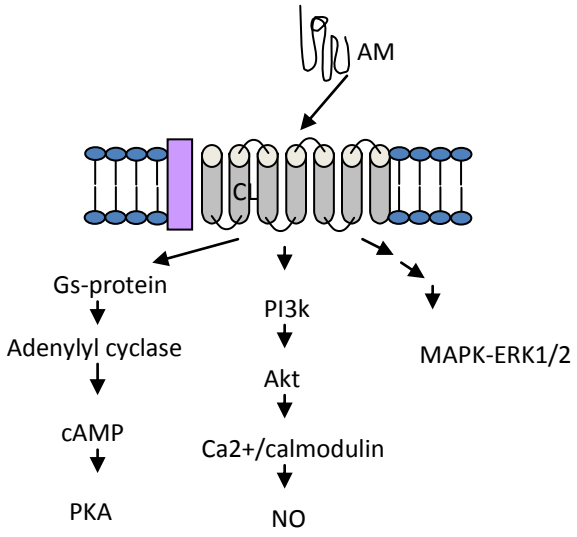


Fig 1.3: Possible intracellular signal transduction mechanism for AM (three main pathways: cAMP pathway, PI3k pathway and MAPK-ERK pathway)

Kim *et al*. have shown that AM carries out the protective and anti-apoptotic functions via the PI3K/Akt pathway which is a well known protective signaling mechanism (Kim *et al*., 2002). In a model of myocardial infarction, adenovirus-mediated gene delivery of AM has protected the myocardium against apoptosis, whereas this effect was abolished in mice containing dominant negative Akt (Yin *et al*., 2004). The mitogen-activated protein kinase/extracellular signal regulated kinases (MAPK-ERK) is a well-characterised stress-induced protective signaling pathway. The angiogenic effect of AM is mediated by activating Akt together with MAPK/ERK 1/2 in endothelial cells (Kim *et al*., 2003). Moreover, in a cardiac hypertrophic model using $AM^{+/-}$ mice subjected to aortic constriction (causing pressure overload) or ATII infusion, it was demonstrated that endogenous AM plays an essential role in protecting the heart from cardiac hypertrophy via suppressing ERK activation by inhibition of protein kinase C (PKC) and activation of protein kinase A (PKA, Niu *et al*., 2004).

1.6 Cardiovascular function of CGRP and AM

1.6.1 CGRP

CGRP shares some vascular activities with AM. The most famous effect of CGRP is being a vasodilator. It has been suggested that CGRP selectively dilates different vascular beds, which include the cerebral, coronary and renal vasculature (Brain and Grant, 2004). However, following studies using CGRP KO mice and antagonists

demonstrate that CGRP does not play an important role in the physiological control of systemic blood pressure in normal individuals. Injection of the selective CGRP receptor antagonists BIBN4096BS or $CGRP_{8-37}$ does not affect the resting heart rate or blood pressure in a range of species, including rats (Arulmani *et al*. 2004; Zeller *et al*. 2008) and humans (Olesen *et al*. 2004). The expression of CGRP together with its receptors are decreased, elevated or unchanged in different experimental models of hypertension (Deng and Li, 2005). In the past, using CGRP KO mice or a hypertensive rat model, several research groups have demonstrated a protective role of CGRP in hypertension (Gangula *et al*., 2000, Wimalawansa 1996, Wang *et al*., 1998). The protective effects of CGRP are believed to be related to its ability to cause vasodilaton and to increase blood flow.

More recently, Li *et al*., by comparing two different hypertensive models, demonstrated that both plasma levels and mRNA levels of CGRP were decreased in spontaneously hypertensive rats (SHR) but increased in a model of 2 kidneys 1 clip (2K1C) hypertensive rats (Li *et al*., 2009). These results suggested that CGRP could play different roles in different circumstances. On the other hand, within our group, by using CGRP KO mice, we demonstrated that CGRP plays a protective role in the hypertension, vascular hypertrophy, vascular inflammation and remodeling induced by ATII (infusion of dose of 1.1mg/kg/day for 14 days, Smillie, 2012).

As discussed before, one of the pathways by which CGRP is released is by activation of neuronal TRPV1 ion channels. Therefore, the activation of TRPV1 and subsequent release of CGRP has been investigated. Deng *et al*. have shown that CGRP synthesis and release following activation of TRPV1, played a protective role in hypertensive models of phenol-induced hypertension (Deng *et al*., 2004) and 2K1C hypertensive rats (Deng *et al*., 2003). More details about the cardiovascular function of TRPV1 will be discussed in a later section.

1.6.2 AM

AM is a known vasodilator. Animal experiments have been carried out in a range of species, such as rat, mouse, cat, sheep and rabbit (Khan *et al*., 1997; Cheng *et al*., 1994; Hjelmqvist *et al*., 1997; Westphal *et al*., 2002), to show that AM can induce a

sustained, dose-dependant vasorelaxation and hypotension. It is also suggested that a bolus dose of AM acts in a functionally different manner to endogenous AM due to the fact that AM normally exists bound to factor-H (Pio *et al*., 2001, Gibbons *et al*., 2007). Therefore it is important to understand more about how endogenous AM contributes to maintain the vascular tone and blood pressure.

As discussed before, expression of AM is increased in patients who have acute and chronic cardiovascular diseases (Ishimitsu *et al*., 1994, Tsuruda *et al*., 2003, Kato *et al*., 1996, Oyar *et al*., 2011 and Suzuki *et al*., 2004). The link between hypertension and the plasma level of AM was shown to be positively related with renal failure and impaired renal function (Ishimitu *et al*., 1994; Kohno *et al*., 1996). In addition, elevated AM levels are also shown to be linked with cardiac and arterial hypertrophy (Morimoto *et al*., 1999). AM induced a substantial decrease in blood pressure in a spontaneous hypertensive and Dahl salt-sensitive deoxycorticosterone acetate-salt (DOCA) rat model of hypertension (Chao *et al*., 1997; Zhang *et al*., 2000). All this evidence suggests that there is a relationship between increased blood pressure and AM release. Such increases are believed to be associated with multiple host protective functions and to be a predictor of future cardiovascular events (Nishida *et al*., 2008). However it has also been shown that circulating levels of AM stay high even in patients treated with effective doses of calcium channel blocker-based anti-hypertensive therapy (Kohno *et al*., 1996). This indicates that the role and regulation of AM in hypertension is complex. Thus, more studies need to be carried out to address the role and benefit of AM in the treatment of hypertension.

As mentioned before, null mutation of the AM gene leads to embryonic lethal mice; therefore several research groups have focused on the $AM^{+/-}$ mice or AM-overexpressing mice as models to study the AM gene. AM performs its cardioprotective effects directly, for example by reducing the ventricular remodeling after myocardial infarction by acting as an endogenous inhibitor of myocyte hypertrophy and fibroblast proliferation (Tsuruda *et al*., 1998, 1999). This finding has been supported by Niu *et al*. who had demonstrated that after aortic constriction or infusion of ATII in $AM^{+/-}$ mice, these mice showed increases in cardiomyocyte size and fibroblast proliferation compared to wild type controls (Niu *et al*., 2004). Moreover AM-overexpression in mice

caused a reduction in blood pressure (Shindo *et al*., 2000). In addition, Nishikimi *et al*. also carried out studies using cultured rat neonatal cardiac fibroblasts and demonstrated that AM played a protective role against fibrosis (Nishikimi *et al*., 2005).Thus, these results suggest that endogenous AM has an essential role in cardiovascular protection. However, little is known about the involvement of its receptors regarding the protection of AM in other cardiovascular conditions, such as hypertension.

In recent years, smooth muscle cell-specific RAMP2 overexpressing mice were generated using a transgene containing an α-actin promoter (details about the transgenic mice are available in the materials and methods section). Using these RAMP2 transgenic mice, our group had previously demonstrated that these mice exhibit no difference in basal or ATII-induced blood pressure despite a greater degree of hypotension is observed following infusion of AM peptide (Tam *et al*., 2006). This study implies that the AM1 receptor component RAMP2 may be involved in mediating blood pressure control under certain conditions. On the other hand, in an *in vitro* study, Kim *et al*. (2003) demonstrated that AM inhibits vascular endothelial growth factor (VEGF) induced by inflammatory cytokines. Furthermore, AM can induce expression of some cell surface adhesion molecules (Hagi-Pavli *et al*., 2004), such as vascular cell adhesion molecule 1 (VCAM-1), inter-cellular adhesion molecule (ICAM-1) and E-selection. Therefore it seems that AM has both inhibitory and pro-inflammatory effects depending on different cell types and disease conditions.

1.7 The Transient Receptor Potential Vanilloid receptor

1.7.1 Discovery of TRPV1

Chilli peppers are eaten on a daily basis all over the world and are one of the oldest cultivated crops in South America. They were first introduced to Europe in the sixteenth century (Perry *et al*., 2007). Capsaicin, (8-Methyl-N-vanillyl-trans-6-nonenamide, molecular formula: $(CH_3)_2CHCH=CH(CH_2)_4CONHCH_2C_6H_3$-4-(OH)-3-($OCH_3$), Fig 1.4), the active ingredient of chilli pepper, was first extracted by Christian Friedrich Bucholz and its almost pure form was first isolated and named by Thresh in 1846. Despite its early discovery, the exact chemical structure of capsaicin was determined by Nelson in

1919; followed by the chemical synthesis 10 years later (Szallasi and Blumberg, 1999). Pure capsaicin is a colourless, odourless and a crystalline to waxy compound. Capsaicin causes a sensation of burning pain when it comes into contact with tissues. Additionally capsaicin can selectively activate sensory nerves, which will cause the release of neurotransmitters such as CGRP, substance P and neurokinin (NKA) (Rubino and Burnstock 1996, Brain 1997, Nagy *et al*., 2004) and these neurotransmitters are linked with cardiac performance and vascular tone. Despite the long history of capsaicin being used in traditional medicine, the capsaicin receptor was not cloned until 1997 from a rat and characterized in the laboratory of David Julius (Caterina and Julius, 1997). Capsaicin evokes these effects by selectively activating its receptor, now known to be transient receptor potential vanilloid 1 (TRPV1). TRPV1 is primarily localised to sensory neurons that transfers information into the central nervous system thus playing a role in the pain sensation (Caterina and Julius; 2001).

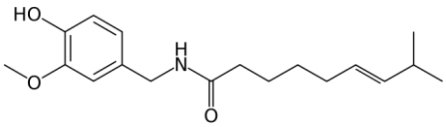


Figure 1.4: The structure for capsaicin

1.7.2 Structure of TRPV1

The transient receptor potential (TRP) channels are a group of non-selective cation channels. The TRP family includes six subfamilies which are different from each other with respect to the amino acid sequences in their C and N termini. The subfamilies are TRPV (1 to 6), transient receptor potential melastatin (TRPM, 1 to 8 members), transient receptor potential canonical (TRPC, 1 to 7 members), transient receptor potential muclolipin (TRPML, 1 to 3 members), transient receptor potential polycycstin (TRPP, 1 to 3 members) and transient receptor potential ankyrin 1 (Nakagawa and Hiura, 2006). In a similar manner to other family members, the TRPV1 receptor is a non-selective cation channel; composed of six complete transmembranous domains, a short, pore region between the fifth and sixth domains and cytoplasmic C and N-Termini (Fig 1.5). TRPV1 is featured with three ankyrin repeat domains at its N-terminus, which is important for the TRPV1 channel to interact with cytosolic proteins, such as calmodulin (Tominiaga and Tominaga, 2005; Baylie and Brayden, 2011). TRPV1 also has a TRP-like domain (like others in the TRP protein family) in its C-

terminus (Ferrer-Montiel, *et al*. 2004; Tominiaga and Tominaga, 2005). The TRPV1 channel is suggested to form both homotetramers (just containing TRPV1 only) and heterotetramers (with other TRPV family, Comway, 2008; Baylie and Brayden, 2011).

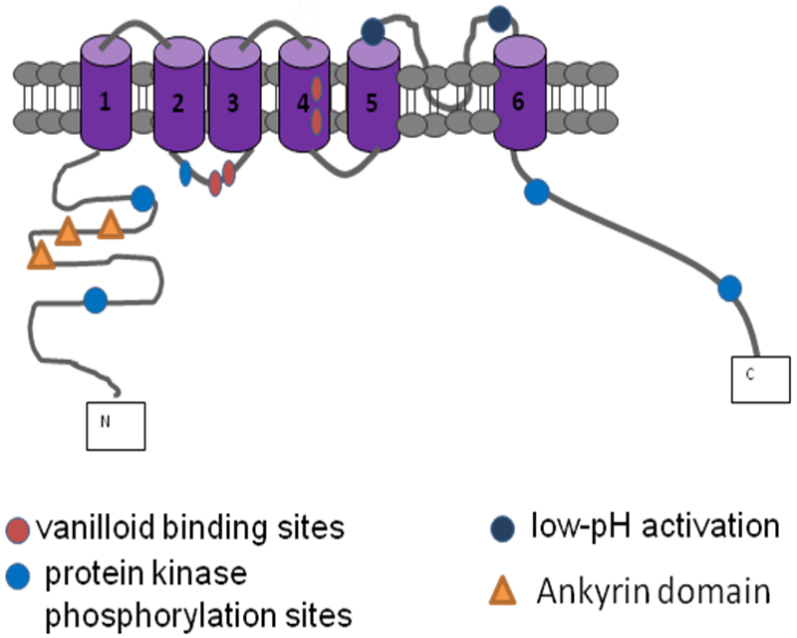


Fig 1.5: Structure of the TRPV1 receptor and different binding sites for a range of stimuli (Ferrer-Montiel, et la., 2004; Tominiaga and Tominaga, 2005).

1.7.3 TRPV1 Expression

TRPV1 is expressed in a range of tissues, predominantly in small to medium size of primary sensory neurons such as myelinated Aδ and unmyelinated C fibres, their dorsal root ganglion (DRG), as well as the trigeminal ganglia (Caterina *et al*., 1997). Activating TRPV1 channels in these neurons results in cations influx (including calcium) into the peripheral terminus (Caterina *et al*., 1997). Studies on WT and TRPV1 KO mice indicated that TRPV1 is also highly expressed in the central nervous system (Mezey *et al*., 2000, Roberts *et al*., 2004). Indeed, TRPV1 has been proposed as a target for treatment not only of pain but also for other conditions such as anxiety (Starowicz *et al*., 2008). However, neonatal capsaicin treatment did not eliminate TRPV1 mRNA in the central nervous system and the mechanism is still poorly understood. Even so, certain studies indicated that some of the TRPV1 expression in the brain must be form the

functional capsaicin receptor (Mezey *et al*., 2000). Many studies had shown significant levels of TRPV1 in the cortex, specific areas of the limbic system (hippocampus, amygdale) as well as thalamus (Holzer 2008) by using different methods. Despite these findings, the existence of TRPV1 in the brain is still controversial as recent study has demonstrated limited expression of TRPV1 in the brain using TRPV1 reporter mice (a line of mice containing certain genes which are under control of the TRPV1 promoter but can be expressed without interrupting TRPV1 coding region (Cavanaugh *et al*., 2011). In addition, TRPV1-expressing fibres innervated blood vessels are found in all layers of viscera (e.g. rat urinary tract and gastrointestinal tract) as TRPV1 immunopositive fibres were shown in the mucosa membrane as well as muscular layer (Avelino *et al*., 2002, Ward *et al*., 2003).

Recent evidence has shown that TRPV1 is expressed in a range of non-neuronal tissue. TRPV1 protein was shown to be present in cultured human keratinocytes by immunohistochemistry. Its functionality was shown by capsaicin activation leading to Ca^{2+} influx in a subpopulation of these cells (Inous *et al*., 2002). TRPV1 is also expressed in vascular tissue, such as endothelium, and smooth muscle (Golech *et al*., 2004; Kark, *et al*., 2008). TRPV1 has been found *in vitro* in cerebrovascular endothelial cells by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (Golech *et al*., 2004) and denervated rat mesenteric arteries by RT-PCR (Poblete *et al*., 2005). TRPV1 may also be expressed in vascular smooth muscle as mRNA for TRPV1 was found in rat pulmonary artery and aorta using RT-PCR, followed by western blot to confirm presence of protein (Yang *et al*., 2006). Furthermore, Kark *et al* (2008) and colleagues revealed expression of TRPV1 in rat skeletal smooth muscle arterioles by immunohistochemistry (Kark, *et al*., 2008). In addition, functional TRPV1 has been found in other non-neuronal cells, such as human bladder urothelium (Lazzeri, M *et al*., 2004), rat pancreatic beta cells (Akiba *et al*., 2004), marcophages (Chen *et al*., 2003). However, the physiological roles in these cells remains to be fully understood (Fernandes *et al*., 2012).

1.7.4 Exogenous agonists of TRPV1

TRPV1 receptors can be activated by certain exogenous stimuli, including chemical agonists/activators, such as acid (proton, pH<6 at room temperature; Jordt *et al*., 2000)

and noxious heat (>43ºC; Caterina *et al*., 1997). TRPV1 is gated open only when the extracellular pH is reduced to less than 6. In contrast, the range of pH 6-7 will increase the sensitization of TRPV1 to other stimuli like capsaicin and heat (Tominage *et al*., 1998, Jordt *et al*., 2000). As a result, the temperature threshold for TRPV1 activation is lower in acidic environments, although the exact mechanism is not clear. However, it is known that activation of TRPV1 by its stimuli leads to a rapid rise in intracellular calcium level (Tominage *et al*., 1998).

Capsaicin, the pungent vanilloid component of chilli peppers, is suggested to be responsible for the burning sensation of the spicy food (Caterina *et al*., 2000). Capsaicin is historically an agonist for TRPV1. At negative holding potentials, the activation of TRPV1 by capsaicin leads to the increase of the influx of cations (both calcium and sodium), and then leads to depolarization of the cell. Capsaicin and its related compounds are collectively named 'vanilloids'. TRPV1 can also be activated by these vanilloids, such as olvanil (the first synthetic capsaicin analogue) and the potent capsaicin analogue resiniferatoxin (RTX; Piper *et al*., 1999).

1.7.5 Endogenous agonists of TRPV1

Under physiological conditions, TRPV1 is unlikely to be triggered by heat or acid. Therefore, the existence of endogenous ligands which act on this receptor or via intracellular signaling has been suggested. These endogenous ligands and activators of TRPV1 are defined as endovanilloids (Di Marzo *et al*., 2001; van der Stelt and Di Marzo, 2004). There are three classes of lipids that have been proposed to act as endovanilloids. Firstly, the fatty acid amide arachidonoyl-ethanolamine, also known as anandamide and known as a cannabinoid receptor 1 (a G-protein-coupled receptor co-expressing with TRPV1 receptor on sensory nerves) agonist, was the first identified endogenous activator of TRPV1 due to its chemical similarity to capsaicin (Smart *et al*., 2000, Ralevic *et al*., 2002). Anandamide is biosynthesized by the phospholipase D mediated hydrolysis of arachidonoyl phosphatidyl-ethanolamine (NAPE, Wang *et al*.,2008a) and was shown to be able to activate human and rat TRPV1 (Bianchi *et al*., 2006, Panlilio *et al*., 2009). Secondly, arachidonic acid is metabolized via the lipoxygenase pathway, producing a range of products including (S)-12-(hydroperoxy)eicosatetraenoic acid (12-HPETE), (S)-15-(hydroperoxy)eicosatetraenoic

acid (15-HPETE) and leukotriene B_4 (LTB_4). 12-HPETE and LTB_4 have both been shown to activate TRPV1 directly. The third class is the long-chain unsaturated fatty acid amides, such as N-arachidonoyl dopamine (NADA) and N-oleoyldopamine (OLDA, Huang *et al*., 2002, Chu *et al*., 2003). NADA is similar to capsaicin in terms of potency as determined by different assays of TRPV1 activity (Huang *et al*., 2002). OLDA is a weak ligand for cannabinoid receptor 1, where it was suggested to be an endogenous agonists as it is similar to capsaicin in its chemical structure and activates TRPV1 (Chu *et al*., 2003).

There are other products that have also been reported to activate TRPV1, such as bradykinin and nerve growth factor (NGF). Bradykinin and NGF are two potent endogenous factors produced during inflammation. The nonapeptide bradykinin has been shown to directly activate TRPV1 as well as sensitize the TRPV1 receptor (Burgess *et al*., 1989). NGF also has been demonstrated to sensitise TRPV1 to capsaicin (Chuang *et al*, 2001). Additionally, TRPV1 can be activated or sensitised by other inflammatory mediators such as prostaglandins (Huang *et al*., 2002).

1.7.6 Activation/sensitization of the TRPV1 receptor

As mentioned before, TRPV1 receptors can be activated directly by vanilloids, such as capsaicin and its analogues. In the rodent form of TRPV1, it is suggested that the two key residues, Arg-114 and Glu-761, in the intracellular N- and C- tails of TRPV1 provide important structural elements by forming a binding pocket to convey sensitivity to capsaicin and its analogues (Jung *et al*., 2002). In addition, Tyr-511 and Ser-512 between the 2^{nd} intracellular loop and 3^{rd} transmembrane domain, and Tyr 550 at the fifth transmembrane domain have also been suggested to be the important sites where vanilloid ligands may interact with TRPV1 (Jordt and Julius 2002, Tominaga and Tominaga, 2005). RTX, the capsaicin analogue, was suggested to be much more (about thousand fold more) potent than capsaicin (Szolcsanyi *et al*., 1990). In addition, the presence of residue Met 547 at the 3^{rd} transmembrane domain is required for the bringing of RTX to TRPV1 (Gavva *et al*., 2004).

TRPV1 is thought to mediate the phenomenon of peripheral sensitization with a reduction in the threshold of activation. Acid (extracellular protons) can increase the

potency of heat or capsaicin by lowering the threshold for TRPV1 activation, as well as acting as direct agonists themselves (Tominaga *et al*., 1998). *In vivo* studies revealed that Glu 600 located within the putative extracellular domain is an important site for proton potentiation of TRPV1. In addition, Glu 648 is implicated in the direct activation of TRPV1 by protons (Jordt *et al*., 2000).

As mentioned before, agents produced during inflammatory events can act to lower the activation threshold of TRPV1 to its chemical and physical stimuli. These agents include bradykinin, prostaglandins as well as others (reviewed by Szallasi *et al*., 2007). Bradykinin can stimulate protein kinase C (PKC) by promoting diacylglycerol formation as well as degrading phosphatidylinositol(4,5)-bisphosphate (PIP_2) by facilitating phospholipase C activity (Chuang*et al*., 2001). Phosphorylation of TRPV1 by PKC is also mediated by prostaglandins. Indeed, PKC can phosphorylate the TRPV1 receptor directly (Numazaki *et al*., 2002) and then increase the probability of channel gating by heat, protons, as well as agonists such endovanilloids ((Premkumar and Ahern, 2000; Vellani *et al*., 2001; Di Marzo *et al*., 2002). Both Ser 502 and Ser 800 have been identified as responsible for the above actions (Numazaki *et al*., 2002). Moreover, prostaglandins, such as prostaglandin E_2, enhance the sensitivity of primary sensory neurons to noxious stimuli, which is believed to involve the cAMP transduction cascade pathway (Szallasi and Blumberg, 1999). Lipoxygenase products, such as 12-HPETE and LTB_4, are by-products of inflammation and are also able to sensitise TRPV1 via phospholipase C or PKC production (Suh and Oh 2005). The binding site for lipoxygenase products is suggested to be intracellular; therefore these products must be produced within the TRPV1 expressing cell or be transported into it (Van Der Stelt M and Di Marzo V., 2004). They have been identified as primary endogenous activators of TRPV1 due to their structural similarity to capsaicin (Jung *et al*., 2002).

1.7.7 Desensitisation of the TRPV1 receptor

Capsaicin, an irritable compound, initiates neuronal excitation which is followed by a refractory rest state that occurs where the previously excited neurons become desensitised to a series of seemingly unrelated stimuli (Szallasi and Blumberg, 1999). This phenomenon is traditionally referred to as desensitisation (Szallasi *et al*., 2007). There are two states of desensitisation. The first phase is an acute phase where

repeated or prolonged capsaicin treatment can result in a decreased response to further capsaicin challenge. As discussed before, activation of the TRPV1 receptor by capsaicin results in calcium influx (Caterina *et al*., 1997). However the prolonged calcium influx may cause acute desensitisation by causing calcium cytotoxicity, producing the acute phase of desensitisation. This desensitisation has been shown to depend on a variety of factors, such as the concentration of capsaicin, duration of application, and the presence or absence of the extracellular calcium. Studies demonstrated that removal of extracellular calcium diminished the desensitisation to capsaicin (reviewed by Szallasi and Blumberg, 1999). However the desensitisation of TRPV1 is complicated because it is also dependent on the agonist used. In contrast to observations with capsaicin, desensitisation to olvanil (another TRPV1 agonist) is apparently not influenced by the removal of extracellular calcium (Liu and Simon, 1998).

The second phase is a chronic phase where capsaicin application can reduce or eliminate responsiveness to other stimuli that range from noxious heat to mechanical pressure to endogenous or exogenous agents, often referred to as functional desensitisation (Bevan & Docherty 1993). High doses of capsaicin selectively destroy C- and Aδ sensory nerves, which will completely prevent nerve activation (Szallasi *et al*., 1995). The capsaicin desensitisation model has been widely used to study the effects of TRPV1, including studies focusing on the study of obesity and hypertension in mice (Gram *et al*. 2007).

1.7.8 Cardiovascular function of TRPV1

As discussed earlier, in the cardiovascular system TRPV1 is expressed in two areas; innervating perivascular nerves and non-neuronal tissues (such as vascular endothelial cells and smooth muscle cells). Wang *et al* (2006) using TRPV1KO mice, demonstrated that electrical field stimulation induced significantly more vasodilation in arteries from WT mice when compared with those from the TRPV1KO mice. The vasodilation was abolished by blocking CGRP release from the sensory nerves but not substance P; such an effect was not seen in the TRPV1KO mice (Wang *et al*. 2006). Thus activation of TRPV1 on peri-vascular sensory nerves leads to release of the potent vasodilatator, CGRP, and results in vasodilation in mesenteric artery. However, it is also suggested that TRPV1 in mesenteric peri-vascular sensory nerves contributes to myogenic

constriction, mediated by releasing substance P after activation of TRPV1 (Scotland *et al*., 2004). They proposed that increased intravascular pressure leads to the release of 20-HETE from VSMC to activate the sensory nerve TRPV1 and then increase neuronal Ca^{2+} entry and cause the release of substance P, which then causes contraction of the smooth muscle.

The function of TRPV1 in endothelial cells and smooth muscle cells is difficult to distinguish due to the presence of peri-vascular nerves. In a chronically denervated mesenteric vascular bed, TRPV1 activation by capsacin leads to endothelium-dependent NO release resulting in vasodilation but such a vasorelaxing effect was not seen with anandamide (Poblete *et al*., 2005). The effect of releasing NO was abolished by applying the TRPV1 receptor antagonist, capsazepine, as well as endothelium removal or NO synthase inhibition (Poblete *et al*., 2005). This confirms the involvement of TRPV1 and endothelium-dependent NO release. Moreover, Kark *et al*. demonstrated the diverse vascular effects of TRPV1 in the arterial bed of the rat hind limb following TRPV1 activation (Kark *et al*., 2008). Activation of neuronal TRPV1 caused vasodilation and was proposed to be due to release of neurotransmitters (such as CGRP and substance P) or endothelial NO synthesis. In contrast, capsaicin also directly stimulated vascular smooth muscle TRPV1 in the isolated skeletal muscle arterioles and this led to vasoconstriction. This effect was not affected by endothelium removal (Kark *et al*., 2008). Moreover, the presence of TRPV1 in the vasculature of skeletal muscles was demonstrated by immunohistochemistry and TRPV1 mRNA was also detected in both aorta and cultured VSMCs (Kark *et al*., 2008). Furthermore, Czikara *et al*. also demonstrated the presence of TRPV1 in isolated arteries and activation by capsaicin caused vasoconstriction (Czikora *et al*., 2012). This effect was absent in the TRPV1 KO mice. All these results suggest that the role of TRPV1 in the cardiovascular system is complex; therefore more research will be of benefit to understand the role of TRPV1 in regulating cardiovascular events.

1.8 Cardiovascular Disease

Cardiovascular diseases (CVDs) include diseases of the heart, blood vessels and vascular diseases of the brain, and are the leading causes of death and disability in developed as well as developing countries. In the 2011 report, the World Health

Organization (WHO) revealed that in 2008 CVDs were responsible for over 17.3 million deaths (31% of the total deaths) (Mendis *et al*., 2011). CVDs include high blood pressure (hypertension, diseases of aorta and arteries), coronary artery disease (e.g. heart attack), stroke, congenital heart disease, myocardial infarction and others. Out of the 17.3 million global deaths in 2008, myocardial infarctions were responsible for 7.3 million and strokes were responsible for 6.2 million deaths. In the developed country of UK the CVD mortality rate in males is about 120-238 (age standardized, per 100,000) while it is 76-180 (age standardized, per 100,000) in females (Mendis *et al*., 2011). In the developing country of China the CVDs mortality rate in male is 239-362 (age standardized, per 100,000) while it is 181-281 (age standardized, per 100,000) in females (Mendis *et al*., 2011). Therefore, CVDs remain a huge challenge and burden to both developed and developing countries.

There are many factors contributing to the development of CVDs. These include behavioural risk factors (such as tobacco smoking, physical inactivity and unhealthy diets) and other major risk factors (such as cancer, diabetes and chronic respiratory disease). Long-term exposure to behavioural risk factors results in raised blood sugar (diabetes), raised blood pressure (hypertension) and obesity, which contribute to the major risk factors for the CVDs. Among those, hypertension is the most common factor which is linked with other CVDs. More details about hypertension and its link with obesity will be discussed in the later sections.

1.8.1 Hypertension

Blood pressure (BP) varies between a minimum (diastolic pressure) and a maximum (systolic pressure) during each heartbeat. Systolic BP is the highest pressure in the arteries, achieved when the ventricles are contracting. Diastolic BP occurs when the ventricles are filled with blood. BP is summarized by systolic and diastolic BP, then it is classified into degrees as shown in table 1.1 (Chobanian *et al*., 2003; Aram *et al*., 2003).

BP classification	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)
Normal	< 120	< 80
Prehypertension	120-139	Or/and 80-89
Stage 1 hypertension (mild hypertension)	140-159	Or/and 90-99
Stage 2 hypertension (moderate/severe hypertension)	≥160	Or/and ≥100
Isolated systolic hypertension	≥ 140	< 90

Table 1.1: Classification of blood pressure in adults.

Classification of hypertension can depend on ethnic origin, gender, age, geographic patterns and socioeconomic status (Oscar and Suzanne, 2000). As mentioned previously, hypertension is a common pathological condition and is the most essential risk factor for the increased cardiovascular morbidity and mortality. The British Heart Foundation reported in 2006 that 31% men and 28% women suffered from hypertension in England. Moreover, the newest data shows that globally the prevalence of hypertension in adults aged 25 and over was around 40% in 2008 (Mendis *et al*., 2011). Indeed, hypertension is estimated to cause 7.5 million deaths, which is about 12.8% of the total of all the annual deaths (WHO, 2010). It is suggested by the WHO that hypertensive patients should lower salt consumption, lower the fat content in their diet and increase physical activity in order to control and reduce their BP.

Hypertension (persistently higher BP over 140/90 mmHg) is one of the main risk factors for many cardiovascular diseases, such as myocardial infarction, heart failure, and stroke (Chobanian *et al*. 2003). In addition, hypertension can also be a consequence of diseases such as obesity and type II diabetes mellitus (Chobanian *et al*. 2003). Indeed, obesity is a growing health problem for both developed and developing countries. It is believed that patients being overweight contribute to about 60% - 70% of hypertensive diseases (World Health Organisation 2003; Wofford & Hall 2004). Although progress

has been made into understanding the pathogenesis of hypertension leading to several different therapeutic approaches, the number of well controlled or treated patients remains low in the world (Franco *et al*., 2004). Therefore, further research into understand the pathophysiological dysfunction and the relation between obesity and hypertension in this area is necessary and essential in order to develop new therapies.

1.8.2 The classification of hypertension

Depending on the causes, hypertension can be classed as either essential (primary) or secondary hypertension. Secondary hypertension, which is much less common than essential hypertension, is caused as a result of another medical condition, such as endocrine disease, kidney disease, heart disease or tumors. Secondary hypertension affects approximately 5% of hypertensive patients and could also be a side effect of a medical treatment. Therefore, it is important to recognize this kind of hypertension because the underlying cause has to be treated. Most of the mechanisms linked with secondary hypertension are well understood. There are common and well recognized secondary causes, such as Cushing's syndrome, renal segmental hypoplasia, pregnancy or sleep disturbances (Magiakou *et al*., 2006; Babin *et al*., 2005; Podymow and August, 2007; Kario *et al*., 2009).

By contrast, essential hypertension often has no identifiable cause and accounts for 90-95% of all hypertensive patients. Even though it has been indicated that there is no direct cause of essential hypertension, a number of known factors lead to the increase of BP, including genetics, increased sympathetic nervous activity, increased renin secretion resulting in increased production of ATII, endothelial dysfunction and deficiencies of vasodilators such as nitric oxide and natriuretic peptides. Moreover, different lifestyles such as high alcohol intake, high salt intake, sedentary lifestyle, stress, low potassium intake and low calcium intake (Oscar and Suzanne., 2000) are other causes contributing to essential hypertension.

The pathophysiology for developing essential hypertension has been researched extensively. One major risk factor is suggested to be sodium sensitivity as nearly one third of essential hypertension is responsive to sodium intake (Katori and Majima, 2006). When sodium intake exceeds the ability of excretion by the body, it results in an

increase in arterial pressure as the cardiac output increases. Indeed a modest reduction in salt intake has a significant and important effect on BP in patients with hypertension (He & MacGregor 2009). The lower the salt intake, the lower the blood pressure, suggesting a correlation between reduction of salt intake and the reduction of blood pressure, studied from the daily intake range of 3–12 grams/day (Brown *et al*., 2009, He and MacGregor, 2009).

Another major pathophysiologic mechanism includes the renin-angiotensin system (RAS - also termed renin-angiotensin-aldosterone system, RAAS). Inappropriately activated systemic or local tissue RAS contributes to the haemodynamic and metabolic abnormalities, which leads to endothelial dysfunction, hypertension and other cardiovascular diseases. Indeed, an overactive RAS leads to vasoconstriction and retention of sodium and water, and then leads to raised blood volume and, in turn, to hypertension (Manrique *et al*. 2009). More details about RAS will be discussed in section 1.8.5.1.

Obesity is another main factor (growing to be the most important factor) as obesity is also the cause of insulin resistance, adult-onset diabetes mellitus, left ventricular hypertrophy and atherosclerotic disease. A weight in kilograms divided by square height in metres (body mass index; BMI) less than 25 kg/m^2 is considered as normal while a BMI of 26 -28 kg/m^2 increases the risk of elevated BP by 180% and the risk of insulin resistance by >1000%. Over 85 % of hypertensive patients have a body mass index greater than 25 (Aram *et al*., 2003). More details about obesity-induced hypertension will be discussed in section 1.8.5.4.

1.8.3 The effects of untreated hypertension

Undetected and uncontrolled hypertension leads to increased cardiovascular diseases, such as myocardial infarctions, stroke, kidney failure as well as blindness (Branca *et al*. 2007). The risk of developing complications with hypertension is higher than with other cardiovascular risk factors. It is suggested that BP variability (both short-term - BP can change within 24 hours, and long-term - marked BP differences are detectable between different days) increases cardiovascular risks, organ damage, risk of coronary heart disease and cerebrovascular events (Grassi *et al*., 2012). The Blood Pressure

Association estimates that there are 16 million people in the UK with hypertension, of which 5.7 million are unaware that they have the condition (Blood Pressure Association 2009).

An analysis of over 1 million adults aged between 40-69 years, indicated that the risk of death from coronary heart disease is doubled when the systolic BP is increased every 20mmHg or diastolic BP is increased every 10mmHg (British Heart Foundation 2008). It has been suggested that if the people who have hypertension in the UK could control their hypertension, the incidence of stroke would reduce by 28-44% while ischemic heart disease would reduce 20-35%. That would equate to a reduction of 42,800 stokes and 82,800 ischemic heart disease incidents (He & MacGregor 2003). Furthermore, direct and indirect costs detecting and treating patients with hypertension and related diseases are increasing every year. Recent figures from the British Heart Foundation suggested that 11% of the entire disease burden in developed countries is from hypertension. Therefore, treatments of hypertension become essential to decrease the morbidity and mortality of different critical cardiovascular events, as well as save treatment expenditure.

1.8.4 Treatments for hypertension

As mentioned ealier, lifestyle plays an important part in developing hypertension Therefore, lifestyle changes such as ensuring a low salt intake, low alcohol intake and stopping smoking, are strongly recommended before drug treatment is started. When blood pressure level becomes mild hypertension (see table 1.1), medication becomes necessary. To date, there are many kinds of antihypertensive drugs available. The most common medications (Table 1.2) are the thiazide diuretics, inhibitors for the RAS system (such as the angiotensin-converting enzyme inhibitors (ACE) and ATII receptor 1 blockers (ARB)), the beta blockers and calcium channel blockers. The effectiveness and choice of treatments depends on both age and race (Baglioni *et al*. 2007, National Institute for Health and Clinical Excellence, 2011). In reality, the treatments are proven, however they do not work all the time especially for some drug-resistant hypertensive patients, as well as due to the complex nature of hypertension development. Therefore, a better understanding of the mechanisms underlying the onset and development of hypertension is required.

In the UK, the National Institute for Health and Clinical Excellence (NICE) published a report in 2011 that stated that the aim of treatment is to maintain BP at 140/90 mmHg for people aged under 80 years and at 150/90 mmHg for people aged over 80 years (NICE, 2011). Figure 1.5 summarises the current therapy decisions to determine the optimum drug combination for hypertension treatment (recommended by NICE, 2011).

Drugs	Examples	Mechanism	Advantages	Disadvantages	Application for hypertensive patients
ACE inhibitors	Captopril, Enalapril	Block the conversion of angiotensin I to angiotensin II under the mediation of ACE.	May have anti-obesity effects. Safe and potent. cause regression of left ventricular hypertrophy	Renal failure, dry cough.	Used with caution in patients with impaired renal function, hypovolemia or dehydration, cardiac outflow obstruction.
AT1 receptor antagonists	Telmisartan, Irbesartan	Block the activity of angiotensin II AT1-receptors	Potent, benefit cardiovascular events	Common adverse drug reactions including dizziness, headache. May increase the risk of heart attack.	Used for the patients who are intolerant of ACE inhibitor therapy.
β receptor blockers	Atenolol, Albetalol	Suggested via reduction of cardiac output and antagonism of peripheral adrenoreceptors.	Reduce stroke in clinical trials, inexpensive; benefit after MI, asthma, heart block.	Fatigue, cold peripheries	Particularly use for patients with angina, cardiac arrhythmia, congestive heart failure, cardioprotection after myocardial infarction and so on.
Calcium Channel blockers	Amlodipine, Diltiazem	Work by blocking voltage-gated calcium channels in cardiac muscle and blood vessels.	Efficacious	Flushing, headache, ankle oedema	Use for patients with atrial fibrillation or flutter in whom control of the heart rate is an issue.
Diuretics (Thiazides)	Bendrofluazide, Chlortalidone	Inhibit sodium re-absorption in distal convoluted tubule	Reduce stroke and inexpensive	Dehydration and impaired glucose intolerance, thrombocytopenia	Use for patient with mild heart failure

Table 1.2: Commonly used antihypertensive drugs and their mechanisms (Oron- Herman *et al*., 2005; Weber 2009; Rang *et al*., 2003).

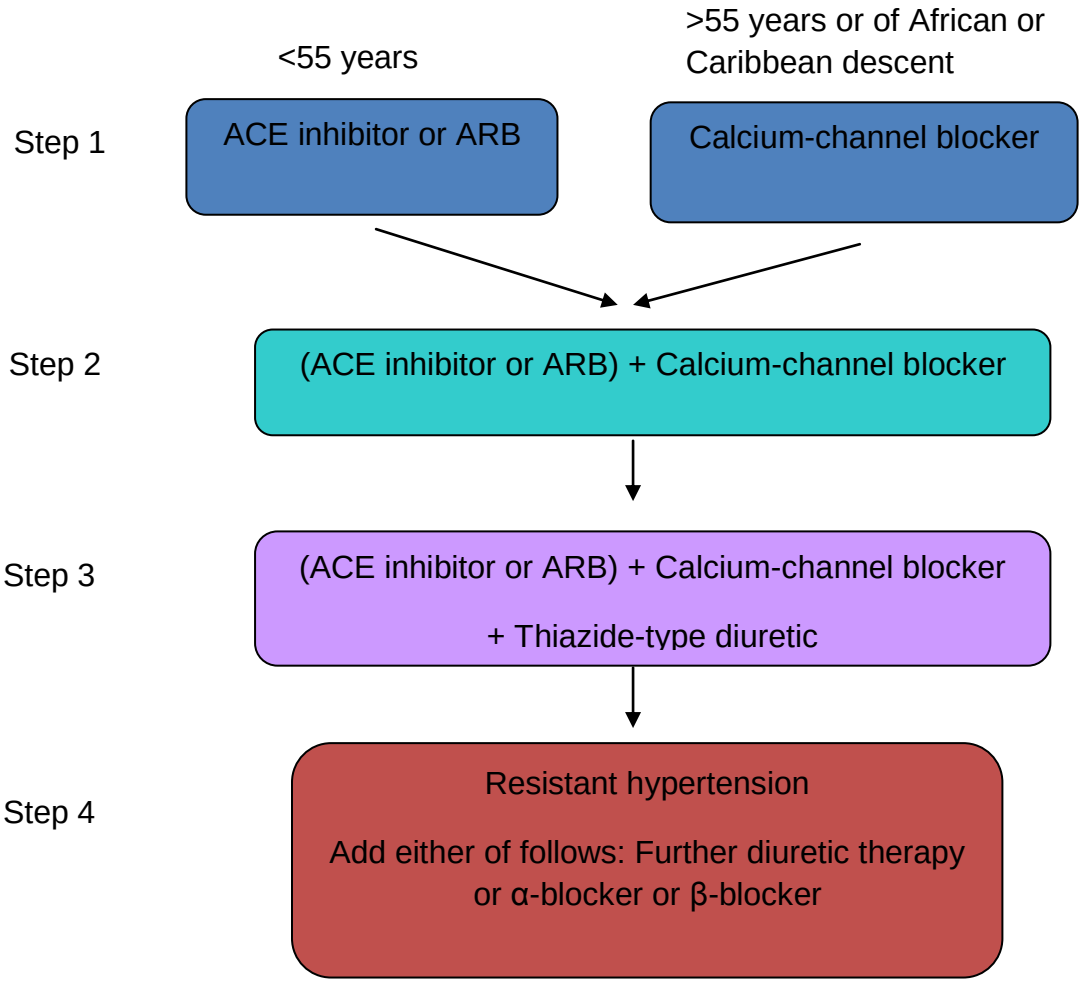


Figure 1.6: Pharmacological treatment of hypertension. Progression of treatment options based on a patients age and ethnicity, ARB=Angiotensin receptor blocker, used when the patient is intolerant to ACE inhibitors (NICE 2011). General treatment principles include offering people aged over 80 years the same antihypertensive drug treatment as people aged 55-80 years, offering people with isolated systolic hypertension the same treatment as people with both raised systolic and diastolic hypertension, and not combining ACE inhibitor with ARB (NICE 2011).

1.8.5 Mechanisms involved in hypertension

As discussed previously, hypertension has been described as the leading cause of morbidity and mortality and is related to the development of co-morbidities. The mechanisms involved in the development of hypertension are multifaceted, ranging from renin-angiotensin system and other humoral contributors to neurogenic mechanisms. This section will focus on discussing constrictor systems in details, which are involved in the animal models that have been utilized in this thesis.

1.8.5.1 Renin-angiotensin system (RAS) pathway

RAS is a hormone system that has an important role in the maintenance of hemodynamic stability via regulation of extracellular fluid volume, sodium balance & cardiac and vascular trophic effects. Figure 1.7 illustrates the major components and function of the RAS. Renin is a circulating enzyme secreted from the granular cells (also called juxtaglomerular cells which synthesise, store and secrete renin) in the kidney under the stimulation of a decrease in arterial blood pressure or sodium chloride level. It helps to maintain extracellular volume and arterial vasoconstriction (Jan Danser, 2012). Thus, it contributes to regulation of blood pressure. Renin regulates the RAS by cleaving angiotensinogen, produced by the liver, to create angiotensin I (ATI) which has no biological activity but exists solely as a stepwise product in the pathway to create ATII. To produce ATII, the ATI is further cleaved in the lung by the angiotensin-converting enzyme (ACE) which can be found at the surface of endothelial cells all over the body but is mainly located in the lung capillaries and renal endothelium due to high density of capillary beds there (Jan Danser J 2012).

ATII is a potent vasoconstrictor that increases peripheral vascular resistance, therein elevating arterial pressure. It also regulates aldosterone release and sodium re-absorption, as well as inducing the expression of many substances, such as growth factors, cytokines, chemokines and adhesion molecules. All these are involved in cell growth/apoptosis, fibrosis and inflammation (Mezzano *et al*., 2001; Sadoshima 2000; Ruiz-Ortega *et al*., 2001; Higuchi *et al*., 2007, Savoia *et al*., 2011). Indeed, in the event of hypertension both ATII-stimulated hypertrophy and inflammation contribute to the vascular remodelling. Delva *et al*. (2002) demonstrated increased collagen I and III mRNA and enhanced collagen protein synthesis in cultured fibroblasts from essential

hypertensive patients (Delva *et al*., 2002). In addition, cardiomyocytes produce active transforming growth factor (TGF) under stimulation of ATII where TGF in turn induces interleukin 6 (IL-6) to promote collagen synthesis (Sarkar *et al*., 2004). Moreover, ATII mediates expression of pro-inflammatory molecules in the vessel wall. In VSMCs ATII induces production of vascular cell adhesion molecule 1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1) as well as interleukin 6 (Phillips & Kagiyama 2002), which contribute to the recruitment of mononuclear leukocytes into the vessel wall. All these effects of ATII are mediated, on the whole, via actions on two seven transmembrane G-protein-coupled receptors: ATII receptor 1 (AT_1R) and ATII receptor 2 (AT_2R).

The AT_1R receptor, primarily expressed on the cardiovascular cells, such as VSMCs, has been shown to mediate most of the physiological and pathophysiological actions of ATII. For instance, the pro-inflammatory actions of ATII are mediated by activation of AT_1R, which causes vasoconstriction, aldosterone synthesis and secretion, fibrosis, increased cardiac hypertrophy, vascular smooth muscle cell proliferation, recruition of proimflammatory cells and so on (Catt *et al*., 1984; Ruiz-Ortega *et al*., 2001). ATII acts on the AT_1R to produce secondary messengers (such as MAPK and PKC) or activates proteins like Rho and Ras, which lead to different downstream pathways (Higuchi *et al*., 2007). Knocking out the AT_1R reduces levels of vascular oxidative stress, endothelial dysfunction and atherosclerosis (Wassmann *et al*., 2004). However, Kato *et al*. showed that the RAS-activated transgenic mice (a mouse model of persistent hypertension) displayed accelerated atherosclerosis and mortality but no change in term of kidney damage after receiving a bone marrow transplantation from an AT_1R KO mouse (Kato *et al*., 2008).

The AT_2R, is expressed at low levels in a few organs (such as adrenal gland, ovary, brain and kidney) and is believed to be associated with the anti-proliferative, vasodilatory and pro-apoptotic action of ATII, which tends to counteract the effects of ATII by AT_1R (D'Amore *et al*., 2005, Savoia *et al*., 2011). Expression of AT_2R has been found in the vascular wall of small resistance-size arteries from hypertensive diabetic patients when treated with ARBs. Moreover, a crosslink between AT_1R and AT_2R is suggested as AT_1R stimulation interferes with AT_2R expression (De Paolis, *et al* ,1999).

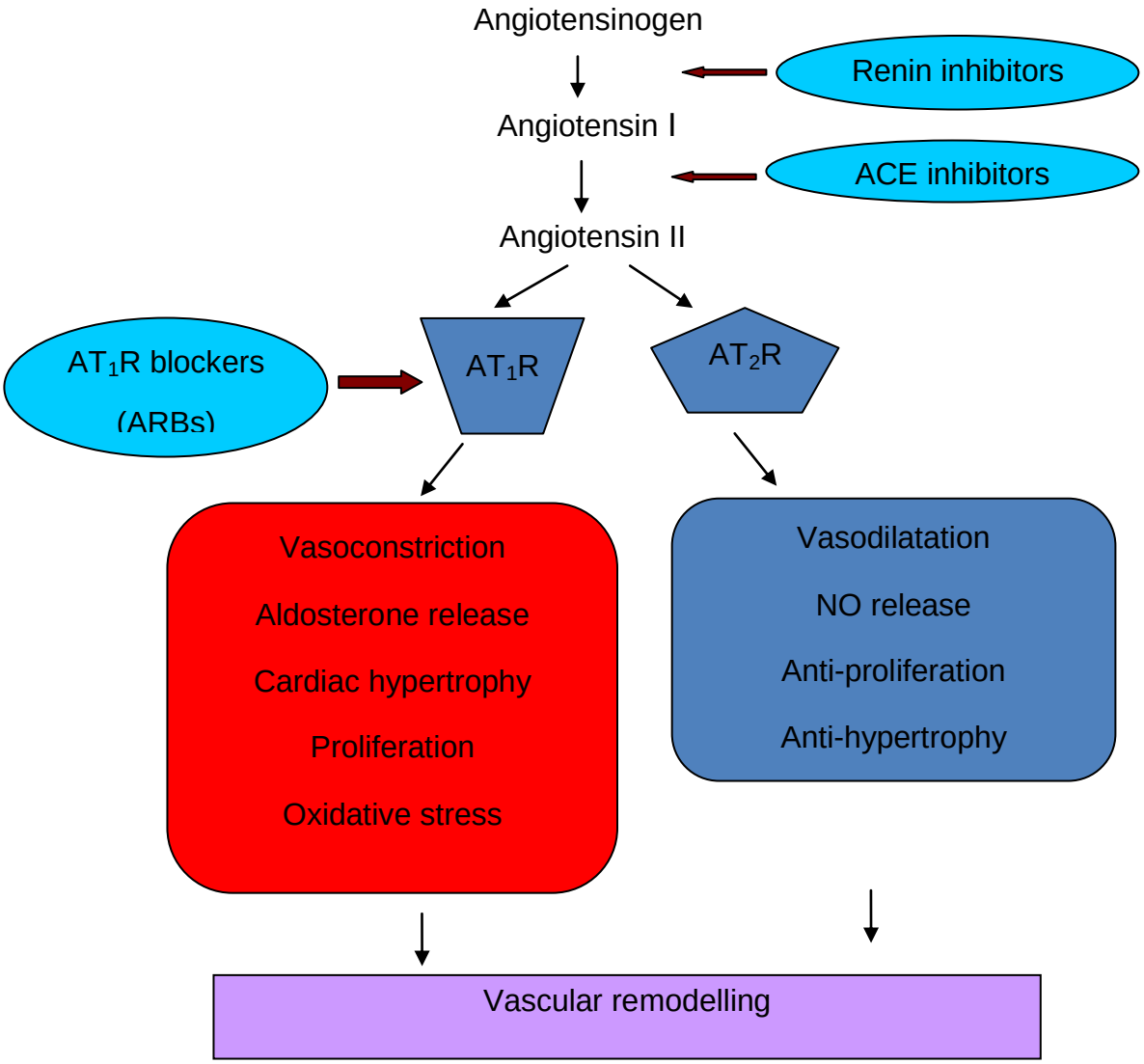


Figure 1.7: Renin-angiotensin pathway and its pharmacological inhibition. Renin inhibitors, ACE inhibitors, and ARB modulate angiotensin activities and inflammatory processes.

It is now noticeable that the RAS plays an essential role in the initiation and maintenance of the vascular inflammatory response which is associated with remodelling of resistance vessels as well as the development of organ damage (such as left ventricular hypertrophy, congestive heart failure, atherosclerosis, stroke, myocardial infarction) in hypertensive patients (McConnaughey *et al*., 1999). Therefore, inactivation of the RAS system, especially targeting the ATII pathway, becomes an essential issue. The removal of the actions of ATII by ACE inhibitors and ARBs leads to

anti-hypertensive and anti-inflammatory effects (Ma *et al*., 2010). ACE inhibitors bind to ACE to limit the ACE-mediated generation of ATII and also to prevent breakdown of bradykinin (Imig, 2004). ACE has therefore been targeted for the treatment of conditions such as hypertension and heart failure. ARBs cause reduction of BP in patients with essential hypertension and left ventricular hypertrophy through blocking the AT_1R (Dahlöf *et al*., 2002). ARBs can stimulate the ATII- AT_2R pathway which then promotes the kinin/NO system. However they can also increase circulating levels of ATII (Csajka *et al*., 1997, Regoli *et al*., 2012). Therefore, ARBs are introduced for the treatment for hypertension for those who are intolerant of ACE inhibitor therapies.

1.8.5.2 Endothelin pathway

Endothelins (ET-1, ET-2 and ET-3) are expressed in humans. ET-1 is a 21 amino acid vasoconstrictor peptide produced in cardiomyocytes and aortic VSMCs, but mainly in endothelial cells. ET-2 is produced primarily by the kidney and intestine while ET-3 is mainly localised in the brain, the intestine, as well as kidney tubule cells (Khimji and Rockey., 2010). Here, we focus on ET-1 whose action in the vascular wall has been the best characterized and is suggested to be implicated in a wide range of diseases, such as cardiovascular diseases (including arterial hypertension) and type II diabetes. ET-1 is released towards the basolateral side of the cells and then typically acts on SMCs. Therefore, normal tissue levels of ET-1 tend to be higher than the plasma levels (range 1-10pmol/L, Haynes and Webb 1994).

ET-1 exerts its action mainly through two major receptor subtypes namely endothelin A receptor (ET_A) and endothelin B receptor (ET_B). Both ET_A and ET_B receptors belong to the GPCR superfamily. In the vascular wall, ET_A receptor is found mainly in smooth muscle cells, which mediates ET-1-induced vasoconstriction under physiological conditions (Huggins *et al*., 1993). For example, ET_A receptor stimulation by ET-1 induces vasoconstriction, production of reactive oxygen species (ROS) and inflammation of the vascular wall (Schneider *et al*. 2007). In contrast to this, the ET_B receptor is found abundantly in endothelial cells and mediates vasodilatation by releasing NO (Huggins *et al*., 1993, Schneider *et al*. 2007). However both receptors have a wide distribution in many other cell types; for example, ET_B receptors are also

found on macrophages and platelets (Dockrell *et al*., 1996). Also, both ET_A and ET_B are distributed in the kidney (Davenport *et al*., 1991).

Generally ET-1 has been shown to be involved in the pathogenesis of a variety of diseases, such as congestive cardiac failure, coronary artery disease, essential hypertension, diabetes and metabolic syndrome (Khimji and Rockey 2010). ET-1 is shown to be correlated to several other inflammatory markers, such as transforming growth factor-beta, TNF, interleukins, insulin, noradrenaline, ATII and thrombin (Yanagisawa *et al*., 1988), which suggests an important role for ET-1 in chronic inflammation in the vascular wall. ET-1 not only can cause vasoconstrictor effects by vascular smooth muscle cellular constriction, but can also indirectly enhance the vasoconstrictor effects of other neurohumoral and endocrine factors, such as ATII and transforming growth factor beta-1 (TGFβ1), which potentiate the progression of essential hypertension (Lariviere and Lebel 2003). In addition, in an *in vitro* model, ET-1 induces conversion of angiotensin to ATII and stimulates the adrenal synthesis of epinephrine and aldsterone (Kaddoura *et al*., 1996). These results suggest that there may be crosslinking mechanisms between ET-1 and RAS, which suggests that ET-1 may contribute to the multisystem complications of hypertension.

ET-1 is suggested to be involved with the development of obesity which has strong links to type 2 diabetes. PreproET-1 mRNA expression and subsequent ET-1 release is mediated by thrombin via activation of PKC and mobilization of intracellular calcium (Emori *et al*., 1992). Circulating levels of ET-1 are raised in obese people with metabolic syndrome (Ferri *et al*., 1997). In addition, it has been shown that adipose tissue produces ET-1 and expresses both ET_A and ET_B receptors. Interestingly, such expression of ET_A receptors and release of ET-1 in adipose tissue is markedly elevated in obesity (Eriksson *et al*., 2009; van Harmelen *et al*., 2008). Moreover, incubation of adipocytes with ET-1 results in increased lipolysis via the ET_A receptor pathway resulting in increased free fatty acid release (Eriksson *et al*., 2009). Furthermore, a recent study in humans demonstrated an increased forearm vasoconstrictor response to exogenous ET-1 in overweight and obese adults when compared with normal weight adults (Weil *et al*., 2011). A selective ET_A receptor antagonist (BQ123) displayed a significant vasodilator effect in overweight/ obese adults but not in normal weight adults

(Weil *et al*., 2011). These results suggest that being overweight or obese is associated with increased sensitivity to ET-1 mediated vasoconstriction, which contributes to the development of endothelial dysfunction. Finally, compared with WT mice, mice with endothelial cell-selective ET-1 overexpression (selectively overexpressing human preproET-1 in the endothelium), cross-bred with apolipoprotein E gene deleted (apoE−/−) mice exhibited significantly enlarged atherosclerotic lesion size following a high fat diet (Simeone *et al*., 2011). These results suggest that endothelial ET-1 contributes to enhanced lipid biosynthesis and accelerates formation of atherosclerosis in the vascular wall. All these observations indicate that there may be a connection between ET-1, obesity and its associated cardiovascular diseases.

1.8.5.3 Sympathetic nervous system pathway

The sympathetic and parasympathetic systems are the two major components of the autonomic nervous system. There are two types of neurons involved in the transmission of sympathetic nervous system. The first type is the relatively short axons of preganglionic neurons which exit the spinal cord and synapse with post-hocganglionic neurons. The second type is the long axons of postganglionic neurons which spread to innervate target organs, like blood vessels, kidney and heart. Acetylcholine is the main neurotransmitter which is produced from sympathetic preganglionic nerves. In contrast, the primary neurotransmitter of postganglionic sympathetic neurons is noradrenaline which activates adrenoceptors (mainly α_1 receptors in the vasculature), causing vasoconstriction (Rang et al., 2012). Today there are several different methods to measure the activity of the sympathetic nervous system, such as microneurography (a method to measure efferent multi-fibre traffic in sympathetic nerves), noradrenaline spillover (a measurement of noradrenaline release to plasma), variability in heart rate/blood pressure and arterial baroreflex sensitivity (Davy and Orr *et al*., 2009, Parati and Esler 2012).

As mentioned above, one of the features of increased sympathetic nerve activity is the increased level of noradrenaline, which will elevate blood pressure. Plasma levels of noradrenaline are increased in the first hours following an acute ischemic or thrombotic cerebrovascular event (Thomas and Marks, 1978). Similarly, an elevation in plasma levels of noradrenaline has been reported in the advanced clinical phases of congestive

heart failure (Cohn *et al*., 1984). However, due to the limited reproducibility of plasma levels of noradrenaline as an indicator of sympathetic nerve activity, regional noradrenaline spillover has been developed (a measurement of organ-specific noradrenaline release to plasma).

It is suggested that the sympathetic nervous system is constantly active at a basal level to maintain homeostasis (Brodal 2004), and plays a key role in the regulation of arterial blood pressure. However, over-active sympathetic nerves may have an important role in the development of hypertension and related cardiovascular disorders. It is suggested that the degree of sympathetic nerve activation is directly related to the severity of the high blood pressure state (Grassi 2009, Grassi 2010). The evidence for the concept that the sympathetic nervous system is activated in hypertension has been investigated long time ago. Elevated levels of sympathetic nerve activity are observed in young, middle-aged and elderly individuals (Anderson *et al*., 1989, Grassi *et al*., 1998, Grassi *et al*., 2000). In addition, Esler *et al*. reported that chronic sympathetic activation is observed in about 50% of patients with essential hypertension (Esler *et al*., 2010). In animal studies, adult spontaneously hypertensive rats (SHR) have demonstrated reduced cardiac parasympathetic nerve activity (Friberg *et al*., 1988) and elevated sympathetic nerve activity with elevated noradrenaline release (Judy and Farrell 1979, Lundin *et al*., 1984). In addition, experimental infusion of noradrenaline elicits vascular remodelling and increased aortic medial thickening (Friberg *et al*., 1988). Therefore, it is certain that sympathetic activation is a hallmark of the essential hypertensive state.

What exactly triggers the increase in sympathetic nerve activity is still unknown. However, evidence indicates that a sedentary lifestyle can lead to mechanisms that trigger increases in sympathetic nerve activities, for example increased visceral abdominal fat mass is associated with increased muscle sympathetic nerve activity (Alvarez *et al*., 2002). In addition, Agapitov *et al*. demonstrated that muscle sympathetic nerve activity in upper and lower limbs was significantly higher in obese subjects when compared with lean normotensive subjects (Agapitov *et al*., 2008). Moreover, cardiovascular and metabolic diseases associated with hypertension are linked to increases in sympathetic nerve activity together with increased noradrenaline levels (Grassi *et al*.,1995, Vaz *et al*., 1997). Overall, these data suggest an important link

between the sympathetic nervous system and obesity. Chronic activation of the noradrenergic system leading to increased noradrenaline levels has been shown to contribute to peripheral sympathetic nerve system activation in heart failure (Lambert *et al*., 1995) and essential hypertension (Ferrier *et al*., 1992).

1.8.5.4 Obesity

In adults, obesity is defined as a BMI ≥ 30kg/m^2 while pre-obese is used to define adults with a BMI of 25-29.9. Overall, an adult is defined as overweight if they have a BMI ≥ 25 kg/m^2 (Branca *et al*., 2007). The UK has one of the highest prevalences of obesity in adults, throughout the world. In addition, a survey of children indicated that in Wales the prevalence of overweight 13-15 year olds is 25% and obese category is even higher at 33% (Branca *et al*., 2007). According to a 2011 National Health Service survey in England, high blood pressure was recorded in 48% of men and 46% of women in the obese group, compared with 30% of those in the overweight and 15% of those in the normal weight category (NHS survey 2011). Obesity is associated with multiple comorbidities, including hypertension, diabetes, sleep apnea and hypercholesterolemia. It has been suggested that 60% - 75% of hypertensive disease is caused by patients being overweight (Allender *et al*., 2006, Wofford and Hall 2004). However the exact mechanisms involved are controversial.

Studies in humans suggest that both bolus oral ingestion and the intravenous infusion of fat result in a significant increase in systolic blood pressure, decreased endothelial function, increased oxidative stress markers and activation of the sympathetic nervous system, assessed by heart rate variability (Gosmanov *et al*., 2010). Indeed, there are many pathways associated with obesity-induced hypertension. An established pathway is increased renal sodium re-absorption with impaired pressure, natriuresis via activation of the RAS and sympathetic nervous systems, and altered intrarenal physical forces. Segura *et al*. (2007) claimed that obesity is a risk factor for developing hypertension because of the activation of RAS in adipose tissue and its link with insulin resistance. They demonstrated that blockade of the RAS with either an ACE inhibitor or ARBs substantially increased adiponectin levels and improved insulin sensitivity. Furthermore, a recent study in humans involved blocking systemic nerve activity with trimethaphan, Shibao *et al*. demonstrated that obesity-induced sympathetic nerve

activation is an important determinant of hypertension and its associated conditions (Shibao *et al*., 2007). This finding suggests an important role of the sympathetic nervous system in the development of obesity associated hypertension. Moreover, in obesity with insulin resistance, NO-mediated vasodilatation is impaired, whereas there is increased levels of endothelin-1, which increases the vasoconstriction (Kotsis *et al*., 2010). This suggests the importance of functional endothelium and the balance between vasodilatators and vasoconstrictors in vascular wall.

Finally the levels of functional hormones (such as insulin, leptin and adipokine) and adipose cell-released ROS play an essential role in the obesity-induced hypertension (Kotsis *et al*., 2010). In addition, pro-inflammatory molecules (such as IL-1β IL-6 and TNFα) also contribute to such an event (Kotsis *et al*., 2010). Obesity is also characterised as impaired glucose tolerance and insulin resistance. High levels of insulin have been shown to enhance sodium retention (Sechi & Leonardo, 1999) and have acute sympathoexcitatory action (Anderson *et al*., 1992). Moreover, adiponectin is the most common adipokine which has vasorelaxant effects on small arteries. Clinical studies demonstrated low levels of adiponectin in hypertensive patients but levels increased with anti-hypertensive treatment (Yilmaz *et al*., 2007). In addition, it has been shown that adiponectin can improve the endothelial dysfunction by increasing NO production in diet-induced obese rats (Deng *et al*., 2010). Furthermore, knocking out the adiponectin, an adipokine, caused severe diet-induced insulin resistance and atherogenesis. This data suggests that lack of adiponectin is one of the factors contributing to cardiovascular symptoms in obesity.

Overall the exact mechanisms involved in the development of obesity-induced hypertension is in need of further research. The possible pathways discussed above are summarised as Figure 1.8.

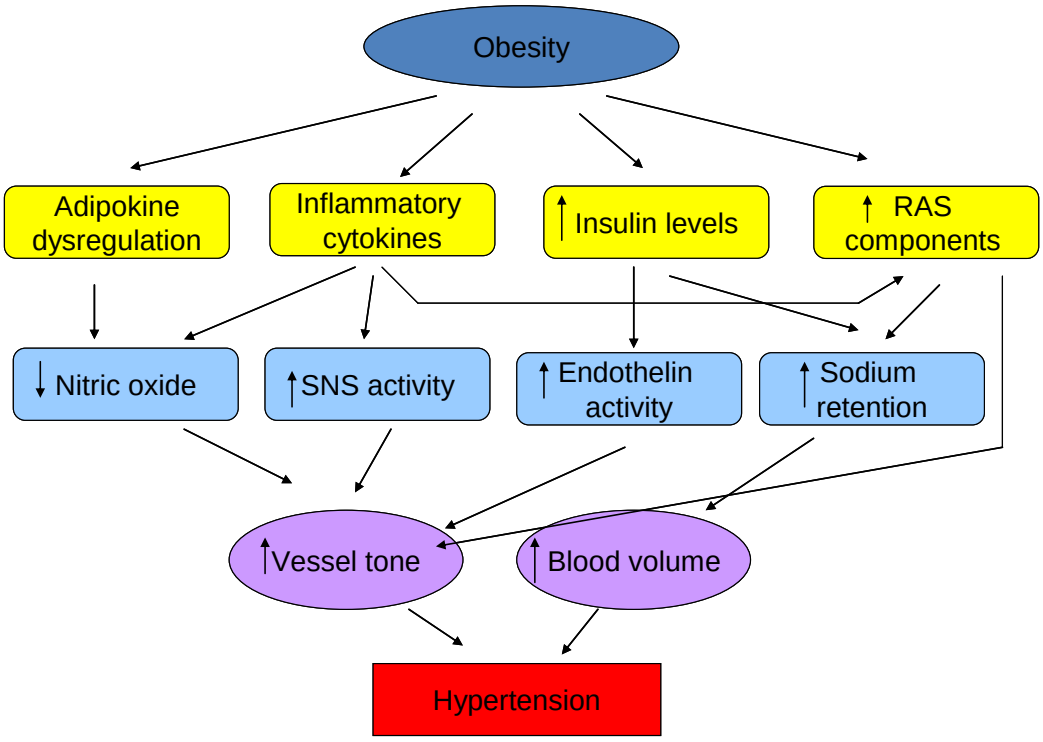


Fig 1.8 The complex pathophysiology of obesity-related hypertension RAS: renin-angiotensin system; SNS: sympathetic nervous system (Aghamohammadzadeh and. Heagerty, 2012).

1.8.6 Experimental models of hypertension

As the mechanisms involved in essential hypertension are complicated, a range of hypertensive models in animals have been developed to investigate new insights into the pathogenesis of hypertension. An ideal animal model of hypertension should be easy to perform and be highly reproducible. It has to be able to predict the potential agent with antihypertensive properties, require minimal quantities of compounds, be comparable to human hypertension and be feasible in a small animal. Several recent literature reviews have reported a range of different models using different animals with different techniques (Badyal, *et al*., 2003; Lerman *et al*., 2005; Nuyt 2008; Dornas and Silva 2011). Each model has its own advantages and disadvantages. As we know that hypertension is a complex disease, no model can be designed to answer all our questions. Therefore, for specific research purposes, different experimental designs and choices of animal models are applied. Generally, animal models of hypertension can be divided into genetic or non-genetic models. Genetic models are usually

designed to study hypertension by altering human and mouse genomes. By comparison, non-genetic models are those using surgery, drug treatments or metabolic disorders by diet feeding, to study the mechanisms involved in hypertension development.

1.8.7 1.8.7 Genetic models: Phenotype-driven models

Essential hypertension accounts for 90-95% of hypertension in humans, in which several genes contribute to the individual phenotype (Dickson *et al*., 2006). Therefore, in humans it is impossible that a single genetic defect can manifest the development of essential hypertension. The understanding of human and mouse genomes has promoted the development of transgenic or gene-targeted animal models to study more about hypertension. Phenotype-driven models are believed to be the most abundant tools for studying hypertension and the potential for exploration of hypertension caused by more than one gene mutation. In general, homozygous hypertensive rat strains are developed by selectively breeding of animals with the desired phenotype for several generations. Once the feature is determined, breeding of relative rats is required for another 20 generations to obtain genetic homogeneity. With this method, the first SHR was introduced in 1963 by Okamoto and Aoki (1963). These SHR rats can become hypertensive at age 4-6 weeks with no physiological, pharmacological or surgical intervention (Zicha and Kunne 1999). This model is not only benefiicial for studying the pathogenesis and therapy for essential hypertension, but also is useful in terms of researching the prevention of the development of essential hypertension and its relative complications. There are other phenotype-driven models such as SHR-stroke prone, Dahl salt-sensitive rat, milan SHR and obesity-related models.

1.8.8 Genetic models: Genotype-driven model

Studies of the molecular basis of hypertension have made significant progress and have revealed several disease susceptible genes. Researchers now know that disruption of certain genes that manage peptides or receptors linked to regulating blood pressure are involved in the pathogenesis of hypertension. The most studied models involve overexpression or deletion of selected genes which are normally involved in regulating vascular tone, renal physiology, and/or electrolyte and fluid homeostasis (Cvetkovic and Sigmund, 2000). This has allowed specific mouse models to be

developed, such as targeting at renin, nitric oxide and ET. For example, selective overexpression of the rat angiotensinogen gene in mice, with accompanying overexpression of rat renin, results in high blood pressure (Ohkubo *et al*., 1990). Moreover, the ET-1 gene is linked to blood pressure in obese patients. Similarly, the ET receptors are also suggested to be related to essential hypertension (discussed previously). Therefore, the ET-1 gene and ET receptor genes have also been targeted. In this thesis, two different transgenic mice have been used. The first one is smooth muscle cell-targeted RAMP2 (an AM1 receptor component) over-expressing mice. As discussed before, AM has shown a protective role in cardiovascular disease, but the influence of the AM1 receptor is unknown. The second mouse is the TRPV1 KO mouse. TRPV1 activation is associated with the release of vasodilator CGRP which is mainly known to be protective.

1.8.9 Non- genetic model: Surgically-induced hypertension

Several experimental non-genetic approaches have led to the development of useful models. These provide the possibility of inducing hypertension and end-organ damage in different species, thus providing a range of models, possibly comparable with the human pathology. These models are based on mechanisms of surgically-induced renal vascular or tissue injury. Goldblatt and co-workers developed the first animal model of hypertension in dogs by unilateral constriction of renal artery (Goldblatt *et al*., 1934). Since then, it became a very commonly used model for hypertension, including development for use in rats (Wilson and Byrom 1939), rabbits (Pickering and Prinzmetal 1937), dogs (Romero *et al*., 1981), pigs (Lerman *et al*., 1999), monkeys (Panek *et al*., 1991) and mice (Wiesel *et al*., 1997). Renovascular hypertension can also be achieved by coarctation of aorta, reduction of renal mass and external compression of renal parenchyma (Badyal *et al*., 2003).

1.8.10 Non- genetic model: Endocrine, metabolic and diet-induced hypertension

Administration of a mineralocorticoid (Selye *et al*., 1942) is the most common endocrine method to evoke hypertension, particularly DOCA. Increased DOCA-induces re-absorption of salt and water resulting in increased blood volume and, thus, increased blood pressure. In rats and dogs, both partial removal of a renal mass and high salt diet feeding are required for generation of hypertension (Selye *et al*., 1942). Administration

of glucocorticoids can also cause hypertension in both rats and mice (Dahl *et al*., 1965), possibly by activating the RAS. However, they are reported to be less effective than DOCA. Moreover, chronic infusion of components of the RAS (such as ATII) has also been shown to induce hypertension successfully (more details will be discussed later).

As discussed before, inherited salt sensitivity is involved in the development of essential hypertension. Thus, an experimental model of hypertension with different dietary interventions was introduced by increasing salt intake. A strain of salt-sensitive rats developed by Dahl (1972), was characterised by hypertension (even with a low-sodium (0.5% NaCl) intake) and low heart rate with elevated sodium and water retention and plasma volume, renal parenchymal lesions as well as elevated activity of the sympathetic nervous system. More recently, dietary the intake of fats and carbohydrate has also been introduced. For example, the intake of simple sugars and the resultant effects of plasma insulin, adipokine and lipid concentrations may have an effect on cardiomyocyte size and function (Sharma *et al*., 2007). In addition, an 11-35% HFD in mice for 12-40 weeks to mimic the western diet-style of human food intake that leads to the development of hypertension has been utilised (more details will be discussed later).

1.9 ATII induced hypertension

ATII, an important mediator of the RAS, plays an essential physiological role in cardiovascular homeostasis. Moreover, ATII is suggested to play a key role in the physiological mediation of vascular tone and BP as well as in pathological conditions such as hypertension and heart failure. ATII is a potent vasoconstrictor of the peripheral vasculature and stimulates the proliferation of smooth muscle cells of blood vessels and in the heart (Itoh *et al*. 1993). Therefore, infusion of ATII induces hypertension and this is a common hypertensive model, which has been used since the agonist became available in pure form. The ATII infusion rate normally does not cause an instant increase in systemic BP but it rather results in a slow development of hypertension over a period of 6–10 days. It is suggested that the gradual development of hypertension by infusion of ATII is to mimic the human high renin (ATII) hypertension (Li and Wang 2005). Such ATII-induced hypertension has a sympathetic component, at least to some extent, even though ATII is a direct constrictor agent. For example, it has been

suggested that different doses of ATII induce distinct degrees of hypertension, which leads to a presumably baroreflex-mediated sympatho-inhibition corresponding to the increased blood pressure (McBryde *et al*. 2007). The ATII-induced hypertension model is now commonly used; however the mechanisms by which ATII chronically leads to hypertension and its associated effects remain unclear.

The primary advantage of the ATII-induced hypertension model is that infusion of ATII results in profound increases in blood pressure, becoming statistically significant within 7-9 days when compared to the vehicle controls (Widder *et al*., 2009). With the same volume of the pumps, the dose of ATII depends on the concentration of ATII and the rate of the mini-pump. Currently there are a range of doses (from 0.3-1.44 mg/kg/day) of ATII used by different research groups (Qin, 2008). Depending on the required end-points of the model, the above doses are administered for different periods, from between 7 to 28 days. The ATII-induced hypertension model established within our group involves the implantation of an osmotic mini-pump under the skin in the scapular region. In 2009 we demonstrated that with a dose of 0.9mg/kg/day and infusion for 13 days, a significant increase in blood pressure is observed, together with vascular inflammation (Liang *et al*., 2009). Also, within our group, a dose of 1.1mg/kg/day has been used and showed a significant increase in blood pressure in addition to vascular inflammation and remodelling in WT and CGRP KO mice (Smillie, 2012).

1.10 Obesity-induced hypertension

As discussed previously, the incidence of obesity and its associated conditions, such as hypertension, are growing worldwide, which makes it essential that animal models sharing characteristics of human obesity and its co-morbidities are developed for novel prevention and treatment. Today, there are many different kinds of obesity models being used by research groups, such as using db/db mouse (Senador *et al*., 2009) and the Zucker rat (Alonso-Galicia *et al*., 1996). A HFD-induced hypertension model is used in this thesis. HFDs have been used to model obesity and insulin resistance in rodents for many decades (Lemonnier *et al*., 1975, Grundleger & Thenen, 1982, Friedman & Halaas, 1998; Reimer & Ahren, 2002). It is clear that exposure of animals to a HFD often results in the development of obesity. Feeding mice a HFD over a period of 12-15 weeks to induce obesity is thought to mimic the development of human obesity in the

Western world and leads to obesity-induced hypertension (Friedman & Halaas, 1998; Reimer & Ahren, 2002). Long-term feeding of mice with HFD (35% fat diet) increased body weight compared to standard chow-fed controls (Lei *et al*., 2007). In addition, in our group, we have demonstrated that feeding mice with 35% fat (lard) causes a significant increase in body weight and blood pressure, in addition to vascular hypertrophy (Marshall *et al*., 2012).

Compared to the ATII-induced hypertension model, this model results in a moderate increase (less extreme than that induced by ATII) in blood pressure in obese animals (approximately 108mmHg to 118mmHg in obese WT animals, Rao *et al*., 2007), which is similar to human obesity-induced hypertension. Obesity-induced hypertension models have become a common model to study hypertension development. In a rat model of obesity-induced hypertension, it was demonstrated that increased oxidative stress and then decreased NO bioavailability did contribute to the development of hypertension induced by HFD (Dobrian *et al*., 2001). In addition, mice fed with HFD beginning at age 3 weeks for 10 weeks, developed hypertension, increased oxidative stress and enhanced vasoconstriction (Smith *et al*., 2006). Interestingly, their results suggest that these effects induced by HFD feeding are not associated with the RAS (Smith *et al*., 2006). However, a recent study in which rats were fed on a HFD for 4, 8 and 17 weeks, the rats exhibited increased plasma levels of aldosterone and/or increased renin activity (Northcott *et al*., 2012). However, they did not investigate the precise mechanisms involved.

A HFD induces endothelial dysfunction which represents an important link between obesity and cardiovascular events. Kobayasi *et al*. demonstrated that HFD feeding in female Swiss mice caused a significant increase in both systolic and diastolic blood pressure and endothelial dysfunction (Kobayasi *et al*., 2010). Their results suggested that decreased anti-oxidant defence mechanisms partially contribute to the impairment of endothelium-dependent relaxation in aorta from obese mice (Kobayasi *et al*., 2010). Furthermore, Wang *et al*. revealed that diet induced obesity is associated with perivascular adipose tissue inflammation (macrophage content and vascular oxidative stress) and endothelial dysfunction in mesenteric arteries, which can be prevented by

deletion of P-selectin glycoprotein ligand-1 (Wang *et al*., 2012). This research suggests that obesity-induced hypertension has a complex aetiology.

1.11 Aim and Hypothesis

As discussed previously, in recent years many different mechanisms underlying hypertension have been suggested. However, considering the rates of obesity in both developed and developing countries, further research is still required in order to provide novel therapeutic targets. Therefore the hypothesis of my PhD project is that both the AM (via upregulating AM1 receptor component RAMP2) and TRPV1 (via release of CGRP) mediate protective roles in models of hypertension as well as hypertension associated vascular hypertrophy induced by either ATII or obesity. The aims of my PhD project are:

1. Investigating the role of the AM1 receptor component RAMP2 in model of ATII-induced hypertension and vascular hypertrophy.
2. Studying the role of TRPV1 in the hypertension model of ATII and its associated vascular hypertrophy.
3. Investigating the role of TPRV1 in the development of hypertension and vascular hypertrophy induced by obesity.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Animals

All the procedures were performed under the UK Animals (Scientific Procedures) Act, 1986. The RAMP2 TG mice were received from the Research Laboratory for Calcium Metabolism in University of Zurich, Switzerland. The RAMP2 TG mice were characterised in 2006 and bred on site. Mice of both sexes were used for these studies and transgenic mice were sex and age-matched with their respective WT controls. Blood pressure results were analysed for gender differences and as no difference were observed, results were pooled from both sexes. WT and TRPV1 KO mice were gifted from Merck, Sharpe and Dohme and also bred in-house. Mice were maintained in a climatically controlled environment with free access to food and water. Mixed gender mice were used for blood glucose tolerance test. Only male mice were used for all the other experiments in TRPV1 projects. In order to keep the experimenter blinded during the studies, mice were put together and labelled as number such as 1,2,3, and so on. Details were kept in the lab books so we could trace back when needed for analysis.

2.2 Generation and genotyping of RAMP2 TG mice

Both WT and RAMP2 TG mice, aged 4-7 months and weighing 25 to 35g, were used. The transgene consisted of an α-actin promoter fused to DNA encoding the CD33 signal sequence, a Mycepitope-tag (Myc) and mouse RAMP2 without the corresponding signal sequence (mRAMP2).

Fig 2.1 The RAMP2 transgene. The RAMP2 transgene consists of a mouse α-actin promoter fragment (1074bp), fused to DNA encoding the signal sequence CD33 (SigCD33), a Myc tag (Myc) and mouse RAMP2 without the corresponding signal sequence (mRAMP2). The binding

position for the oligonucleotide primers used for PCR genotyping are shown as black horizontal arrows on the bottom line.

The α-actin promoter is designed for the transgene targeting primarily to smooth muscle cells. The Myc tag, a human gene, is the target gene to identify the presence or absence of the transgene and the signal sequence CD33 targets the whole gene product to the membrane. To generate, the cDNA construct was inserted into a pSMP8 plasmid using restriction enzymes BamHI at the 5' end and EcoRI at the 3' end, followed by the ligating enzyme T4-ligase. The transgene was microinjected into a male pronucleus which was mated with a female pronucleus. Finally the zygote was surgically injected into a female WT (C57BL6) mouse, which will generate heterozygous positive mice. Expression of myc-mRAMP2 was examined by Prof. Born's group (University of Zurich) in a range of smooth muscle cells, where myc-mRAMP2 predominantly expresses (Fig 2.2).

After obtaining the RAMP2 TG mice from Prof. Born's group, genotyping was carried out regularly. Genotypes were determined by end-point PCR analysis of ear biopsies. In order to create a similar level of up-regulation of RAMP2 in each mouse, a heterozygous TG mouse is bred with a WT mouse to produce offspring that are 50% WT and 50% heterozygous positive for the transgene. The mice are not positive for the transgene are used as WT controls.

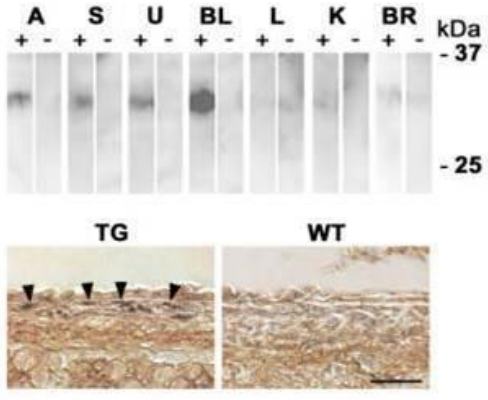


Fig 2.2: Myc-mRAMP2 expression (top) in mouse aorta (A), stomach (S), uterus (U), bladder (BL), liver (L), kidney (K) and brain (BR) from WT (-) and TG (+). Immunohistochemistry of cross-sections of aorta with anti-myc antibodies reveal myc-mRAMP2 expression (arrowheads) in smooth muscle cells. Scale bar: 50μM. (Provided by Prof Born's group)

To genotype, ear samples were collected using metal ear clippers from mice under 2% isoflurane and 2% O_2 (Abbott Laboratories, Kent, UK) anaesthesia. Genomic DNA was

extracted using the REDEXtract – N – AMP Tissue polymerase chain reaction (PCR) kit (Sigma, UK) and a PCR amplification of the target regions was performed on the genomic DNA using the following primers () :

RAMP2 forward: 5'AGC AAA AGC TCA TTT CTG AAG AGG 3'
RAMP2 Reverse: 5' GTG GGA AGG ATG AGA GTA AGT GG 3'

These RAMP2 primers are used to amplify the copies number of DNA that is present in the isolated genomic DNA, which will allow the visualization of absence and presence of the RAMP2 transgene. Details of the PCR mixture reagents are shown in table 2.1.

Component	Volume/sample
5x High fidelity PCR green GoTaq® buffer (600mM Tris-SO4, pH8.5, 180mM ammonium sulphate and blue dye and yellow dye)	4 µl
dNTP mix (2.5mM)	0.8 µl
RAMP2 Forward Primer (50µM)	0.4 µl
RAMP2 Reverse Primer (50µM)	0.4 µl
Mg2+ (50mM)	1.6 µl
Platinum Taq DNA polymerase (Invitrogen)	0.2 µl
DEPC H2O	10 µl
Total/sample	16.6 µl

Table 2.1: The PCR mixture for RAMP2 genotyping

Amplification of the DNA was carried out in a thermal cycler (Techne, Cambridge, UK) using the conditions listed in table 2.2:

No. Cycles	Temperature	Time	Phase
1	95°C	5 mins	Initial melting the DNA
30 cycles	95°C	1 min	Melting the DNA
	56°C	1 min	Annealing of primers
	72°C	2 min	Elongation
1	72°C	7 mins	Final elongation

Table 2.2 PCR programme for RAMP2 genotyping

Following the final elongation, the samples were stored at 4ºC until use. The PCR products were visualised using gel electrophoresis on a 1.8% agarose gel in running buffer (0.5% tris-borate-EDTA buffer) with 4µl ethidium bromide (to get final concentration 0.5 ug/ml). The gel was covered in running buffer and 10µl of the PCR product mixing with 5µl loading buffer (0.1% bromphenol blue, 15% w/v Ficoll in running buffer) and 5µl of DNA ladder (Sigma, UK) were loaded into the wells. The gel was run at 90V for 20 minutes and the DNA was visualised under UV light to allow identification of WT and RAMP2 transgene bands.

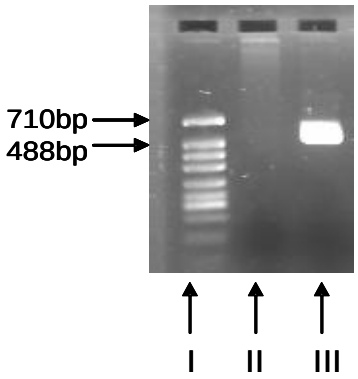


Fig 2.3: Representative image of gel with PCR products. (I) = DNA size maker; (II) = negative for the transgene; (III) = positive for the transgene; bp=base pairs. In the molecular maker, the gap between the uppermost (710bp long) and the band beneath (488bp long) indicates the gene product which is 578bp long.

2.3 Generation and genotyping of TRPV1 KO mice

TRPV1 KO mice were generated by replacing an exon encoding part of the fifth and entire sixth transmembrane domains of the channel, including the interconnecting pore-loop, with a pGK-neo (phosphoglycerine kinase-neomycin) casette as described by Caterina and colleagues (Caterina *et al*., 2000). This genetic alteration was shown to render the channel unable to conduct ions and respond to selective agonists (Cateriba *et al*., 2000). Heterozygote TRPV1 mice were mated to produce homozygous WT, KO and heterozygote offsprings. After five generations, following genotyping, homozygous breeding pairs were set up to give homozygous WT and TRPV1 KO offspring. Subsequent generations of the WT and TRPV1 KO breeding colonies were genotyped to confirm the presence or absence of the functional TRPV1 receptor gene. An ear biopsy was collected under 2% isoflurane and 2% O_2 anaesthesia from mice. Genomic DNA was extracted using the REDEXtract – N – AMP Tissue polymerase chain

reaction (PCR) kit (Sigma, UK) and a PCR amplification of the target regions was performed on the genomic DNA using the following primers:

TRPV1 - Forward 5' CGA GGA TGG GAA GAA TAA CTC ACT G 3'

TRPV1 – Reverse 5' GGA TGA TGA AGA CAG CCT TGA AGT C 3'

NEO – Forward 5' TTT TGT CAA GAC CGA CCT GTC C 3'

NEO – Reverse 5' CCC TCA GAA GAA CTC GTC AAG AAG 3'

The TRPV1 WT primers were used to amplify a PCR product of approximately 188 base pairs that confirmed the presence of the WT TRPV1 receptor gene. The neomycin primers were used to amplify a PCR product of approximately 700 base pairs that corresponded to the neomycin construct found in the disrupted gene in the KO mice. The PCR reaction mixture was composed of the materials listed in table 2.3

	Volume (µl)
REDEXtract – N – AMP PCR Reaction Mix	10
TRPV1 – 1 (5pmol/µl)	2
TRPV1 – 2 (5mol/µl)	2
NEO - 1 (5pmol/µl)	2
NEO – 2 (5pmol/µl)	2
Genomic DNA sample	5
Total volume	25

Table 2.3 The PCR mixture for TRPV1 genotyping

Amplification of the DNA was carried out in a thermal cycler (Techne, Cambridge, UK) using the conditions listed in table 2.4.

No. Cycles	Temperature	Time	Phase
1	95°C	14.5 mins	Initial melting the DNA
35 cycles	95°C	45 sec	Melting the DNA
	60°C	60 sec	Annealing of primers
	72°C	60 sec	Elongation
1	72°C	60 sec	Final elongation

Table 2.4 PCR programme for TRPV1 genotyping

The PCR products were visualised using gel electrophoresis, as described in 2.2.

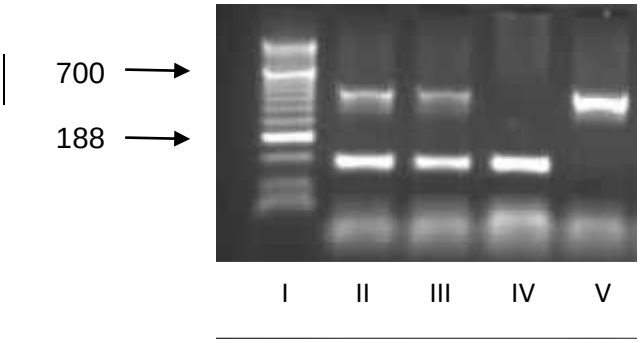


Figure 2.4: Representative image of gel with PCR products under UV light (I) = DNA size maker; (II) and (III) TRPV1 heterozygote; (IV) TRPV1 WT (product size=188bp); (V) TRPV1 KO (product size=700bp).

2.4. Mini-pump Implantation

Sex and age matched mice were used for these experiments. Experimental hypertension was introduced using osmotic minipumps (Model 1002, Alzet, USA) containing angiotensin II (ATII; infusion rate 0.9 mg/kg/day, Sigma, UK) or vehicle (0.01% 10 mmol/l acetic acid in saline) were implanted into the mid-scapular region of WT and RAMP2 TG mice (25-35 g, 12 – 16 weeks old, matched for age and sex). The body weight of each individual mouse was obtained on the same day of the surgery. Calculations for the amount of ATII were based on the body weight and the pumping rate, and then osmotic mini-pumps were filled carefully. Surgical procedure was carried out using aseptic techniques under isoflurane anaesthesia (2.5%, Abbott Laboratories, UK, in 3L/minute O2) following administration of buprenorphine hydrochloride (Reckitt Benckiser, UK, 50 μg/kg) intramuscular for post-hoc-operative pain relief. The outer incision was sealed with absorbable sutures (4.0, Ethicon, Johnson and Johnson) in a discontinuous pattern. Then mice were kept under a heating lamp to provide warmth until recovery. Mice were singly housed. Thorough daily checking was provided post-hoc-surgery until termination.

2.5 High Fat Diet induced hypertension model

Age and sex matched male and female TRPV1 WT and KO mice (3-4 weeks old) were either fed with a HFD, 35% of fat from lard, 58.4% Kcal from fat, custom diet ref: TD03584, was bought from Harlan Teklad, USA). The 35% fat was comprised of approximately 50% monounsaturated fat and 40% saturated fat and 10%

polyunsaturated fat. It also contained sodium level: 0.15% and potassium level: 0.5%. The normal diet consisted of 4% fat (mainly from fatty acids but also from plant or grain sources) with 6.8% Kcal from fat with the sodium level: 0.25% and potassium level: 0.67%, Rodent Diet RM 1E, SDS, Essex, England) for 12 weeks. Paired mice were also matched by body weight at the beginning of the study to reduce variability. Mice were housed in groups of 2-3 until termination or telemetry surgery. Body weight and food intake for individual mice was monitored twice weekly. When the mice were singly housed, a box maze and wooden bars were provided to reduce stress.

The relative components of these two diets are detailed in fig 2.5.

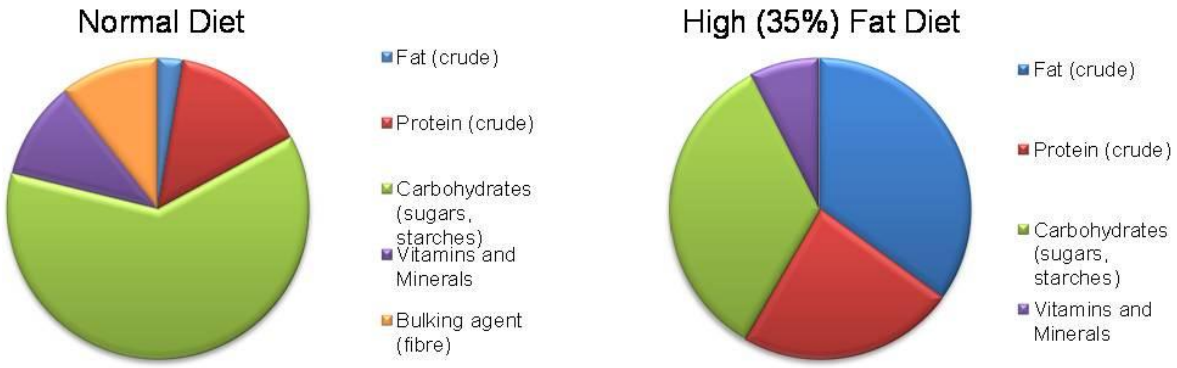


Figure 2.5: Relative components of the normal diet (4% fat diet) and the high fat diet (35% fat diet). The high fat diet contains much less carbohydrates and bulking agents when compared to the normal diet. For human, nutritionists advise us that a maximum of 35% of our calorie intake should come from fats, with only 10% derived from saturated fats.

2.6 Blood Pressure Measurement Methods

Blood pressure was monitored either using tail cuff plethysmography (CODA6 System, Kent Scientific, USA) in conscious restrained mice or through telemetry probe (PA-C10, DSI, NL)) in conscious unrestrained mice. Mice were handled as gently as possible to reduce any stress.

2.6.1 Tail cuff plethysmography

Tail cuff plethysmography is a well established method in our group (Clark *et al*., 2007; Liang L *et al*., 2009) using the CODA 6 non-invasive BP acquisition technique system for mice (Kent Scientific, Torrington, CT, USA). Briefly, this system uses volume-

pressure recording to measure blood pressure. Mice are restrained in the specially designed tubes. An occlusion cuff is placed at the base of the tail followed by a v-cuff. The occlusion cuff halts the blood flow into the tail where it is slowly deflated, and the v-cuff that is contacted to a pressure sensor measuring the returning blood flow. Return of blood causes the tail to swell which is when the systolic blood pressure is measured. Once the tail has completely ceased swelling the diastolic blood pressure is calculated. Then the mean arterial blood pressure is calculated based on systolic and diastolic measurements.

For this study, mice were trained for the tail cuff method for two weeks during which mice were acclimatized to the procedure. All the measurements were carried out in a 25ºC, semi-dark, quiet room. For each measurement session, five acclimatization cycles were performed before collecting data. Any cycles with animal moving disruption or with an inadequate waveform were discarded. At least five good cycles were collected for each mouse every day.

For mice used in the mini-pump implantation experiments, blood pressure was measured before the implantation as baseline and at day 3, 6, 9 and 13. For the HFD study, different sets of studies were carried out. For one set of study, blood pressure was measured on the last week of the feeding study, to determine the blood pressure change after 12 weeks of HFD compared to normal diet. In a separate study, blood pressure was measured for the last four weeks of the study to investigate when the blood pressure changes began.

2.6.2 Telemetry

Blood pressure was also measured using a telemetry probe (PA-C10, DSI, NL). Together with the blood pressure, telemetry also provides data on the heart rate and spontaneous activity. The implantation surgery procedure was carried out using aseptic techniques under isoflurane anaesthesia (2.5%, Abbott Laboratories, UK, in 3L/minute O_2) followed by intramuscular administration of buprenorphine hydrochloride (Reckitt Benckiser, UK, 50 μg/kg) for post-hoc-operative pain relief. A catheter tip was inserted into the left carotid artery and advanced towards the aortic arch. The catheter was secured to the carotid artery using non-absorbable surgical braided silk sutures (5.0,

waxed, Pearsalls sutures, Pearsalls Ltd) and the transmitter body was implanted subcutaneously into the right flank. The outer wound was closed with absorbable sutures (4.0, Ethicon, Johnson and Johnson) in a discontinuous pattern. The mice were kept under a heating light until fully recovered. Mice were singly housed and special care was provided post-hoc-surgery until termination. Mice were allowed to fully recover for two weeks before mini-pump implantation or collection of the data. Data was recorded every 15 min for 30 seconds.

For ATII treated mice, baseline data was collected following day 13 post-hoc- mini-pump implantation for 4 days. Then blood pressure was collected on day 3 and 6 onwards until termination at day 13. For the HFD model, telemetry surgery was carried out following 8 weeks feeding, when the normal diet fed mice were big enough while the HFD mice were not too fat for the surgery. Data was collected at week 13. All collected data was calculated by the DSI software (DSI Dataquest A.R.T.) and analysed in Microsoft Excel and GraphPad Prism 5.

2.7 Methodology of other physiological parameters measured *in vivo*

2.7.1 Body Weight and food intake

Body weight was recorded twice per week throughout the study where mice were handled as gently as possible. Food intake was measured for a few mice twice a week. During this recording period the HFD was changed for fresh after each measurement point.

2.7.2 Glucose tolerance test

A glucose tolerance test was carried out the day prior to termination, after measuring blood pressure in the morning. Mice were fasted for 6 hours followed by a bolus glucose (2mg/kg, Sigma) i.p injection. Blood samples were taken prior to glucose administration as baseline and then every 15 mins for 135 mins post-hoc-injection. Blood samples (approximately 3µl) were obtained by creating a small incision in the tail of conscious mice using a needle allowing measurement of blood glucose. The blood glucose was measured using an Acu-Chek Compact Plus glucose meter (Roche, Diagnostics, UK).

2.8 Experimental procedures performed *in vitro* following termination

2.8.1 Preparation of plasma samples

On the day of termination, under isoflurane anaesthesia (2.5%, Abbott Laboratories, UK, in 3L/minute O_2), blood sample was collected by cardiac puncture, using a 25G needle (Therumo Coperation) and 1ml syringe (BD Plastipak, UK) washed with heparin (500 I.U/ml heparin sodium, LEO Pharma, UK) to prevent coagulation. The blood sample was then centrifuged at 2000rpm at 4ºC for 20 min. Plasma (the supernatant) was collected, aliquoted and stored at -80ºC for further analysis.

2.8.2 Homogenisation of kidney samples

One of the two kidneys was frozen in liquid nitrogen whilst the other was protected by RNAlater (Ambion) in the fridge overnight and then stored at -80ºC, until use for RT-qPCR. To prepare for endothelin-1 ELISA and CGRP ELISA, the frozen kidney samples were homogenized. Appoximately 50mg of kidney was homogenised in 5ml/g lysis buffer (10 mM Tris, pH 7.4) using a Qiagen TissueLyser®LT (at 30Hz for 2 mins, Qiagen, UK). Following centrifugation (1,600 g for 15 min at 4°C) the supernatants were collected and used for peptide extraction. To homogenise the kidney samples for noradrenaline ELISA, 50mg of kidney sample was placed in 900µl RIPA buffer (Sigma) containing protease inhibitor cocktail (one tablet in 50ml of buffer, Roche diagnostics), followed by mixing for two hours on an orbital shaker. Finally, the homogenates were centrifuged at 13000rpm for 10 min at 4°C and the supernatant was collected for extraction.

2.8.3 Endothelin-1 & CGRP ELISA

Peptide extraction from plasma or homogenised tissue supernatant is required before measurement using ELISA. This CGRP (rat and mouse) ELISA kit measures both the α and β isoforms (Phoenix Pharmaceuticals Inc, USA). The antibody also has 78.6% cross-reactivity with rat CGRP II, 0.01% cross-link with rat amylin and 0% link with rat calcitonin. The endothelin-1 ELISA was also purchased from Phoenix Pharmaceuticals Inc.

The plasma or supernatant was acidified with an equal amount of buffer A (TFA, 1% trifluoroacetic acid, Phoenix Europe GmbH, Europe), mixed well and centrifuged at

10,000 rpm for 20 min at 4ºC. The SEP-COLUMN containing 200mg of C18 was equilibrated by washing with 1ml of buffer B (60% acetonitrile in 1% of TFA, Phoenix Europe GmbH, Europe), followed by washing three times with 1ml buffer A. The acidified plasma or supernatant was loaded onto the pre-equilibrated C-18 SEP-Column (Phoenix Europe GmbH, Europe), where it was washed with 3ml of buffer A (3 times). Eluant was collected following addition of 3ml of buffer B. Eluant was dried overnight for 24h in a freeze dryer.

Plasma endothelin 1 and CGRP, and endothelin-1 from kidney homogenates were assayed following peptide extraction. Extracted peptide powders were resuspended in 250µl of 1xassay buffer (Phoenix Europe GmbH, Europe). Standards were also prepared using 1xassay buffer to provide concentrations ranging from 0.04–25ng/ml for endothelin-1 and 0.01–100ng/ml for CGRP. The immunoplate is pre-coated with secondary antibody and nonspecific binding sites are pre-blocked. The secondary antibody can bind to the Fc fragment of primary antibody (CGRP antibody or endothelin-1 antibody) whose Fab fragment will be competed for by the biotinylated peptide, and then by peptide standard or the targeted peptide in the samples. The biotinylated peptide interacts with streptavidin-horseradish peroxidase (SA-HRP) which catalyses the substrate solution, producing a yellow colour. The intensity is directly proportional to the amount of biotinylated peptide-SA-HRP complex but inversely proportional to the amount of the peptide in the standard solutions or samples. The reaction was terminated using 100µl/well of 2N HCL and the plate was read in a microtiter plate reader (SpectraMAX 190, Meso Scale Discovery, Maryland, USA; used with SOFTmaxPRO software, version 3.13 ©1999, Molecular Devices Corporation, California, USA) at 450nm.

2.8.4 Noradrenaline ELISA

Supernatants from kidney homogenates were purified according to the manufacturer's instructions (IBL international GMBH, Germany). Briefly, 20µl of each standard, positive control, negative control or the supernatant samples were pipetted into respective wells of the extraction plate (provided with the ELISA kit) which is coated with boronate affinity gel. Samples were extracted with the extraction buffer containing an acylation

reagent. The release buffer (0.1M HCL and colour indicator) was added and the extracted samples were immediately quantified using the ELISA.

The standard concentration ranges from 0ng/ml to 500ng/ml. The wells of the plate were coated with a goat anti-rabbit antibody. The added liquid antibody, directed towards an epitope of an antigen molecule binds to the plate within the incubation time. The antigen of the sample is incubated in the well coated with enzyme conjugated second antibody, directed towards a different region of the antigen molecule. After the substrate reaction, the intensity of the developed colour is proportional to the amount of the antigen. At the end of the experiment, the plate was read in a microtiter plate reader (SpectraMAX 190, used with SOFTmaxPRO software, version 3.13 ©1999, Molecular Devices Corporation, California, USA) at 405nm. Results of samples were determined directly using the standard curve.

2.8.5 Protein assay

The bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, USA) was used. To determine the protein content in cells, 10μl of BSA protein standards (0.1-1.2 mg/ml BSA in distilled water) were added in duplicate into wells in a 96-well plate and 50μl /well of 0.5M NaOH added to these. 10μl of MilliQ water was added to the 50μl of lysed cell samples to maintain a consistent volume in all the wells. The 'blank' was measured in duplicate where it consisted of 10μl of MilliQ water and 50μl of 0.5M NaOH. 200μl of BCA reagent was added which comprised of reagent A (sodium carbonate, sodium bicarbonate BCA detection reagent and sodium tartrate in 0.1N NaOH) and reagent B (4% copper sulphate) in a ratio of 50:1, respectively. The plate was covered and incubated for 30min (37^{o}C) before the absorbance was read using a plate reader (SpectraMAX 190, used with SOFTmaxPRO software, version 3.13 ©1999, Molecular Devices Corporation, California) at 562nm. Protein assay did not carried out for the ELISA experiment in chapter 4.

To determine the protein content of tissue homogenates or plasma, 10μl of standards (0.1-2mg/ml) or homogenate/plasma samples were added into their respective wells. 200μl of BCA reagent was added to each well which consisted of reagent A (sodium carbonate, sodium bicarbonate BCA detection reagent and sodium tartrate in 0.1N

NaOH) and B (4% copper sulphate) in a ratio of 50:1, respectively. The plate was covered and incubated for 30min (37^{o}C) and read at 562nm.

2.8.6 Smooth muscle cell culture

Primary culture of vascular smooth muscle cells (VSMCs) was obtained from mouse aorta explants of WT and RAMP2 TG mice. Mouse aorta was quickly collected under isoflurane anaesthesia (2.5%, Abbott Laboratories, UK, in 3L/minute O_2) and was stored in ice-cold sterilized-PBS. In a cell culture hood, the aorta was opened longitudinally, cut into 2-mm cubes and placed on the surface of a 25-cm^2 culture flask (BD Biosciences). The whole procedure was carried out under sterile conditions. Tissue was incubated in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 20% foetal calf serum (FCS; Sigma) and 1% of (100 U penicillin / 0.1 mg/ml streptomycin) in a 37^{o}C, 5% CO_2 incubator. The flask was placed upright for two hours then was laid down slowly to make sure that the medium was covering all the tissue cubes. When the VSMCs started growing and reached 80% of a confluent monolayer in the flask, they were sub-cultured into a 75-cm flask The VSMC were cultured to third passage before experiments.

To study the proliferation, the VSMCs were trypsinised and placed into 24-well plates at a density of 10^4 cells/well. The cells were kept undisturbed for 24h and the baseline was obtained before addition of drugs by cell counting. Cell counts were performed using haemocytometer. The cells were treated with PBS as control, ATII (10^{-7} M) or ATII (10^{-7} M) with or without AM_{22-52} (10^{-7} M) in DMEM containing 5% FCS for 24, 48 or 72 hours. Cells were trypsinised and cell counts performed for each treatment group.

2.8.7 Tissue processing for histology

Aortic tssues, mesenteric tissues or adipose tissue were fixed in 10% paraformaldehyde (Sigma) for 24 hours to prevent autolysis and necrosis, and to preserve the antigenicity of the tissue. Tissues were processed overnight using a Tissue-Tek VIP Vacuum Infiltration processor (Sakura Tissue Tek VIP 1000), dehydrated using graded alcohol, cleared with xylene (100%) and then infiltrated the tissue with paraffin. Aortic tissue (standing longitudinally upright) and mesenteric tissue

were put into a mould and was embedded with paraffin. It was then set onto a cold panel until the blocks were removed from the mould, approximately 10-15 min later. Paraffin blocks were then stored at room temperature until further use. To prepare slides for staining, 4 µm thick transverse sections were cut from the paraffin blocks using a microtome (Reichert-Jung 2030 Biocut microtome). APE (3-aminopropyletriethoxysilane) -coated slides were mounted with four 4 µm thick sections. The mounted slides were then dried for a minimum of 18h. Slides were stained for haematoxylin and eosin (H&E) and Masson's trichrome.

2.8.8 Haematoxylin and eosin (H & E) staining

The H&E stain: This standard stain uses two separate dyes, hematoxylin (dark purple) staining the nucleus and eosin (orange-pink) staining the cytoplasm and connective tissue. The slices were taken through changes of xylene, alcohol and distilled water to de-paraffinize and re-hydrate sections (table 2.5). The slices were then stained with hematoxylin and rinsed, then placed in water and differentiated in acid alcohol (0.5%, HCL in 70% ethanol), and then placed in the eosin (1% eosin in ethanol). After haematoxylin and eosin staining, slices were dehydrated with alcohol, cleared in xylene and finally covered with a cover-slip using DPX as mounting media, then dried overnight in a fume hood.

Deparaffinize & rehydrate		→	Hematoxylin stain		→	Eosin stain	
Xylene 1	10 mins		Hematoxylin	30 sec		Eosin	3 mins
Xylene 2	2 mins		Rinse			Ethanol 1	2 mins
Ethanol 1	2 mins		Tap water 1	2 mins		Ethanol 2	2 mins
Ethanol 2	2 mins		Tap water 2	2 mins		Ethanol 3	2 mins
Ethanol 3	2 mins		Acid ethanol	10 fast		Xylene 1	2 mins
Ethanol 4	2 mins		Tap water 3	2 mins		Xylene 2	2 mins
Distill water	2 mins		Blot excess water				
Blot excess water							

Table 2.5: Procedures of H&E stain

The sections were analysed for aortic wall width, aortic wall area, aortic minimum wall width, arteriolar diameter and arteriolar wall width. To quantify the aortic wall thickness, radial lines were drawn to determine the distance from internal to external elastic

lamina at 8 sites which were chosen to be 45° apart around the circumference of each aortic section and a mean value and a minimum wall width were obtained (three aortic sections per mouse). To determine the aortic cross-sectional wall area, the perimeters of the internal and external elastic laminas were traced of each section. The area inside each respective perimeter was determined and the difference between these two areas was defined as the aortic wall area (mean of six aortic sections per mouse). To assess the arteriolar diameter from mesenteric tissue, the perimeter of each vessel was traced and the diameter was determined (average of 9 measurements per mouse). To quantify the arteriolar wall width from mesenteric tissue, radial lines were drawn to determine the distance from internal to external elastic lamina at 4 symmetrical sites and a mean value was obtained (average of 9 measurements per mouse). To measure the size of the adipocytes, after H&E staining, 3 pictures were randomly taken from each mouse. Three different people randomly measured 100 cells from each slide (different people started from different corner of each picture). The mean of the measurements from these three people are presented in this thesis.

2.8.9 Masson's trichrome staining

In order to further differentiate between collagen and smooth muscle, Masson's trichrome staining was used. Masson's Trichrome stain uses three dyes selectively: haematoxylin stains the nucleus, beibrich scarlet (Sigma) stains the cytoplasm and muscle and aniline blue stains collagen/fibrin. After the sections were de-paraffinised in xylene and re-hydrated in alcohol, sections were placed in Bouin's solution (a compound fixative, Sigma) at 65^{o}C to intensify the final coloration of the tissue, and then washed in running tap water and rinsed in distilled water (table 2.6). After that, sections were stained with haematoxylin (Sigma) and rinsed, then placed in Phospho solution (phosphomolybdic/phosphotungstic acid solution; volume ratio: phosphomolybdic: phosphotungstic: distilled water = 1:1:2, Sigma), followed by aniline blue staining and rinsed in distilled water. The sections were then placed in 1% acetic acid and dehydrated in ethanol (VWR BDH Prolab International Ltd) and cleared in xylene (VWR BDH Prolab International Ltd). Slides were covered with a coverslip using DPX as mounting media and dried overnight in a fume hood.

Deparaffinize & rehydrate		→	Masson trichrom stain	
Xylene 1	10 mins		Bouins solution	15 mins
Xylene 2	2 mins		cool and wash out the yellow color in water	
Ethanol 1	2 mins		Hematoxylin	5 mins
Ethanol 2	2 mins		wash in the water for 5 mins and rinse in	
Ethanol 3	2 mins		Biebrich scarlet	5 mins
Ethanol 4	2 mins		Rinse in distill water	
Distill water	2 mins		Phospho solution	5 mins
Blot excess water			Aniline blue	5 mins
			Ethanol acid	2 mins
			Ethanol	5 mins
			Xylene	5 mins

Table 2.6: Procedures of Masson Trichrome stain

With the Masson's trichrome stain, the medial thickness and perivascular fibrosis thickness were measured. To assess the medial thickness and the perivascular fibrosis thickness, the perimeters of lumen, the boundary between media and fibrosis (between the red and blue) and the vessel were traced. The diameter of every respective perimeter was obtained and the different distance between the lumen and the media was reported as the medial thickness while the difference between the media and the fibrosis was defined as the perivascular fibrosis thickness (average of six measurements per mouse).

2.8.10 Immunohistochemistry

The principle of immunohistochemical staining uses a primary antibody raised against the antigen of the protein being investigated, and a biotinylated second labeled antibody raised against the primary antibody. The biotinylated secondary antibody is coupled with steptavidin horseradish peroxidase, which reacts with 3, 3' diaminobenzidine (DAB) to produce a brown staining. This process is termed as DAB staining.

The presence of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1) was examined by immunohistochemistry in thoracic aorta. For VCAM-1 staining, isolated aortic samples were immersed in OCT compound (Cryo-M-Bed, Bright, UK) snapped frozen in liquid nitrogen and stored at -80ºC until further analysis. A cryostat cut frozen aortic cross 10 µm sections where they were fixed in acetone for 10 min before staining or storage at -20ºC. On the staining day, slides were warmed and dried at room temperature before applying blocking serum (3% of goat serum, from the same species in which the secondary antibody is made, Dako, UK) followed by incubation with a rabbit anti-human VCAM-1 antibody at room temperature for one hour (1:50 dilution; sc-8304, Santa Cruz Biotechnology, USA) and visualised with Envision plus System-HRP (DAB; Dako Cytomatic, Denmark). A negative control was also used in all the staining procedures to verify the specificity of the labeling of the target antigen by the primary antibody. The absence of specific staining in the negative control tissue confirmed the lack of antibody cross-reactivity to the cells/cellular components. Staining was stopped by immersing the slides in running tap water for 5 minutes. In order to counterstain the sections, slides were immersed in haematoxylin (Sigma) for 1 minute followed by washing in running tap water for a further 5 minutes. Slides were then rehydrated through a series of ethanol and xylene washes before mounted with pertex xylene based mountant (Sigma). Slides were covered with a coverslip.

For MCP-1 expression, paraffin aortic sections (5µm) were cut and dried before staining. Antigen unmasking was achieved by incubating the slides in Tris-EDTA (pH7.5) and microwaving for 5 min at 500W. Slides were allowed to cool down after which they were immunostained with goat anti-MCP-1 antibody (1:50, sc-1785, Santa Cruz Biotechnology, USA). The MCP-1 staining was amplified using the VECTASTAIN Elite ABC kit (pk-6105, Vector Laboratories) and visualised by DAB staining. Then slides were counterstained and dehydrated as above.

The percentage of the overall area of brown stained sites representing VCAM-1 or MCP-1 compared to the total area of the field of vision was quantified by Image Pro Plus software or Cell^P Olympus software. The mean area percentages were

calculated for each section by measuring three sites. This was done on two tothree sections from each group.

2.8.11 Myography

First-order mesenteric artery branches (approximately 100-200µm diameter) were isolated in a modified ice cold Krebs'-Henseleit (118mM NaCl, 24mM $NaHCO_3$, 1mM $MgSO_4$, 4mM KCl, 0.5mM NaH_2PO_4, 5.5mM glucose, and 2.5mM $CaCl_2$, all salts from Sigma, UK). They were cleaned of fatty tissue, mounted and normalized to normal peripheral arterial tension (13.3kPa) on a wire myograph (DMT 610M or 620, Danish Myo Technology, Denmark) using 25µm tungsten wire. Vessels were maintained throughout the experiment in 37°C Krebs' solution gassed with air/5% CO_2. The maximum contraction was assessed using 2 minute incubations in 'high (80mM) K' solution (38mM NaCl, 24mM $NaHCO_3$, 1mM $MgSO_4$, 80mM KCl, 0.5mM NaH_2PO_4, 5.5mM glucose, and 2.5mM $CaCl_2$). Endothelial function was assessed using carbachol (10µM diluted in water, Sigma, UK), where a relaxation of >60% over 2 minutes was regarded as an endothelial-intact tissue. For contraction studies, tissues were contracted with accumulative doses of phenlyephrine (10nM, Sigma, UK) For relaxation studies, tissues were sub-maximally pre-constricted with phenylephrine then given accumulative concentrations of CGRP (cambridge bioscience, UK) and carbachol with % relaxation at 3 minute intervals were determined. Time and vehicle controls were collected for all vessels to ensure comparative functioning. Results from multiple vessels per mouse were averaged to produce each n. The pEC_{50} was calculated for each mouse using curve fitting software (GraphPad Prism 5).

2.8.12 Real-Time Quantative Polymerase Chain Reaction (RT-PCR)

RT-qPCR is a technique to quantitatively detect gene expression through creation of cDNA transcripts from mRNA. Total RNA was extracted from approximately 30mg of aorta samples (collected immediately after termination and stored in RNAlater (Ambion)) using a commercial kit, where tissue was disrupted by the phenol-based reagent QIAzol (Qiagen RNeasy Microarray tissue mini Kit, Qiagen, USA) and mechanically lysed (Qiagen's TissueLyser LT). The homogenate was then treated with chloroform (Sigma) before being centrifuged, purified and eluted using a spin column based

procedure. RNA quality was then assessed using a Nanodrop Spectrophotometer which provides a ratio of A260/A280 indicating whether there is any ethanol and phenol contamination; a ratio between 1.8 and 2.2 was considered as good purity. 500ng of extracted RNA was used for the reverse transcription to create cDNA. This was performed using the high capacity RNA to cDNA, reverse transcription enzyme kit with RNAase inhibitor (Applied Biosciences) on a thermal cycler (Applied Biosystems) set to temperatures recommended in the manufacturer's instructions.

To perform the RT-qPCR, stock cDNA was diluted 1:5 in RNAse/DNAse free water (Fisher, UK). PCR was conducted in 10µl reactions using SYBR-green based PCR mix (Sensi-Mix, SYBR-green No ROX, Bioline), together with 1µl cDNA, 0.4µl of each primer (10µM) and 3.6µl RNAse/DNAse free water. This was added to 100 well gene discs (Qiagen) by an automated robot (CAS1200, Corbett Robotics). Discs underwent PCR (hold: 10 minute at 95^{o}C; cycling: 45 cycles: 10s at 95^{o}C, 15s at 57^{o}C and 5s at 72^{o}C; melt: 68-90^{o}C) in a Corbett Rotorgene 6000. Data was collected as copies/µl and standardised against B2M and HPRT1 expression using a normalisation factor produced by GeNorm software (v3.4). Primers were purchased from Sigma (UK) as below:

Gene	Forward Primer	Reverse primer	Amplicon Size
CGRP	AGCAGGAGGAAGAGCAGGA	CAGATTCCCACACCGCTTAG	71
TRPV1	CAACAAGAAGGGGCTTACACC	TCTGGAGAATGTAGGCCAAGAC	77
SOD	CTGGACAAACCTGAGCCCTA	GATAGCCTCCAGCAACTCTCC	61
HO-1	GGTCAGGTGTCCAGAGAAGG	CTTCCAGGGCCGTGTAGATA	70
eNOS	GACCCTCACCGCTACAACAT	GTCCTGGTGTCCAGATCCAT	62
ET-1	GTGTCTACTTCTGCCACCTGGACAT	GGGCTCGCACTATATAAGGGATGAC	474
VCAM-1	TGGTGAAATGGAATCTGAACC	CCCAGATGGTGGTTTCCTT	86
Nox 2	TGCCAACTTCCTCAGCTACA	GTGCACAGCAAAGTGTTGG	73
HPRT1	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC	90
B2M	CCTGCAGAGTTAAGCATGCC	GATGCTTGATCACATGTCTCG	74

Table 2.7: primers of each gene and amplicon size (base pairs) used for Q-PCR.

2. 9 Data interpretation and statistical analysis

Results are shown as mean ± standard error of the mean (SEM) except for histology (as mentioned in the section 2.8.8). Statistical analysis was undertaken with one way and two way ANOVA + Bonferroni's post-hoc t test, the unpaired Student t test of mean area under curve as appropriate, using Graph Pad Prism version 5.02 (Graph Pad Software, USA). A significant difference from the null hypothesis was accepted when $p \leq 0.05$.

CHAPTER 3: THE PROTECTIVE ROLE OF SMOOTH-MUSCLE TARGETED RAMP2 TG MICE IN ATII INFUSION MODEL.

3.1 Introduction

As discussed in the introduction, AM is a member of the CGRP family of peptides (Brain *et al*., 2004). AM is a potent vasodilator peptide which is expressed in cardiovascular tissue, including vascular wall and heart (see general introduction for details). Levels are elevated in patients with different cardiovascular conditions (Ishimitsu *et al*., 1994, Kato *et al*., 1996, Suzuki *et al*., 2004 and Oyar *et al*., 2011). Therefore, it is believed to be a predictor of future cardiovascular events (Nishida *et al*., 2008). Despite this, studies in AM transgenic and heterozygous knockout (partially knocking out 50% of the AM gene, in this chapter, AM KO mice refer to this heterozygous knockout mice unless stated) mice have demonstrated a protective role of AM (Kato *et al*; 2005, Bell *et al*., 2010). Thus increased attention has been focused on AM and its importance in cardiovascular events. However, little is known about the involvement of its receptor components.

AM acts via a unique family of GPCRs, composed of CLR and RAMP1, RAMP2 or RAMP3. Heterodimerization of the CLR with RAMP1, RAMP2 and RAMP3 produces CGRP, AM1 or AM2 receptors, respectively (McLatchie *et al*., 1998). AM can act on CGRP, AM1 and AM2 receptors, whilst CGRP primarily activates the CGRP receptor. However, CLR together with RAMP2 forms the functional receptor AM1, which is primary target of AM and can be antagonised by the AM peptide fragment AM_{22-52}. It should be noted that the AM-related peptide intermedin also is an agonist at all the receptors complexes. Receptors for CGRP and AM are widely distributed but the RAMP2 is expressed to a greater degree than RAMP1, producing functional AM1 receptors (Hay *et al*., 2006). Indeed RAMP2 is found to be expressed in most tissue types, such as rat lung, liver, spleen by northern blot analysis, and also be expressed in human aortic smooth cells, human aortic endothelial cells determined by qPCR (Hay *et al*., 2006). RAMP2 expression has been studied not only in healthy individuals, but also in patients with a range of cardiovascular conditions. For instance, increased gene expression of RAMP2 is reported in left ventricular hypertrophy and even to a higher level in heart failure (Nishikimi *et al*., 2003). Therefore, the levels of RAMP2 expression,

as a consequence of functional AM1 receptor may be related to the development and progression of some cardiovascular diseases. However receptor activities and signalling are different depending on the vascular cell type and beds (Brain and Grant, 2004). As a consequence, little is known regarding the cardiovascular relevance of the functional AM1 receptors *in vivo*.

Previously in our group, we have developed and investigated smooth muscle cell specific, overexpressing RAMP2 TG mice (Tam *et al*., 2006). It was the first time, to our knowledge, the AM1 receptor rather than the peptide itself, has been targeted for *in vivo* study. In our previous findings, the RAMP2 TG mice showed similar profiles in baseline blood pressure and heart rate, recorded by tail cuff in conscious mice or by carotid artery cannulation of anaesthetised mice. Interestingly the RAMP2 TG mice showed increased sensitivity to AM and subsequently an increase in functional AM1 receptor was shown, as i.v. administration of AM induced a greater hypotensive response in RAMP2 TG mice. Furthermore studies with isolated aortic vessels and mesenteric microvessels showed increased responses to AM in RAMP2 TG compared with WT tissue, while both RAMP2 TG and WT mice displayed similar responses to CGRP. The combined *in vivo* and *in vitro* data indicates the potentially important role of RAMP2 in the sensitivity of AM-induced hypotensive responses via the selective up-regulation of the functional AM1 receptor. This implies that the potency of the anti-hypertensive effect of AM can be affected by up-regulating levels of RAMP2.

3.2 Hypothesis and aims

3.2.1 Hypothesis

RAMP2 TG mice will be protected against hypertension and vascular hypertrophy induced by ATII.

3.2.2 Aims

The aims of this chapter were:

- Investigate WT and RAMP2 TG mice in the ATII-induced hypertensive model
- To measure blood pressure by tail cuff plethysmography
- To measure vascular hypertrophy in the aorta by histology
- To investigate vascular inflammation by immunohistochemistry
- To assess proliferative modulatory effects of RAMP2 on VSMC from mouse aorta.

3.3 **Results**

3.3.1 Effect of ATII on body weight, blood pressure and cardiac hypertrophy in WT and RAMP2 TG mice

Both WT and RAMP2 TG mice (25-35 g, 12-16 weeks old, matched for age and sex) were treated with ATII (0.9mg/kg/day) or vehicle (0.01% 10mM acetic acid in saline) for 13 days by mini-pump infusion. Body weight was monitored on day 0, 3, 6, 9 and 13. Figure 3.1 shows that the body weight remains stable for both WT and RAMP2 TG mice throughout the entire period (n=9-12).

Figure 3.2 demonstrates blood pressure measured by tail cuff plethysmography before surgery (taken as baseline) and day 3, 6, 9 & 13 post-hoc mini-pump implantation. Fig 3.2a indicates both WT and TG have similar systolic blood pressure when they were treated with vehicle. However ATII (0.9mg/kg/day) increased the systolic blood pressure in a similar manner in both WT and RAMP2 TG mice, and became significantly different on day 13 when compared to vehicle-treated mice. Fig 3.2b demonstrates diastolic blood pressure and exhibited the same trend as systolic blood pressure. Basal blood pressure did not differ between WT (117.8±3.2mmHg) and RAMP2 TG mice (119.7±4.2mmHg; n=8-16), as previously reported. Again, ATII (0.9 mg/kg/day) increased mean arterial blood pressure over 13 days in both WT and RAMP2 TG mice (Fig 3.2c).

Fig 3.3 displays that ATII (0.9mg/kg/day for 13 days) also induced significant and similar left cardiac and ventricular hypertrophy in both WT and RAMP2 TG mice when compared to the vehicle treated mice (Fig 3.3a and 3.3b).

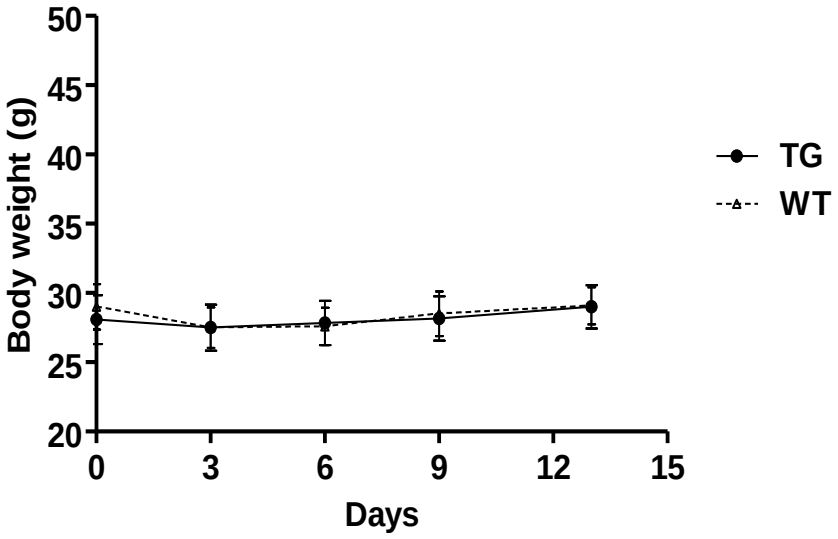


Figure 3.1. The body weight of WT and RAMP2 TG mice over the 13 days implantation peroid. N=9-12. There is no significant difference between WT and TG. Statistics by student's unpaired tests.

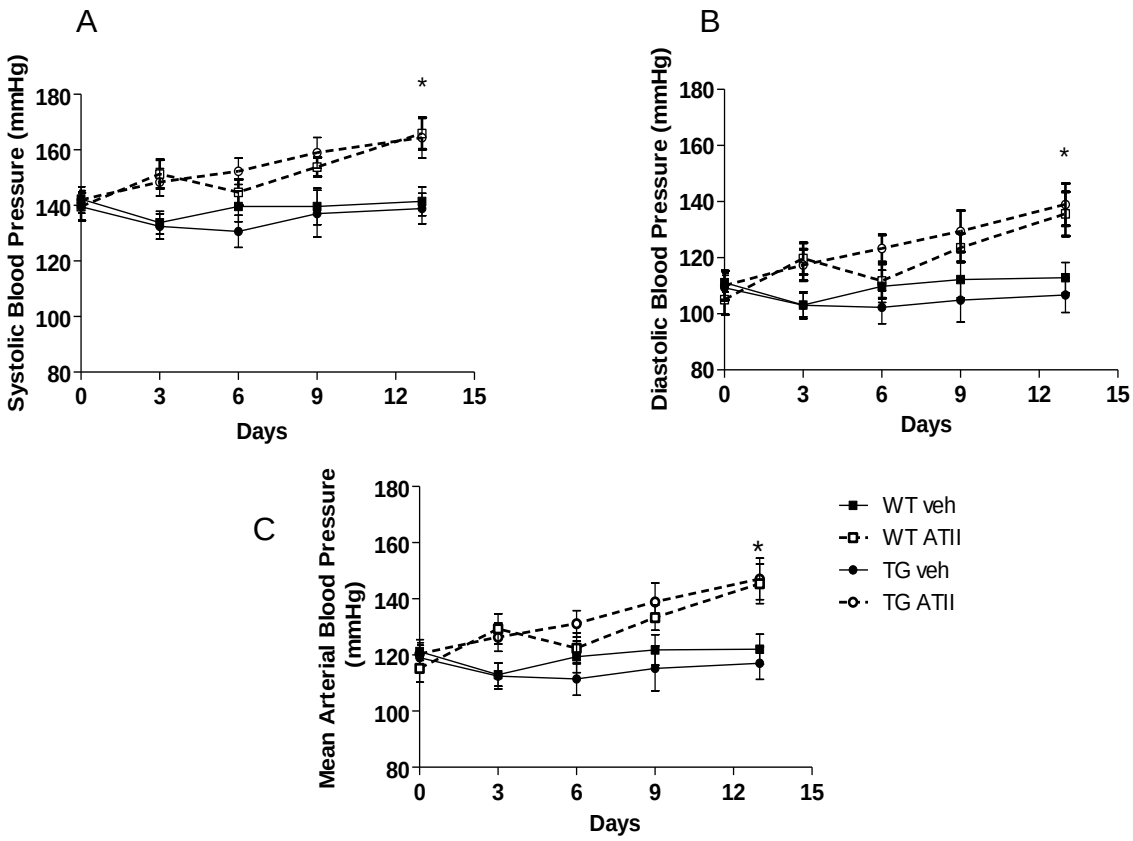


Figure 3.2. Effect of ATII (0.9 mg/kg/day for 13 days) on blood pressure in WT and RAMP2 TG mice. A. Systolic blood pressure, B. Diastolic blood pressure and C. Mean arterial pressure measured in conscious mice by tail cuff plethysmography, n=8-16. Data are presented as mean ± SEM. * $p<0.05$ WT and TG mice after ATII treatment compared to their vehicle-treated mice respectively. Statistics by two-way ANOVA with Bonferroni post-hoc-tests

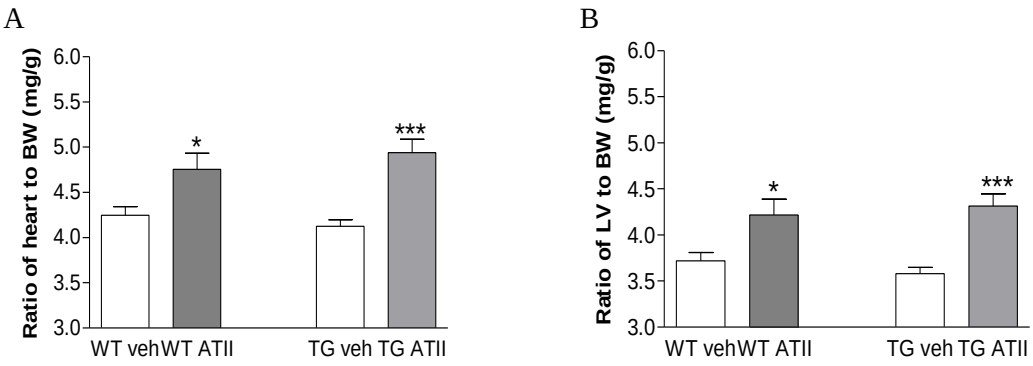


Figure 3.3. Effect of ATII (0.9 mg/kg/day for 13 days) on cardiac hypertrophy in WT and RAMP2 TG mice. (A) Heart to body weight (BW) ratio, (B) Mouse left ventricle (LV) to body weight (BW) ratio, n=10-12. Data are presented as mean ± SEM. * $p<0.05$ and *** $p<0.001$ when WT and TG mice after ATII treatment were compared to their vehicle-treated mice respectively. Statistics by two-way ANOVA with Bonferroni post-hoc-tests

3.3.2 Effect of ATII on vascular hypertrophy in WT and RAMP2 TG mice

Aortic sections were collected from both WT and RAMP2 TG mice after 13 days infusion either with ATII (0.9mg/kg/day) or vehicle (0.01% of 10mM acetic acid in saline). Aorta tissues were then processed into paraffin blocks. 5μm of aortic sections were used for haemotoxylin and eosin (H&E) staining and Masson's Trichrome staining. Vascular hypertrophy was determined by histological assessment using both staining methods.

Fig 3.4 displays representative H&E staining images of aortic wall at 20X and 100X magnification. In the images, haematoxylin stains the nuclei as blue while the eosin Y stains the other components as pink/red. Although, both WT and RAMP2 TG mice demonstrated similar hypertension when compared to the vehicle-treated mice, the WT ATII-treated mice did show an increase in aortic wall width when compared to WT vehicle-treated mice. Such differences were not seen between ATII-treated and vehicle-treated RAMP2 TG mice. Indeed, Fig 3.5a and 3.5b indicate that ATII caused hypertrophy of the aorta in WT mice, this was not observed in RAMP2 TG mice. Analysis of H&E-stained cross-sections revealed a significant increase in thickness of aortic wall of ATII-treated WT mice as compared to the vehicle-treated WT mice; such a difference was not seen in the RAMP2 TG mice. Furthermore, ATII also induced a 1.5-fold increase in aortic wall area when compared to the vehicle-treated WT mice ($p<0.01$; n=8-9 sections from 3 mice in each case); again ATII-treated RAMP2 TG mice displayed no significant difference from vehicle-treated RAMP2 TG mice regarding the aortic wall area.

Fig 3.6 demonstrates the representative images for Masson's trichrome staining with aortic wall at 20X and 100X magnification. Masson's trichrome staining is a three-colour staining protocol to reveal the muscle fibers as red (cytoplasm as light red or pink), the collagen/fibrin and bone as blue, and cell nuclei as purple. With Masson's Trichome staining, it displays more convincingly that the smooth muscle layer in the ATII-treated WT mice is much thicker than the vehicle-treated WT mice. Such a difference is not seen between ATII-treated RAMP2 TG mice and vehicle-treated RAMP2 TG mice. Fig 3.7 indicates the ATII caused no significant difference in fibrosis/collagen thickness

between different treatment groups (Fig 3.7a). However, significant differences were observed in medial thickness between the ATII-treated WT and vehicle-treated WT mice, between ATII-treated WT mice with ATII-treated RAMP2 TG mice (Fig. 3.7b). Furthermore, significant increases were also obtained in aortic wall area when comparing ATII-treated WT with vehicle-treated WT mice, and comparing ATII-treated WT mice with ATII-treated RAMP2 TG mice (Fig 3.7c).

Due to such a significant differences being detected with the aortic tissue, it was of interest to investigate if there was an effect in much smaller vessels, such as mesenteric arteries. Therefore, mesenteric tissues were stained with H&E and analysed as above. Fig 3.8 demonstrates that ATII caused no significant difference in mesenteric arteriolar vessels between ATII-treated and vehicle-treated mice for both WT and RAMP2 TG mice. Measurements were carried out on H&E staining.

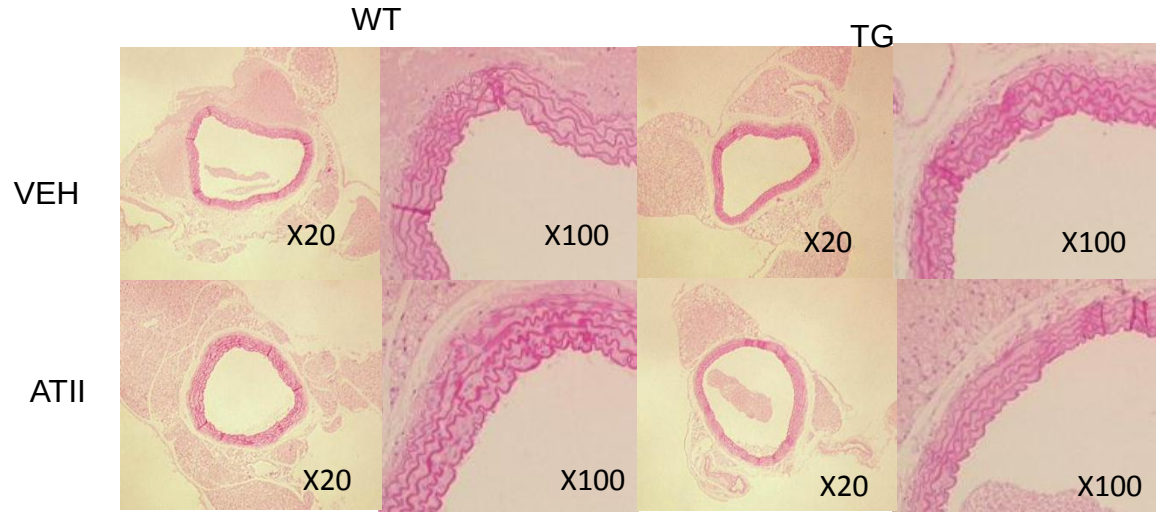


Figure 3.4: Effect of ATII (0.9 mg/kg/day for 13 days) compared with vehicle (0.01% 10 mM acetic acid in saline) on vascular hypertrophy in WT and RAMP2 TG mice. Representative H&E staining of thoracic aorta wall at 20x and 100x magnification as indicated.

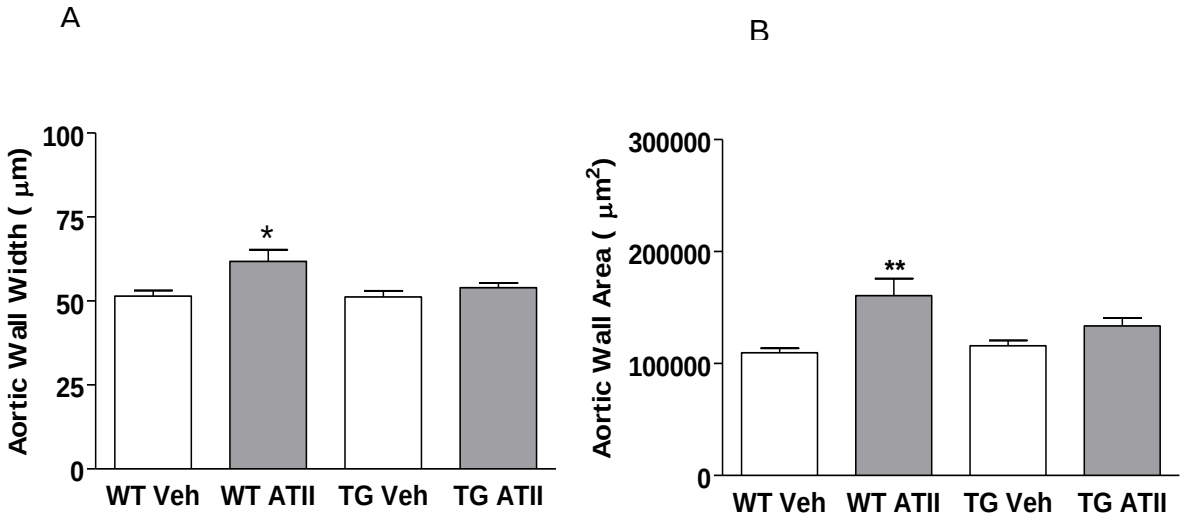


Figure 3.5: Effect of ATII (0.9 mg/kg/day for 13 days) compared with vehicle (0.01% 10 mM acetic acid in saline) on vascular hypertrophy in WT and RAMP2 TG mice. (A) aortic wall width (n=7-9 sections from 3 mice in each case) and (B) aortic wall area (n=7-9 sections from 3 mice in each case). Data are presented as mean ± SEM. *$p<0.05$ and **$p<0.01$ when WT ATII-treated mice were compared to the WT vehicle treated. Statistics by two-way ANOVA with Bonferroni post-hoc-tests

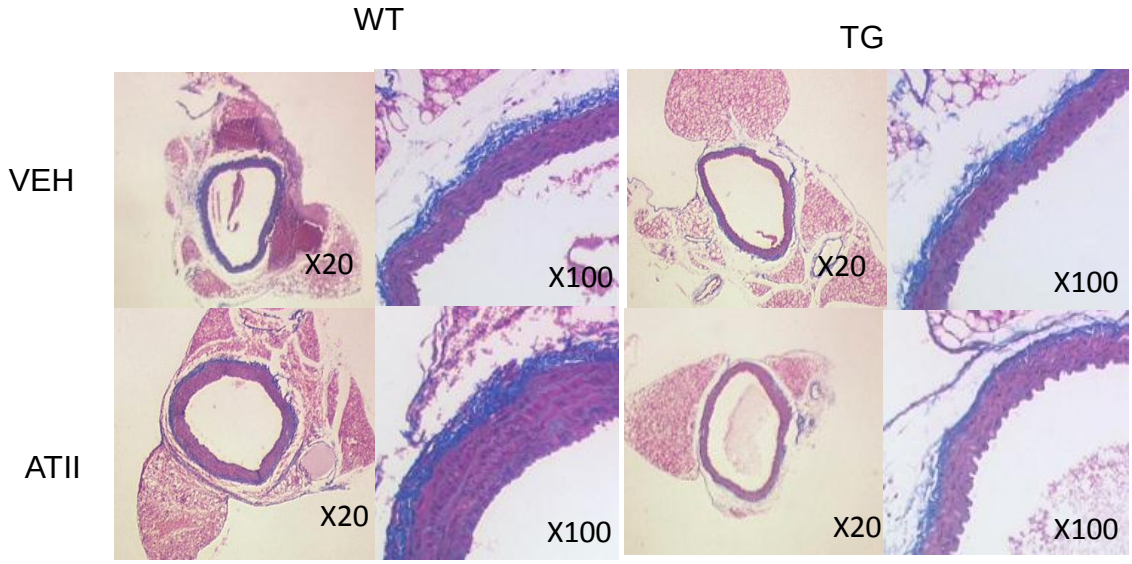


Figure 3. 6: Effect of ATII (0.9 mg/kg/day for 13 days) compared with vehicle (0.01% 10 mM acetic acid in saline) on vascular hypertrophy in WT and RAMP2 TG mice. Representative Masson's Trichrome staining of thoracic aorta wall at 20x and 100x magnification as indicated. Dark pink shows the staining of muscle, the blue shows collagen fibers & fibrin, while the purple shows the nucleus.

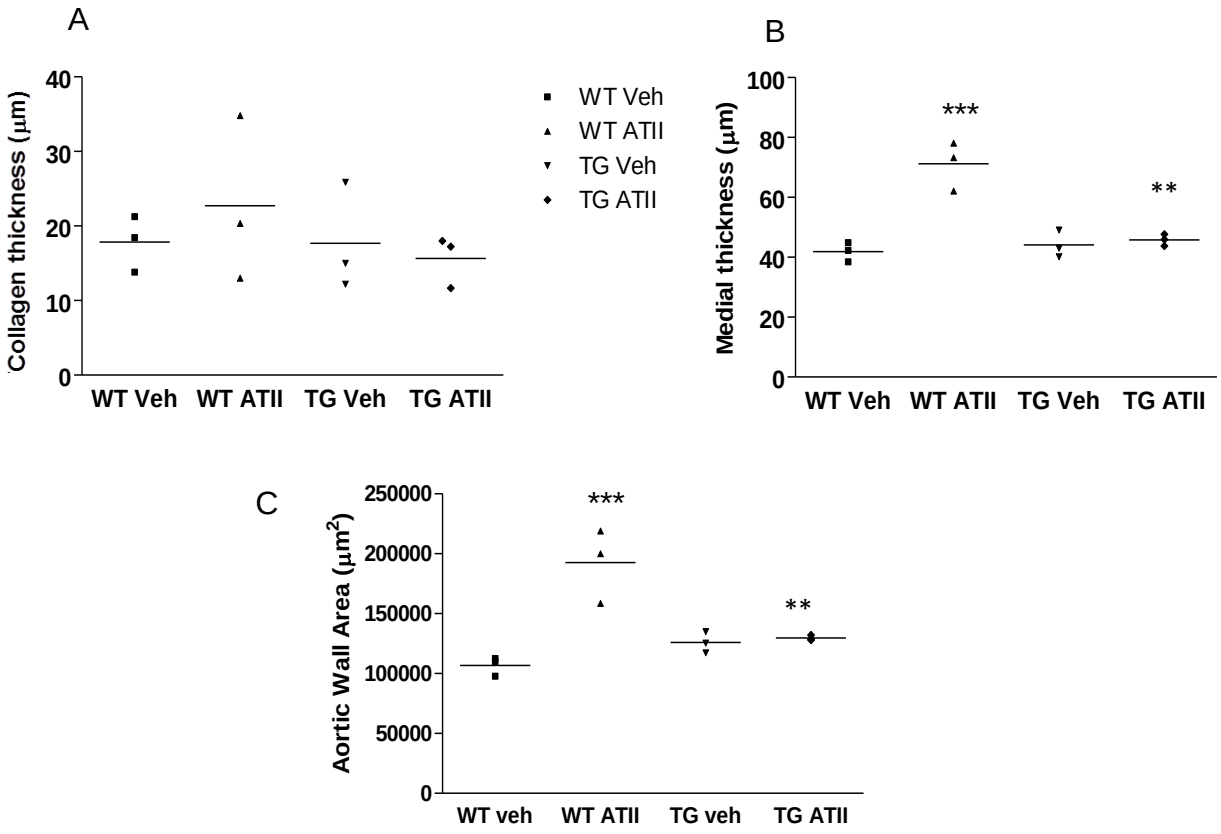


Figure 3.7: Effect of ATII (0.9 mg/kg/day for 13 days) compared with vehicle (0.01% 10 mM acetic acid in saline) on vascular hypertrophy in WT and RAMP2 TG mice. (A) Thickness of collagen, (B) medial thickness in the aortic wall right; and (C) aortic wall area. n=8-9 sections from 3 mice in each case. Data are presented as mean ± SEM. ** $p<0.01$ when the RAMP2 TG ATII-treated mice were compared with the WT ATII-treated mice. ***$p<0.001$ when WT ATII-treated mice were compared with the WT vehicle-treated mice. Statistics by two-way ANOVA with Bonferroni post-hoc-tests

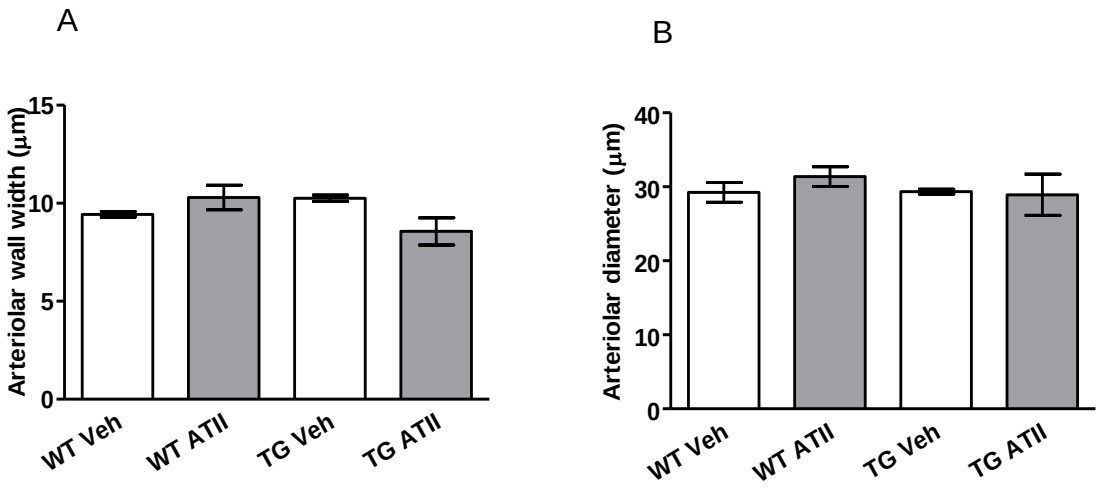


Figure 3.8: Effect of ATII (0.9 mg/kg/day for 13 days) compared with vehicle (0.01% 10 mM acetic acid in saline) on mesenteric arteriolar wall width (A) and arteriolar diameter (B). The whole mesenteric tissue was embedded into a paraffin block and cross sections of the whole block were collected. Therefore the mesenteric arteriole can be from any order. Data are expressed as mean±SEM, n=2-3 sections from 3 mice. Statistics by two-way ANOVA with Bonferroni post-hoc-tests

3.3.3 Effect of ATII on vascular inflammation in WT and RAMP2 TG mice

To examine the expression of vascular inflammatory markers on the aortic wall, immunohistochemical staining of VCAM-1 and MCP-1 was performed.

For VCAM-1 staining, fresh isolated aortic sections (10μm) were stained with rabbit anti-human VCAM-1 antibody (1:50) and positive staining was visible as brown. Fig 3.9a reveals the representative images for VCAM-1 staining by immunohistochemistry on aortic wall at 20x and 650x magnification. A negative control was carried out using the same staining protocol but with no primary antibody incubation. The pictures were taken at 20x magnification. Staining was quantified by comparing the area of the brown staining to the area of the whole aortic area. As indicated in Fig 3.9b, ATII significantly ($p<0.001$) increased the VCAM-1 expression on the aorta in the WT mice when compared to the vehicle-treated WT mice. It was also significantly increased in the WT mice after ATII treatment when compared with the RAMP2 TG.

For MCP-1 expression, paraffin aortic sections (5μm) were immunostained with goat anti-MCP-1 antibody (1:50) and positive staining was also shown as brown. Fig 3.10a reveals the representative images for MCP-1 staining by immunohistochemistry of the aortic wall at 100X and 400X magnification. The negative control (same method as VCAM-1 negative control, where aortic sections were stained as others but without incubating with primary antibody) was also displayed at 100x magnification. As in the image of the aortic section from ATII-treated WT mice, the red arrow shows the positive staining on the endothelial cells. Following the assessment of the quantification showed in Fig 3.10b, ATII significantly ($p<0.001$) increased the MCP-1 expression on the aorta in the WT mice when compared to the vehicle-treated WT mice. In contrast, no difference is seen in the RAMP2 TG mice between ATII and vehicle treatment. Thus, a significant difference is observed when the WT mice were compared with the RAMP2 TG mice after ATII treatment for 13 days.

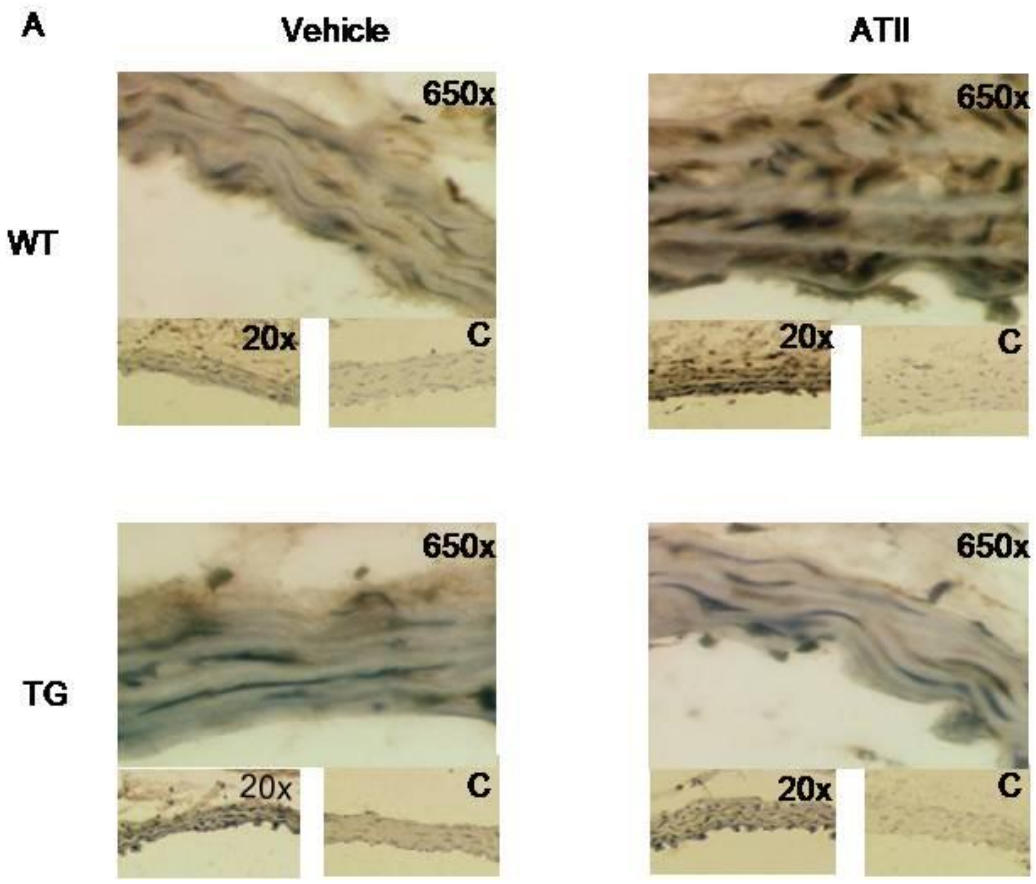
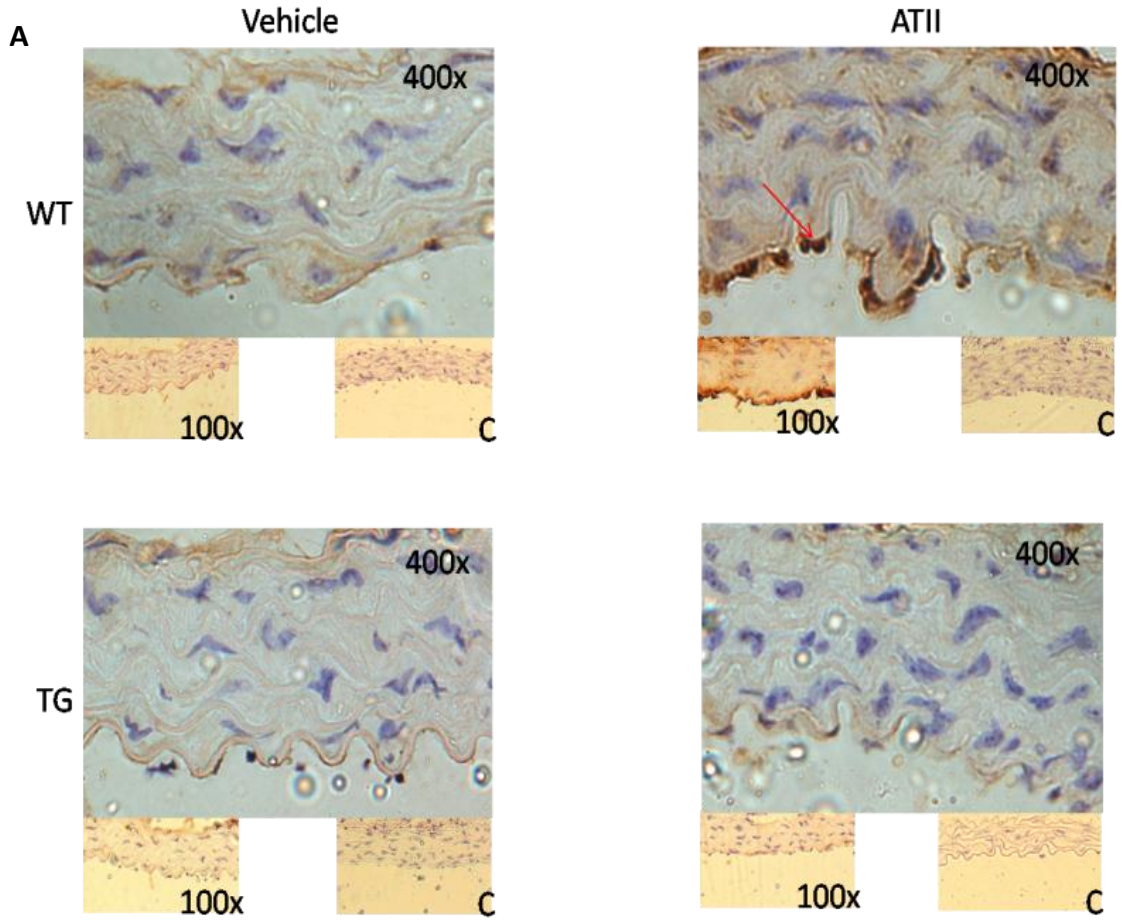


Figure 3.9: Effect of ATII (0.9 mg/kg/day for 13 days) on the vascular inflammatory marker VCAM-1 expression in aortic wall in WT and RAMP2 TG mice. (A) Representative immunohistochemical staining at 20x and 650x, and negative control (B) Percentage area of VCAM-1 (n=3-6) expression compared with total area of the aorta. Data are presented as mean ± SEM. *** $p<0.001$ WT ATII-treated mice were compared with WT vehicle-treated mice. ### $p<0.001$ WT ATII-treated mice were compared with TG ATII-treated mice. Statistics by two-way ANOVA with Bonferroni post-hoc-tests



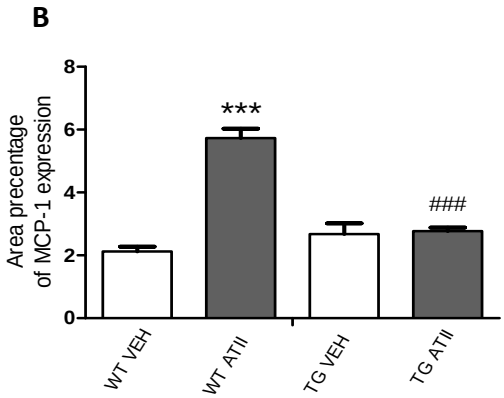


Figure 3.10: Effect of ATII (0.9 mg/kg/day for 13 days) on MCP-1 expression in aortic wall (red arrow: positive MCP staining on the endothelial cell) in WT and RAMP2 TG mice. (A) Representative staining at 100x and 400x, and negative control (c). (B) Percentage area of MCP-1 (n=8-9 section from 3 mice for each group) expression to total area of the aorta. Data are presented as mean ± SEM. *** $p<0.001$ WT ATII-treated mice were compared with WT vehicle-treated mice. ### $p<0.001$ compared WT ATII-treated with TG ATII-treated mice. Statistics by two-way ANOVA with Bonferroni post-hoc-tests

3.3.4 Effect of ATII and AM_{22-52} on WT and RAMP2 TG vascular smooth muscle cells

To determine the effect of RAMP2 overexpression on cell proliferation, studies were carried out in cultured VSMCs from aortic explants in both WT and RAMP2 TG mice. Using the explant technique, VSMCs were cultured upto the third passage before the cells were used for experiments. Cell proliferation was assessed by cell counting using the haemocytometer after trypsinisation and placed into 24-well plates at a density of 10^4 cells/well for 1 to 3 days. Fig 3.11 reveals that the VSMC from RAMP2 TG mice grew significantly slower than WT cells from as early as day 1.

Then to investigate the effect of ATII on the VSMCs from WT and RAMP2 TG mice, VSMCs were stimulated with ATII (10^{-7} mol/L) for 1 to 3 days For this procedure, the cells were kept undisturbed for 24 hours prior to treatment Furthermore, the possible involvement of endogenous AM in VSMC growth was investigated using the AM1 receptor antagonist AM_{22-52} (10^{-7} mol/L). As indicated in Fig 3.12, ATII induced significant increases in the cell number in WT (Fig. 3.12a) as early as 24h, but not in TG cells (Fig 3.12b). Interestingly, the antagonist AM_{22-52} was found to significantly enhance the growth of RAMP2 TG cells in the presence of ATII, as shown in Fig 3.12b. But such enhancement induced by AM_{22-52} was not observed with WT cells (Fig 3.12a). Results are also depicted as a ratio of proliferation induced by ATII + AT_{22-52} alone, to that with ATII alone (Fig 3.12c).

Initially, it was considered that protein content would match the cell proliferation results. Therefore we carried out the protein assay on day three of ATII treatment after determination of the cell numbers. Protein content together with cell number in both WT and RAMP2 TG mice (Fig 3.14) were determined after 3 days of treatment with ATII (10^{-7} mol/L). Fig 3.14a indicates that ATII significantly increases cell proliferation in WT cells when compared to vehicle-treated cells. But such a difference was not seen with RAMP2 TG cells. However, the protein content in ATII-treated WT cells is increased but not significantly, which may be due to low n number for this protein assay method.

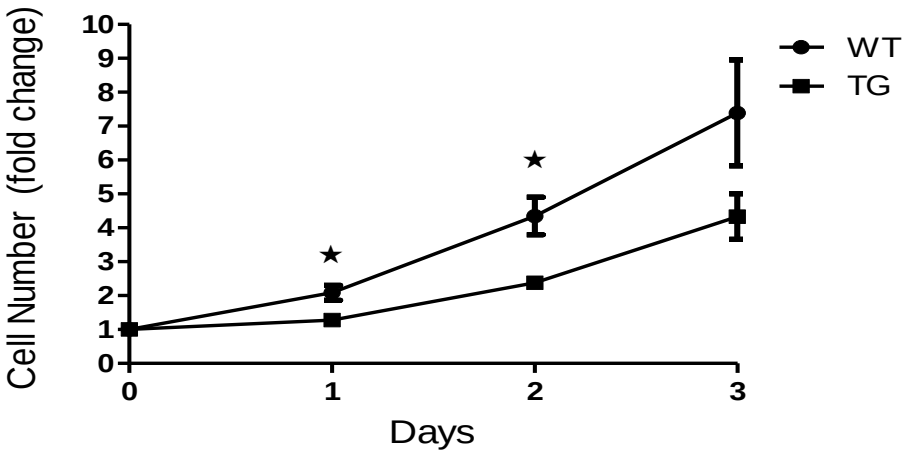


Figure 3.11: Growth of VSMC of WT and TG mice in DMEM supplemented with 5% FCS. Each point presents the mean of three separate experiments run in triplicate on three separate cultures. Data are presented as mean ± SEM. *$p<0.05$ compared to the RAMP2 TG mice at the same time point. Statistics by student's unpair T-test.

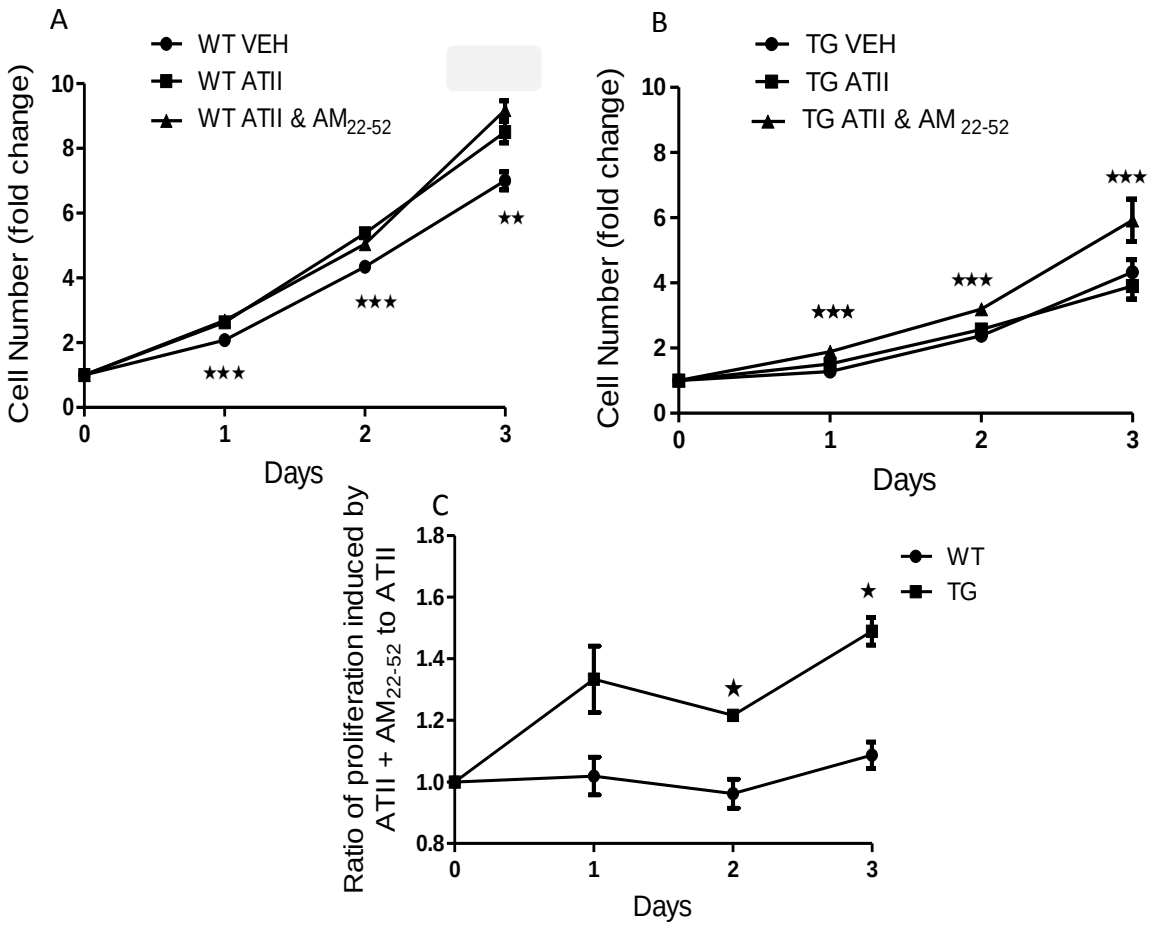


Figure 3.12: Effect of ATII in the present or absence of AM_{22-52} compared with PBS on the growth of VSMC cultured from WT and TG mice. Cell numbers were determined at 24, 48 and 72 hours. (A) WT VSMC were treated with PBS or ATII (10-7 mol/L) ± AM_{22-52} (10-7 mol/L); (B) TG VSMC were treated with PBS or ATII (10-7 mol/L) ± AM_{22-52} (10-7 mol/L); (C) Ratio of proliferation induced by ATII + AM_{22-52} compared to ATII. Each point presents the mean of three separate experiments run in triplicate on three separate cultures. Data are presented as mean ± SEM. In Fig A **p<0.05 and *** p<0.001 WT veh cells were compared with WT ATII-treated cells. In Fig B, *** p<0.001 TG ATII cells were compared with TG ATII + AM_{22-52} cells. In Fig C, *p<0.05 VSMC from WT mcie were compared with those from TG mice. Statistics by two-way ANOVA with Bonferroni post-hoc-tests or T-test.

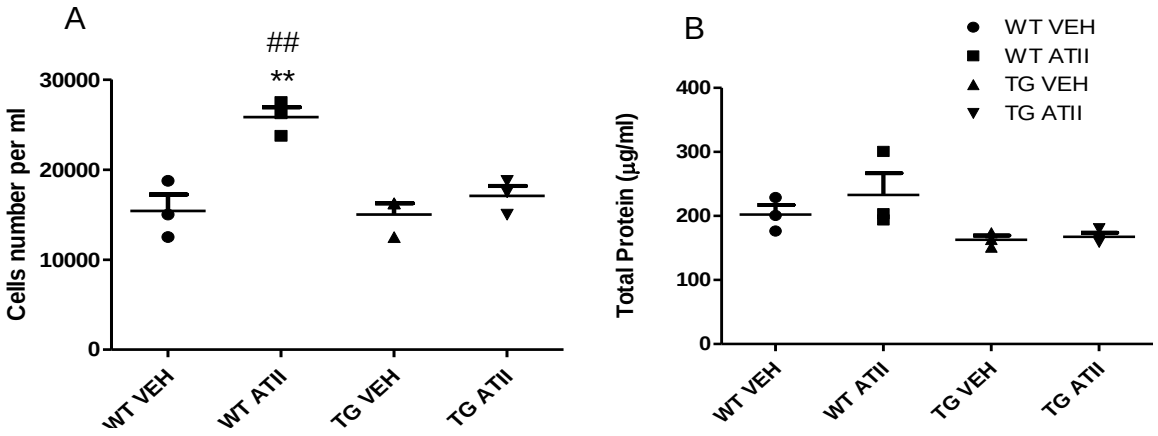


Figure 3.13: Effect of ATII on the growth of VSMC cultured from WT and TG mice. Cell numbers were determined at 72 hours post-hoc treatment. (A) total cell number and (B) Protein content after treatment either with ATII or PBS for 72 hours. Each point presents the mean of three separate experiments run in triplicate on three separate cultures. Data are presented as mean ± SEM. **$p<0.01$ when compared WT ATII with WT veh, ## $p<0.01$ when compared WT ATII with TG ATII. Statistics were by two-way ANOVA with Bonferroni post-hoc-tests.

3.4 **Summary**

- ATII (0.9mg/kg/day) caused no change in body weight in either WT or RAMP2 TG mice.

- Tail cuff plesythmography indicated that both WT and RAMP2 TG mice showed similar basal blood pressure and increase in BP following ATII treatment.

- 13 days ATII infusion produced cardiac hypertrophy in both WT and RAMP2 TG mice when compared to the vehicle-treated mice.

- WT ATII-treated mice demonstrated vascular hypertrophy when compared to their vehicle-treated mice. However the RAMP2 TG mice were protected against the vascular hypertrophy.

- Mesenteric arterioles were investigated for evidence of vascular hypertrophy but no significant difference was found between ATII- or vehicle-treated groups.

- Consistent with the vascular hypertrophy, following ATII treatment WT mice had increased expression of inflammatory markers (VCAM-1 and MCP-1) on aortic tissue when either compared to the vehicle-treated mice or compared with the ATII-treated RAMP2 TG mice.

- Primary VSMCs were prepared from WT and RAMP2 TG mice for cell proliferation study. We have demonstrated that VSMCs from RAMP2 TG mice proliferated slower compared to WT VSMCs in the presence or absence of ATII. Furthermore, the AM1 receptor antagonist AM_{22-52} was able to induce cell proliferation significantly in RAMP2 TG cells after ATII treatment, but such an effect was not seen in the WT cells.

3.5 **Discussion**

3.5.1 Use of RAMP2 TG mice and effect on blood pressure and vascular remodelling

In this study, we have used smooth muscle cell-targeted RAMP2 overexpressing mice in order to investigate of the consequence of the increased function of the AM1 receptor levels in tissue rich in vascular smooth muscle cells (Tam *et al*., 2006). To our knowledge, it is the first time that these mice have been used to investigate the role of the AM1 receptor in a model of hypertension and hypertrophy induced by ATII. In the present study, despite up-regulated RAMP2 level in smooth muscle cells, RAMP2 TG mice showed no significant difference in raised blood pressure and cardiac hypertrophy after 13 days infusion of ATII (0.9mg/kg/day). Our results were supported by those of Caron *et al*. (2007), who generated genetically controlled transgenic mice with levels of AM mRNA ranging from 50% (one copy of AM gene) -140% (4 copies of AM gene) compared to the WT. They demonstrated that those genetically controlled mice showed no difference in the basal blood pressure measured by tail cuff plesythmography. This also agreed with a previous study carried out in our group using the RAMP2 TG mice (Tam *et al*., 2006). Moreover, Caron and co-workers determined the affect of the down-regulation of AM level in models of hypotension from pregnancy or septic shock. They revealed that genetic reduction of AM in mice had no affect on the early hypotension observed during induced septic shock (Caron *et al*., 2007). This also agreed with previous studies from our laboratory that investigated the possibility that RAMP2 is involved in LPS-induced endotoxic shock (Liang *et al*., 2009). Here it was demonstrated that vascular smooth muscle RAMP2 and subsequently the AM1 receptor are unlikely to affect the LPS-induced hypotensive response and associated inflammatory events. Conversly, by crossing AM knockdown mice (one copy of AM gene) with renin gene overexpressing mice, Caron *et al. (2007)* demonstrated that the AM level does not affect the blood pressure in a chronic hypertension condition with overexpression of renin transgene (Caron *et al*., 2007). All the results from Caron *et al*., (2007) support our results that the RAMP2 TG mice show no difference in blood pressure after 13 days of ATII treatment when compared to the WT mice.

The results in our study using RAMP2 TG mice show a similar degree of hypertension compared to WT mice. This is supported by other research groups. Heterozygous AM gene KO mice (plasma and organ AM concentrations that are 50%) exhibited no difference in basal blood pressure compared to WT controls, as determined by tail cuff (Shimosawa *et al*., 2002). At the same time, both heterozygous AM gene KO and WT mice were treated with ATII (0.9mg/kg/day) combined with a high salt diet for 12 days. A significant reduction in the AM concentration in the hearts of heterozygous AM KO mice was observed. Additionally, the heterozygous AM gene KO mice exhibited a similar degree of hypertension compared to WT mice, although significantly increased damage to coronary arteries was observed in the AM KO mice. Such damage was suggested to be mediated by oxidative stress (Shimosawa *et al*., 2002). Furthermore, more recent studies showed that with human gene delivery of AM (by systemic administration via the tail vein into rat) no progression of hypertension induced by the chronic administration of the NO synthesis inhibitor L-NAME, when compared to the control rat, measured by tail cuff. However, ventricular cardiomyocytes from these rats were protected against L-NAME-induced hypertrophy, as determined by ventricular cardiomyocyte width and mRNA level of hypertropic marker gene (skeletal-α-actin). This group later demonstrated that this protection was associated with oxidative stress (Bell *et al*., 2010). Niu and his colleagues demonstrated that following high dose ATII (3.2 mg/kg/day) treatment for 14 days, the heterozygous AM KO mice had significantly increased blood pressure and cardiac hypertrophy when compared to the WT mice, However, it is noted that the significant increase in blood pressure may be due to the high dose of ATII administered (Niu *et al*., 2004). Moreover, our RAMP2 TG mice were not protected against the cardiac hypertrophy, which could be due to the tissue selection of the RAMP2 up-regulation. This suggests that the up-regulated RAMP2 levels, and as a consequence, the functional AM1 receptor in the heart may not influence the high blood pressure and ventricular hypertrophy induced by ATII.

RAMP2 TG mice were protected against the vascular hypertrophy induced by ATII as a significant increase of aortic wall thickness and wall area was observed in the WT but not in the TG mice. This indicates that a local up-regulation of RAMP2 in aortic VSMCs is sufficient to protect against the vascular proliferation response in this hypertensive model. Our results were supported by Imai and his colleagues who established

transgenic mice with significant overexpression of AM (2-4 fold overexpression in major organs and 2-8 fold in aorta) in a vessel-selective manner (Imai *et al*., 2002). They examined the effect of AM on the formation of vascular lesions by using two different models - cuff placement (on the left femoral artery) induced or hypercholesterolemia-induced fatty streak formation. With the cuff injury model, they demonstrated that AM up-regulated mice were protected against high blood pressure and vascular injury, but this protection was diminished following chronic L-NAME treatment suggesting that the protective action of AM is NO-dependent. Moreover, after treatment with hydralazine (which increases cGMP levels and decreases the phosphorylation of smooth muscle myosin light chains, resulting in blood vessel relaxation) the WT mice were protected against the high blood pressure but not the vascular injury when compared to the AM up-regulated mice. This indicated that the protective properties of AM are independent of blood pressure reduction. Furthermore, Imai and colleagues also demonstrated a protective role of AM in atherosclerosis by comparing AM overexpressing/ApoE KO-crossed mice with ApoE KO mice. This protective effect is partially mediated by NO (Imai *et al*., 2002). These studies supported the current results in this dissertation. Upregulation of the AM1 receptor, and consequently the function of AM, is able protect against ATII induced vascular hypertrophy observed by a significant augmentation of the smooth muscle layer but not to the collagen layer, as determined and analysed using the standard method - Masson's Trichrome staining (as used similarly by others, Shimosawa *et al*., 2002, Zhan *et al*., 2005). However, the exact mechanism involved in our model requires further investigation.

Vascular inflammation and remodelling accompanies several diseases, including atherosclerosis and hypertension. Therefore, the anti-inflammatory effect of AM via the AM1 receptor was also examined in this study by immunohistochemistry to locate whether two established inflammatory markers, VCAM1 and MCP1 were present and up-regulated with ATII. In the current study, VCAM-1 and MCP1 were found to be significantly upregulated in the aortic wall of the WT mice after infusion of ATII for 13 days compared to the vehicle-treated group. However, this was not seen in the RAMP2 TG mice. Therefore, our results indicate that the protective function of AM via upregulation of functional AM1 receptors is achieved by inhibiting the smooth muscle proliferation and development of vascular inflammation in response to hypertension

induced by chronic infusion of ATII. Indeed, data from a previous PhD student (2009-12) Sarah-Jane Smillie of our research group supports these results that ATII treatment for 13 days significantly increases the protein and mRNA expression of VCAM-1 in the aorta, when compared to the vehicle-treated mice. She used immunohistochemistry techniques that trained by me and then extended them to also measure gene expression using RT-qPCR (Smillie, 2012). She also demonstrated increased levels of MCP-1 in the aorta by using RT-qPCR. Moreover, Zhan and colleagues have also revealed that levels of inflammatory markers (both VCAM-1 and MCP-1) are increased in the mouse aortic smooth muscle wall following chronic stimulation with 1.4mg/kg/day of ATII for 2 weeks (Zhan *et al*., 2005). The up-regulation of VCAM-1 and MCP-1 in these studies appears to correlate with vascular remodeling in the models and in cardiovascular diseases specifically (Smillie, 2012; Zhan *et al*., 2005).

3.5.2. Study of VSMC and protective mechanisms *in vitro*

I then wanted to learn more about the possible protective mechanism of RAMP2 in our model by observing proliferation of VSMCs *in vitro*. For this I developed the technique of isolation and culture of primary VSMCs from mouse aorta. This technique had not previously been used in our group. VSMCs from both WT and RAMP2 TG mice were extracted using aortic explants. For all the experiments, primary cells from passage 3 were used. We have demonstrated that VSMCs overexpressing RAMP2 proliferated significantly slower than the WT VSMC, either with foetal calf serum (20%) or ATII stimulation. In addition, to investigate the involvement of AM1 receptor, the selective AM1 receptor antagonist, AM_{22-52}, was able to elevate the ATII-induced proliferation significantly in the RAMP2 TG VSMC. These results indicate that AM can inhibit VSMCs proliferation induced by ATII via increasing the functional AM1 receptor signalling.

In this study, the mechanisms involved were not studied. However, several mechanisms have been identified by other researchers that may explain such a protective function of AM. Liu and co-workers revealed the protective effect of AM against ATII-induced proliferation of cultured rat aortic smooth muscle cells (ASMC), in which AM directly inhibits the intracellular reactive oxygen species production induced by ATII in a concentration-dependent manner. Activation of Src plays a major role in

growth signalling (Liu *et al.,* 2006). Based on these findings, Liu *et al*. (2006) further demonstrated that ATII-enhanced COOH-terminal Src kinase (a key enzyme that inactivates Src) phosphorylation inhibits Src phosphorylation. The inhibitory role of AM in cell proliferation was shown by up-regulating the COOH-terminal Src kinase in ASMCs to inhibit Src activity (Liu *et al*., 2006). Furthermore, another report implicated AM in regulating human ASMC proliferation as a growth promoter under quiescent state via ERK1/2 activation (Rossi *et al*., 2006). This group also revealed that AM acts as potent inhibitor of a proliferative state where they demonstrated that AM inhibited the ATII-induced proliferation at a concentration of 100nM and greater showing a concentration-dependent effect. Such an inhibition was mainly via the cAMP/PKA pathway. It is worth noting that the AM effects can be inhibited by the AM1 receptor antagonist AM_{22-52}, and also blunted by $CGRP_{8-37}$, which indicates the presence of specific AM receptors and some link with the CGRP receptor (Rossi *et al*., 2006).

3.5.3. Relevance of findings with respect to the CGRP family of peptides

As discussed previously, the results indicate that AM plays a protective role in cardiovascular events via a functional AM1 receptor. However, AM may also act via the CGRP receptor (Rossi *et al*., 2006). Moreover, in an L-NAME-induced hypertension model, the concentrations of both AM and the AM-related peptide, intermedin, have been shown to be increased, at protein and mRNA level. Interestingly, the anti-hypertension drug, hydralazine/hydrochlorothiazide, abolished the up-regulation of AM and its receptor components but not intermedin or RAMP1 (Zhao *et al*., 2006). Furthermore, a recent report demonstrated that intermedin plays a protective role in an ischaemia-reperfusion injury model in cultured cells However, this protection was abolished following treatment with siRNA for CLR and RAMP2, but not RAMP1 and RAMP3, indicating that the protection is via an AM1 receptor-dependent pathway (Bell *et al*., 2012). All these reports indicate that the functional AM1 receptor activity may be influenced by more than one agonist. Indeed, in this dissertation the results of these studies may involve either adrenomedullin or intermedin as an agonist.

In conclusion, for the current study, by using smooth muscle cell targeted RAMP2 overexpressing mice with both *in vivo* and *in vitro* methods, we demonstrate a novel

and important role of vascular smooth muscle AM1 receptors in terms of protecting against vascular inflammation and hypertrophy induced by ATII.

CHAPTER 4 : A ROLE OF TRPV1 ON ATII-INDUCED CARDIOVASCULAR DISEASE

4.1 **Introduction**

We have shown in last chapter that local upregulation of the RAMP2 gene in smooth muscle cells protected mice from vascular hypertrophy and vascular inflammation induced by ATII (0.9mg/kg/day), that was not related to blood pressure changes. This supports the hypothesis that AM is protective in many cardiovascular diseases (see chapter 3 for details). As a member of the CGRP family of peptides, AM shares similar vascular activities with CGRP. Both AM and CGRP are vasodilators *in vivo*, although CGRP is 3-30 times more potent, depending on the tissue (Brain and Grant, 2004). Additionally, AM and CGRP share some components for their receptors (discussed in section 1.3.2). CGRP and its receptor are expressed at elevated levels in experimental models of hypertension (Deng and Li 2005). There is evidence to support CGRP also possesses protective attributes in hypertension (Deng *et al*., 2004; Li *et al*., 2009; Smillie, 2012). However, the study of CGRP in the model of ATII-induced hypertension in the mice has not been previously investigated. Furthermore, it is noted that the role of CGRP depends on the experimental model chosen. Therefore, *in vivo* studies are essential to learn more about the role of CGRP in cardiovascular regulation.

Thus within our group, we aimed to determine whether CGRP is protective in ATII-induced hypertension and hypertrophy in a similar manner to AM. The effect of CGRP in an ATII model was investigated using WT and global αCGRP KO mice. This study was carried out by S-J Smillie (Smillie, 2012), who administered ATII (1.1mg/kg/day) to both WT and αCGRP KO mice for 13 days by implantation of an osmotic mini-pump, using the same technique described in this thesis. Blood pressure was monitored by tail cuff plethysmography. Vascular hypertrophy and inflammation were investigated at the end of the study by histology and immunohistochemistry. She demonstrated that WT mice became hypertensive (Fig 4.1) where they exhibited vascular hypertrophy (Fig 4.2) after 13 days of infusion of ATII, when compared to the vehicle-treated mice. Moreover, αCGRP KO mice demonstrated significantly increased hypertension (Fig 4.1) and vascular hypertrophy (Fig 4.2) when compared to the WT ATII-treated mice, with enhanced expression of the inflammatory marker VCAM-1 in the aortic vascular wall

(Fig 4.3). These results strongly suggest a protective effect of CGRP, in addition to AM, in protecting against the hypertension and vascular remodeling in this ATII model (Smillie, 2012).

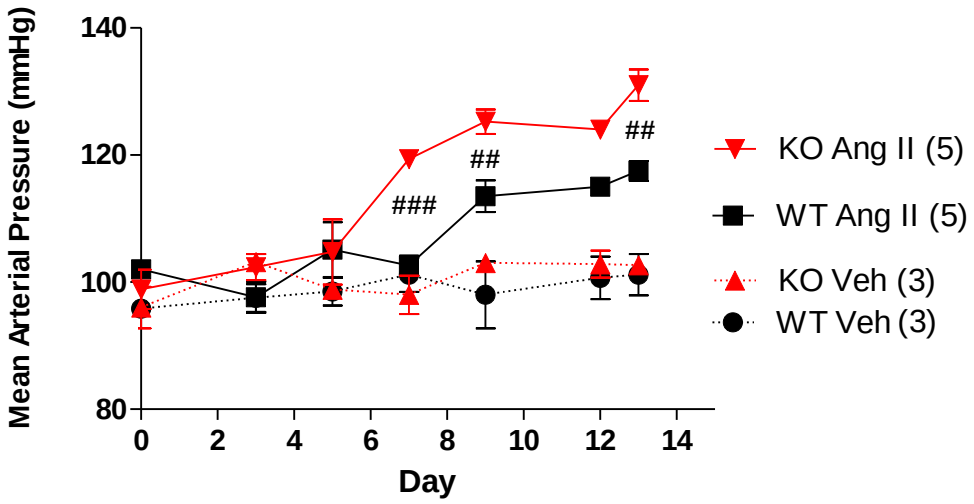


Fig 4.1. Mean arterial pressure (of mixed gender WT and αCGRP KO mice treated with either Vehicle (saline) or ATII infusion for 14 days, measured by tail cuff plethysmography Statistical evaluation of mean ± SEM where ##=p<0.1 and ###=p<0.01 when compared to WT ATII treated animals by two-way ANOVA + Bonferroni's test (from SJ Smillie PhD thesis, Smillie, 2012).

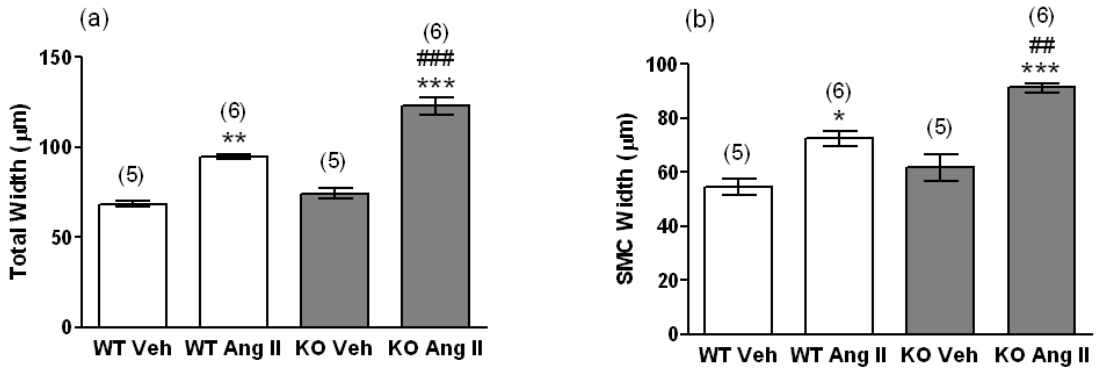


Fig 4.2. Total and SMC (µm), measured from aortic sections taken from mixed gender WT and αCGRP KO mice treated with either Vehicle (saline) or ATII infusion for 14 days. (a) Total width (SMC + collagen) in WT and αCGRP KO mice. (b) Total SMC width (µm) in WT and αCGRP KO mice, (n=5-6) in WT and αCGRP KO mice. Statistical evaluation of mean ± SEM by 2 way ANOVA and Bonferroni's test, where *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$. ##=$p<0.01$ and ###=$p<0.001$ when comparing the ATII treated groups (from SJ Smillie PhD thesis, Smillie, 2012).

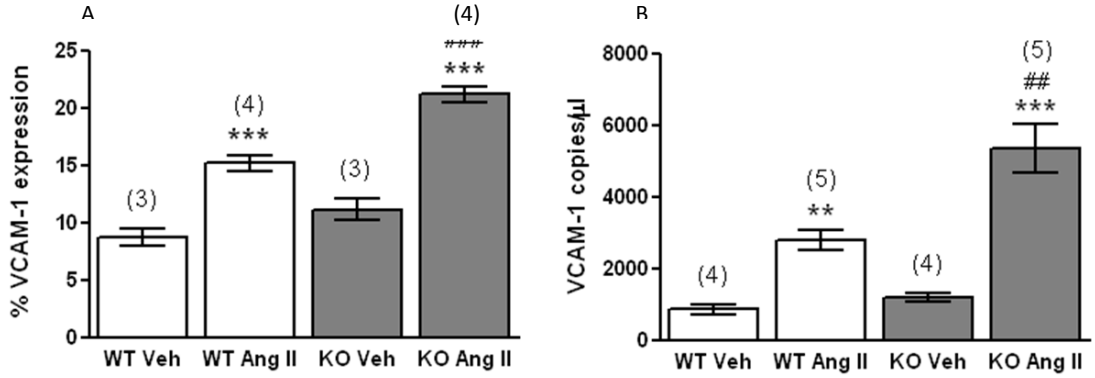


Fig4.3 Effect of ATII (1.1 mg/kg/day for 14 days) compared with vehicle (saline) on VCAM-1 protein and mRNA expression in WT and αCGRP KO mice. Quantitative analysis of aortic VCAM protein expression (A) by immunohistochemistry and representative mRNA expression measured by RT-qPCR, expressed as copies/µl and normalised to SDHA, HPRT-1 and PLA2 (g). **p<0.01 and ***p<0.001 compared to vehicle. ##p<0.01 and ###p<0.001 compared to WT ATII-treated animals (from SJ Smillie PhD thesis, Smillie, 2012).

Indeed, studies have been carried out to investigate the role of CGRP in other models of hypertension. Li and his colleagues performed studies in two different animal hypertensive models, SHR and 2K1C hypertensive rats (Li *et al*., 2009). Systolic blood pressure was significantly increased in both SHR and 2K1C, measured by tail cuff. Plasma CGRP levels were decreased dramatically in SHR, when compared to the controls. A similar phenomenon was also documented for human essential hypertension, performed by the same group. However, plasma levels of CGRP were significantly increased in 2K1C hypertensive rats. The mRNA levels of both αCGRP and βCGRP were also significantly decreased in the SHR but increased in 2K1C hypertensive rats (Li *et al*., 2009). By using the different models, this example shows how CGRP has the potential to play different roles depending on circumstance.

Due to the diverse role of CGRP in hypertension, one of our primary considerations was to understand how the family of CGRP related peptides are endogenously released. There is a range of agents regulating the tissue levels of both AM and CGRP, such as cytokines and TNFα (Sugo *et al*., 1995, Schafers *et al*., 2003). However, we are unaware of any selective agents. ATII has been shown to directly affect the plasma levels of both AM and CGRP (Eto *et al*., 2003; Harada *et al*., 2009). At the same time, the ATII infusion model was established in our group (Liang *et al*., 2009).

The TRPV1 receptor is a non-selective cation channel, composed of six putative transmembrane domains, including a pore region between the fifth and sixth transmembrane domain (Alexander *et al*., 2008). TRPV1 is expressed in a range of tissues, but primarily on the peripheral terminals of polymodal unmyelinated C-fibres (Alexander *et al*., 2008). TRPV1 is also thought to be expressed in some non-neuronal tissue, such as smooth muscle cells (Kark *et al*., 2008) and keratinocytes (van der Stelt and Di Marzo, 2004). It is well established that activation of TRPV1 on sensory neurons can trigger the release of neuropeptides, including CGRP and substance P (Brain 1997). Therefore, together with the evidence that both AM and CGRP show certain protective roles in ATII-induced hypertension and hypertrophy, we investigated whether TRPV1 can act via the release of CGRP to protect in this hypertensive model.

It has been suggested that rutaecarpine, a major quinazolinocarboline alkaloid isolated from a well-known Chinese medicine called Wu-Chu-Yu, depressed the phenol-induced hypertension by synthesis and release of CGRP from sensory nerves via the TRPV1 receptor (Deng *et al*., 2004). Deng and colleagues injected 50µl of 10% phenol to induce hypertension. Fifteen days later rats were divided into different groups receiving different dose (3 or 6 mg/kg/day) of rutaecarpine, with or without capsaicin or capsazepine (Deng *et al*., 2004). Blood pressure was monitored by tail cuff and indicated that rutaecarphine did significantly reduce the high blood pressure induced by phenol. Interestingly, high doses of capsaicin, used to deplete CGRP from the sensory nerve, abolished the majority of the depressor effect of rutaecarphine. A similar effect was observed by injection of the TRPV1 antagonist, capsazepine (Deng *et al*., 2004). They also investigated the protein and gene expression of CGRP (both αCGRP and βCGRP) by radioimmunosssay and qPCR, from which they revealed that CGRP was upregulated in rutaecarpine-treated groups in a dose-dependent manner. But such up-regulation was lost following treatment with high dose capsaicin or with capsazepine. Moreover, the same research group demonstrated that following surgery there was increased sensory nerve-mediated CGRP release in the 2K1C hypertensive model, where CGRP is possibly counteracting the increased blood pressure; pre-treatment with high dose capsaicin significantly decreased the expression of CGRP and increased the blood pressure (Deng *et al*., 2003). These reports indicate the protective potential of TRPV1 in hypertension models by up-regulating sensory nerve-mediated CGRP levels.

After TRPV1 KO mice were generated, different phenotypes between WT and TRPV1 KO mice have been investigated. TRPV1 KO mice exhibit normal responses to noxious mechanical stimuli, but impaired nociception to heat (Caterina et al., 2000). Moreover compared to the WT mice, TRPV1 KO mice also show less anxiety-related behaviour in the light-dark test but more anxiety-related behaviour in the plus maze (Marsch et al., 2007). Interestingly there is no difference in locomotion (Marsch et al., 2007). However a recent study has shown that compared to the WT mice, TRPV1 KO mice have higher locomotor activity when they are young, but lower locomotor activity and overweight when they are old (Wanner et al., 2011). Furthermove in term of autonomic regulation,

TRPV1 KO mice show hypometabolic and hypervasoconstricted effects, when compared to WT mice (Garami et al., 2011).

I wanted to investigate the role of TRPV1 in hypertension. Therefore in my studies, I utilised the TRPV1 KO mice previously characterized by our group by the studies of Dr Anna Starr who applied capsaicin in ethanol on the ear in WT mice to show significantly increased blood flow when compared to the ethanol-treated ear. The capsaicin-increased blood flow was not observed in the TRPV1 KO mice (Starr *et al*., 2008). Further characterisation of these mice was performed by Dr Jeelie Keeble who induced Freund's complete adjuvant (CFA)-induced inflammation by intra-articular injection to both WT and TRPV1 KO mice. Knee swelling and hyper-permeability were assessed in this study where it was demonstrated that TRPV1 KO mice were protected against the CFA-induced joint inflammation with significantly swelling and thermal hyperalgesic sensitivity compared to the WT mice (Keeble *et al*., 2005).

Here we used these TRPV1 KO mice to investigate the role of TRPV1 in the ATII-induced hypertension model as developed by myself that is now a well established model in our group (Liang *et al*., 2009, Smillie, 2012).

4.2 Hypothesis and aims

4.2.1 Hypothesis

TRPV1 protects against ATII-induced hypertension and hypertrophy via release of CGRP

4.2.2 Aims

The aims of this chapter were:

- Investigate WT and TRPV1 KO mice in the ATII-induced hypertensive model
- To measure blood pressure by use of the tail cuff technique and telemetry
- To measure vascular hypertrophy in the aorta by histology
- To investigate plasma and kidney CGRP levels by ELISA

4.3 Results

4.3.1 Effect of ATII (1.1mg/kg/day) on body weight and blood pressure on WT and TRPV1 KO mice

Both WT and TRPV1 KO mice (25-35 g, 12 – 16 weeks old, male) were treated with ATII (1.1mg/kg/day) or saline as vehicle for 13 days by mini-pump infusion. Body weight was monitored on day 13 (the last day of the study before termination). Figure 4.4 shows that ATII (1.1mg/kg/day) caused no change in body weight for both WT and TRPV1 KO mice compared to the vehicle-treated mice (n=6-7).

For this study blood pressure was measured by two methods; tail cuff plethysmography and radiotelemetry. For the first set of studies, blood pressure was measured by tail cuff where two weeks of training was provided to the mice prior to mini-pump surgery. Blood pressure was taken before surgery (and used as baseline) and also on day 3, 6, 9 and 13 post-hoc mini-pump implantation as showed in Fig 4.5 A. In this figure systolic, diastolic and mean arterial blood pressure measurements are shown. Both WT and TRPV1 KO mice show similar baseline blood pressure. There is no significant difference between ATII and vehicle treated WT mice in systolic blood pressure (Fig 4.5A) but statistical significance was detected from day 3 in both diastolic and mean arterial blood pressure results (Fig 4.5B and C). Such differences were not seen between ATII and vehicle-treated TRPV1 KO mice. Again in Fig 4.6, blood pressure on day 13 (the last day of study) shows that there was no significant difference in the blood pressure (systolic, diastolic and mean blood pressure) between WT and TRPV1 KO vehicle mice (Fig 4.6A, B and C). In summary, the WT mice showed a significantly enhanced systolic blood pressure and mean arterial pressure compared to the KO mice following ATII treatment.

To further investigate the blood pressure, telemetry was introduced into this study. This is suggested to be a more sensitive measurement system and it allows measurement in the conscious-unrestrained mouse as discussed in the general introduction. In this study, no vehicle treatment was used; blood pressure before mini-pump implantation was taken as baseline. Telemetry surgery was carried out two weeks before mini-pump

surgery to ensure recovery from the telemetry surgery and provide sufficient time to collect baseline blood pressure. Fig 4.7 shows the blood pressure trace (A: systolic, B: diastolic and C: mean arterial pressure) recorded by telemetry. No significant difference in basal blood pressure between WT and TRPV1 KO mice, whether measured during the day or night, supporting the tail cuff data. Fig 4.8 shows daily systolic, diastolic and mean blood pressure differences between WT and TRPV1 KO mice on day 3 and from day 6 to day 13 post-hoc-administration of the ATII-containing mini-pump. It shows that the WT mice developed higher blood pressure than TRPV1 KO mice after ATII, although no significant difference was observed. This may due to a low n number in this study. But when the data was analysed from the last day (day 13 after ATII infusion) of the study as shown in Fig 4.9, both WT and TRPV1 KO displayed similar baseline blood pressure. However, ATII-treated WT mice displayed significantly higher systolic, diastolic and mean blood pressure compared to their baseline. Conversely, TRPV1 KO mice were protected against the increase induced by ATII (1.1mg/kg/day), as the TRPV1 KO mice displayed significantly lower systolic and mean arterial blood pressure than the WT mice.

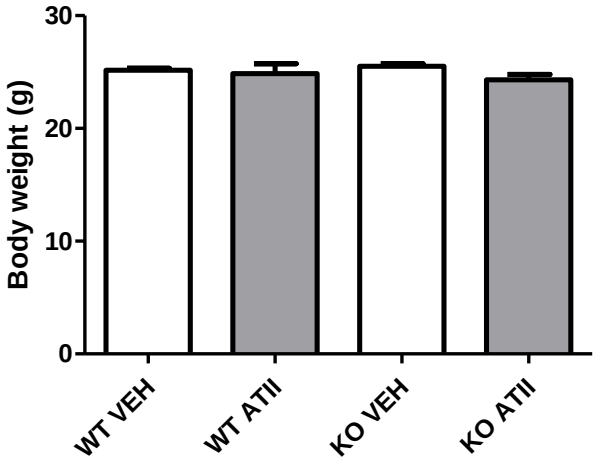


Figure 4.4. The body weight of WT and TRPV1 KO mice after infusion with ATII by mini-pump for 13 days. No significant difference was observed before and after ATII treatment between WT veh and KO veh, N=6-7. Statistics by two-way ANOVA with Bonferroni post-hoc-tests.

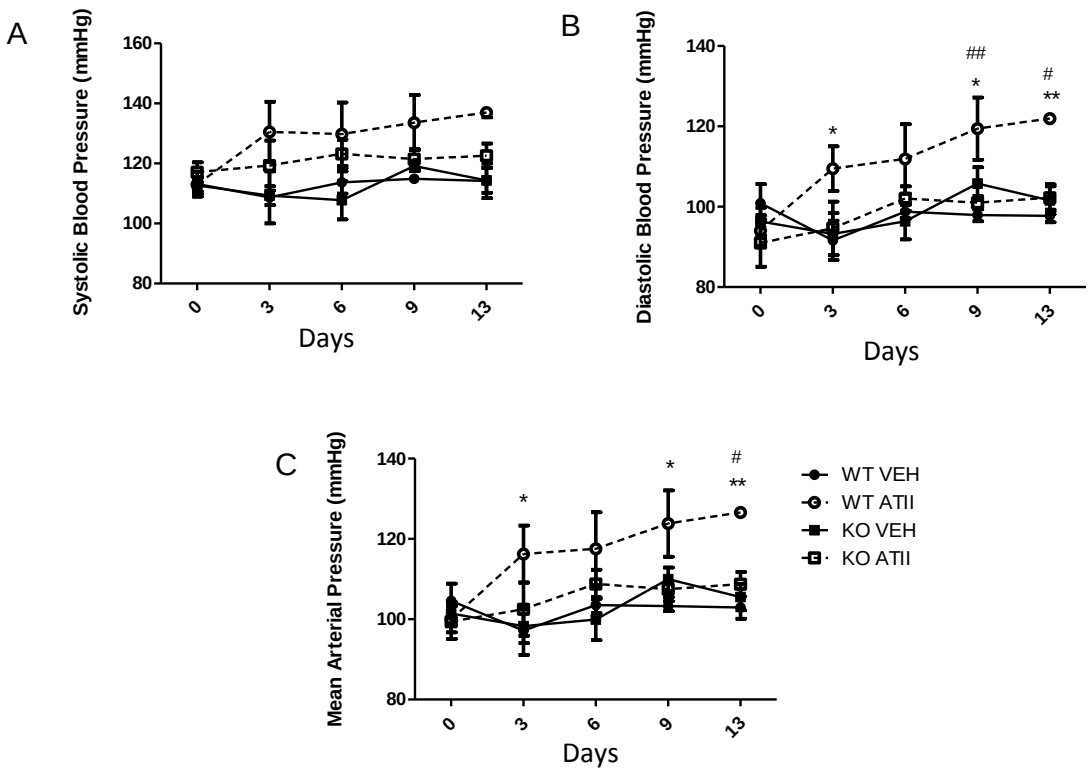


Figure 4.5: Effect of ATII (1.1mg/kg/day) on blood pressure in TRPV1 WT and TRPV1 KO mice, measured by tail cuff on day 3,6,9 and 13 post-hoc mini-pump implantation. A, systolic; B diastolic and C mean blood pressure. *=$p<0.05$ and **=$p<0.01$ when the WT ATII-treated mice were compared to the WT veh mice. #=$p<0.05$ and ##=$p<0.01$ the WT ATII-treated mice were compared to the TRPV1 KO ATII mice. N=3-4. Statistics by two-way ANOVA with Bonferroni post-hoc tests

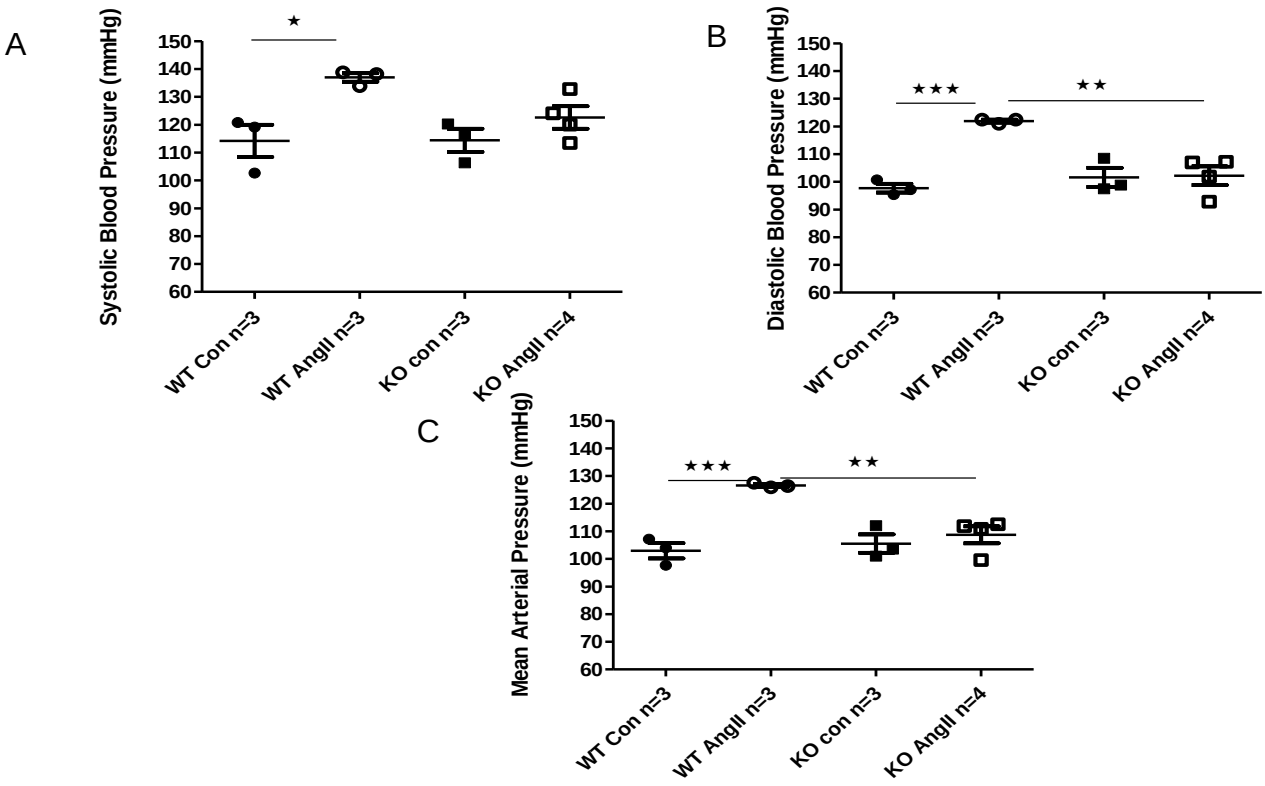


Figure 4.6: Effect of ATII (1.1mg/kg/day) on blood pressure in WT and TRPV1 KO mice, on day 13 post-hoc mini-pump implantation, measured by tail cuff. A, systolic; B diastolic and C mean blood pressure. *=$p<0.05$ and ***=$p<0.001$ when the WT ATII-treated mice were compared to the WT veh mice. **=$p<0.01$ when compared the WT ATII-treated mice were compared to the TRPV1 KO ATII mice. N=3-4. Statistics by two-way ANOVA with Bonferroni post-hoc-tests.

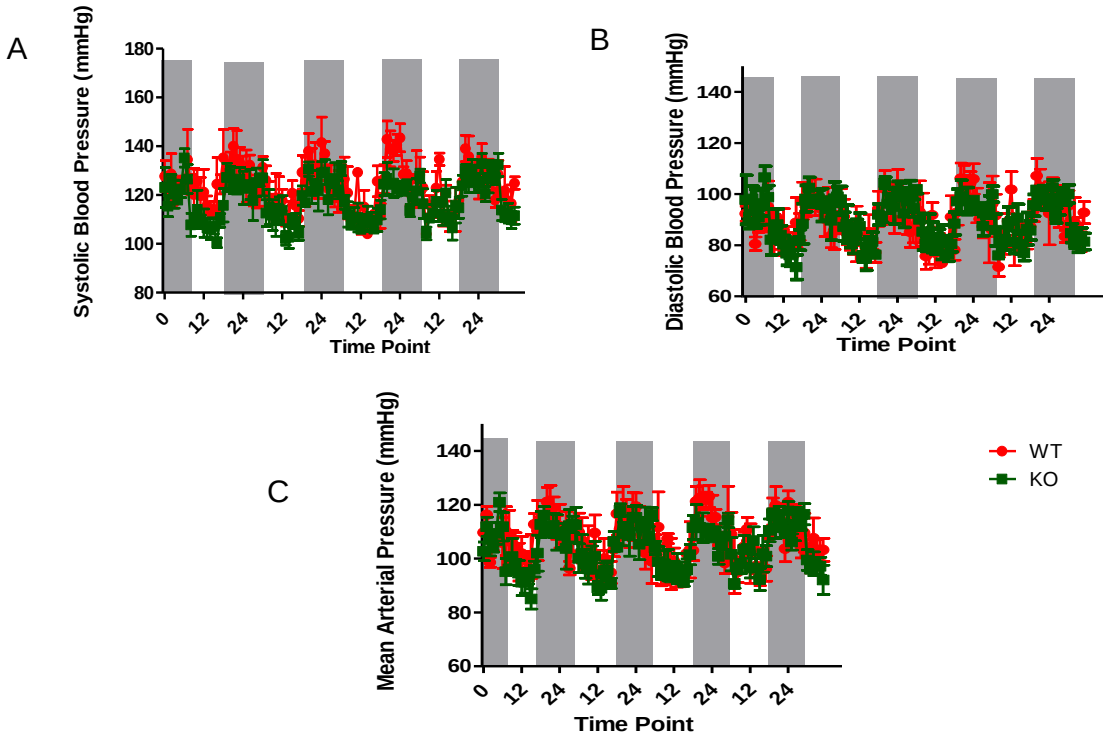


Figure 4.7: Blood pressure trace from telemetry recording. Results from WT and TRPV1 KO mice before ATII treatment (n=4) are recorded as baseline. Grey shade shows night time (7pm-7am).

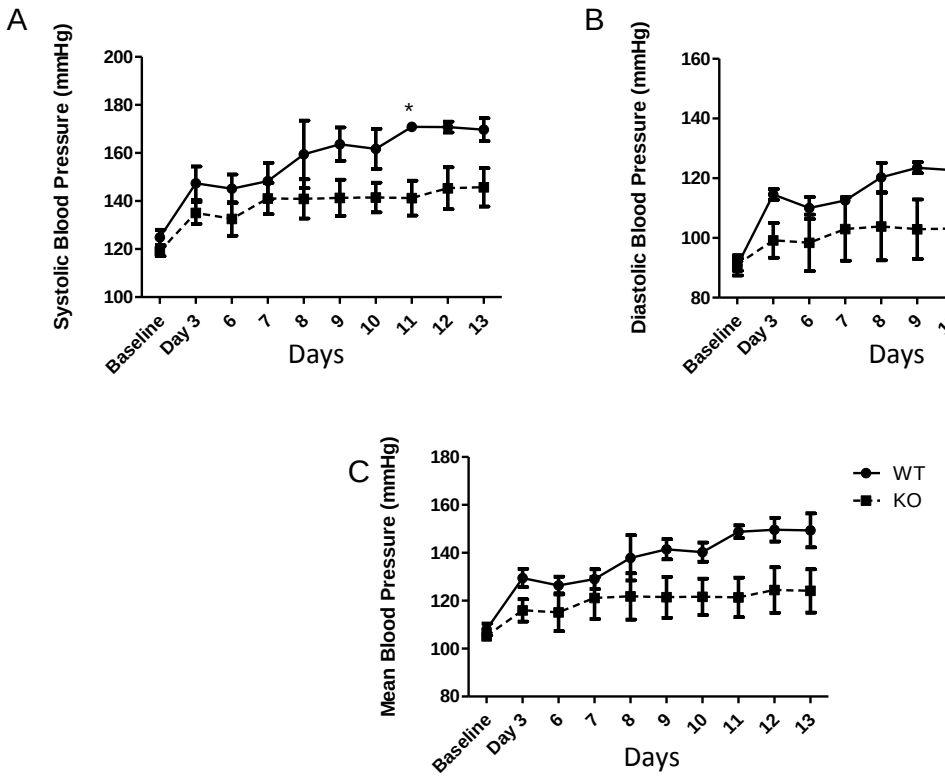


Figure 4.8: Effect of ATII (1.1mg/kg/day) on blood pressure in TRPV1 WT and TRPV1 KO mice, measured by telemetry for the baseline before the surgery and from day 3 to day 13 post-hoc mini-pump implantation. A, systolic; B diastolic and C mean blood pressure. *=$p<0.05$ when the WT mcie were compared to the TRPV1KO mice. N=3-4. Statistics by two-way ANOVA with Bonferroni post-hoc-tests

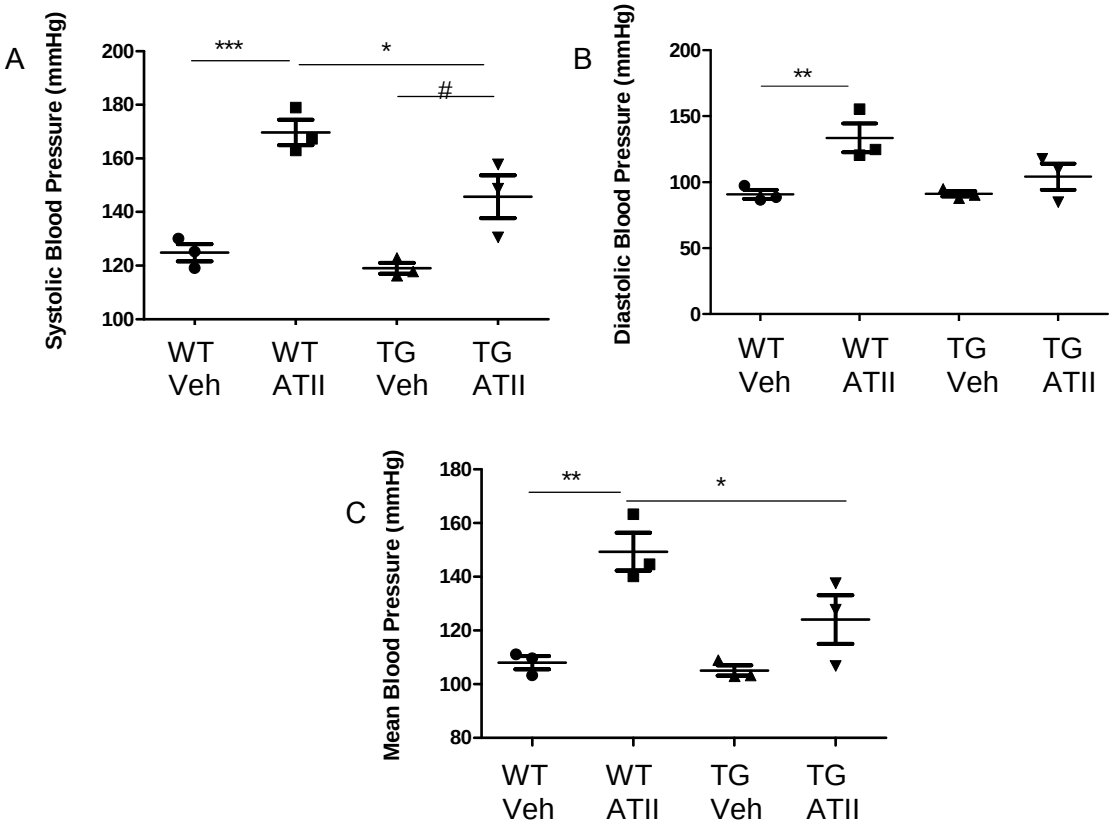


Figure 4.9: Effect of ATII (1.1mg/kg/day) on blood pressure in WT and TRPV1KO mice on day 13 post-hoc mini-pump implantation measured by telemetry. A. systolic; B. diastolic and C. mean blood pressure. **=$p<0.01$ and ***=$p<0.001$ when the WT ATII-treated mice were compared to the WT vehicle (VEH) mice. *=$p<0.05$ when the WT ATII-treated mice were compared to the TRPV1 KO ATII mice. #=$p<0.05$ when the TRPV1KO veh mcie were compared with the TRPV1 ATII-treated mice. Statistics by two-way ANOVA with Bonferroni post-hoc-tests. N=3.

4.3.2 Effect of ATII (1.1mg/kg/day) on heart to tibia ratio and vascular hypertrophy on WT and TRPV1 KO mice

Thirteen days of treatment with ATII (1.1mg/kg/day) did not cause significant differences in heart to tibia ratio between WT and TRPV1 KO mice (Fig 4.10) suggesting there is no difference between WT and TRPV1 KO mice in terms of developing cardiac hypertrophy. To investigate the vascular hypertrophy induced by ATII, aortic sections were collected from both WT and TRPV1 KO mice after 13 days infusion either with ATII or vehicle (saline). The aorta was then processed into paraffin blocks (please see the section 2 for further details). Aortic sections (5μm thickness) were used for H&E staining and Masson's trichrome staining. Vascular hypertrophy was determined based on histological assessment from using both staining methods

Fig 4.11 displays the representative H&E staining images of aortic wall at 100X and 400X magnifications. Measurements based on H&E staining are shown in Fig 4.12. Measurements show that in vehicle-treated groups, mice deleted of TRPV1 developed thicker aortic wall thickness when compared to the WT mice. However, after ATII (1.1mg/kg/day) treatment, WT mice showed significant increases in aortic wall width when compared to their vehicle-treated mice. But such a difference was not seen in the TRPV1 KO mice. Moreover, the WT ATII-treated mice displayed significantly thicker aortic wall width than the TRPV1 KO ATII-treated mice.

Fig 4.13 demonstrates the representative images for Masson's trichrome staining with aortic wall at 100X and 400X magnifications. With Masson's trichrome staining, measurements of smooth muscle layer and collagen thickness were performed separately. Fig 4.14A indicates that ATII caused a significant increase in collagen area in both WT and TRPV1 KO mice compared to their vehicle-treated mice respectively. However, ATII did increase medial thickness (Fig 4.14B) and aortic wall area to perimeter ratio (Fig 4.14C) significantly in WT ATII treated mice when compared to the WT control mice, a difference not seen in the TRPV1 KO mice.

A

The raw data of body weight, heart weight and tibia length of the ATII treated TRPV1WT and TRPV1KO mice						
Mouse number	1(KO)	2(KO)	3(WT)	4(WT)	5(KO)	6(WT)
Body Weight(g)	25.9	23.2	26.8	23.8	25.2	26.4
Heart weight(g)	0.14	0.14	0.155	0.15	0.18	0.15
Tibia length(mm)	17.5	16.7	18.2	16.8	17.1	18.1

B

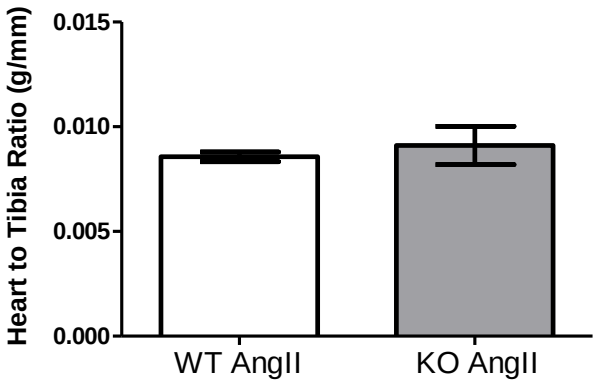


Figure 4.10: Effect of ATII (1.1mg/kg/day for 13 day) in WT and TRPV1 KO mice 13 days after mini-pump implantation. A. raw data of body weight, heart weight and tibia length. B. Heart to tibia ratio.

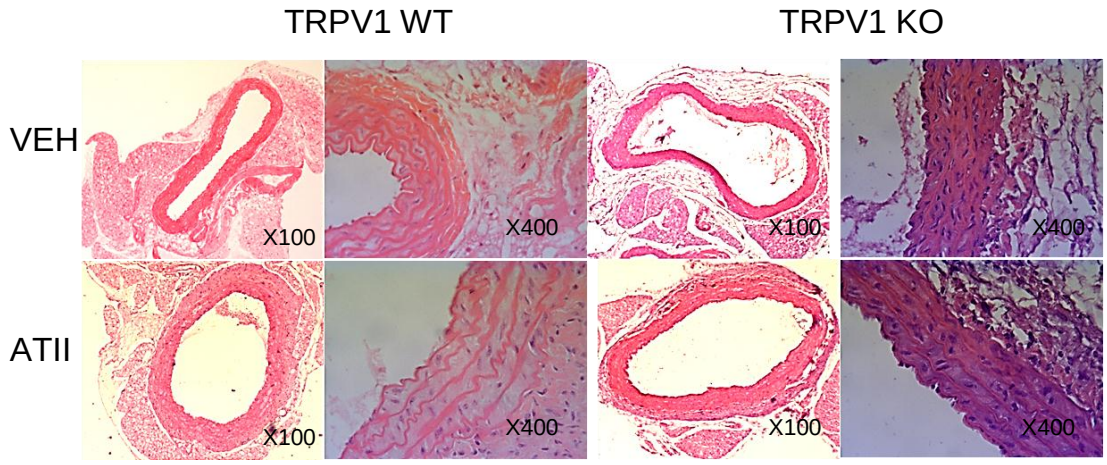


Figure 4.11: Effect of ATII (1.1 mg/kg/day for 13 days) on vascular hypertrophy in WT and TRPV1 KO mice. Representative H&E staining of thoracic aorta wall at indicated magnification. Purple shows nucleus while the pink shows all other tissue.

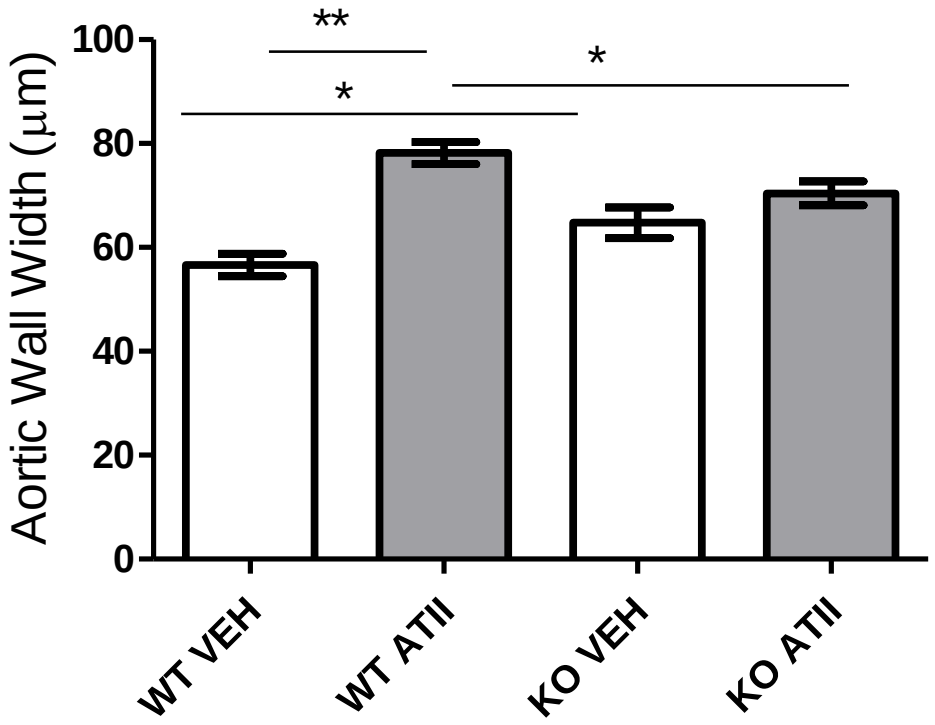


Figure 4.12: Effect of ATII (1.1 mg/kg/day for 13 days) on vascular hypertrophy in WT and TRPV1 KO mice aortic wall width (n=9-18 sections from 3-6 mice in each case). Data are presented as mean ± SEM. *=$p<0.05$ and **$p<0.01$. Statistic was done by two-way ANOVA with Bonferroni post-hoc-tests.

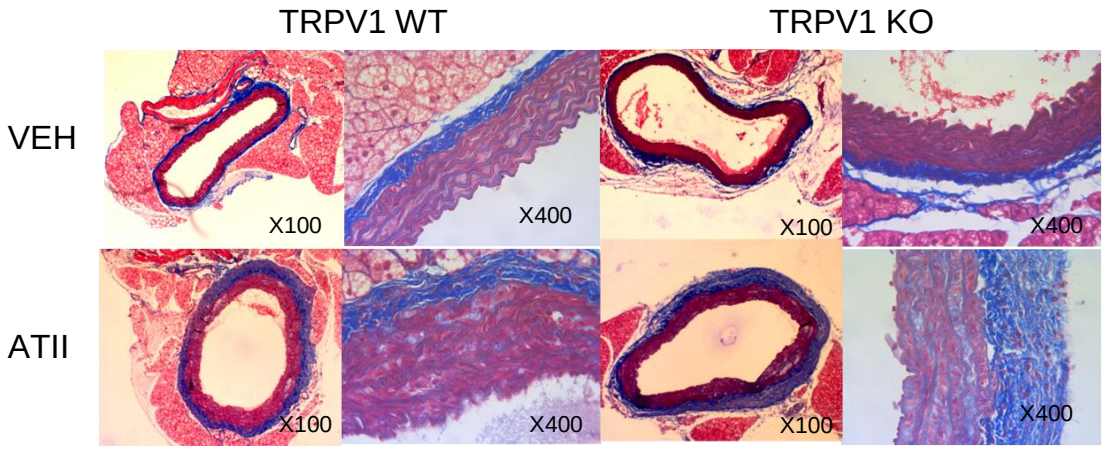


Figure 4.13: Effect of ATII (1.1 mg/kg/day for 13 days) on vascular hypertrophy in WT and TRPV1 KO mice. Representative Masson's Trichrome staining of thoracic aorta wall at indicated magnification. Dark pink shows the staining of muscle, the blue shows collagen fibers & fibrin, while the purple shows the nucleus.

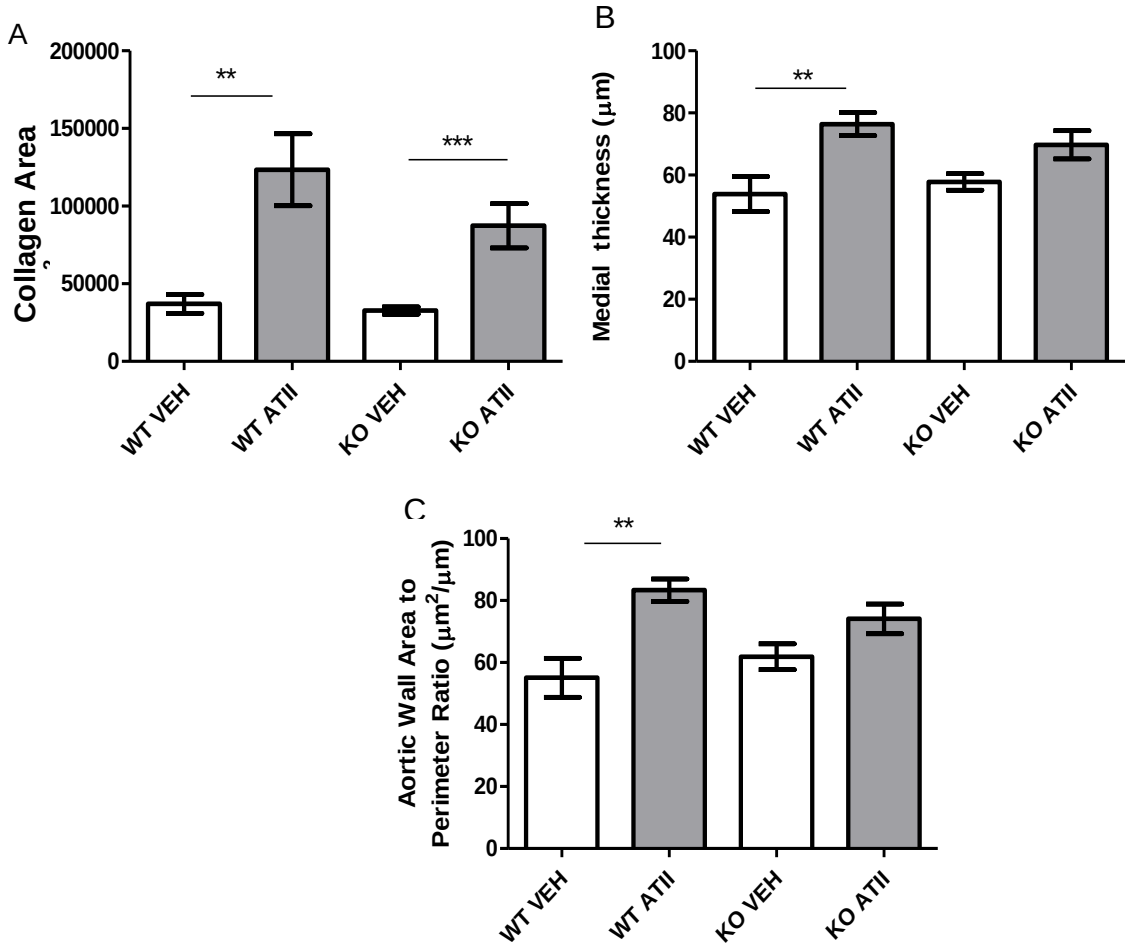


Figure 4.14: Effect of ATII (1.1 mg/kg/day for 13 days) on vascular hypertrophy in WT and TRPV1 KO mice, measured from Masson's Trichrome staining. A. collagen layer area, B. Smooth muscle wall width (medial thickness), and C. Smooth muscle wall width to perimeter (PRM) (n=9-18 sections from 3-6 mice in each case). Data are presented as mean ± SEM. **=$p<0.01$ when the WT ATII-treated mice were compared to the WT vehicle (VEH) mice. ***=$p<0.001$ when the TRPV1KO ATII-treated mice were compared to the TRPV1 KO veh mice. Statistic was done by two-way ANOVA with Bonferroni post-hoc-tests.

4.3.3 Effect of ATII (1.1mg/kg/day) on plasma and tissue levels of CGRP on WT and TRPV1 KO mice

Plasma and tissue levels of CGRP were also investigated by rat and mouse enzyme immunoassay. Both plasma and tissue homogenized supernatant were extracted before quantifying CGRP levels using ELISA. As it was noticed that TRPV1 KO were protected from ATII-induced hypertension and partially protected against the vascular hypertrophy, we were interested in measuring plasma and kidney CGRP levels to see whether the protection in TRPV1 KOs was due to a possible compensatory up-regulation of CGRP. The analysis of the samples was not straightforward, due to technical problems with the freeze dryer. However, we were able to show that plasma CGRP levels were similar between groups despite the different treatments (Fig 4.15, n=2-4). Interestingly, the kidney CGRP tissue levels were elevated in the WT ATII-treated mice, albeit non-significantly possibly due to the small n number and high degree of variation (Fig 4.16, n=3-6).

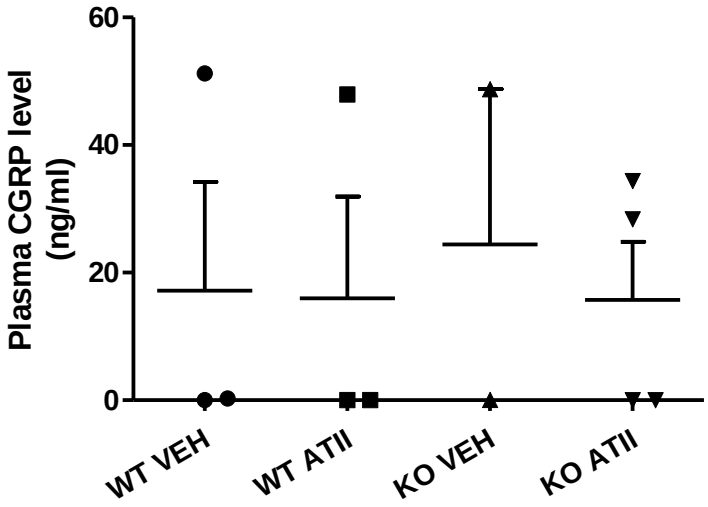


Figure 4.15: Effect of ATII (1.1 mg/kg/day for 13 days) on plasma CGRP levels in WT and TRPV1 KO mice. n=2-4.

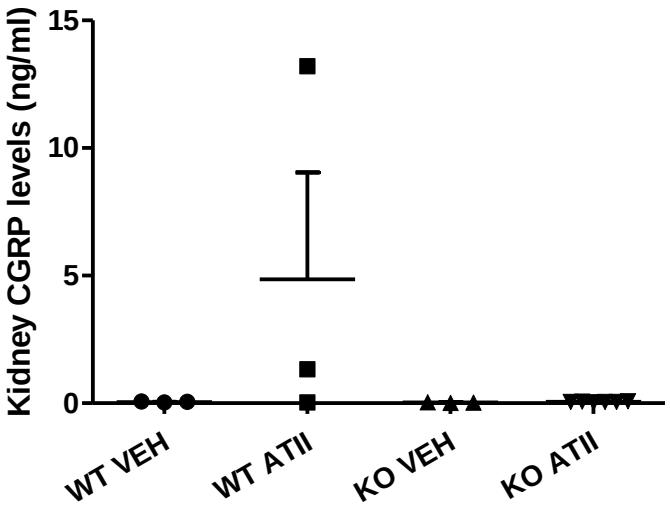


Figure 4.16: Effect of ATII (1.1 mg/kg/day for 13 days) on kidney levels of CGRP in WT and TRPV1 KO mice. n=3-6. Statistics was done by two-way ANOVA with Bonferroni post-hoc-tests

4.4 **Summary**

- ATII (1.1mg/kg/day) had no effect on the body weight when WT and TRPV1 KO mice were compared.

- Tail cuff plesthymography indicated that both WT and TRPV1 KO mice had similar basal blood pressure. ATII treatment (1.1mg/kg/day for 13 days) increased blood pressure significantly in the WT compared to the control, but this increase was reduced in the TRPV1 KO mice.

- Radio-telemetry was used to record blood pressure also. The data collected through this method supported those from tail cuff plesthymography, indicating that the TRPV1 KO mice were protected against the hypertension induced by ATII but not the WT mice.

- WT ATII-treated mice demonstrated vascular hypertrophy when compared to their vehicle-treated mice. However the TRPV1 KO mice were significantly protected against the vascular hypertrophy induced by ATII to a certain extent.

- Plasma CGRP levels and kidney tissue CGRP levels between the ATII-treated WT and TRPV1 KO groups were investigated but a conclusion can not be drawn due to the the low number.

4.5 **Discussion**

In this study, we have used WT and TRPV1 KO mice to investigate the role of TRPV1 in mediating hypertension and hypertrophy induced by ATII (1.1mg/kg/day). As expected, the WT mice developed hypertension but the TRPV1 KO mice were protected against the hypertension induced by 13 days infusion of ATII. Thus, the original hypothesis was incorrect. The degree of hypertension in the WT mice was substantial where the systolic blood pressure was significantly more elevated than compared to TRPV1 KO mice. The results were determined by two different blood pressure recording methods, tail cuff and telemetry, where both showed the same result. This was accompanied by vascular hypertrophy in the ATII-treated WT mice but not the ATII-treated TRPV1 KO mice. Previously the plasma CGRP levels had been investigated, which showed no difference between control rat (35.3 ± 3.4 pg/ml) and treatment rat (37.6 ± 2.2 pg/ml) after infusuin of ATII with 144mg/kg/day for 10 days (Li and Wang, 2005). In humans, ACE activities can increase CGRP plasma levels from 13.4±4.4pg/ml to 27.0±20.4pg/ml but no significant difference was found (Fusayasu et al., 2007). In my study, in order to understand more about the mechanisms involved, we also measured the plasma and kidney levels of CGRP. The result from my study shown the mean value of the plasma levels of CGRP was higher than those from above publications, which may due to one really high reading from each group. That may due to the measurement of the majority of the samples was affected by equipment malfunction and no further samples were available for analysis. The preliminary results suggested the involvement of CGRP was not sufficient to protect against a role for TRPV1 in ATII-mediated hypertension.

TRPV1 when activated leads to the release of neuropeptides that include CGRP in sensory neurones (Brain and Grant, 2004). CGRP is a potent vasodilator where it would be expected to combat the increased blood pressure. Indeed, as discussed in the introduction (section 1), Dr Sarah Smillie (Smillie, 2012) has shown that ATII-CGRP KOs exhibit a greater hypertensive phenotype than the ATII-WT mouse. In addition, CGRP gene expression is increased in ATII-treated WT mice in the present study. Therefore, I hypothesised that TRPV1 may play a protective role in regulating the hypertension. TRPV1 has been shown to be protective in regulating blood pressure in

hypertensive models by releasing CGRP. As discussed in the introduction (section 1), ruteacarpine was shown to play a protective role in a rat model of phenol-induced hypertension by activating TRPV1 and releasing CGRP (Deng *et al*., 2004).

Moreover, in order to investigate whether spinal CGRP in afferent renal nerves affects the development of one-kidney renal wrap (1K-WRAP) hypertension, Burg and his colleagues applied the technique of selective long-term partial depletion of spinal CGRP within small primary afferents nerve fibres by intrathecal administration of high dose capsaicin (Burg *et al*., 1994). Interestingly, they revealed that systolic arterial pressure was greater in 1K-WRAP rats after depleting CGRP than in vehicle-treated 1K-WRAP rats (Burg *et al*., 1994). This shows the expected protective effect of CGRP following TRPV1 activation.

A separate study provided evidence for a protective effect of CGRP downstream of TRPV1 activation. In this study, baseline arterial blood pressure was significantly increased after feeding with high salt (4% by weight) diet for 3 weeks when compared to the normal sodium diet intake (0.4% by weight), measured following cannulation of carotid artery in anaesthetized rats. These authors then proceeded to measure blood pressure in conscious and un-restrained rats when capsazepine (3mg/kg), a selective TRPV1 antagonist, was administrated by i.v. injection. They demonstrated that capsazepine significantly increased the mean arterial pressure in the high salt fed but not in the normal salt fed rats. Plasma levels of CGRP were significantly increased in the high salt fed rats when compared to the normal salt fed rats. The results indicated that the TRPV1 channels were activated to release CGRP during the high salt intake, where CGRP counteracted the salt-induced elevation of blood pressure presumably through the vasodilator action of CGRP (Wang *et al*., 2007).

All these studies contrast with the findings we report that TRPV1 deletion was associated with a lower blood pressure than in the TRPV1 WT mice. These results suggest that TRPV1 may be able to release CGRP, but that the CGRP was not able to protect against ATII-induced hypertension. The exact reasons for this are presently unknown and require further investigation. Possible mechanisms may include the following two possibilities.

First of all, substance P is known as a vasodilator as it has been reported to induce relaxation through the production of NO via tachykinin NK1 receptors in man (Bossaller et al., 1992). However in non-contracted rabbit intrapulmonary arteries, substance P mediated constrictor effects via different receptors depending on its concentrations (NK1 receptors at low concentrations and NK2 receptors at higher concentrations (Shirahase et al., 1995). Moreover it is reported that myogenic constriction is mediated by activation of TRPV1 and consequent action of substance P, which acts on NK1 receptors on vascular smooth muscle, causes constriction (Scotland *et al*., 2004). *In vitro*, elevation of intraluminal transmural pressure of mesenteric small arteries caused a myogenic response. This could be inhibited by 73% with capsaicin desensitisation. Moreover, activation of C-fiber nerve terminals by pressure ending myogenic constriction was due to stimulation of TRPV1, which was inhibited by capsazepine (Scotland *et al*., 2004). By blocking the tachykinin NK_1 receptors, they also demonstrated that the myogenic constriction was due to neuropeptide substance P release (Scotland *et al*., 2004). Thus activation of TRPV1 and, release of substance P, could contribute the myogenic constriction.

Secondly, there is growing evidence that vascular tissue contains non-neuronal TRPV1 receptors associated with vasoconstriction (Keeble *et al*., 2006; Kark *et al*., 2008 and Czikora *et al*., 2012). TRPV1-mediated vasoconstriction has been observed in the WT mouse knee joint, but not in TRPV1 KOs (Keeble *et al*., 2006). Using an isolated rat hind limb preparation, Kark *et al* (2008) demonstrated increased vascular resistance together with decreased skeletal muscle perfusion in response to capsaicin. Such an effect was unaffected by surgical sensory denervation (Kark *et al*., 2008). Furthermore, in isolated rat skeletal muscle arterioles, which are devoid of sensory innervation, high concentrations of capsaicin (0.1 – 1 μM) displayed endothelium-independent vasoconstriction (Kark *et al*., 2008). More recently Czikora *et al (2012)*., also demonstrated capsaicin evoked a constrictor response in isolated arteries similar to the effect mediated by noradrenaline. However, such constriction was absent in the arteries from TRPV1 KO mice and competitively inhibited by TRPV1 antagonist AMG9810 (Czikora *et al*., 2012). Therefore, it is possible that the reduced magnitude of hypertension in TRPV1 KO mice in the present study may be due to the loss of function

of the non-neuronal TRPV1 and could be due to the adaptive phenotype of the TRPV1 KO mice.

In conclusion, these results lead to the proposal that TRPV1 deletion (and potentially blockade) may be beneficial in ATII-induced hypertension and vascular hypertrophy. However the exact mechanisms of observed protection in TRPV1 KO mice are unknown but may involve non-neuronal TRPV1 receptors.

CHAPTER 5: A ROLE OF TRPV1 IN OBESITY-INDUCED CARDIOVASCULAR DISEASE

5.1 **Introduction**

Results in the previous study with WT and TRPV1 KO mice in an ATII-induced hypertension model (chapter 4), demonstrated that TRPV1 KO mice were protected from hypertension and partially from the vascular hypertrophy induced by ATII (1.1mg/kg/day). We had hypothesised that TRPV1 was protective in hypertension, due to release of CGRP, but it was apparent that this was not correct. Even with low N number, the CGRP levels in the kidney showed a trend towards increasing CGRP levels in the WT ATII treated mice but not in the TRPV1 KO mice. Overall, the results indicated that global deletion of TRPV1 is beneficial in the model of ATII-induced hypertension. The mechanisms are unknown, however, possibly CGRP levels can be up-regulated in tissues following TRPV1 activation (as CGRP kidney levels in the ATII-WT mice were increased, but not in the TRPV1 KO mouse). Nevertheless, the increased hypertension observed in TRPV1 WT mice indicates a novel mechanism that requires further investigation. We therefore sought a second model to investigate the role of TRPV1 in hypertension.

Recently, attention has been drawn to investigating the role of TRPV1 in regulating the development of obesity and type 2 diabetes (T2D). In 2007, it was demonstrated that when compared with lean counterparts, visceral adipose tissue from obese mice and humans displayed a reduced TRPV1 expression associated with reduced capsaicin-induced calcium influx (Zhang *et al*., 2007) Moreover, activation of TRPV1 by oral administration of capsaicin for 120 days prevented obesity in WT mice but not in TRPV1 KO mice (Zhang *et al*., 2007). This led to an understanding that TRPV1 is protective in obesity.

It is thought that approximately 60% to 70% (World Health Organisation 2003; Wofford & Hall 2004) of hypertensive disease is caused by the patient being overweight. Even though the relationship between obesity and hypertension has been well documented, the exact mechanisms remain uncertain. However, it is likely to be due to a combination of factors promoting sodium retention and vasoconstriction via the RAS

(Montani *et al*., 2002). We therefore thought that this high fat model may suit our studies as obesity-induced hypertension (Fellmann L. *et al*., 2012) may allow us to learn more about the role of TRPV1. The HFD model is less acute and less extreme in terms of the hypertension observed than the ATII model (Erdos B *et al*., 2009; Liang L. *et al*., 2009).

5.2 **Hypothesis and aims**

5.2.1 Hypothesis

TRPV1 plays a protective role in HFD-induced obesity and its associated hypertension

5.2.2 Aims

The aims of this chapter were:

- Investigate WT and TRPV1 KO mice in the HFD-induced hypertensive model
- To monitor blood pressure by both the tail cuff technique and telemetry
- To investigate the glucose tolerance state
- To measure vascular hypertrophy in the aorta by histology
- To assess the vascular inflammation in the aorta by immunohistochemistry
- To investigate the resistance vessel in response to constrictor (phenylephrine) and vasodilators (carbachol and CGRP)
- To measure the plasma CGRP levels and ET-1 levels by ELISA
- To determine the mRNA expression of TRPV1, CGRP and other genes such as oxidized stress markers.

5.3. **Results**

5.3.1 Effect of HFD on body weight, cardiac hypertension and adipocyte size in WT and TRPV1 KO mice.

Both WT and TRPV1 KO mice (3-4 weeks old, matched for bodyweight, age and sex) were fed with HFD (35% of fat from lard) or normal diet (4% of fat; control) for 12 weeks. Body weight was monitored weekly. There was no significant difference in the body weight between WT and TRPV1 KO mice when they were fed with the normal diet. The HFD caused similar weight gain between WT and TRPV1 KO mice (Fig 5.1). Heart to tibia ratio was examined where there was no significant difference between WT normal diet and TRPV1 normal diet fed mice. However, HFD fed for 12 weeks caused significant increases in both WT and TRPV1 KO mice in heart to tibia ratio when compared to their normal diet fed mice (Fig 5.2). This suggests that both WT and TRPV1 KO mice, after fed with HFD for 12 weeks, developed cardiac hypertrophy. Moreover, assessment of adipocyte size revealed that mesenteric adipose tissue from TRPV1 KO HFD fed mice contained a significantly higher percentage of small adipocytes (1-20 μm) than that from WT HFD fed mice (Fig 5.3). This assessment was done by three different people in a blind manner (please see the methods section for details).

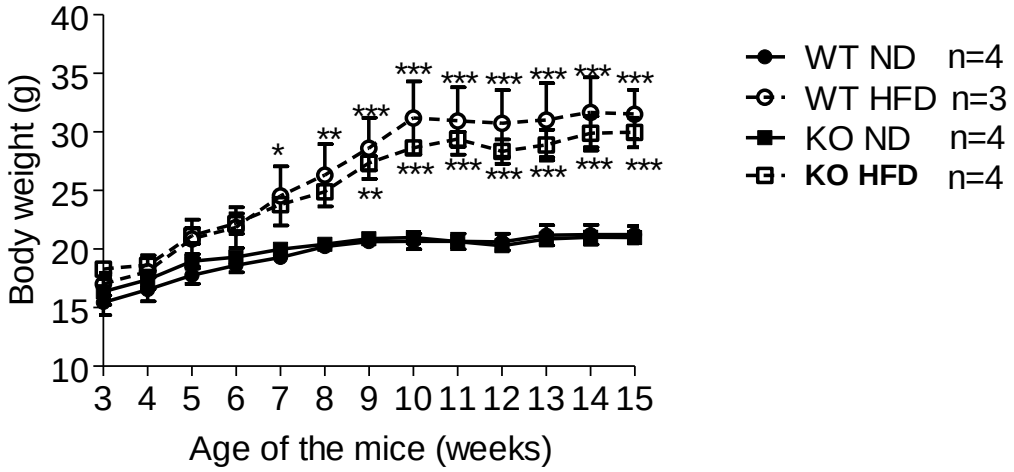


Figure 5.1. Effect of HFD (35%) on WT and TRPV1 KO mice. Body weight from 3 to 15 weeks. N=3-4 *=p<0.05, ** = p<0.01, ***=p<0.001, compared to respective normal diet control. Statistics by two-way ANOVA with Bonferroni post-hoc-tests.

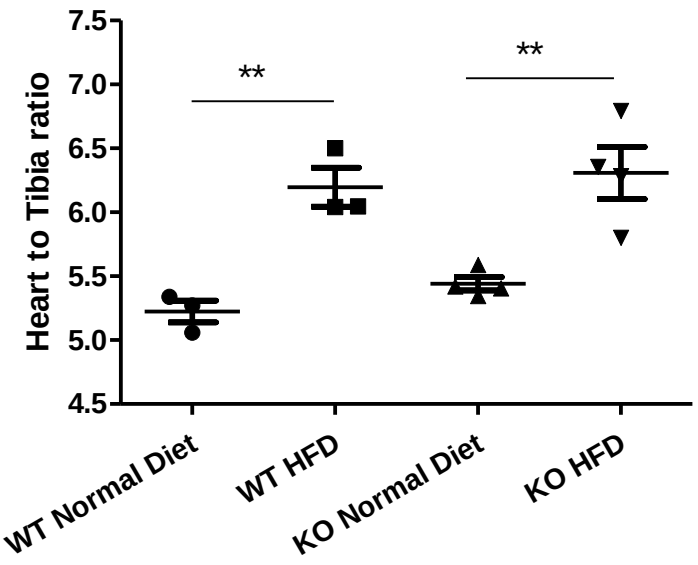


Figure 5.2: Heart to tibia ratio of WT and TRPV1 KO mice after feeding with HFD or normal diet for 12 weeks. **=p<0.01 Statistics by two-way ANOVA with Bonferroni post-hoc-tests.

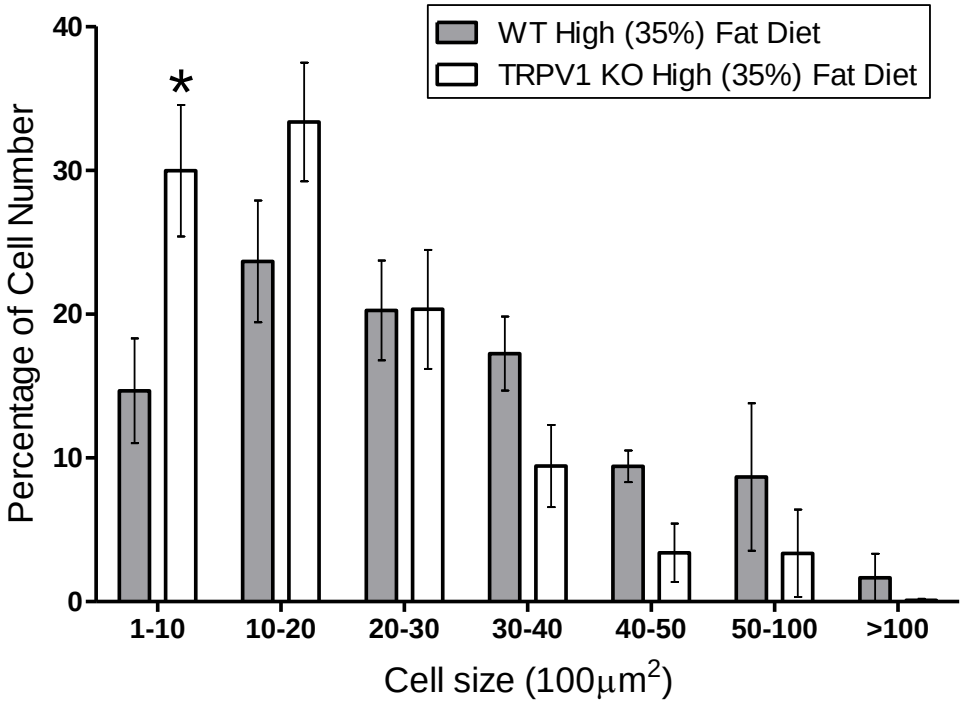


Figure 5.3. Effect of HFD (35%) on WT and TRPV1 KO mice on the adipocytes. Percentage of adipocytes based on cell size (μm2), n=4-5. *=$p<0.05$ (student's unpaired-test) when compared with WT HFD.

5.3.2 Effect of HFD on blood pressure and vascular hypertrophy in WT and TRPV1 KO mice.

For this study, blood pressure was determined by both tail cuff and radio-telemetry. Initially, blood pressure was assessed in the last week of the feeding over the last three days before termination. Data was presented as mean of the three days (Fig 5.4) of systolic (A), diastolic (B) blood pressure and mean arterial pressure (C). HFD induced a significant increase in blood pressure in WT mice when compared to their normal diet fed mice but not in TRPV1 KO mice. Based on these results and the fact that this was a long term study (12 weeks), I was interested in the development of the hypertension in the WT mice. Therefore, a second study was performed where measurement of the blood pressure was monitored from 9 weeks feeding by tail cuff. As shown in Fig 5.5, a significant increase in diastolic and mean arterial pressure occurred after 8 weeks HFD feeding and for systolic blood pressure after 10 weeks, where these increases continued until the experiment was terminated. Despite the low n, we have clearly demonstrated the trend of consistent hypertension in WT HFD-fed mice but not in TRPV1 HFD-fed mice. To further investigate the effect of the HFD on blood pressure, a third set of studies was carried out using radio-telemetry to measure the blood pressure. The telemetry probe was implanted after 8 weeks of feeding when the normal diet fed mice were large enough and the HFD fed mice were not completely obese to compromise the surgical procedure. Data was collected only on the last three days of the study. Blood pressure traces are shown in Fig 5.6 with n=3-4 for normal diet and n=6 for HFD fed mice. The mean value of those three days was plotted in graph form (A systolic, B diastolic and C mean arterial pressure, Fig 5.7) and showed a similar trend with those obtained in the tail cuff studies. The data from telemetry demonstrated that both WT and TRPV1 KO normal diet fed mice had similar blood pressure. However HFD significantly induced high blood pressure in the WT mice but not in the TRPV1 KO mice.

Due to the interesting results for the blood pressure, I moved to investigate the aortic histology using Masson's trichome staining. Fig 5.8 shows the representative staining pictures of aortic section (5μm thickness) under 40x or 400x of magnifications. This staining was done by a previous PhD student (Marshall) who worked with me on some

aspects of the HFD feeding study. Assessment of vascular hypertrophy was performed and results are shown in Fig 5.9A (aortic wall width) and Fig 5.9B (fibrosis/collagen width). These assessments reveal that HFD significantly increased the aortic wall width and fibrosis/collagen width in the HFD WT mice when compared with WT normal diet fed mice. These differences were not seen in the TRPV1 KO mice between HFD and normal diet fed mice. On the other hand, the gene expression of collagen III in the aortic tissue showed no significant difference between different treatment groups (Fig 5.9 C).

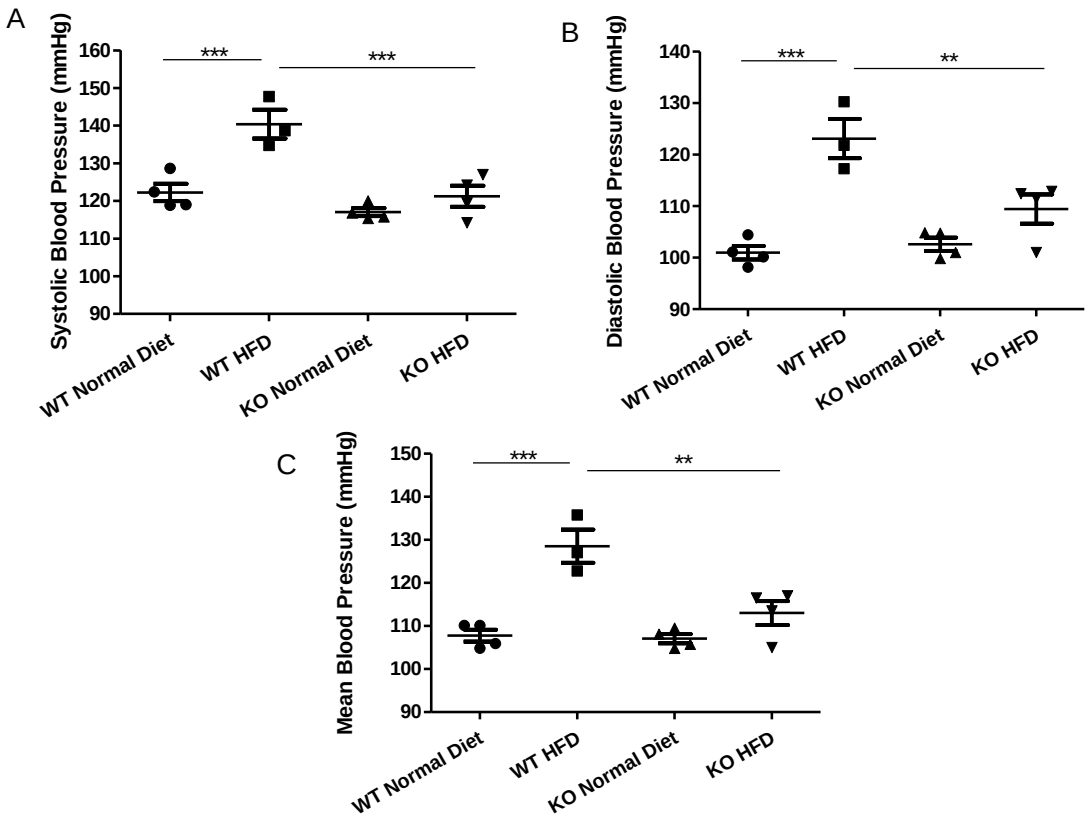


Figure 5.4: Effect of HFD (35%) on blood pressure measured by tail cuff after feeding with either HFD or normal diet for 12 weeks. Readings were taken in the week 12 of the feeding study. A. systolic, B. diastolic and C mean blood pressure. **=$p<0.01$ and ***=$p<0.001$. N=3-4. Statistics by two-way ANOVA with Bonferroni post-hoc-tests.

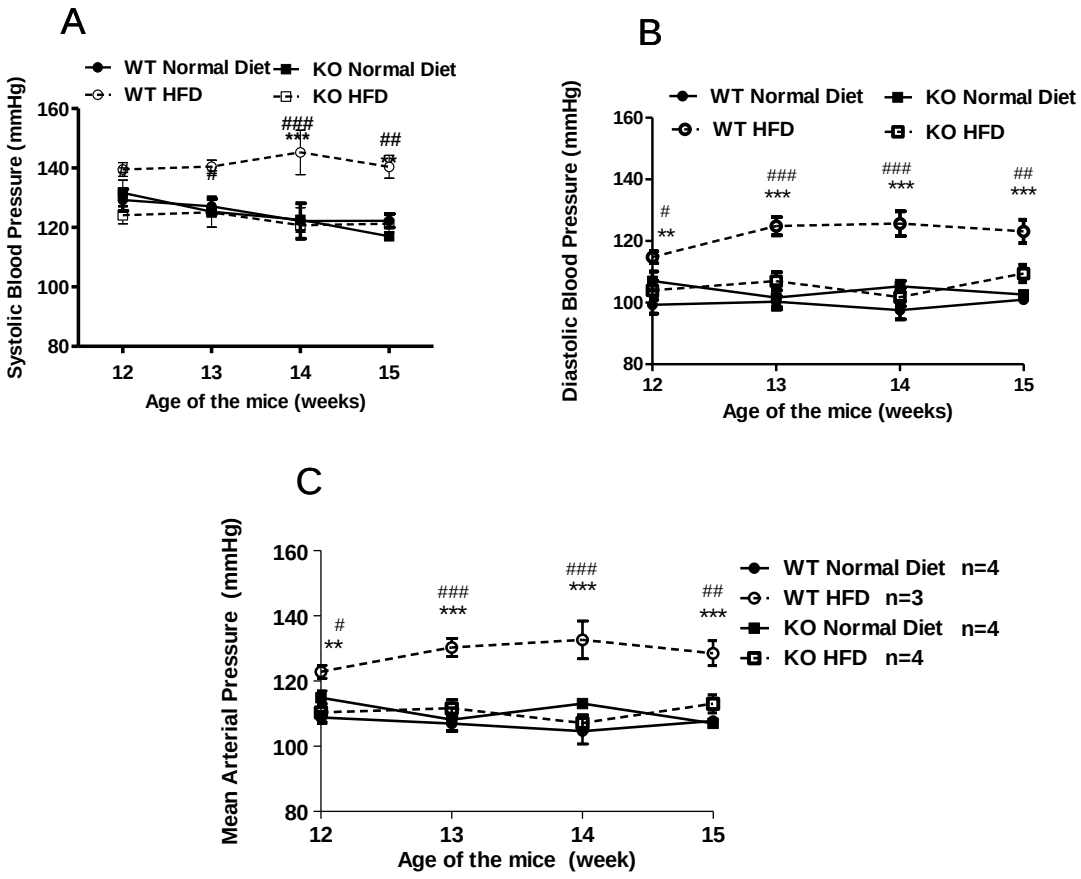


Figure 5.5: Effect of HFD (35%) on blood pressure measured by tail cuff for four weeks after feeding with either HFD or normal diet. A. systolic, B. diastolic and C mean blood pressure. **=p<0.01 and ***=p<0.001 when compared to the normal diet. #=p<0.05, ##=p<0.01 and ###=p<0.001 when compared to the KO HFD mice. N=3-4. Statistics by two-way ANOVA with Bonferroni post-hoc-tests.

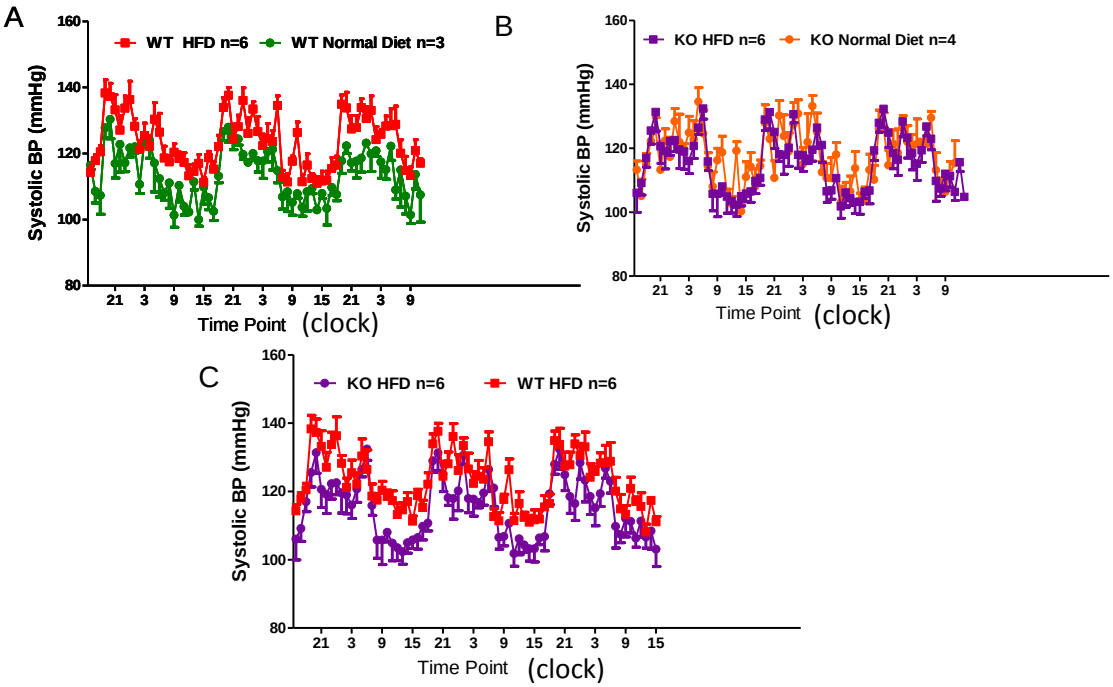


Figure 5.6: Effect of HFD (35%) on systolic blood pressure measured by telemetry for the last three days in the week 12 of HFD feeding study. Blood pressure trace comparing WT normal diet mice with WT HFD mice (A), KO normal diet mice with KO HFD mice (B) and WT HFD mice with KO HFD mice (C).

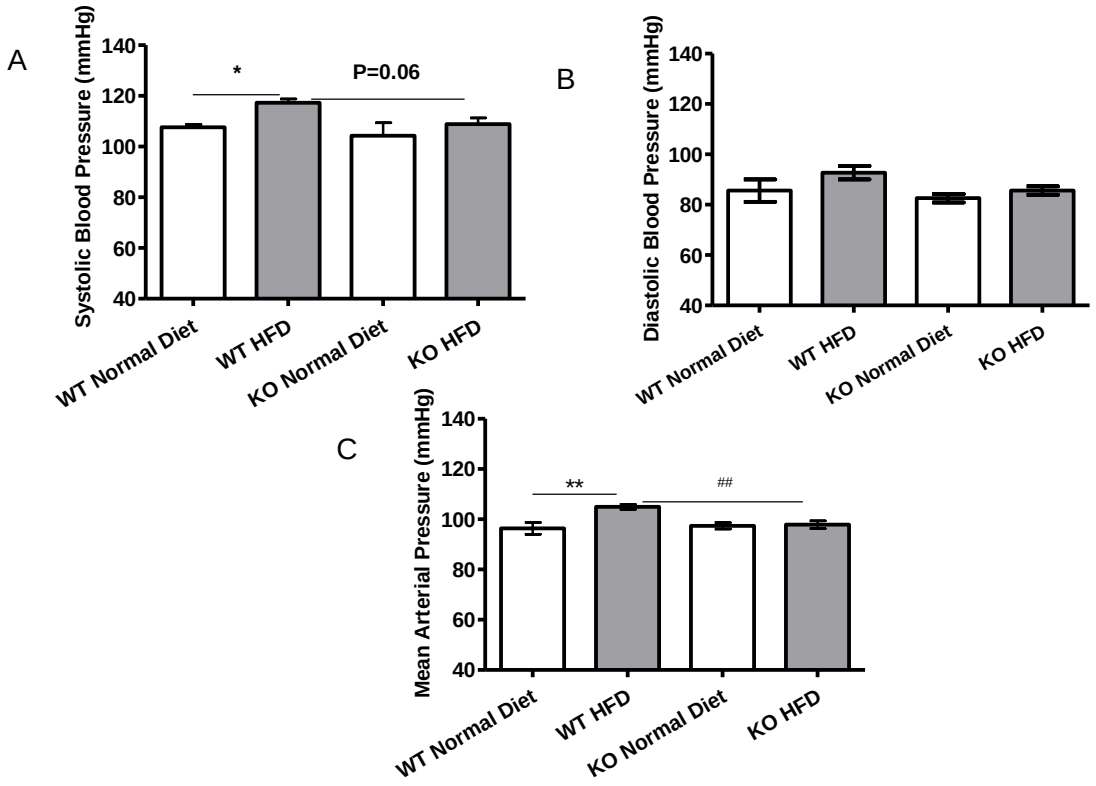


Figure 5.7: Effect of HFD (35%) on blood pressure measured by telemetry after feeding with either HFD or normal diet. Readings were taken in the last three days of the week 12 of the feeding study.A. systolic, B. diastolic and C mean blood pressure. *=$p<0.05$, **=$p<0.01$ and ##=$p<0.01$. WT normal diet n=3, WT HFD n=6, KO normal diet n=4 and KO HFD n=6. Statistic was done by two-way ANOVA with Bonferroni post-hoc-tests.

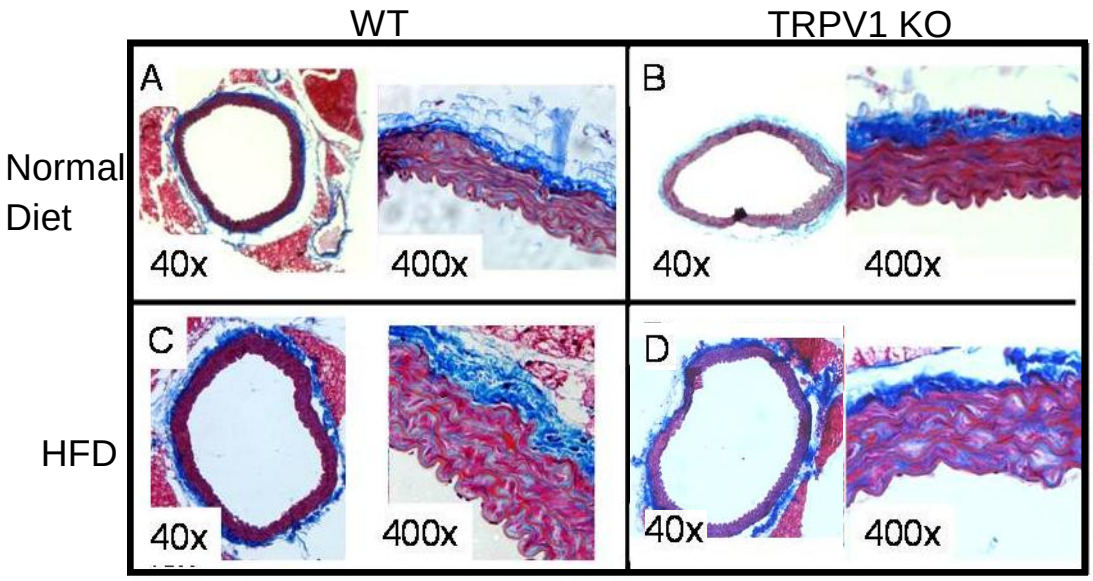


Figure 5.8. Effect of HFD (35%) on vascular hypertrophy after feeding with either normal diet or HFD for 12 weeks. Representative Masson's Trichrome staining of thoracic aorta wall at 40x and 400x magnification as indicated. Dark pink indicates the staining of muscle; the blue indicates collagen while the purple indicates the nucleus.

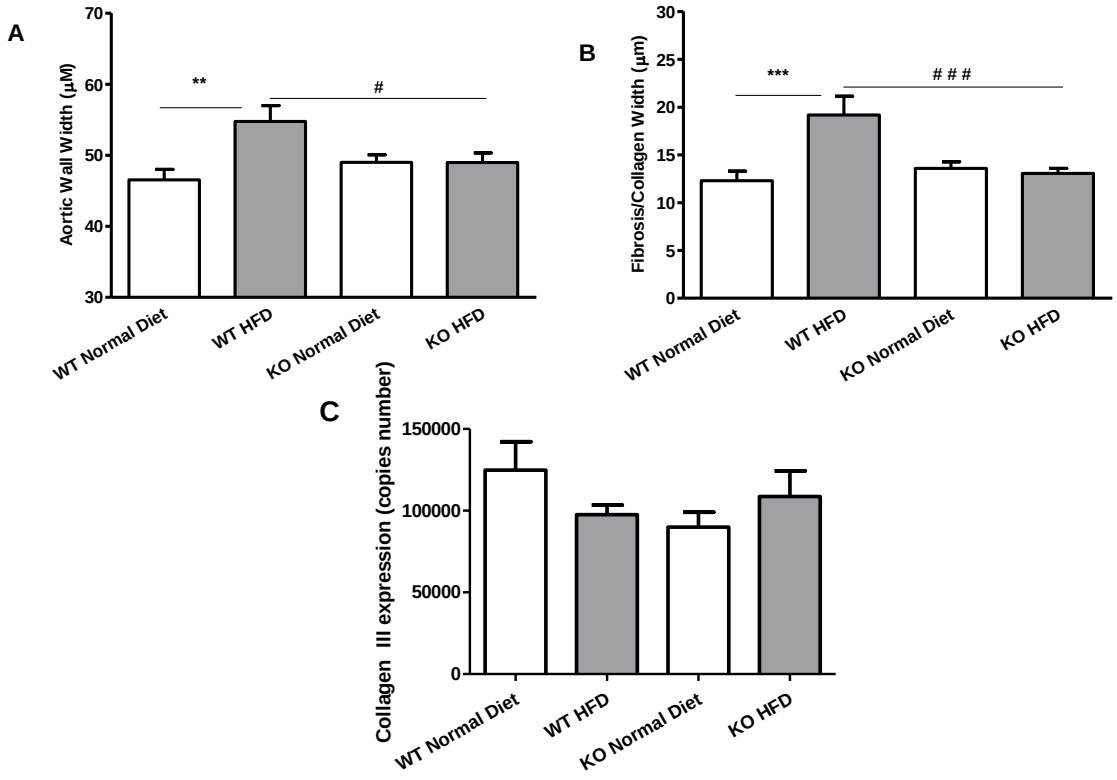


Figure 5.9. Effect of HFD (35%) on aorta remodelling after fed either normal diet or HFD for 12 weeks. A. Aortic wall width; B. Fibrosis and collagen thickness; and C. collagen III gene expression by QPCR. Samples were measured from Masson's trichrome stained sections (n=10-14 with 3-4 sections from 3-4 mice in each group). **=p<0.01 and ***=p<0.001 WT HFD mcie were compared with WT normal diet control mice. #=p<0.05 and ###=p<0.001 when WT HFD mice were compared with TRPV1KO HFD mice. Results are analysed by two-way ANOVA with Bonferroni post-hoc-tests.

5.3.3 Effect of HFD on blood glucose and vascular inflammation in WT and TRPV1 KO mice.

The effect of HFD on glucose tolerance was also investigated in the final week of the experiment, before the termination. Baseline blood glucose was measured following a 6 hours fasting period and bolus i.p. administration of a 2 mg/kg glucose. Both WT and TRPV1 KO displayed similar baseline blood glucose with or without HFD (Fig 5.10 and Fig 5.11A). Although it seemed that the mice with HFD showed higher baseline blood glucose than the normal diet fed mice, there was not a significant difference between the different diets. The blood glucose levels of WT and TRPV1 KO mice fed a normal diet increased and peaked at a similar level (Fig 5.10). When glucose was administered to HFD fed WT and TRPV1 KO mice, blood glucose peaked rapidly 60 minutes after, before returning close to baseline. For both genotypes, the HFD resulted in significantly higher glucose levels, which indicates a reduction in glucose tolerance. However, the blood glucose of TRPV1 KO mice returned to baseline significantly quicker than WT mice suggesting that TRPV1 KO mice retained more glucose tolerance than WT when fed a HFD (Fig 5.10 and Fig 5.11B).

Vascular inflammation (expression of VCAM-1 in aortic tissue) was also assessed in this study by immunohistochemical staining. Fig 5.12A shows the representative pictures of the VCAM-1 staining in frozen tissue at 200x magnification. Based on the staining, VCAM-1 expression was assessed as percentage of VCAM-1 in the aortic wall area (Fig 5.12 B). There is low expression of VCAM-1 on the aortic wall and there is no significant difference between different treatment groups. However the VCAM-1 mRNA expression on aorta tissue shows that the VCAM-1 gene expression was significantly lower in the WT HFD fed mice than their normal diet fed control mice (Fig 5.13). There was no difference between HFD fed and normal diet fed TRPV1 KO mice, normalized by two reference genes (B2M and HPRT1).

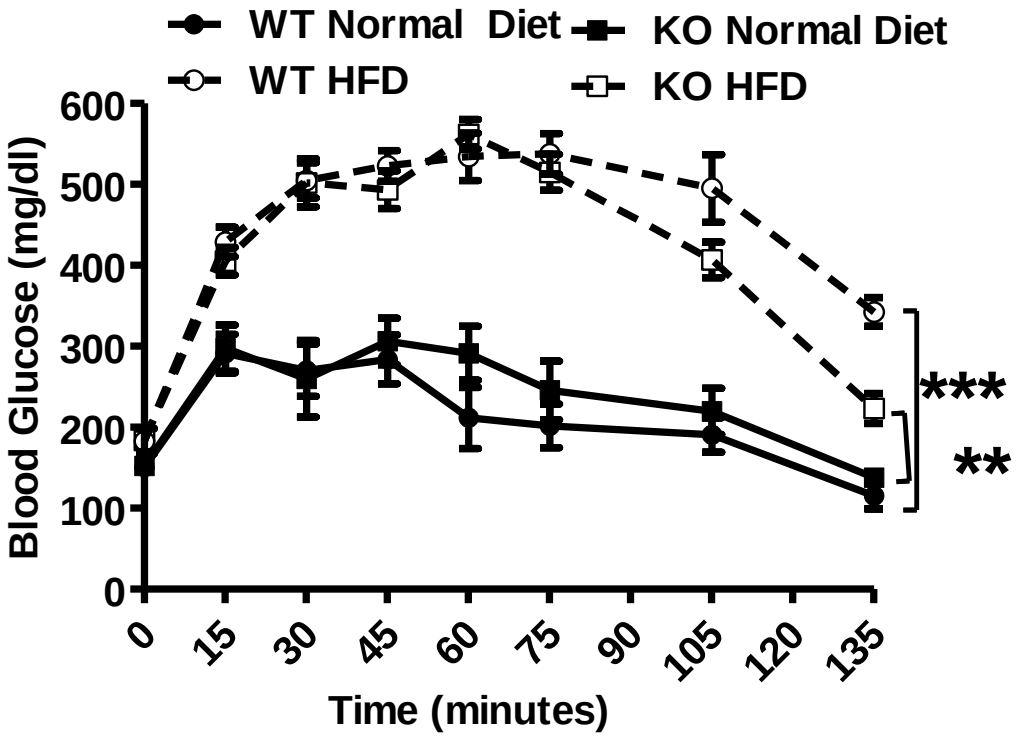


Figure 5.10: Effect of HFD (35%) on blood glucose levels of WT and TRPV1 KO mice after feeding with HFD or normal diet for 12 weeks, following a 2mg/kg glucose bolus (n=5-13). Results are analysed by comparison of mean area under curve with two-way ANOVA with Bonferroni post-hoc-tests.**=$p<0.01$ and ***=$p<0.001$ when compared to the normal diet.

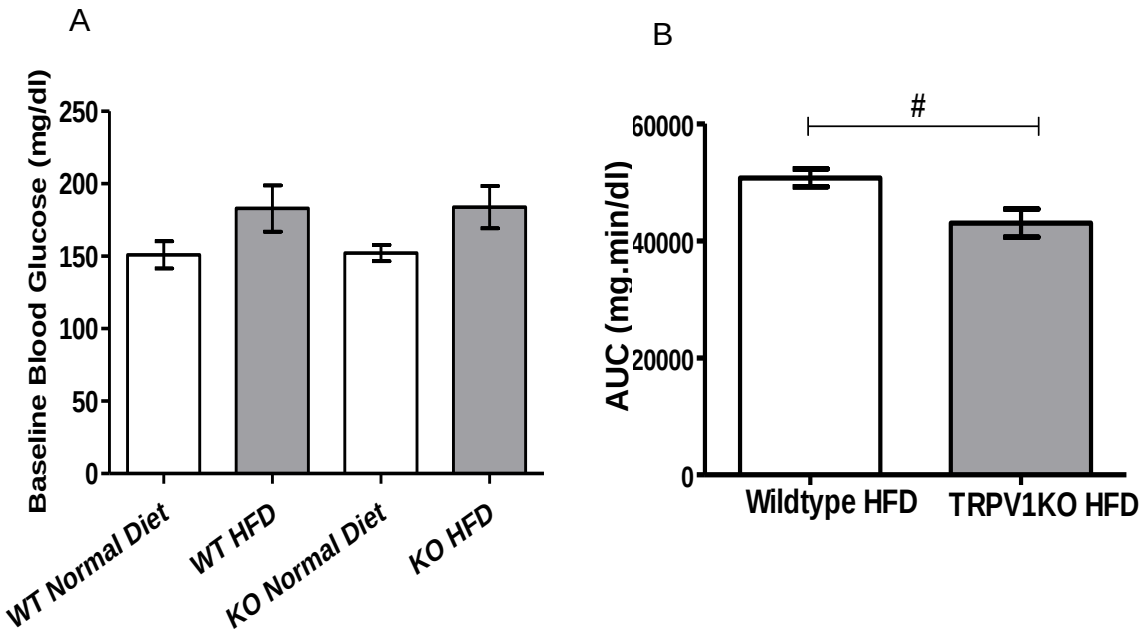


Figure 5.11: Effect of HFD (35%) on blood glucose levels of WT and TRPV1 KO mice after feeding with HFD or normal diet for 12 weeks, A. baseline blood glucose and B. area under the curve of plasma glucose following a 2mg/kg glucose bolus. n=5-13. #=$p<0.05$. Results are analysed by two-way ANOVA with Bonferroni post-hoc-tests for baseline and student t-test for area under the curve of plasma blood glucose.

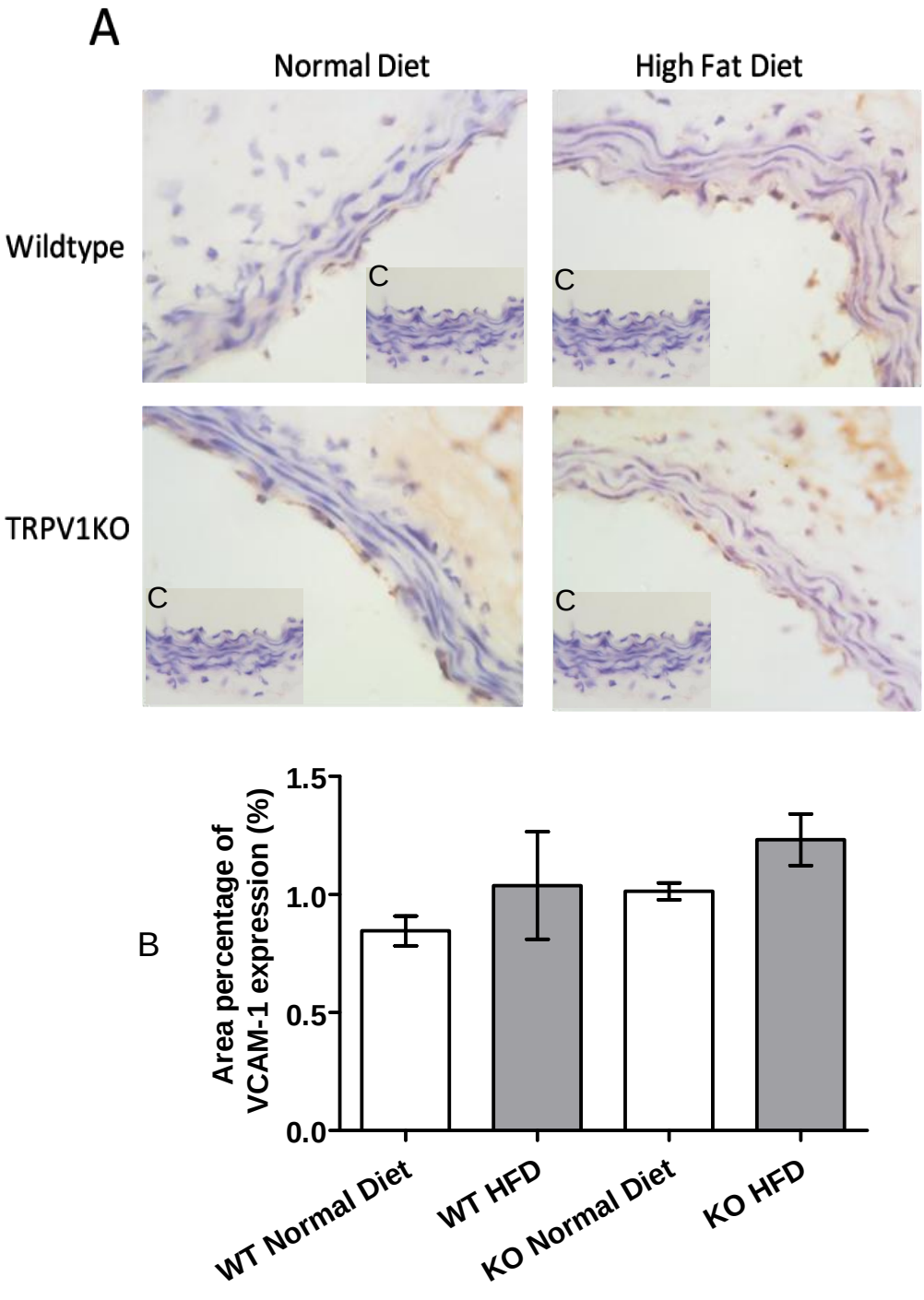


Figure 5.12 Effect of high (35%) fat diet on expression of VCAM-1 on aortic wall. A) Representative immunohistochemical staining of VCAM-1 in the thoracic aorta wall at 200x magnification. (B) Percentage of VCAM-1 expression in relation to total aortic area, n=3-4

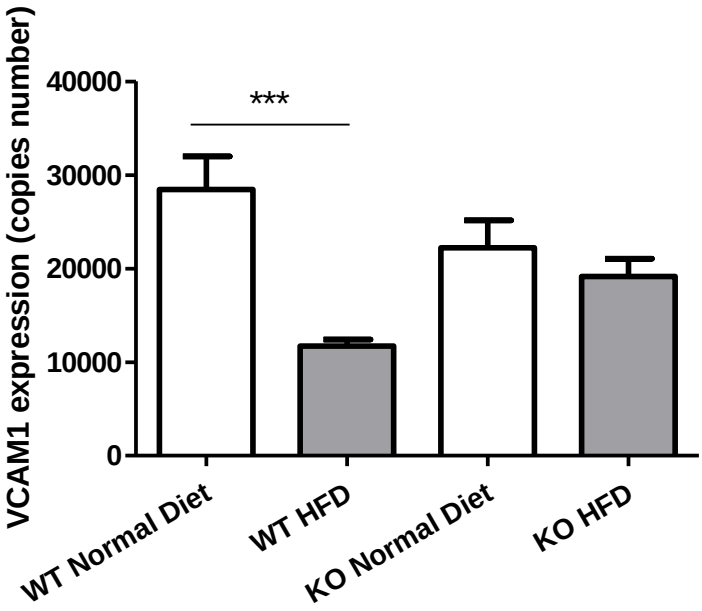


Figure 5.13. Effect of high (35%) fat diet on expression of VCAM-1 on aortic tissue using qPCR. Two reference genes (HPRT1 and B2M) were used to analysed data. ***=$p<0.001$. N=6-7. Results are analysed by two-way ANOVA with Bonferroni post-hoc-tests.

5.3.4. Effect of HFD on resistance vessel responsiveness and eNOS mRNA expression in WT and TRPV1 KO mice.

The vascular responsiveness was investigated in resistance vessel (first order mesenteric arterioles) determined by wire myography. Following cumulative addition of phenylephrine (0.1-10μM), no significant change was observed between different treatment groups (Fig 5.14 and Fig 5.15). We also investigated the vasodilatation ability of these mesenteric arterials using CGRP (0.33-33nM) and endothelium-dependent vasodilator carbachol (1nM-10μM) following preconstriction with 10μM phenylephrine. Interestingly, there was no significant change seen between different treatment groups. Data were presented into two different ways: as percentage of contraction/relaxation (Fig 5.14) or as force (Fig 5.15). These results indicate that the HFD caused no significant effect on resistance vessels from both WT and TRPV1 KO mice in terms of their responsiveness to either constrictor (phenylephrine) or vasodilators (CGRP and carbachol) when compared to the normal diet treated mice.

In addition eNOS expression in aortic tissue was investigated by RT-qPCR (normalised by two reference genes: HPRT1 and B2M). Both WT and TRPV1 KO mice had similar levels of eNOS mRNA levels after feeding with normal diet. Moreover, HFD increased the eNOS expression in both WT and TRPV1 KO mice at a similar level. But such an increase was not significantly different when compared to their normal diet fed mice (Fig 5.16).

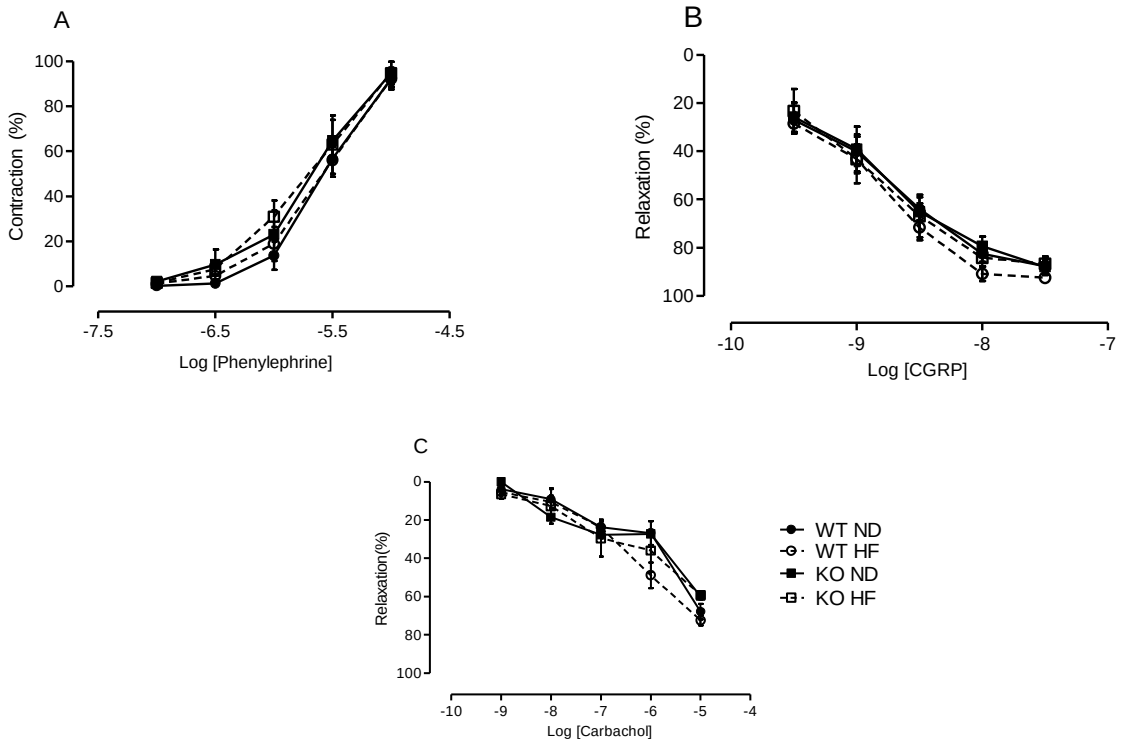


Figure 5.14. The effect of phenylephrine, CGRP and carbahol on ND-WT and ND-TRPV1 KO mouse and also HFD-WT and HFD-TRPV1 KO mouse mesenteric arteries, as determined by myograph and data was present as percentage of contraction or relaxation. (A) Effect of phenylephrine (0.1-10μM) and (B) CGRP (0.33-33nM) on mesenteric arteries (including endothelium intact and denuded).n = 5-7. (C) Effect of carbachol (1nM-10 μM) on endothelium intact mesenteric arteries. n=3-6. Dilator responses were examined in the presence of phenylephrine (10μM). Results are analysed by two-way ANOVA with Bonferroni post-hoc-tests.

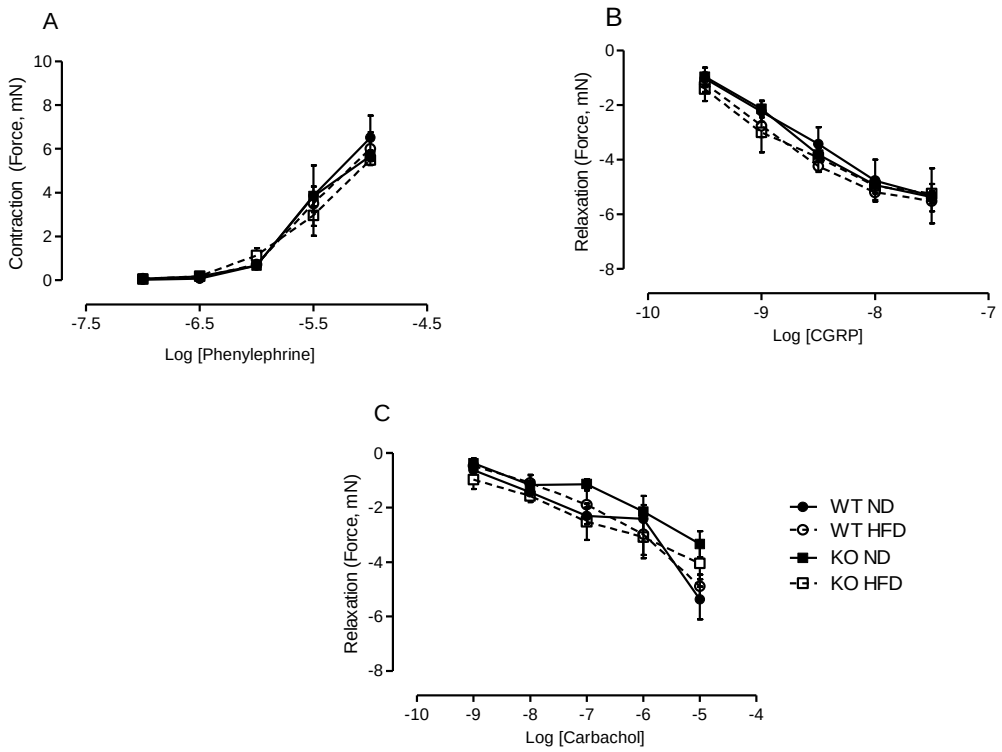


Figure 5.15. The effect of phenylephrine, CGRP and carbachol on ND-WT and ND-TRPV1 KO mouse and also HFD-WT and HFD-TRPV1 KO mouse mesenteric arteries, as determined by myograph and data was present as force. (A) Effect of phenylephrine (0.1-10μM) and (B) CGRP (0.33-33nM) on mesenteric arteries (including endothelium intact and denuded). n = 5-7. (C) Effect of carbachol (1nM-10 μM) on endothelium intact mesenteric arteries. n=3-6. Dilator responses were examined in the presence of phenylephrine (10 μM). Results are analysed by two-way ANOVA with Bonferroni post-hoc-tests.

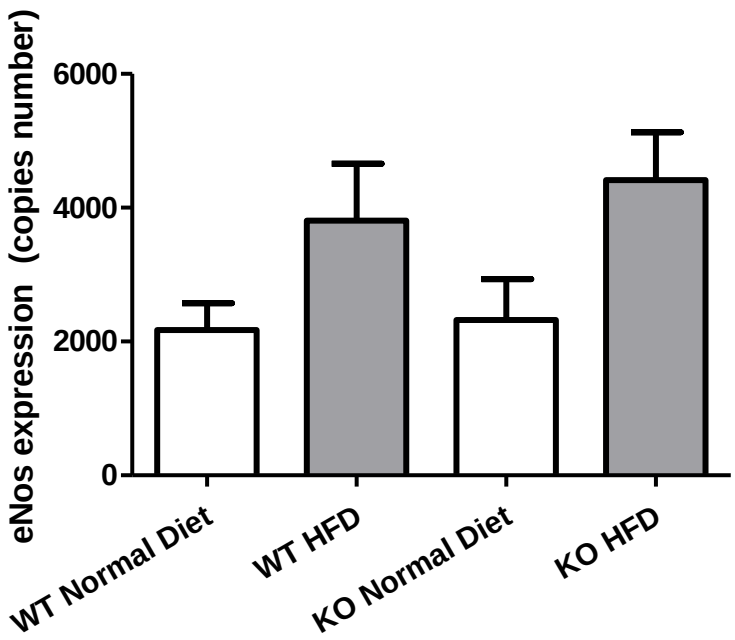


Figure 5.16. Effect of high (35%) fat diet on expression of eNos in aortic tissue samples using QPCR. Two house keeping genes were used to analyse data. N=6-7. Results are analysed by two-way ANOVA with Bonferroni post-tests.

[5.3.5. Effect of HFD on expression of TRPV1, CGRP, ET-1, Oxidized Stress markers and noradrenaline in WT and TRPV1 KO mice.

In order to understand more about possible mechanisms of the protection we have observed in the TRPV1 KO mice in this study, RT-qPCR was used to evaluate selected genes in aortic tissue and followed with assessment of protein expression, using ELISA assays, to determine potential mediators.

TRPV1 mRNA showed no significant difference in the WT mice between HFD and normal diet fed mice (Fig 5.17A) and as expected there was no expression in the TRPV1 KO mice. HFD reduced levels of CGRP mRNA in the WT mice when compared to the normal diet fed mice but with no significant difference. Interestingly, both HFD and normal diet fed TRPV1 KO mice showed similar CGRP mRNA levels to the HFD fed WT mice (Fig 5.17B). Moreover, plasma levels of CGRP were determined by ELISA and normalised by protein content, showed that both normal fed WT and TRPV1 KO mice had similar levels of plasma CGRP. Furthermore, the HFD did not cause any significant change in the plasma CGRP levels between WT and TRPV1 KO mice (Fig 5.17C).

The measurement of both plasma and mRNA levels of ET-1 (endothelin 1) were assessed in this study. Plasma levels of ET-1 were determined by ELISA after peptide extraction and normalised by protein content. After HFD there was a non-significant trend towards increased plasma levels of ET-1 in the WT mice (16.0±2.7pg/mg of protein) when compared to the normal diet fed mice (15.1±2.4 pg/mg of protein). However, such an increase was not seen in the TRPV1 KO mice between HFD (14.7±2.0 pg/mg of protein) and normal diet fed mice (14.9 ±2.2 pg/mg of protein, Fig 5.18A). Interestingly, the mRNA levels of ET-1 in the aortic tissue was significantly increased following HFD when compared to the normal diet fed mice (Fig 5.18B). However, there was no such difference in the TRPV1 KO mice between HFD and normal diet fed mice. When comparing the WT and TRPV1 KO mice after the HFD, WT mice had significant increases in levels of ET-1 expression (Fig 5.18B).

Superoxide dismutase (SOD) exists as three isoforms (SOD1, SOD2 and SOD3) where all are important for the antioxidant defense. In this study, I focused on SOD1 as it has been studied and proved to be involved in inflammatory conditions by both others (Segui *et al*., 2004 and Gongora *et al*., 2006) and within our group (Smillie, 2012). SOD mRNA expression was investigated by RT-qPCR, where both WT and TRPV1 KO normal diet fed mice had similar levels of SOD mRNA levels. However, HFD significantly increased the SOD mRNA levels in the WT mice but not in the TRPV1 KO mice when compared to their normal diet control mice (Fig 5.19A). Moreover, we also assessed the mRNA expression of NOX2 (a member of the NADPH family of enzymes which primarily produce ROS) and HO1 (heme oxygenase composed of two forms HO1 and HO2, we focused on HO1 in this study). For NOX2 no significant difference was seen in expression in the aorta between either WT and TRPV1 KO mice, or between HFD and normal diet fed mice (Fig 5.19B). HO1 mRNA was expressed at a similar level between WT and TRPV1 normal diet fed mice. The HFD did not affect the expression of HO1 in the WT mice. However, an increased level of HO-1 was observed in the TRPV1 KO mice after being fed the HFD (Fig 5.19C).

At the end of the study, we investigated the noradrenaline level in the kidney tissue via ELISA and then normalised by protein content. Interestingly, the HFD significantly increased the noradrenaline levels in the kidney in the WT mice when compared to the normal diet fed mice (Fig 5.20). All the TRPV1 KO mice (both HFD and normal diet fed mice) had low levels of noradrenaline level in the kidney when compared to the WT mice. The HFD WT mice showed significantly more noradrenaline levels than the HFD TRPV1 KO mice ($p<0.001$, Fig 5.20).

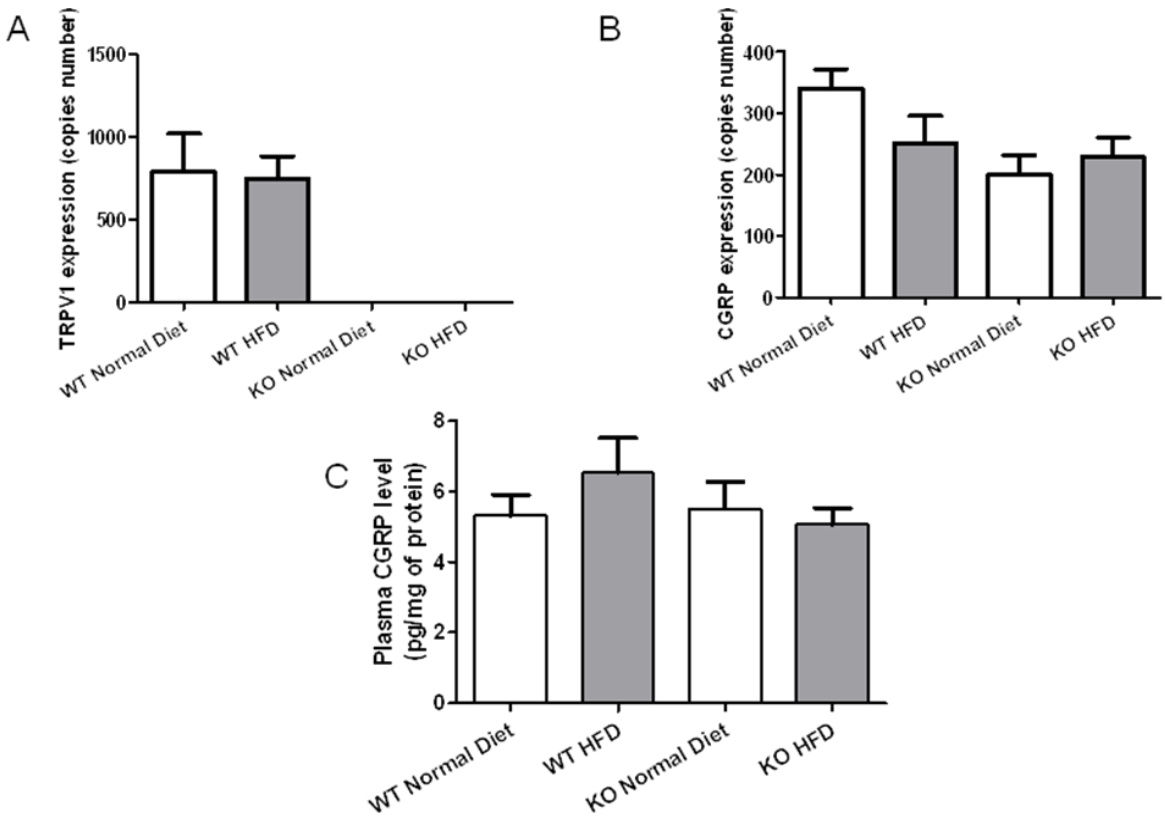


Figure 5.17. Effect of high (35%) fat diet on expression of TRPV1 (A) and CGRP (B) on aortic tissue using QPCR. Two reference genes (HRPT1 and B2M) were used to analysed data. N=6-7. C. plasma level of CGRP by CGRP ELISA (WT normal diet n=7, WT HFD n=8, KO normal diet n=5 and KO HFD n=8). Results were analysed by two-way ANOVA + Bonferroni's post-hoc-test.

A	WT normal diet, n=7	WT high (35%) fat diet, n=8	TRPV1KO normal diet, n=5	TRPV1KO high (35%) fat diet, n=8
Endothelin-1 (pg/mg of protein)	15.1 ± 2.4	16.0 ± 2.7	14.7 ± 2.0	14.9 ± 2.2

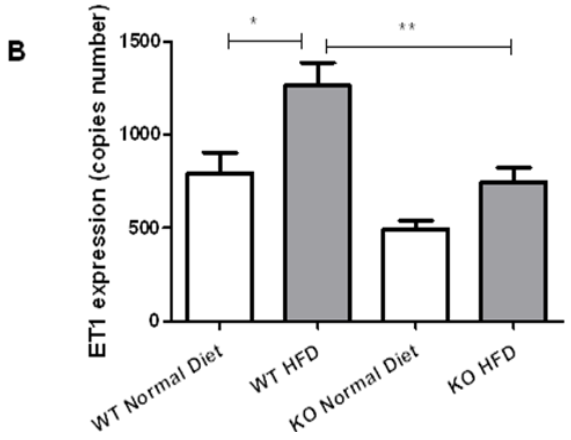


Figure 5.18. The effect of a HFD on (A) plasma levels of endothelin-1 taken from WT and TRPV1 KO mice fed either a normal or HFD from 3-15 weeks of age (WT normal diet n=7, WT HFD n=8, KO normal diet n=5 and KO HFD n=8) and (B) gene expression of ET1 on aortic tissue. *=$p<0.05$ and **=$p<0.01$. Results were analysed by two-way ANOVA + Bonferroni's post-hoc-test.

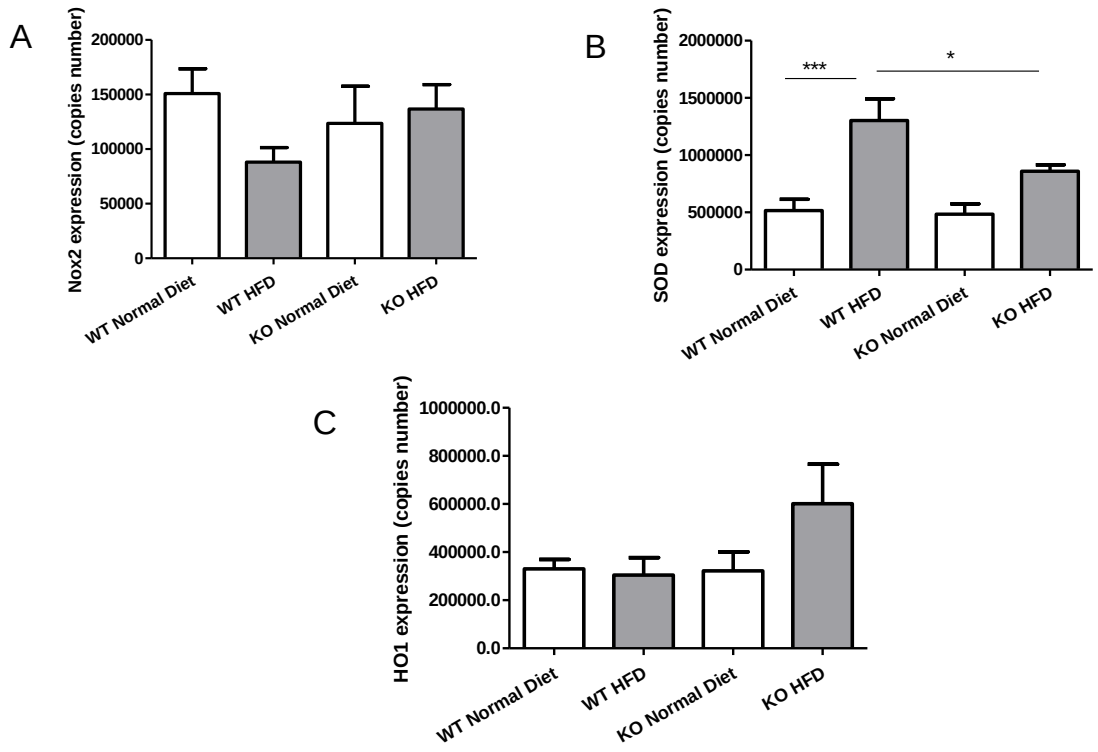


Figure 5.19. Effect of high (35%) fat diet on expression of oxidase stress markers (Nox2, SOD and HO1) on aortic tissue using QPCR. Two reference genes (HRPT1 and B2M) were used to analysed data. *=$p<0.05$ and ***=$p<0.001$. N=6-7. Results are analysed by two-way ANOVA with Bonferroni post-hoc-tests.

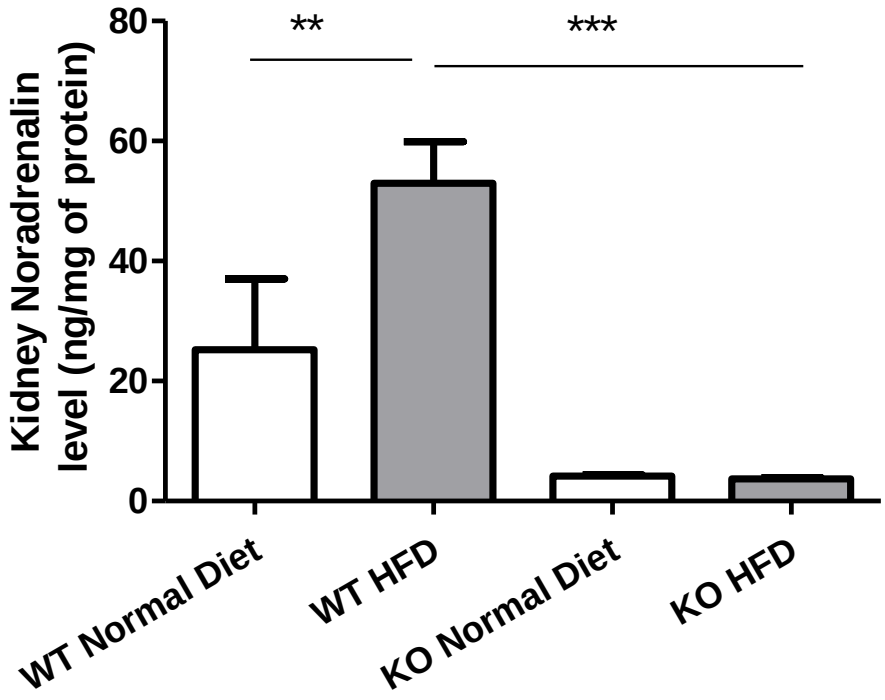


Figure 5.20. The effect of a HFD on noradrenaline levels in kidney tissue taken from WT and TRPV1 KO mice fed either a normal or HFD from 3-15 weeks of age. WT normal diet n=4, WT HFD n=4, KO normal diet n=4 and KO HFD n=8. Analysed by two-way ANOVA + Bonferroni's t-test. **=$p<0.01$ and ***=$p<0.001$.

5.4 **Summary**

- There was a similar weight gain in both WT and TRPV1 KO mice after feeding with a normal diet. However HFD (35% of fat) for 12 weeks significantly induced obesity in both WT and TRPV1 KO mice when compared to their normal diet fed mice. There was no difference between HFD-WT and HFD-TRPV1 KO mice in terms of weight.

- The HFD significantly increased heart to tibia ratio in both WT and TRPV1 KO mice, indicating cardiac hypertrophy when compared with mice on a normal diet. In addition, the HFD induced enlargement of the size of mesenteric adipocytes in the WT mice but not in the TRPV1 KO mice, indicating an effect on adipocyte differentiation.

- Both tail cuff plesthymography and telemetry revealed the same trend that both WT and TRPV1 KO have similar blood pressure levels under normal conditions, but HFD induced a hypertensive phenotype in the WT mice but not in the TRPV1 KO mice.

- It was also demonstrated that HFD significantly induced aortic vascular hypertrophy in the WT mice but not in the TRPV1 KO mice.

- WT and TRPV1 KO mice had similar blood glucose levels after HFD for 12 weeks, when compared to those on normal diet. Both WT and TRPV1 KO mice, fed with HFD, resulted in significant glucose intolerance. However, the blood glucose of TRPV1 KO mice returned to baseline significantly faster than that of WT mice. This indicates that HFD-TRPV1 KO mice possess improved glucose tolerance when compared with HFD-WT mice.

- Both immunohistochemistry and QPCR indicated that there was no significant difference in term of the expression of the inflammatory marker VCAM-1 between the different treatment groups.

- Use of myograph to study resistance vessel reactivity demonstrated no significant difference between the treatment groups in terms of their responsiveness to a vasoconstrictor agent phenylephrine, and vasodilators carbachol and CGRP.

Moreover, no significant difference was observed in term of the eNOS mRNA expression between the different treatment groups.

- Feeding with HFD for 12 weeks did not cause significant change in the mRNA expression of TRPV1, CGRP, NOX2 and HO-1. A significant increase in mRNA level of ET-1 in aortic tissues in HFD-WT mice were seen compared to both normal diet and HFD-TRPV1 KOs. Moreover, HFD induced greater SOD1 mRNA expression on aortic tissue compared to WT but not in the TRPV1 KO mice. Furthermore, HFD-WT mice demonstrated significantly higher levels of noradrenaline in the kidney when compared to the normal diet fed WT mice. Interestingly, the basal noradrenaline levels in the kidney were low in the TRPV1 KO mice.

5.5 **Discussion**

This study was designed to investigate the role of TRPV1 in the obesity associated hypertension induced by 12 weeks of HFD feeding in WT and TRPV1 KO mice. The reason for this was to determine whether HFD-WT mice would be protected from hypertension due to endogenous release of CGRP in this model as it is considered to be less 'aggressive' than the ATII model used in Chapter 4. Here we demonstrate that the HFD-WT mice displayed an enhanced metabolic syndrome, in terms of hypertension and glucose handling, when compared to the HFD TRPV1 KO mice. Thus, we did not observe a protective role for TRPV1.

Our current results confirmed that WT and TRPV1 KO mice developed similar weight gain between ages 3 to 15 weeks after feeding with HFD containing 35% of fat from lard. However, other studies have suggested that TRPV1 does affect weight gain. There are several studies where it has been concluded that TRPV1 is associated with weight loss (Zhang *et al*., 2007; Gram *et al*., 2007). For example, capsaicin-treatment (doses of capsaicin at 20, 30 and 50mg/kg, administered over three days) of Zucker rats (a model of the metabolic syndrome and T2D) led to less weight gain than the vehicle-treated Zucker rats over a three week period following treatment. However, after 3 weeks of the treatments, the body weight of capsaicin-treated rats increased significantly more than those of the vehicle-treated rats (Gram *et al*., 2007). This treatment regime of capsaicin is likely to cause desensitisation of the TRPV1 channels on sensory nerves which may imply that loss of functional neuronal TRPV1 in rats leads to a greater increase in body weight than the vehicle-treated rats. Therefore, our current results agreed that depletion of TRPV1 did not protect mice from gaining weight with HFD feeding compared to the normal diet.

Secondly, our results revealed that HFD caused no significant effect on heart weight to tibia ratio between WT and TRPV1 KO mice. However, there was significant difference between HFD and normal diet fed mice. These results indicate that both WT and TRPV1 KO mice developed cardiac hypertrophy compared to their normal diet-fed counterparts. However, this study did demonstrate that WT mice showed a greater percentage of hypertrophied adipocytes, which agrees with the concept that functional

TRPV1 is implicated in adipocyte differentiation (Zhang *et al*., 2007). In the current study, both WT and TRPV1 KO mice had similar adipose tissue weight but the WT mice possessed a higher percentage of larger adipocyte cell size, which indicated that the TRPV1 KO mice had more (albeit smaller) adipocytes than the WT mice. However the enlarged adipocytes in the WT mice could be problematic as hypertrophied adipocytes is one of the characteristics of dysfunctional adipose tissue in obesity (Hajer *et al*., 2008). Moreover, recently a study in humans has shown that enlarged adipocyte cell size is one of the characteristics of adipose tissue metabolic dysfunction in non-obese individuals with low insulin sensitivity (Hammarstedt *et al*., 2012).

Generally, this study provided surprising results as TRPV1 KO mice were protected against the HFD-induced increase in blood pressure compared to WT mice. In this study, blood pressure was measured by two methods. The tail cuff data suggested that hypertension had developed, while the telemetry data suggested that increased blood pressure was present at the end of the study, but had not reached the hypertensive threshold. It was discussed in chapter 4 that TRPV1 is believed to be protective by releasing vasodilator neuropeptides, such as CGRP, resulting in peripheral vasodilation (Fernandes *et al*., 2011). Therefore, I hypothesised that TRPV1 KOs would be lacking this protective vasodilator effect and suffer a worse hypertension after HFD. However this was not the case. The increase in HFD-induced blood pressure increase was mirrored by an effect of HFD on vascular hypertrophy in the WT mice but not in the TRPV1 KO mice. Moreover, the protein expression in the plasma and the mRNA expression in aorta showed that the CGRP was expressed similarly between HFD-WT and HFD-TRPV1 KO mice. These results indicated that the HFD-WT mice did not appear to have the TRPV1-CGRP protective pathway activated. These results led us to think about other mechanisms via which TRPV1 could be acting. Following on from the discussion of TRPV1 potentially mediating vascular constrictor activity (please see the discussion at Chapter 4), it is possible that this is one of the reasons for the increased blood pressure observed in HFD-WT but not TRPV1 KO mice. However, there are other possibilities.

In this study, it was also revealed that mRNA levels of ET-1 were significantly increased in the aorta from the HFD-WT mice than the WT normal diet mice or HFD-KO mice.

This could be one of the mechanisms why the HFD -WT mice developed hypertension but not the TRPV1 KO mice. There is evidence from other studies that link ET-1 levels with hypertension. The endothelin pathway is suggested to become functionally significant at an early stage in spontaneously hypertensive rats in which insulin resistance leads to impaired NO production in the vascular endothelium and enhanced ET-1 secretion (Potenza *et al*., 2005). This may reflect one pathophysiological mechanism in vascular beds that contributes to elevated peripheral vascular resistance and hypertension. Moreover, a recent study has revealed a mechanism by which TRPV1 activation leads to upregulation of the endothelin/ET_A-mediated pathway, which results in vasoconstriction leading to increased mean arterial pressure. Results showed that capsaicin increased mean arterial pressure in a dose-dependent manner (1 μg/kg: 5.7±1.6, 10 μg/kg: 11.7±2.1, 20 μg/kg: 25.4±3.4, 100 μg/kg: 51.6±3.9%) in the WT mice when compared to the control mice, measured in anaesthetised mice following artery catheterization. This effect was not seen in the TRPV1 KO mice (Ohanyan *et al*., 2011). Inhibition of endothelin A receptors abolished the increase in blood pressure induced by capsaicin (Ohanyan *et al*., 2011). However in this chapter, the myography results revealed that constrictor and dilator responses to phenylephrine, CGRP and the endothelial-dependent dilator carbachol were similar, suggesting little vascular dysfunction at the mesenteric resistance vessel level at least. This was mirrored by the result of eNOS mRNA expression, which showed no significant difference between the treatment groups. However, when these experiments were being performed, I had yet to determine that ET-1 gene expression was affected by TRPV1 in our HFD model, where unfortunately I did not save tissue from the myography studies to determine the role of ET-1 directly.

In this study, we also demonstrated that using the glucose tolerance test the HFD-TRPV1 KO mice were able to reverse the high plasma glucose levels significantly quicker than the HFD WT mice (analysed by area under the curve). Our results are supported by those of Gram *et al*. who demonstrated that sensory nerve desensitisation by resiniferatoxin (a TRPV1 agonist, used at a concentration that causes desensitisation) improved oral glucose tolerance, which was accompanied by elevated plasma insulin levels (Gram *et al*., 2005). The same group showed that desensitisation with capsaicin maintained the non-fasting glucose levels and 24 hour blood glucose

profile in the physiological range. Furthermore, in an oral glucose tolerance study, the capsaicin-desensitised rats had lower fasting glucose levels but higher plasma insulin levels. Interestingly, such enhanced insulin secretion in capsaicin-treated rats was associated with reduced serum CGRP (Gram *et al*., 2007). Thus, they suggested that the loss of CGRP-containing sensory fibres could prevent the deterioration of glucose homeostasis, mainly by reducing CGRP-mediated inhibition of insulin secretion. These results are supported by this current study as they suggested that the presence of TRPV1 enhanced glucose levels, which implies that removing the TRPV1-CGRP pathway may be beneficial in term of glucose tolerance.

We also investigated mRNA levels of oxidative stress markers (SOD, NOX2 and HO1) in the aorta. Significantly higher levels were found in HFD-WT extracts compared with HFD-TRPV1 KO mice in term of expression of SOD. Such an increase in SOD may be due to this anti-oxidant attempting to counteract oxidative stress which is known to increase blood pressure (Welch *et al*., 2006).

Finally, I investigated the noradrenaline level within the kidney. We revealed that there was significantly higher level of noradrenaline in the HFD WT mice when compared to the WT normal diet mice. High levels of noradrenaline indicate high levels of sympathetic activity, which may contribute to the increased blood pressure in the WT mice. My results are partially supported by Moreira-Rodrigues *et al*. (2012) who demonstrated that mice fed with high fat high simple carbohydrate diet had insulin resistance and increased sympathetic tone with increased plasma noradrenaline levels (Moreira-Rodrigues *et al*., 2012). Furthermore, recently Santoni *et al*. (2013) demonstrated a functional and structural cross-talk in cancer cells (PC3 cells) between α_{1d}-adrenergic receptor and TRPV1 receptor via a functional assay with different antagonists and confocal microscopy (Santoni *et al*., abstract 2013). They demonstrated that noradrenaline stimulated proton release, calcium influx and cell proliferation in PC3 cells were completely reverted by the WS433 (α_{1d}-adrenergic receptor antagonist) and capsazepine (TRPV1 antagonist) given combination. This indicates that TRPV1 is working, in part, upstream of noradrenaline activities.

In conclusion, these results lead to the proposal that TRPV1 deletion (and potentially blockade) is beneficial in obesity-induced hypertension and vascular hypertrophy. Although I originally hypothesised a protective role of TRPV1, this is not supported by my findings. The precise mechanisms of the pro-hypertensive mechanisms are unknown but may involve non-neuronal TRPV1 channels, endothelin-1 and sympathetic nerves.

CHAPTER 6: GENERAL DISCUSSION

6.1 Hypertension: the present situation

Hypertension, as discussed in the introduction (chapter 1), remains a major public health problem and is a risk factor for many secondary cardiovascular diseases such as for atherosclerosis, one of the biggest killer in the western world (British Heart Foundation 2011). Therefore, it is important to understand the mechanisms underpinning the development of hypertension so that the underlying cause of the disease can be detected and treated more effectively than presently possible. Although there is no direct cause of essential hypertension, many factors lead to the increase in blood pressure. Obesity is one of the main factors where more than 85% of patients with hypertension also have body mass index greater than 25 (Aram *et al*., 2003). At present, several lines of evidence show that current blood pressure control methods are unsatisfactory in treated hypertensive patients. This is due to a variety of factors, for instance patients' poor compliance, insufficient use of combination drug treatment and real difficulties in achieving well controlled BP. Moreover, understanding the mechanisms involved in the development of hypertension is essential. Therefore, it is hoped that the investigation of mechanisms will allow a deeper insight and novel pharmacological approaches into the detection and treatment of hypertension. This thesis has hopefully provided more information into the development of hypertension and also suggested mechanisms by which we can protect against hypertension and vascular hypertrophy.

6.2 Major novel findings from the studies in this thesis

This thesis involved the study of vascular components of murine models of hypertension with two main topics. Firstly, the protective role of the CGRP-like peptide AM was investigated using smooth muscle selective RAMP2 (AM1 receptor component) up-regulated mice. Secondly, TRPV1 was studied, activation of which is known to release CGRP, using WT and TRPV1 KO mice.

The data obtained in chapter 3 of this thesis has shown for the first time that RAMP2 TG mice with up-regulation of the vascular smooth muscle RAMP2 levels, which correlates with raised levels of functional AM1 receptor, were protected against ATII

induced aortic vascular hypertrophy although the level of hypertension was similar to that in WT mice. The current study is novel also because in chapter 4 and 5 of this thesis I have demonstrated for the first time that the TRPV1 KO mice were protected against the hypertension and aortic vascular hypertrophy induced either by ATII or HFD feeding (which will be discussed in details in the later part of this discussion section). These findings provide evidence of new mechanisms involved in the pathophysiology of hypertension and of factors involving the CGRP like peptide AM and the sensory nerve receptor TRPV1 (that when activated releases CGRP).

6.2.1 AM

AM, a member of the CGRP family of peptides, is a vasodilator. This project provides an important insight in that the vascular smooth muscle AM1 receptor (CL/RAMP2) plays a key protective vascular role *in vivo* in the ATII-induced hypertension model. After infusion with ATII, the RAMP2 TG mice did not differ from WT mice in terms of the magnitude of the raised systemic blood pressure observed. However, the RAMP2 TG mice were protected against ATII–induced aortic vascular hypertrophy mainly via preventing smooth muscle cell proliferation. This finding was supported by the study of VSMCs *in vitro*. The cell proliferation study demonstrated that ATII significantly increased cell number in the WT VSMCs but not in RAMP2 TG cells, which provides evidence of an anti-proliferative function of AM by raising levels of functional AM1 receptor. Moreover, the observed vascular hypertrophy in this part of the thesis was also accompanied by increased inflammatory markers (VCAM-1 and MCP-1). Overall this novel data provides evidence that up-regulation of RAMP2, and therefore raised levels of the AM1 receptor plays a novel protective role in ATII induced aortic vascular remodeling.

6.2.2 Role of AM in hypertensive models induced by ATII

The role of AM in ATII induced hypertensive models has been studied before using AM transgenic mice with lower endogenous AM levels ($AM^{+/-}$) or with overexpressing AM level (Niu *et al*., 2003; Niu *et al*., 2004; Caron *et al*., 2007). Those studies illustrated that AM plays a protective role in ATII infusion induced diseases, such as cardiovascular hypertrophy, renal dysfunction, cardiac hypertrophy, and fibrosis without affecting the blood pressure. Here, in this thesis for the first time, by introducing smooth

muscle targeted RAMP2 overexpression we demonstrate the protective function of the AM1 receptor against aortic vascular hypertrophy and inflammation induced by ATII infusion. Thus, the current study has revealed the important role of the smooth muscle AM1 receptor, which may represent a useful new target for the treatment of cardiovascular diseases.

6.2.3 Potential therapeutic role of AM

AM is known as a vasodilator and plays a generally protective and beneficial role to the cells and tissues. Indeed, in *in vitro* experimental studies, AM suppressed the hypertrophy of cardiomyocytes and proliferation of fibroblasts (Tsurude *et al*., 1998; Tsurude *et al*., 1999). The current study demonstrated that AM inhibited VSMC proliferation *in vitro* induced by ATII mainly via upregulating RAMP2 and as a consequence raised functional AM1 receptor levels. *In vivo* experiments have also showed that the infusion of AM plays a protective role in left ventricular remodeling after ischemia (Nakamura *et al*., 2002; Okumura *et al*., 2003). These findings indicate that AM administration may benefit patients who have myocardial infarction. However, AM has a short half-life in the plasma that this limits the systemic infusion of AM as therapy. Therefore, the strategy of AM gene delivery hopefully will extend the beneficial actions of AM as discussed above using transgenic mice (Niu *et al*, 2003, 2004 and Caron *et al*., 2007). More studies are required in order to better utilise the protective role of AM for therapy.

Although AM has an important therapeutic role, few studies have been able to associate with its RAMP receptor components in specific cell or tissue types. In this thesis, my data provided evidence that smooth muscle AM1 plays an important protective role against vascular hypertrophy caused during the development of hypertension, therefore providing evidence that the vascular AM1 receptor may be a potential and novel target for the treatment of vascular hypertrophy by tissue-specific targeting of the AM1 receptor via gene delivery or smooth muscle-specific means.

6.2.4. CGRP in ATII-induced hypertension

The role of CGRP (αCGRP), one of the CGRP family peptides like AM, in mediating the blood pressure under both normal physiological conditions and during the onset of

hypertension has been investigated extensively and is well documented (Smillie & Brain SD, 2011). In our group, the role of CGRP in the ATII-induced hypertension has been investigated (Smillie, 2012) using αCGRP KO mice. We, for the first time, demonstrated that αCGRP KO mice are more susceptible to the development and severity of hypertension induced by ATII infusion for 14 days. Such elevated increased blood pressure in the CGRP KO mice was also associated with an enhanced aortic inflammation and vascular remodeling. These results provide evidence for a protective role of αCGRP in the development of hypertension induced by ATII, which provides evidence for the potential development of new drugs for treatment of hypertension.

6.2.5. The mechanisms of AM and CGRP in protecting against hypertension

AM is 3-30 times less potent vasodilator than CGRP. The mechanisms involved in the vascular relaxation and anti-proliferation of AM against hypertension are different with regards to both species and vascular beds. Although they are not completely understood, it is suggested that it involves smooth muscle cell located CGRP receptors, in addition to AM receptors. As mentioned in the introduction (chapter 1), the CGRP receptor is composed of CL and RAMP1, while CL with RAMP2 produces the AM1 receptor and CL with RAMP3 produce a CGRP/AM2 receptor. It was shown that AM can act via CGRP receptors located on vascular smooth muscle cells to cause vasodilatation. This would lead to increasing cAMP levels and activation of a protein kinase A-dependent pathway (Nakamura *et al*., 1995). Interestingly, this pathway is also thought to mediate AM1 receptor-mediated effects that lead to activation of the cAMP/protein kinase A–dependent pathway. Moreover, AM can also act via the phosphatidylinositol 3-kinase (PI3K)/Akt-NO synthase dependent pathway (Brain & Grant 2004, Ishimitsu *et al*., 2006). These AM responses could then be the mechanisms observed in my studies with vascular smooth muscle localised AM1 receptors, rather than any effect of AM on CGRP receptors.

CGRP is the most potent vasodilator to date (Smillie & Brain SD, 2011), with its vasodilator activity predominantly mediated by CGRP binding to its receptor on the membrane of both VSMCs and the endothelium of blood vessels. Such binding stimulates either endothelium dependent or independent mechanisms triggering a rise in cAMP thereby causing vasodilatation (Brain and Grant, 2004). It has been suggested

that CGRP suppresses the proliferation of VSMC together with an increase of the cAMP (Li *et al*., 1997). More recently CGRP has been shown to inhibit VSMC proliferation induced by ATII (Qin *et al*., 2004; Fang *et al*., 2011), which was believed to be blocking the MAPKs and NF-κB pathways. However the exact mechanism of CGRP regulating the proliferation of VSMCs remains unclear. Certainly from the studies carried out in this group with the ATII model, evidence was found for an effect of CGRP on blood pressure but not for AM acting via the AM1 receptor.

Overall, there is similarity in the mechanisms of CGRP and AM induced relaxation and anti-proliferation. This may be because they all belong to CGRP family peptides and act via the same G protein-linked receptor (CL).

6.2.6. Release of endogenous AM and CGRP

AM is widely expressed in a range of organs and cell types (see the chapter 1 introduction). The stimulation and release of AM has been examined in isolated and/or cultured endothelial cells, vascular smooth muscle cells and cardiomyocytes (Ishimitsu *et al*., 2006). AM production is up-regulated by several factors including oxidative stress, pro-inflammatory cytokines (such as IL-1 and TNFα), hypoxia, RAS and the sympathetic nervous systems (Beltowski and Jamroz 2004; Ishimitsu *et al*., 2006). However, there is not a clear single mechanism that primarily upregulates AM, all these factors are very general.

CGRP is distributed widely throughout the central and peripheral nervous systems. In the periphery, it is primarily expressed within sensory neurons, specifically unmyelinated C-fibres and myelinated Aδ-fibres (Brain and Grant, 2004, Smillie & Brain SD, 2011). It is well charaterised that TRPV1 activation on sensory neurons leads to the downstream release of CGRP (Brain SD 1997).

Therefore, it is difficult to selectively stimulate endogenous AM release in our models. However, activation of TRPV1 receptor provides the possibility of stimulation of the endogenous release of CGRP. It may be one of the reasons why there has been a large interest to investigate the role of TRPV1 in different conditions in recent years.

6.3 TRPV1

Previous novel findings from first part of this thesis (protective role of AM1 receptor) and Smillie's PhD project (Smillie, 2012, protective role of αCGRP in ATII-induced hypertension) form my basis for the interest of the second part of my PhD – to investigate an endogenous mechanism via which either AM or CGRP may be released. An evaluation of these mechanisms lead me to consider that activation of TRPV1 may be the most direct mechanism to study, especially as WT and TRPV1 KO mice were available.

6.3.1 TRPV1 activation and neuropeptides release

As discussed in the general introduction (chapter 1), TRPV1 can be activated/sensitised by a number of different endogenous compounds, such as anandamide, protons (pH<6), nerve growth factor (NGF) tachykinins (Deng & Li 2005), lipoxygenase products also known as endovanilloids and other fatty acids (van der Stelt and Di Marzo, 2004). Activation of afferent neuronal TRPV1 causes the opening of the central pore which is permeable to the influx of extracellular cations and calcium. This results in the depolarization of the neuronal membrane and causes the subsequent release of sensory neuropeptides, including CGRP and substance P. As discussed previously, CGRP is a potent vasodilator and plays a protective role in several hypertension disease models (Smillie and Brain 2011). Therefore I believed that activation of TRPV1 leading to the release of CGRP would cause vasodilatation, and thus should protect in the hypertensive models.

6.3.2 TRPV1 in hypertension

The peripheral vascular system is widely innervated both by sympathetic and capsaicin-sensitive sensory nerves, which play an important role in mediating vascular tone via releasing vasoactive neurotransmitters. Among the vasodilators involved (e.g. nitric oxide and prostacyclin etc), CGRP released following TRPV1 activation may down-regulate the raised blood pressure in hypertensive models. Indeed it has been shown that rutaecarpine (a well known Chinese herbal medicine Wu-Chu-Yu) was able to stimulate the release of CGRP by activation of TRPV1 to protect in phenol-induced or 2K1C hypertensive rats (Deng *et al*., 2004). Furthermore, in high salt diet fed rats, capsaicin induced a dose-dependent decrease in blood pressure (Wang and Wang

2006). The effect of capsaicin in this model has been supported by further studies which showed that i.v. infusion of capsazepine increases systolic blood pressure in those rats (Wang and Wang 2006). These observations suggest that in rats, TRPV1 protects against the hypertension, probably through release of CGRP.

The contribution of TRPV1 to the hypotensive effect *in vivo* is still equivocal especially as observed in studies using TRPV1 KO and WT mice. Hypotension was also observed with the administration of the proposed TRPV1 agonist anandamide, which were almost completely abolished in the presence of capsazepine (Wang *et al*., 2007). However Pacher *et al*. (2004) demonstrated that the predominant hypotensive effects of anandamide were similar in TRPV1 KO and TRPV1 WT mice, and were completely prevented by a CB1 receptor antagonist. These results suggest that the hypotensive effect of anandamide *in vivo* is regulated by the CB1 receptor but not by TRPV1. Moreover, in a separate study where TRPV1 KO and WT mice were made hypertensive with DOCA salt, no difference in blood pressure was seen between genotypes despite more extensive renal end organ damage in TRPV1 KO mice compared to WT counterparts (Wang *et al*., 2008b). Thus, the general hypothesis that TRPV1 activation results in the release of vasodilator CGRP and this is central in hypertension may be flawed, although this was my hypothesis for the studies designed to investigate models involving TRPV1 where endogenous CGRP may be involved.

6.3.3 Role of TRPV1 in hypertensive models induced by ATII or HFD

The investigation of the role of TRPV1 in hypertensive models induced by ATII or HFD in this thesis, using TRPV1 KO and WT mice, showed surprising and interesting results. My data showed that TRPV1 KO mice were partially protected against the hypertension and vascular hypertrophy induced by ATII. This ‘partial’ effect may be due to the nature of the ATII model (extreme and aggressive). Data indicated up-regulation of kidney tissue levels of CGRP in the WT mice but we assume that it was not able to protect against such an aggressive increase in blood pressure in response to ATII.

Therefore, we moved to a less extreme hypertensive model – obesity-induced hypertension. Here we demonstrate that global knock out of TRPV1 indicated a beneficial phenotype against hypertension and vascular hypertrophy induced by HFD.

Such protection in the TRPV1 KO mice was accompanied by an observed protection in term of glucose tolerance.

Overall the findings from this thesis were against my original hypothesis (TRPV1 protects in hypertension) but provided evidence for a novel insight into the complex role of TRPV1. Further investigations are required in order to fully understand the mechanisms. However, these findings lead to a further insight as follows.

6.3.4. Comparison of the finding with published literature.

As previously mentioned, the results discussed in this thesis are novel. To our knowledge, no other group has yet published on the role of TRPV1 on ATII-induced or obesity-induced hypertension using TRPV1 KO mice.

6.3.4.1. TRPV1 and ATII-induced hypertension

It is well documented that because CGRP is a potent vasodilator, that activation of TRPV1 causes release of CGRP which should theoretically protect against the augmenting effects of the RAS system on blood pressure (Deng & Li *et al*, 2005, Szallasi *et al*., 2007). Indeed, a study had shown that rats with capsaicin-depleted sensory nerves exhibited a higher mean arterial pressure than control rats when ATII (0.216 mg/kg/day) was administered via chronic mini-pump infusion (Wu *et al*., 2000) for 14 days, which suggested a protective role of sensory nerves in attenuating the elevated blood pressure initiated by ATII. In our model, we observed that global TRPV1 KO mice were protected against hypertension and vascular hypertrophy following ATII infusion for 14 days. We did observe an increase in kidney levels of CGRP in the WT mice after ATII infusion but apparently this was insufficient to mediate protection. Moreover, there is increasing evidence for the presence of non-neuronal TRPV1 and its constrictor properties. This may contribute to the increased blood pressure and compensate for CGRP-induced vasodilator activity in this model. However, the mechanism of how TRPV1 mediates the blood pressure in ATII model is still unclear. More experiments are required.

6.3.4.2 TRPV1 and obesity-induced hypertension

Several studies have investigated the role of TRPV1 in obesity and/or diabetic conditions via long term treatment with capsaicin (Gram *et al*., 2007,Yang *et al*,., 2010), a TRPV1 antagonist (Tanaha *et al*., 2011) or TRPV1 KO mice (Motter *et al*., 2008, Yang *et al*,., 2010 Ma *et al*., 2011). However, we have data demonstrated novel data where TRPV1 deletion is associated with protection against obesity-induced hypertension and aortic vascular hypertrophy by HFD feeding. Our results on body weight gain agree with Motter *et al*. (2008) that both WT and TRPV1 KO mice had similar body weight gain after HFD feeding. Moreover, we showed that TRPV1 KO have improving glucose tolerance. This finding was supported by Gram *et al.,(*2007) and Tanaka *et al*., (2011) who applied capsaicin denervation in Zucker diabetic rat and TRPV1 antagonist in ob/ob diabetic mice respectively to show the anti-diabetic pharmacological effect of blocking TRPV1 signal. Yang *et al* (2010) showed that dietary capsaicin consumption can reduce blood pressure in normal conditions and such an effect was not seen in the TRPV1 deficient mice. However, it is noted that chronic dietary capsaicin will increase plasma levels of capsaicin and consequently result in strong agonism of all accessible nerve located TRPV1 receptors. There is also the complicating factor that chronic capsaicin pre-treatment can desensitise TRPV1 receptors. Therefore, such an effect of reduced blood pressure may involve other neuronal TRPV1 co-located receptors such as arterial baroreceptors. Further studies are required to understand the multiple TRPV1 actions and their role in mediating blood pressure. Our study focused on the role of TRPV1 in obesity induced hypertension which is specific to the disorder of endocrinology system. Therefore it is very difficult to accurately compare results with Yang *et al*.,(2010).

6.3.5. Possible mechanisms of TRPV1 in mediating increased blood pressure

The findings from this thesis have led me to suggest possible mechanisms by which TRPV1 may be mediating increased blood pressure.

The first possible mechanism could be via the non-neuronal TRPV1 pathway (Fig 6.1). There is increasing evidence showing the constriction function caused by non-neuronal TRPV1 (Keeble *et al*., 2006; Kark *et al*., 2008 and Czikora *et al*., 2012). As mentioned before, Keele *et al*. (2006) have demonstrated TRPV1-mediated vasoconstriction in the

WT mouse knee joint, but not in TRPV1 KOs. Moreover both Kark *et al*., (2008) and Czikora *et al.,* (2012) demonstrated the exciting and constriction function of non-neuronal TRPV1 using skeletal muscle with surgical sensory denervation on skeletal muscle arterioles obtained from WT or TRPV1 KO rats. Those studies support my findings that the observed increased blood pressure in the WT may be mediated by non-neuronal TRPV1. Therefore, the lost function of non-neuronal TRPV1 may be beneficial for the hypertension induced by HFD (please refer to the chapter 4 and 5 for discussion in details).

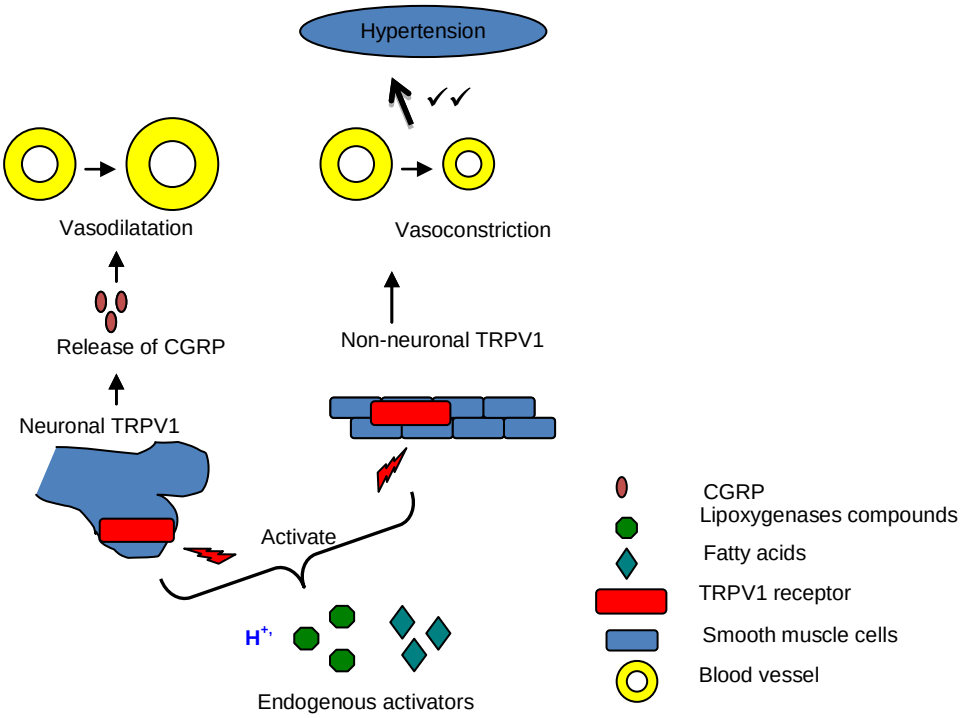


Figure 6.1: Proposed mechanism by which blood pressure is mediated via the non-neuronal TRPV1 pathway as compared with the neuronal pathway.

The up-regulation of ET-1 mRNA levels in aorta suggest the second possible mechanism, which could be via the endothelin pathway (please refer to the discussion in chapter 5 for details). It was shown that endothelin potentiates TRPV1 via a protein kinase C dependent mechanism (Plant *et al*., 2007). It was also demonstrated that

activation of TRPV1 by capsaicin caused increased blood pressure in anaesthetised mice following artery catheterization, which was abolished by antagonising the endothelin A receptor (Ohanyan *et al*., 2011). These findings were further supported when cultured endothelial cells exposed to capsaicin, it increased endothelin production, which was attenuated by inhibiting TRPV1 or endothelin-converting enzyme (Ohanyan *et al*., 2011). These findings support the proposal of the second possible mechanism that activation of TRPV1 may lead to release of endothelin from endothelial cells, which causes vasoconstriction via endothelin A receptor. (Fig 6.2)

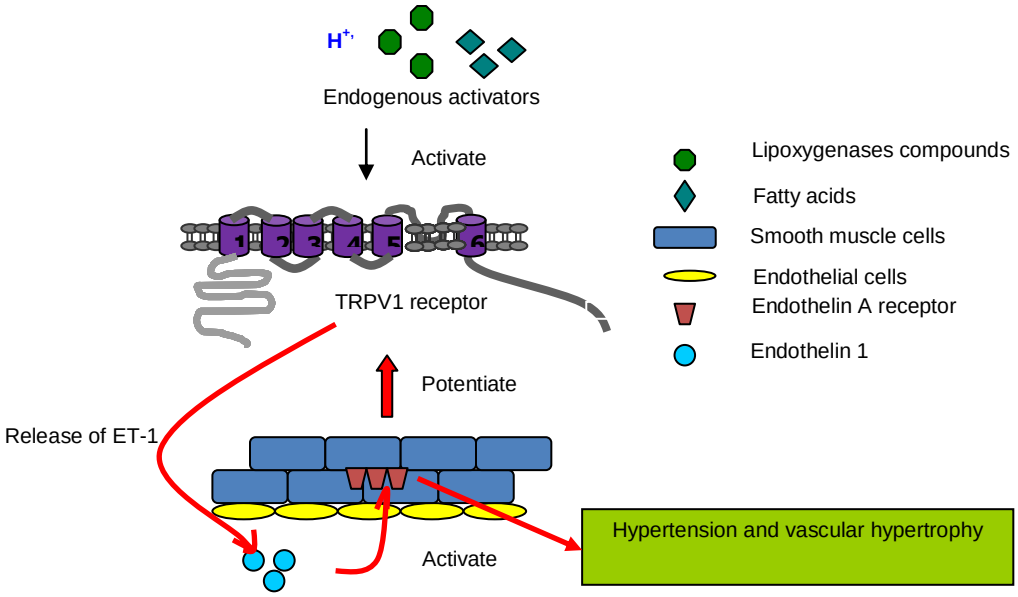


Figure 6.2: Proposed mechanism by which blood pressure is mediated via the endothelin pathway.

A third possible mechanism may involve the sympathetic system. Indeed in obese patients, total noradrenaline spillover is increased, which indicates elevated levels of sympathetic activity (Esler, 2011). Studies indicate that excess weight gain is associated with increased sympathetic nervous system activation (especially renal sympathetic activity) and that it contributes to the development of hypertension in obese humans and animal models of diet induced obesity (Davy *et al*., 2009, Hall *et al*., 2000). Moreover Scott *et al.,* (2000) demonstrated that blockage of the sympathetic nervous system with hexamethonium (ganglionic blocker) caused a significant

decrease in mean arterial pressure in obese zucker rats (Scott *et al*., 2000). This literature supports a proposed sympathetic pathway mechanism. My findings of low kidney noradrenaline levels in the TRPV1 KO mice indicate low renal sympathetic activities, which may help to prevent the increased blood pressure induced by obesity.

In conclusion, obesity is the most common cause of human hypertension and it is a complex autoimmune mediated disease. Therefore the above suggested mechanisms may play a part but further investigations are required.

6.3.6. Future work

In order to better understand the exact mechanism behind the findings presented in this thesis, several experimental suggestions are now made. Firstly, as mRNA data indicates increased ET-1 levels in the aorta, investigation of protein expression of ET-1 in the aorta and other organs like the kidney will provide more convincing evidence of its presence. The ET_A antagonist can be applied to block the constrictive effect induced by ET-1, and to investigate whether ET_A antagonist can reduce the evelated blood pressure and vascular inflammation induced by HFD feeding in the WT mice. If ET-1 does play a functional role in the hypertension induced by obesity, ET_A antagonists should be able to prevent or attenuate the increased blood pressure in the WT mice with the HFD feeding.

Secondly, it has been discussed before that the metabolic mechanisms are sympathetically driven. Therefore further investigation of the involvement of the sympathetic nervous system in the development of hypertension induced by HFD feeding is important. The activities of sympathetic nervous system can be investigated through study of noradrenaline levels in different organs such as heart and aorta. The involvement of the sympathetic nervous system can also be tested by applying blockers of the sympathetic nervous system to the WT mice to check whether the increased blood pressure in the WT can be prevented. If the sympathetic nervous system does contribute to the development of hypertension in the WT mice after HFD feeding, it indicates an important link between TRPV1 activities and sympathetic activaties. Therefore we may consider whether or not removing the TRPV1 activities can influence/control the activities of the sympathetic nervous system in metabolic

conditions, which may be novel and benefical for other metabolic-linked sympathetic drived diseases, such as renal failure in diabetic patients.

Thirdly and most importantly, it is important to test for involvement of non-neuronal TRPV1. I believe that the smooth muscle TRPV1 expression is likely to be responsible for the constriction, which contributes to the development of hypertension during the ATII infusion and HFD feeding. This hypothesis could be confirmed with conditional (tissue selective) TRPV1 KO mice. If the VSMC selective TRPV1 KO mice could be developed, then they can be characterised after HDF study. If my hypothesis is correct then those VSMC selected TRPV1 KO mice should show similar or maybe a better control of blood pressure when compared with the global TRPV1 KO mice. Moreover conditional TRPV1 antagonists which should exert the same effects as conditional TRPV1 KO mice, Potentially this may become a breakthrough in terms of the pharmacological prevention of obesity-induced metabolic syndrome with lower vascular inflammatory side effects than normally happens after a prolonged period of HFD feeding.

6.3.7. Potential therapeutic role of TRPV1

TRPV1 is an interesting therapeutic target due to its complex role in mediating diseases. There may be two directions for TRPV1 in drug development, namely TRPV1 agonists to activate and desensitise the channel, and inactivation using antagonists (Wong & Gavva 2009, Knotlkova *et al*., 2008). Agonists can block the whole nerve terminal, which may be more potent and beneficial for pain treatment (such as with capsaicin creams), while antagonists selectively block TRPV1 function. Indeed, choice of agonist or antagonist therapy may be disease dependent. Recently many TRPV1 antagoinsts are currently under investigation, especially for treatments for a range of painful conditions via blocking the TRPV1 activation. For example, A-995662, a potent and selective TRPV1 antagonist, shows its potential for treatment against osteoarthritic pain in rat by reduction of CGRP and glutamate production from spinal cord (Puttfarcken et al., 2010). In vivo experiements have demonstrated that JTS-653, a potent TRPV1 antagonist, showed an antihyperalgesic effect but with transiently increased temperature in rat (Kitagawa et al., 2012). Indeed acute increase in body temperature (hyperthermia) had been known as adverse effects of TRPV1 antagonist (Gavva et al.,

2007, Othman et al., 2011, Kitagawa et al., 2012). This suggests that TRPV1 plays an important role in the maintenance of body temperature. However some TRPV1 antagonists did not show the hyperthermia effect in rats studies, such as AMG8562 (Lehto et al., 2008) and AS1928370 (Watabiki et al., 2010). It suggests the possibility of selective TRPV1 antagonist may solve the hyperthermia problem. At the same time, the results presented within this thesis suggest that pharmacological down-regulation of non-neuronal TRPV1 receptor may be therapeutically useful. If my hypothesis is correct, antagonists to selectively block the TRPV1 receptor may be beneficial for controlling the increased blood pressure and vascular inflammation induced by obesity. Therefore the therapeutic potential of TRPV1 is high, but more investigations are needed to understand more about the role of TRPV1 in different conditions.

6.4 Comparison with hypotheses

As previously mentioned, my original hypothesis was that both AM1 (via smooth muscle cell selective up-regulation of RAMP2) and TRPV1 (via vasodilator action of released CGRP) would play protective roles in the ATII-induced hypertension model. Interestingly, I found that up-regulation of the AM1 receptor was able to protect mice from vascular hypertrophy but not hypertension induced by ATII. This finding is consistent with the current literature (see also the discussion in the chapter 3). However, TRPV1 was not able to protect in the ATII model with WT and TRPV1 KO mice. In contrast, deletion of TRPV1 revealed a beneficial effect. I also investigated the role of TRPV1 in a less extreme hypertension model (obesity-induced hypertension), where again TRPV1 KO mice but not the WT mice were protected against the hypertension and vascular hypertrophy induced by HFD. However, there are some indications in the literature that this may occur (see above), also that TRPV1 may have directly deleterious effects on blood pressure, independently of CGRP.

6.5 Limitation of the study

There are possible limitations of the studies in my thesis. My studies were based on using transgenic mice, either tissue selective upregulating RAMP2 or globally knocking out the TRPV1. Therefore if suitable agonists or antagonists could be also investigated to test the hypotheses, it will potentially help to draw the conclusion in this thesis. On another hand, there are some methodological limitations. Sample size was low in

certain analysis, such as the ELISA for CGRP. Therefore the increase of the sample size would have provided more convincing data.

6.6 Conclusion

In conclusion, this thesis provides interesting and novel findings about functional AM1 and TRPV1 in hypertension models induced either by ATII or HFD. The findings reveal a protective role of functional smooth muscle AM1 receptor against the vascular inflammation and hypertrophy which occurs during the development of ATII induced hypertension. Moreover, the results of this thesis are novel in that they reveal a protective role of deletion of TRPV1 in the development of hypertension and vascular hypertrophy in ATII-induced hypertension or obesity-induced hypertension models, which is against my original hypothesis. Further experiments are required to elucidate the mechanisms behind each of these findings specifically as to why a potential protective effect of CGRP is not observed as a consequence of probable TRPV1 activation. However, it is hoped that these findings have advanced our knowledge of both the AM1 receptor and TRPV1 receptor, and can be utilised in the development of fundamental knowledge with respect to mechanisms involved in hypertension and also in the future in novel approaches for the treatment of hypertension.

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