THE ROLE OF KININ RECEPTORS IN ABDOMINAL AORTIC ANEURYSM

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CONTENTS

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DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines of the James Cook University Statement and Guidelines on Research Practices which is based on the NHMRC Australian Code for Responsible Conduct of Research (2007). The proposed research methodology received approval from the James Cook University Animal Ethics Committee (A1455).

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Communications & Awards

1. CONFERENCES

Mechanisms of inflammation mediated through the Kinin B2 receptor – A therapeutic target for abdominal aortic aneurysms.
T.Dougan VBU, School of Medicine, James Cook University, Townsville, Australia, 4811.Oral presentation for Celebrating Research - FMHMS 3 minute competition, JCU, Townsville, Australia, August 2010.

Mechanisms of inflammation mediated through the Kinin B2 receptor – A therapeutic target for abdominal aortic aneurysms.
T.Dougan VBU, School of Medicine, James Cook University, Townsville, Australia, 4811.
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Silencing the Silent Killer.
Tammy Dougan, Lynn Woodward, Corey Moran, Catherine Rush and Jonathon Golledge, VBU, School of Medicine, James Cook University, Townsville, Australia, 4811.
Poster Presentation, Townsville Festival of Life Science, JCU, Nov 2010.

The relationship between blood pressure and aortic dilation in the angiotensin II mouse model of AAA
Tammy Dougan, Sai-Wang Seto, Corey S Moran, Catherine Rush, Lynn Woodward, Jonathan Golledge VBU, School of Medicine, James Cook University, Townsville, Australia, 4811.
Poster Presentation, Townsville Festival of Life Science, JCU, Sep 2011.

The relationship between blood pressure and aortic dilation in the angiotensin II mouse model of AAA
Tammy Dougan, Sai-Wang Seto, Corey S Moran, Catherine Rush, Lynn Woodward, Jonathan Golledge VBU, School of Medicine, James Cook University, Townsville, Australia, 4811.
Poster Presentation, TTH & JCU Research symposium, 14 Oct 2011.
Abstract published in Annals of the ACTM, Official Journal of The Australasian College of Tropical Medicine, Vol 12, supplement 1. Oct 2011.

Blocking the kinin pathway: A potential new therapy for aortic aneurysm
T.Dougan VBU, School of Medicine, James Cook University, Townsville, Australia, 4811.Oral presentation for Celebrating Research - FMHMS 3 minute competition, JCU, Townsville, Australia, Sep 2012.

The role of kinins in abdominal aortic aneurysms
Tammy Dougan, Corey Moran, Catherine Rush, Lynn Woodward, Jonathan Golledge
From: The Vascular Biology Unit, School of Medicine & Dentistry, James Cook University, Townsville, Queensland, Australia.
Poster Presentation. Australian Vascular Biology Society 20th National Scientific Conference September 13th - 16th, 2012 Hyatt Regency Sanctuary Cove Gold Coast, Queensland.

The role of kinins in abdominal aortic aneurysms
Tammy Dougan, Corey Moran, Catherine Rush, Lynn Woodward, Jonathan Golledge
From: The Vascular Biology Unit, School of Medicine & Dentistry, James Cook University, Townsville, Queensland, Australia.
Poster Presentation. Australian Society Immunology Conference, December 2012 Melbourne, Victoria.

The role of kinins in abdominal aortic aneurysms
Tammy Dougan, Corey Moran, Catherine Rush, Lynn Woodward, Jonathan Golledge
From: The Vascular Biology Unit, School of Medicine & Dentistry, James Cook University, Townsville, Queensland, Australia.
Poster Presentation. Townsville Festival of Life Science, JCU, Oct 2012.

The role of kinins in abdominal aortic aneurysms
Tammy Dougan, Corey Moran, Catherine Rush, Lynn Woodward, Jonathan Golledge
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Talk/ Presentation. Townsville Festival of Life Science, JCU, Oct 2012.

2. PAPERS

Kinin deficiency and the progression of abdominal aortic aneurysms in the angiotension II infused mouse model.
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The relationship between blood pressure and kinin deficiency in the angiotensin II mouse model.
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The role of the kinin receptors in pathology of AAA and value as a therapeutic target.
Dougan T[1], Rush C[2], Woodward L[1], Moran C[1] & Golledge J[1]
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3. AWARDS

- James Cook University International Research Scholarship- three years.
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Abstract

Abdominal aortic aneurysm (AAA) is an important cause of mortality in older adults. An AAA is an abnormal dilatation of the body's main blood vessel, the aorta, within the abdominal region. If left undetected rupture is one of the end-stage results of aortic expansion. There is currently no drug treatment available for AAA, and surgery remains the only option. AAA is often associated with atherosclerosis. Aortic infiltration of cells of both the innate and adaptive immune systems is believed to play a critical role in the pathogenesis of AAA. Chronic inflammation can be observed in human AAA tissue sections.

The kallikrein-kinin system has long been recognised for its role as part of the innate immune system but its role in AAA has been little studied. Bradykinin is the principle effector of the KKS, acting via two types of Bradykinin receptors, B1 and B2. Under physiological conditions, Bradykinin signalling through the B2 receptor has a central role in the modulation of cardiovascular function, but the B1 receptor is barely detectable under normal physiological conditions and its expression is up-regulated in injured or inflamed tissue.

The focus of this study was to elucidate the importance of the kinin receptors in AAA development and atheroma severity in a mouse model of vascular disease. The angiotensin II (AII) mouse model has been used extensively by many groups to study the contribution of AII-induced hypertension to several vascular pathologies, such as atherosclerosis and the formation of AAAs. It was hypothesised that:

1. Bradykinin receptor deficiency in the apolipoprotein E-deficient ($ApoE^{-/-}$) mouse limits angiotensin II (AII)-induced AAA and atherosclerosis.

2. Kinin receptor deficiency promotes cardiac hypertrophy in an AII mouse model.

3. Activation of neutrophils via kinin receptor signalling stimulates release of the serine protease myeloperoxidase (MPO)

Specifically, the aims of this project were:

1. Assess aortic expression of kinin B1r and B2r in Apo $E^{-/-}$ mice

2. Determine if kinin receptor deficiency inhibits aneurysm development and atheroma severity in the AII-infused $ApoE^{-/-}$ mouse model

3. Compare the circulating profile of monocytes, neutrophils, and pro-inflammatory cytokines in control and kinin receptor-deficient AII-infused $ApoE^{-/-}$ mice

4. To assess the effect of kinin receptor deficiency on cardiac function in the AII-infused $ApoE^{-/-}$ mice model, this includes cardiac hypertrophy, the regulation of blood pressure and heart rate, and to assess the B1 and B2 kinin receptor expression in mice hearts and whether up regulation of B1 receptor expression compensates for lack of B2 receptor expression in the mice

5. Determine the pattern of B1 and B2 kinin receptor expression in $ApoE^{-/-}$ mice neutrophils

6. Assess the action of kinin receptor-agonists and antagonists on neutrophil activation and MPO production, *in vitro*

We demonstrate that in a mouse model of AAA kinin receptor deficiency, inhibited AAA development and progression. Study outcomes demonstrated that both B2r and B1rB2r

deficiency decreased aortic dilatation. Deficiency in both B1 and B2 receptors promoted model survival.

The effect of kinin receptor deficiency on atheroma severity was also investigated. In the current study it was noted that $ApoE^{-/-}$ control mice had a significantly greater development of atheroma in the aortic arch compared to either of the $B2r^{-/-}ApoE^{-/-}$ or $B1rB2r^{-/-}ApoE^{-/-}$ mice .

A significant effect of kinin receptor deficiency was observed in systolic blood pressure (SBP), prior to AII infusion, with an increased SBP in mice deficient in both receptors. SBP over time in $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice, was significantly higher in $B1rB2r^{-/-}ApoE^{-/-}$ mice compared to both $B2r^{-/-}ApoE^{-/-}$ mice and ApoE-/- controls throughout AII infusion. In addition, a greater degree of cardiac hypertrophy was observed in $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice compared to $ApoE^{-/-}$ controls.

In vitro, the B2 receptor agonist stimulated neutrophil activation and myeloperoxidase production. Plasma concentration of MPO was markedly lower in mice that were deficient in kinin B2 receptors compared to control mice, suggesting that the inhibition of aortic dilatation in response to AII in these mice was potentially associated with a reduced state of neutrophil activation within the vessel wall.

Together, these findings implicate kinin receptors in the promotion of AAA via activation of neutrophils for the first time. Targeting the kinin signalling pathway may be a novel therapeutic strategy for AAA.

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Abbreviations

μg/kg	Microgram/ kilogram
μl	Microlitre
AAA	Abdominal aortic aneurysm
ACE	Angiotensin converting enzyme
AII	Angiotensin II
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
BK	Bradykinin
bp	Base pairs
B1rB2r	Bradykinin receptors 1 and 2
$CaCl_2$	Calcium Chloride
CCL2	Chemokine cc motif ligand-2
CD11b	Cluster of differentiation 11b
CHD	Coronary heart disease
CI	Confidence intervals
Cm	Centimetre
CXC	Chemokine receptors
DAPI	Diamidino-2-phenylindole
DF	Degrees of Freedom
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleos
DPPI	Dipeptidyl peptidase I
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
EVAR	Endovascular aneurysm repair
F	Forward
FACs	Fluorescence activated cell sorting
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HAE	Hereditary angioedema
HBSS	Hanks Buffered Salt Solution
H&E	Haematoxylin and eosin
HPLC	High-pressure liquid chromatography
HMWK	High-molecular-weight kininogen

HR	Heart rate
ICAM	Intercellular adhesion molecule
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitonal
IRA	Infrarenal
KKS	Kallikrein-kinin system
KO	Knock out
LDL	Low density lipoprotein
LMWK	Low-molecular weight kininogen
LPS	Lipopolysaccharide
M	Month
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemo-attractant protein-1
MPO	Myeloperoxidase
MM	Millimetre
mM	Millimolar
MMD	Mean maximum diameter
MMP	Metalloproteinase
MS	Mean squares
NaCl	Sodium chloride
NC	Negative control
NEO	Neomycin
NETS	Neutrophil extracellular traps
NK-κB	Nuclear Factor kappa B
nM	Nanomolar
NO	Nitrous oxide
OCT	Optimal cutting temperature compound
OD	Optical density
PAD	Peripheral arterial disease
PBS	Phosphate buffered saline
PC	Positive control
PCR	Polymerase chain reaction
PI	Propidium iodine
PK	Plasma kallikrein

PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear
P13K	Phosphinositide 3 Kinase
qRT-PCR	Quantitative real time PCR
R	Reverse
RANTES	Regulated on activation, normal T cell expressed, and secreted
RAS	Renin angiotensin system
RBC	Red blood cells
RFU	Relative fluorescence units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
SRA	Suprarenal aorta
SS	Sum of squares
SSC	Side scatter
TA	Thoracic aorta
TGF	Tumour growth factor
Th1	Helper T-cell type-1
Th2	Helper T-cell type-2
TK	Tissue kallikrein
TK	Thymidine kinase
TNF	Tumour necrosis factor
US	Ultra sound
VCAM	Vascular cell adhesion molecule
VSMC	Vascular smooth muscle cell
W	Week
WT	Wild-type

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

An abdominal aortic aneurysm (AAA) is an abnormal dilatation of the main blood vessel in the body, the aorta. Aneurysms can vary in size but the most accepted definition is based on a diameter of 3.0 cm or larger of the infra-renal aortal wall (Johnston KW 1991; Moll, Powell et al. 2011). AAA is a common, late age-at-onset disorder that affects ~1.6 to 7.2 % of the older population in developed countries (Conway, Malkawi et al. 2012; (Lenk, Tromp et al. 2007; Norman, Spilsbury et al. 2011) and is a common cause of mortality in men in the Western world (Tilson 2005; Golledge, Muller et al. 2006; Norman, Spilsbury et al. 2011). Degeneration, weakening and progressive dilatation of the infrarenal aortic wall ultimately leads to vessel rupture and fatality in 80-90% of cases if undetected or left untreated (Moll, Powell et al. 2011). Aneurysms are often asymptomatic until rupture. At present, there are no accurate non-imaging methods of diagnosis and current treatment for this condition is limited to repair by open or endovascular surgery in the absence of accepted drug therapy (Golledge, Muller et al. 2006). The development of an effective medication would not only facilitate the management of established AAA in patients unsuitable for surgical repair, but could potentially be employed in the treatment of small aortic aneurysms.

The pathogenic mechanisms precipitating AAA are poorly understood. Current evidence indicates that a series of events involving inflammation, proteolysis, and vascular smooth muscle cell loss are important for disease progression (Ernst 1993). Immune cells such as neutrophils, T cells, and macrophages have been found to be important in the formation of AAAs in animal models and these are likely to be similarly involved in humans (Daugherty and Cassis 2004). Inflammatory cells are present in end stage human AAA tissue biopsies, which are characterised by chronic inflammation (Pagano, Bartoli et al. 2007). These immune cells

release a cascade of chemokines and cytokines that have pleiotropic effects, including stimulating the activation of proteases linked to the degradation of the vessel wall. Studies have suggested that the up regulation of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor -α (TNF−α), as well as chemokines such as chemokine cc motif ligand 2 (CCL2/MCP-1), are associated with the development of AAA (Middleton, Lloyd et al. 2007). Some studies have linked the presence of a so-called TH1 biased acquired immune response to AAA (Shimizu, Shichiri et al. 2004; Galle, Schandené et al. 2005) with other studies also linking TH2 (Platsoucas, Lu et al. 2006). Understanding the mechanisms stimulating and driving the initial acute inflammation and the eventual transformation to chronic aortitis may improve early diagnosis and treatment (Golledge and Norman 2011). There are likely multiple mechanisms in addition to inflammation that contribute to the development of AAAs, including inherited and environmental factors, but inflammation is the focus of this study.

The Kallikrein-kinin system comprises of a group of blood and tissue proteins that play a role in inflammation, blood pressure control and coagulation. Interestingly, this system has been implicated in AAA through a murine angiotension II (AII) infusion model (Merino, Todiras et al. 2009) and also analysis of AAA human biopsies (Biros, Norman et al. 2011). Other links between the kallikrein system and AAA include; evidence of vascular remodelling by the kallikrein system (Carretero 2005) and congenic kininogen deficiency and aneurysm formation (Kaschina, Stoll et al. 2004). Kallikreins are short-lived peptides with associated serine protease activity that are generated as a consequence of tissue injury (Hecquet, Tan et al. 2000). The principle effectors of the kallikrein system are plasma kallikrein (Bhoola, Elson et al. 1992) and tissue kallikrein (Leeb-Lundberg, Marceau et al. 2005). These proteases liberate bradykinin (BK) from high-molecular-weight kininogen (HMWK), (Espinola, Uknis et al. 2004). BK is a vasoactive peptide that influences many physiological processes, including vascular permeability, vasodilatation, smooth muscle contraction, pain, and the synthesis of IL-1 and

TNF-α by monocytes (Duchene, Lecomte et al. 2007). BK also plays an important role in leukocyte recruitment and can induce the secretion of neutrophil elastase and other serine proteases such as myeloperoxidase (Gustafson, Schmaier et al. 1989), and additionally it is thought to be a regulator of endothelial cell function.

There are two known bradykinin receptors known as B1 and B2. BK and Lys-BK stimulate the constitutively expressed B2 receptor, which has been identified in many tissues, including endothelia, smooth muscle, fibroblasts, mesangial cells and neutrophils (Couture, Harrisson et al. 2001). The des- arginine metabolites of B2 (des-Arg^9-BK and Lys-des-Arg^9-BK), are produced by cleavage of the C-terminal arginine of BK and Lys-BK respectively by carboxypeptidases, and these stimulate the B1 receptor (Huang and Player 2010). The B1 receptor is induced by pro-inflammatory cytokines and peptides, such as angiotensin II (AII) (Pesquero, Araujo et al. 2000; Uknis AB 2001; Ceravolo, Fernandes et al. 2007). Expression of the B1 receptor also appears to play a role in neutrophil recruitment, as B1 receptor deficiency inhibits neutrophil accumulation (Fernandes, Passos et al. 2005). B2 receptor activation is thought to promote a TH1 response via activation of the IL-12 pathway (Aliberti, Viola et al. 2003) and dendritic cells (Bertram, Baltic et al. 2007). Other functions of BK include the promotion of increased intracellular Ca^{2+} and NO production (Leeb-Lundberg, Marceau et al. 2005), and stimulation of MAP kinase cascade during activation.

The human neutrophil plays a central role in acute inflammation. Neutrophils also possess the complete components of the kallikrein-kinin system, including membrane bound kinin-forming substrates (kininogens) (Gustafson, Schmaier et al. 1989; Henderson, Figueroa et al. 1994), and express both the B1 and B2 receptors (Bhoola, Elson et al. 1992) . Neutrophils are activated by plasma kallikrein and are also producers of tissue kallikrein (Selwyn, Figueroa et al. 1989), ensuring a role for kinins in inflammation (Bhoola, Elson et al. 1992). Neutrophils are present within the adventitia and mural thrombus of human AAA tissue suggesting a role of neutrophils

and potentially their associated serine proteases in aneurysmal degeneration (Cohen, Keegan et al. 1991).

Preliminary studies performed at the James Cook University, Vascular Biology Unit using the AII- infused apolipoprotein E deficient (Apo $E^{-/-}$) mouse model strongly implicated a key role for kinins in AAA pathology. Consequently, the focus of this study was to assess whether neutrophils play a critical role in the ability of kinins to promote abdominal aortic aneurysm development and rupture. It is hypothesised that:

1. Bradykinin receptor deficiency in the $ApoE^{-/-}$ mouse limits AII induced AAA and atherosclerosis.

2. Inflammatory leukocytes contribute to AAA and atheroma.

3. Kinin receptor activation of neutrophils stimulates the release of the potent serine protease myeloperoxidase

4. Kinin receptor deficiency promotes cardiac hypertrophy in an AII mouse model.

Specifically, the aims of this project are:

1. Determine if kinin receptor deficiency inhibits aneurysm development and atheroma severity in a mouse model of AAA

2. Assess the mechanisms involved in AAA and atheroma, by measuring markers of inflammation
3. Assess aortic expression of kinin B1r and B2r in Apo $E^{-/-}$ mice

4. Assess the action of kinin receptor-agonists, and antagonists on MPO production, *in vitro*

5. Determine the pattern of B1 and B2 kinin receptor expression in Apo $E^{-/-}$ mice neutrophils

6. To assess the effect of kinin receptor deficiency on cardiac hypertrophy in an AII mouse model.

7. To assess the role of kinin receptors in the regulation of blood pressure and heart rate.

8. To assess the relationship between B1 and B2 kinin receptor expression in Apo $E^{-/-}$ mice hearts and whether upregulation of B1 receptor expression compensates for lack of B2 receptor expression in ApoE mice.

The current study was undertaken to examine the potential link between the bradykinin receptors and neutrophils in the pathogenesis of AAA. The basis of this research was to develop a further understanding of the mechanisms that drive the inflammation leading to aneurysm development. This research can contribute to an understanding of AAA disease progression, by exploring the role of the bradykinin receptors and neutrophil activation to establish if aneurysm formation can be inhibited. Currently surgery is the only treatment option available for AAA, with rupture of the aorta wall the end stage, if treatment fails. It is vital that the mechanisms of AAA are understood if we are to improve therapy, and the development of a non-invasive intervention aimed at inhibiting the progression of AAA is urgently required. Exploring the role of kinins and the B1 and B2 receptors in the pathology of AAA and the use of blockers of these receptors to treat this disease may demonstrate their potential as pharmacological targets.

CHAPTER 2

LITERATURE REVIEW

2.1 ABDOMINAL AORTIC ANEURYSMS

Abdominal aortic aneurysms (AAA) are localised abnormal dilatations of the entire abdominal aortic wall (Figure 2.1) that occur in ~1.6 to 7.2 % of the general population age 50 years and older (Conway, Malkawi et al. 2012; Lenk, Tromp et al. 2007). The prevalence of AAA appears to increase with age and differs substantially by sex. For example, it affects 1.6% to 8.8% of males and 0.2% to 6.2% of females >60 years (so the ratio is generally 4 to 15 times greater in men than women), leading to ~1000 deaths per year in Australia. (Golledge, Muller et al. 2006; Norman and Powell 2007; Schermerhorn, Zwolak et al. 2008; Wanhainen 2008). Individual aneurysms vary in size and severity but the most accepted definition is based on a diameter of 3.0 cm or larger of the infra-renal aortal wall (Moll, Powell et al, 2011). Aneurysms are often asymptomatic until rupture and can be fatal if not detected or left untreated, for example there is a $\geq$ 70% mortality rate when rupture occurs before surgery (Gillum 1995). Open surgery is one treatment option for this condition, with a 5% peri-operative mortality rate in elective patients (Arko, Lee et al. 2002). More widely used therapies, such as endovascular aneurysm repair (EVAR), (Golledge, Muller et al. 2006) have a lower peri-operative mortality rate of 1-2% (Greenhalgh 2004). Up to 20% of treated patients require additional surgery within 5 years, further increasing risks and costs (Jones, Taylor et al. 2007). No direct drug therapy is currently available for this disease and is therefore urgently required. Such medication would allow not only the management of established AAA patients unsuitable for surgical repair but also the treatment of developing aortic aneurysms. At present small AAAs are monitored by periodic ultrasound until the aortic diameter reaches about 5cm, then treatment is carried out by endovascular repair or in some cases open surgery.

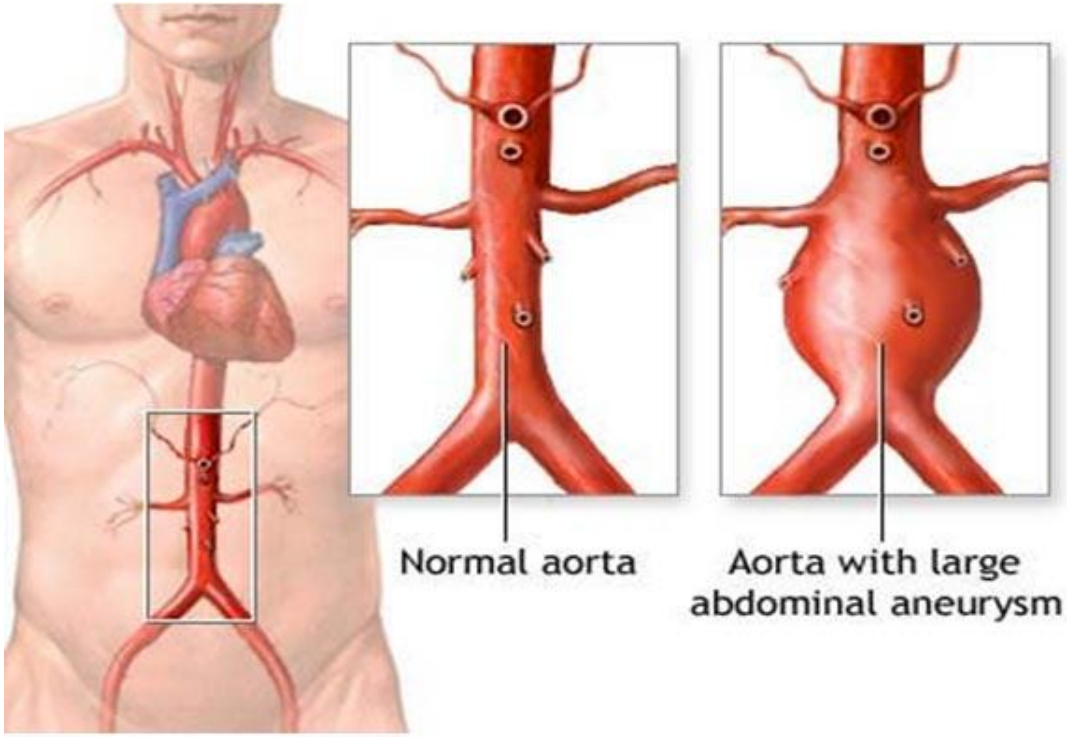


Figure 2.1 A diagram of the abdominal aorta *in situ*, comparing a normal aorta versus an aorta with a large aneurysm (http://www.health-res.com/EX/08-01-02/AAA.jpg).

There are number of well established risk factors for AAA, these include: low LDL, family history, sex (male), history of coronary heart disease (CHD) and history of peripheral arterial disease (PAD) (Moxon, Parr et al. 2010) (Figure 2.2). There is evidence for a genetic predisposition to AAA, exacerbated by the normal ageing processes and environmental risk factors (Fleming, Whitlock et al. 2005) such as smoking, hypertension and obesity (Vardulaki, Walker et al. 2000). For example, AAA are more common in the white northern European population then in African or Asian populations but the reasons for this have not been linked to genetics (Golledge, Muller et al. 2006). Other risk factors include atherosclerotic disease and renal dysfunction, although diabetes Mellitus is thought to be a protective factor (Shantikumar, Ajjan et al. 2010). AAA are primarily associated with atherosclerosis.

2.2 PATHOGENESIS OF AAA

A normal aortic media consists of organised elastin, collagen, smooth muscle cells and extra cellular matrix but in AAA there is a persistent proteolytic imbalance that results in excess matrix destruction and progressive weakening of the arterial wall (Abdul-Hussien, Soekhoe et al. 2007). All three layers of the aortic wall (intima, media and adventitia) (Figure 2.2) are involved in the pathology at a cellular level (Fontaine, Jacob et al. 2002). The resident cells in the aorta, which include endothelial and smooth muscle cells produce proteolytic enzymes which are involved in tissue remodelling.

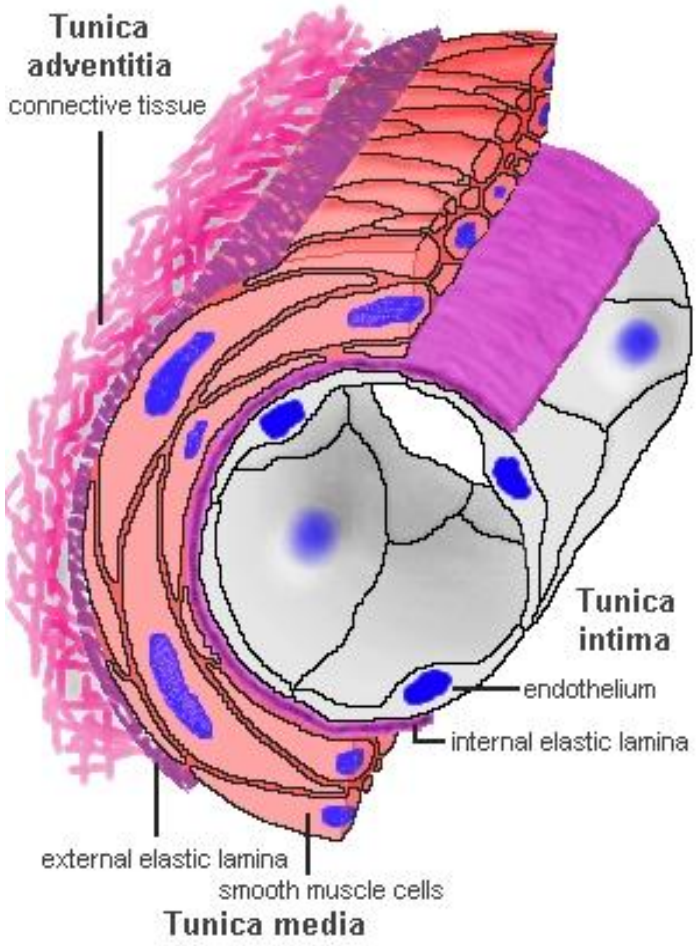


Figure 2.2 A diagram demonstrating the different layers of an aorta, including some of the cell types found.(http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Vascular/Images/VesWall.jpg)

The evidence for the pathogenesis behind the development of AAA, obtained from histology of AAA biopsies (Figure 2.3 and 2.4) and the use of animal models has indicated that chronic vascular inflammation, proteolytic degradation of elastin and collagen and vascular smooth muscle cell loss (VSMC) are all important in disease progression (Ernst 1993; Hellenthal, Buurman et al. 2009). Evidence such as circulating levels of markers of extracellular matrix degeneration supports the involvement of matrix metalloproteinases (MMP) in the process of degradation (Galis, Sukhova et al. 1994), elastin peptides, tissue plasminogen activators, leukocyte elastases (Houard, Leclercq et al. 2006), and cysteine proteases such as cathepsin K (elastase) in the vascular remodelling (Bossard, Tomaszek et al. 1996),

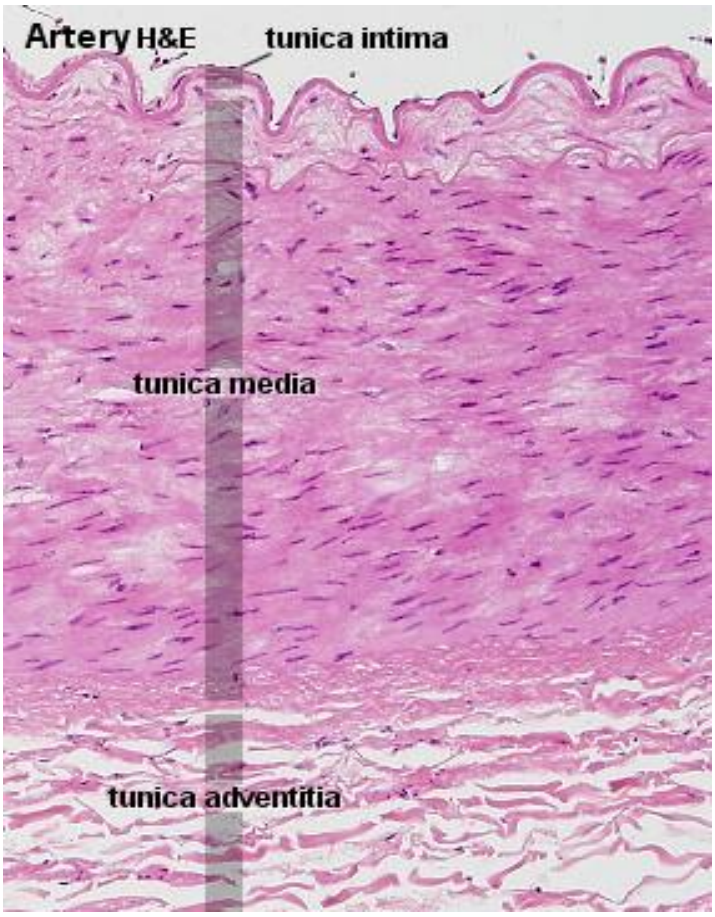


Figure 2.3 A haemotoxylin and eosin stain of a cross section of a human aorta, indicating the tunica intima, tunica media and the tunica adventitia (http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Vascular/Vascular.htm).

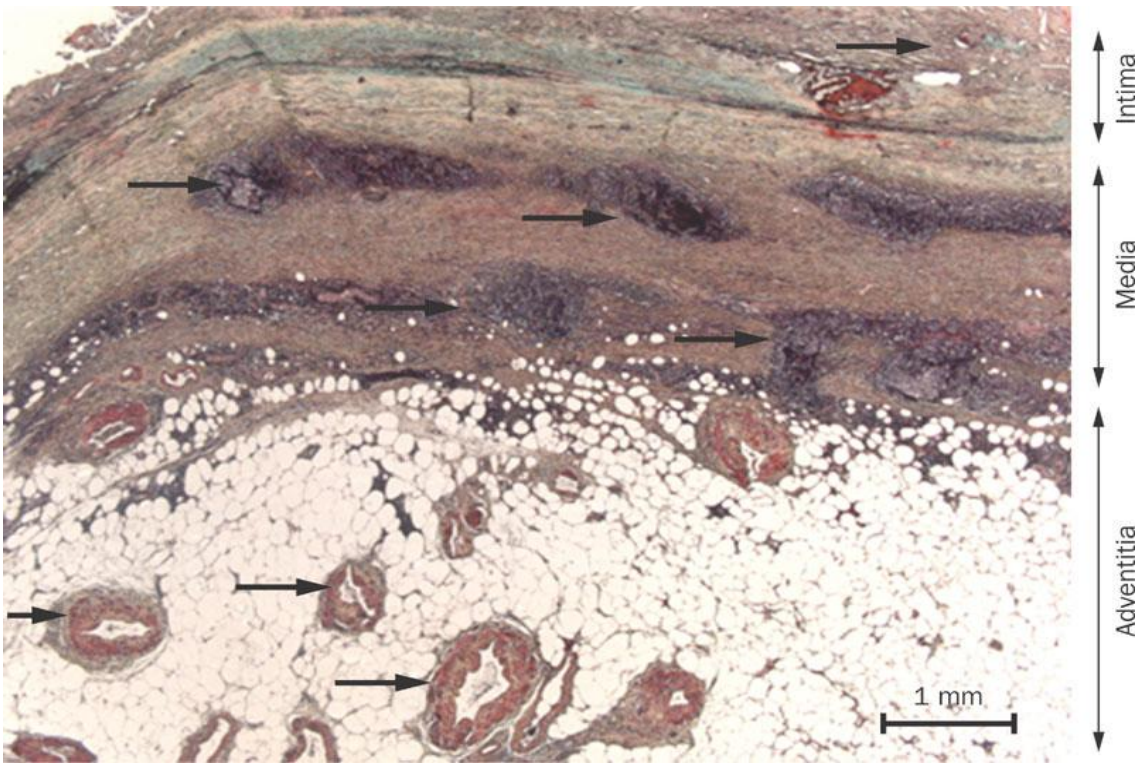


Figure 2.4 A histological section of an abdominal aortic aneurysm, stained with Movatt's pentachrome (25x), characterising fibrous tissue (arrow, intima), inflammatory cell infiltrates in the center of the medial layer and the adventitia densely populated with arterioles (Femke A. M. V. I. Hellenthal, Nature Reviews Cardiology 6, 464-474 (July 2009)

In reality many interacting immune and non-immune mediated mechanisms may be involved in the pathogenesis of AAA (Szekanecz, Shah et al. 1994). Associated with destructive remodelling of aortic wall connective tissue, end-stage human AAA tissue samples are characterised by chronic aortic wall inflammation (Pagano, Bartoli et al. 2007). The inflammatory infiltrate typically includes neutrophils, monocytes, macrophages and lymphocytes which move into the media and adventitia after transmigrating through the endothelium (Maiellaro and Taylor 2007). This inflammatory process in AAA is the focus of this research.

Several studies have suggested that an up regulation in the aortic wall of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor alpha (TNF-α), as well as chemokines such as CCL2/MCP-1 are associated with the development of AAA (Golledge, Tsao et al. 2008). Interferon gamma (IFN-γ) and CCL2/MCP-1 may contribute to the recruitment of leukocytes and other immune cells (Pearce, Sweis et al. 1992; Koch, Kunkel et al. 1993). Aortic aneurysms also appear to be an important source of circulating IL-6, for example in explant cultures aneurysm biopsies secrete large amounts of IL-6 and the circulating concentration of IL-6 in aneurysm patients appears to increase during disease progression (Jones, Brull et al. 2001). A role for the acquired immune response in the form of both helper T-cell type-1 (TH1) and type-2 (TH2) cytokines has been confirmed in both human AAA and animal models (Platsoucas, Lu et al. 2006).

Evidence has implicated a role for circulating neutrophils in early experimental AAA formation. Neutrophils are an important source of the matrix degrading enzymes MMP-2 and MMP-9, which may play a direct role in tissue damage and destabilisation (Wilton, Bland et al. 2008). However, neutrophil depletion can inhibit experimental AAA development through non-MMP-2/9 mediated mechanisms associated with an overall attenuation of inflammatory cell recruitment. (Eliason, Hannawa et al. 2005). Progressive dilation of the aortic wall is associated with extracellular matrix breakdown and this may involve proteolytic enzymes in addition to MMP-2 and MMP-9, as overall levels of proteases are increased in AAA tissue.
Neutrophil activation also triggers the release of neutrophil granules, several of which are implicated in damage to aortic tissue, particularly in the destruction of the collagen matrix (Wright, Moots et al. 2010) (Table 2.1).

Also of interest, platelet-derived RANTES and neutrophil-derived IL-8 are also involved in attracting further neutrophils to the luminal layer, which further stimulates the release of large amounts of proteases (Houard, Touat et al. 2009). Dipeptidyl peptidase I (DPPI), a cysteine protease that regulates neutrophil recruitment at sites of inflammation, could play a critical role

in the development of elastase induced experimental AAA as diminished recruitment of neutrophils to the aortic wall and impaired local production of CXC chemokines protected DPPI -/- mice from AAA development (Pagano, Bartoli et al. 2007).

Table 2.1 Neutrophil granules contain a variety of proteases that are implicated in damage to host tissue in inflammatory disease

Granule	Granule enzyme	Role in tissue damage	Reference
Azurophilic (primary)	Myeloperoxidase Elastase, Proteinase 3,	Production of HOCl Degradation of matrix Degrades elastin and collagen	(Wright, Moots et al. 2010) (Kessenbrock, Fr et al. 2008)
Specific (secondary)	Lactoferrin Collagenase	Degradation of matrix Degrades collagen	(Wright, Moots et al. 2010)
Gelatinase (tertiary)	Gelatinase Cathepsin G	Degradation of matrix Vascular permabilty	(Wright, Moots et al. 2010) (Korkmaz, Horwitz et al. 2010)

Neutrophils are not the only cell type involved in AAA progression. Macrophages also play a major role. Macrophages have been linked to the initial inflammatory response in murine experimental AAA progression (Lindholt 2006). Macrophages contribute to ECM degradation through the production of proteolytic enzymes, these include elastases and collagenases, MMP-9, MMP-2 and MMP-12, which play a major role in vessel weakening, leading to rupture (Thompson 2002). B and T cells can also be found within the cellular infiltrate of AAA (Forester, Cruickshank et al. 2006). T cells produce cytokines such as TNF-α, IL-6, IL-5 and IFN-γ, which contribute to the inflammation of the aortic wall (Pearce, Sweis et al. 1992). B

cell antibody production may also influence inflammation in AAA (Forester, Cruickshank et al. 2006).

2.2.1 Animal models for investigating AAA

There are many limitations using human samples alone to investigate disease progression, these include aneurismal tissue being difficult to obtain, and the restriction of end stage disease analysis from AAA samples (Daugherty and Cassis 2004). These limitations are overcome by the use of animal models, mainly rodents (Table 2.2) with the view of later translating these findings, if applicable to patients. A number of mouse models have been developed to study the mechanisms of AAA formation and progression and these have highlighted many important mechanisms, including the potential importance of inflammatory cytokines in pathogenesis (Cassis, Gupte et al. 2009). Daugherty *et al*, noted that an infusion of Angiotension II enhanced formation of abdominal aortic aneurysms in ApoE deficient mice, with males being more susceptible to AAA formation, just as in humans. Mice also develop atherosclerotic plaques in the aorta wall. The key mechanisms contributing to the progression of AAA in this mouse model include inflammation, extracellular matrix (ECM) degradation, angiogenesis and VSMC loss, (Daugherty and Cassis 2004). The use of animal models has proven to be particularly useful in the study of the early phase development of AAA, including the analysis of inflammation and cell recruitment. The subcutaneous infusion of Angiotension II into C57/B6 mice induced aortic production of the proinflammatory cytokines IL-6 and CCL2/MCP-1. Thus, leukocyte-fibroblast interactions in the aortic adventitia could potentiate IL-6 production, inducing local monocyte recruitment and activation, as well as promoting further CCL2/MCP-1 secretion, vascular inflammation, ECM remodelling, and aortic destabilisation (Tieu, Lee et al. 2009). Mice harbouring double knockout null mutations impacting on CD4 and IFN-γ production ($CD4^{-/-}$ $IFN\gamma^{-/-}$) do not normally develop significant aneurysms but this phenotype can be partially reversed by the administration of IFN- γ (Xiong, Zhao et al. 2004).

Table 2.2 Examples of some common mouse models of abdominal aortic aneurysm (Daugherty and Cassis 2004)

Mouse model		Characteristics	Comments
Genetically Determined	LDL receptor-/-	Medial degeneration	Supra-renal aorta aneurysm, high fat diet
	ApoE-/-	Medial degeneration, atherosclerosis	Supra-renal aorta aneurysm
	MMP-3 deficiency	Medial degeneration	Thoracic and abdominal aorta aneurysm
AngII infusion	LDL receptor-/-	Medial degeneration, atherosclerosis Inflammation, thrombus	AAA form in supra-renal aorta
	ApoE-/-	Medial degeneration Inflammation, thrombus atherosclerosis	AAA form in supra-renal aorta
	C57BL/6	Medial degeneration Inflammation, thrombus	Low aneurysm rate
Chemically Induced	Calcium chloride	Medial degeneration Inflammation	Inflammation, dilation at site of application
	Elastase	Medial degeneration Inflammation	Inflammation, dilation at infra-renal infusion site

Another murine model of AAA involves the chemical application of calcium chloride solution to the infra-renal aorta, directly between the renal branches and iliac bifurcation (Figure 2.5).

Pearce et al, showed that a large inflammatory infiltrate was observed in the intima and media layers of calcium chloride treated mice, including neutrophils, lymphocytes, monocytes, and multinucleated giant cells (Chiou, Chiu et al. 2001). In another chemically induced model the elastase- induced rat model, animals were protected from aneurysm formation by blocking the action of TNF-α (Hingorani, Ascher et al. 1998). Another rat study indicated that TNF-α was increased in aneurysmal compared with normal aortic tissue.

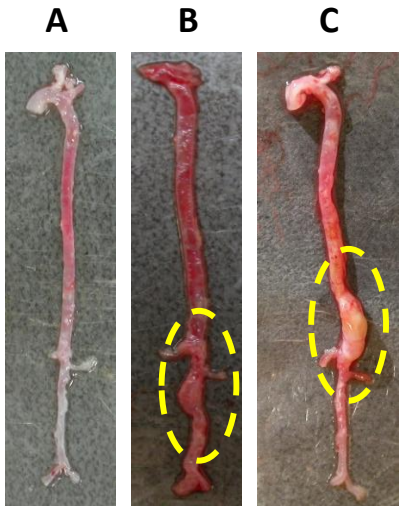


Figure 2.5 Examples of AAA formation in different mouse models
(A) Untreated ApoE$^{-/-}$ mouse, (B) Calcium chloride treated aorta, indicating infra-renal aneurysm on a C57BL/6 mouse, (C) AII infused ApoE$^{-/-}$ mouse, indicating supra-renal aneurysm.

The use of animal models to determine the mechanisms of disease progression are vital in research, as these findings could later be translated to patients if applicable. Micro array data from a AII infused ApoE$^{-/-}$ mouse model identified many genes relevant to aneurysm development or protection, those up-regulated in non aneurysm forming areas include Kallikrein 10, indicating a potential protective role (Rush, Nyara et al. 2009).

2.3 THE KALLIKREIN-KININ SYSTEM

Kallikreins are classified into plasma (PK) and tissue kallikrein (TK) (Cassim, Shaw et al. 2009). Plasma kallikrein, which is far more complex and activates the coagulation pathway, is called the contact system of plasma. It has chemotactic properties, can prime neutrophils for superoxide production (Bhoola, Elson et al. 1992) and can induce the secretion of neutrophil elastase (Gustafson, Schmaier et al. 1989). Plasma kallikrein can also stimulate the proliferation of a number of cells, such as neutrophils and monocytes (Stadnicki, Sartor et al. 1998). The plasma kinin forming system triggers the kallikrein-kinin cascade and liberates bradykinin from high-molecular-weight kininogen (HMWK) (Wilhelm 1971). It is composed of Hageman factor, factor XI, HMWK and prekallikrein (Figure 2.6).

A simpler pathway of kinin generation involves tissue kallikrein, which is synthesized in many tissues, such as the kidney and pancreas (Moreau, Garbacki et al. 2005). Active tissue kallikrein acts on LMWK to release Lys-bradykinin (Sharma 2006). Tissue kallikrein is also expressed in the aorta, kidney and brain. It can also generate bradykinin from HMWK) (Leeb-Lundberg, Marceau et al. 2005), (Marceau, Hess et al. 1998). Tissue kallikrein is widely distributed throughout the body in the kidney, blood vessels, central nervous system, pancreas, gut, salivary and sweat glands, spleen, adrenal, and neutrophils and are believed to act locally on their tissue of origin (Bhoola, Figueroa et al. 1992).

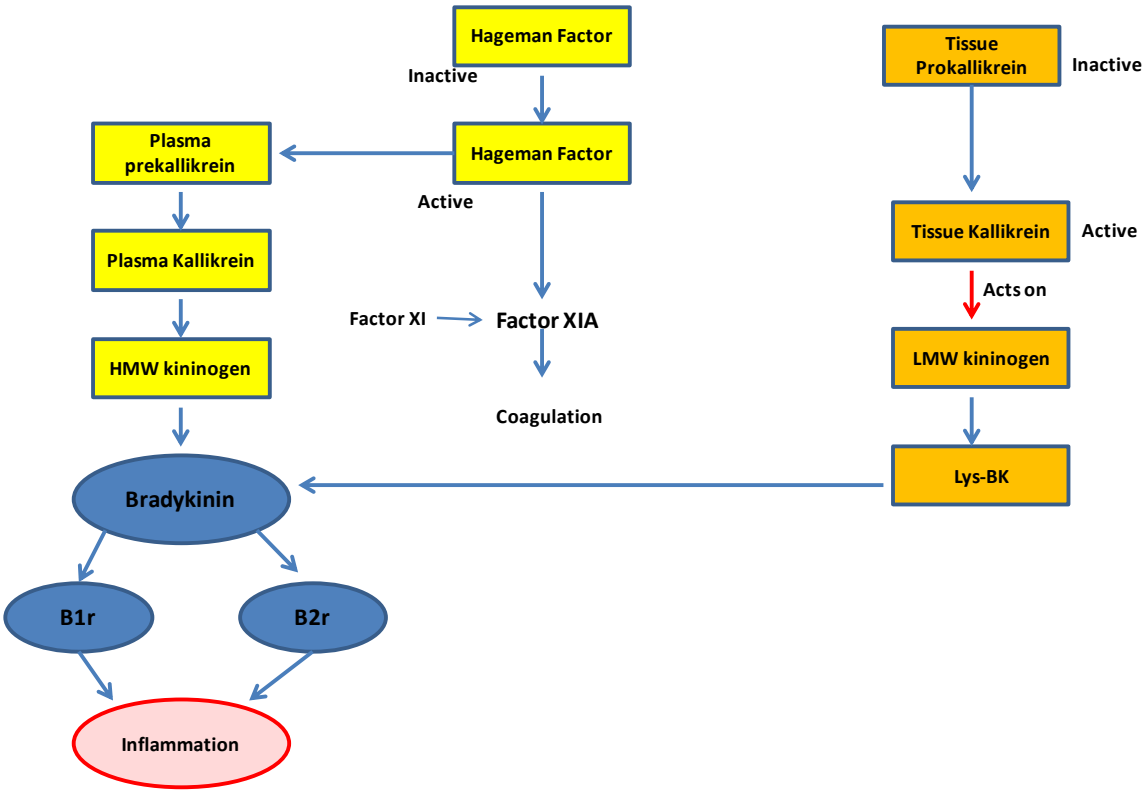


Figure 2.6 Schematic illustrating the formation of kinins by kallikreins: diagram of the production of kinin from kallikrein *Adapted from a figure by: (Sharma 2006)*

Another mechanism for initiation of the activation of the kallikrein-kinin system depends on the binding of components of the contact activation cascade on the surface of cells such as leukocytes, platelets and endothelial cells (Zhao, Qiu et al. 2001). This can occur when a neutrophil releases its granule content, such as MPO, the MPO can leak into the lumen of the vessel (Figure 2.7). The endothelial cell then binds the MPO with the cell- surface protein CK1. MPO can activate the action of the plasma kallikrein system and also associate with HMWK. In the absence of MPO the endothelial surface proteins such as CK1 bind KMWK. This leads to plasma kallikrein cleaving Bradykinin (Astern‡, Pendergraft et al. 2007). Multiple studies have confirmed the presence of kininogens, kallikreins, and kinin receptors in immune cells (Calixto, Medeiros et al. 2004).

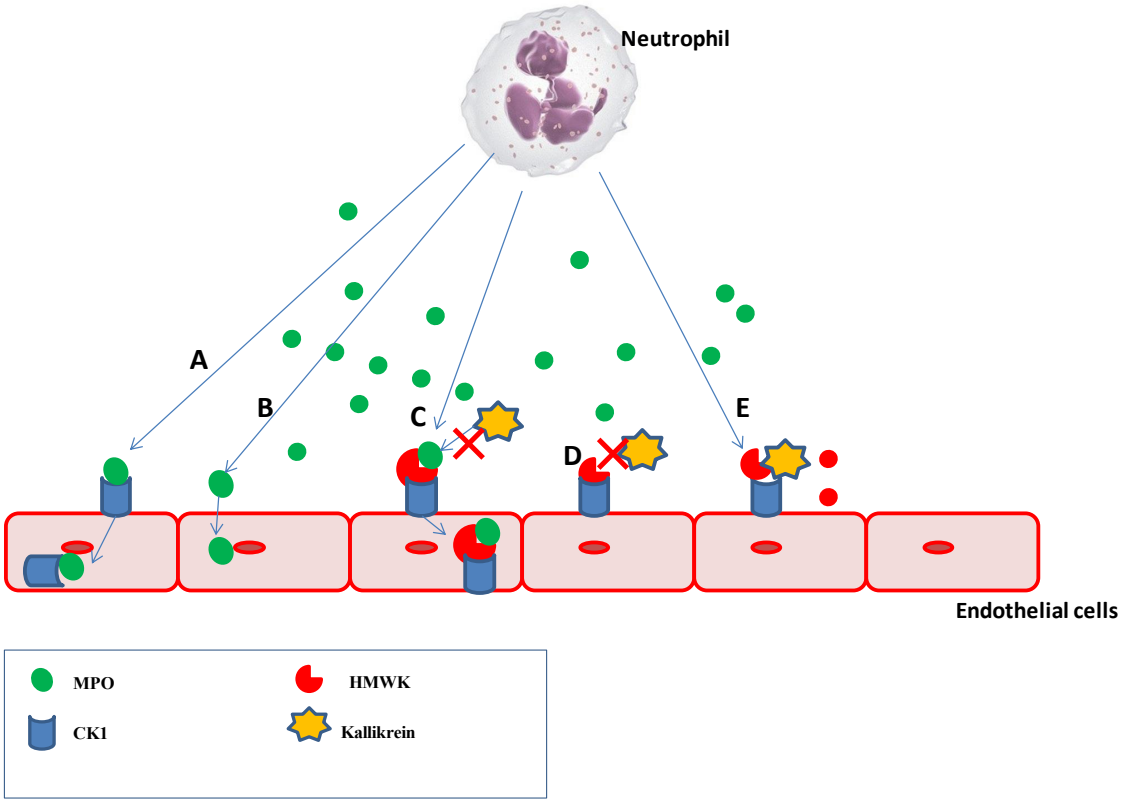


Figure 2.7 Schematic diagram of the interactions of MPO with endothelial cells and the plasma kallikrein- kininogen system. The neutrophil degranulates releasing MPO at the site of inflammation and the MPO leaks into the lumen. (A) Endothelial cell binds MPO with CK1 cell surface protein, the complex enters the cell. (B) MPO enters cell. (C) MPO associates with HMWK and CK1and this complex enters the cell, when MPO and kininogen couple, kallikrein cannot cleave HWMK and MPO produces hypochlorous acid. (D) Hypochlorous acid can inactivate HMWK by altering the kallikrein cleavage site. (E) In absence of MPO, CK1 bind KMWK, plasma kallikrein cleaves Bradykinin, which binds endothelial cells and induces nitric oxide. *Adapted from a figure by* (Astern‡, Pendergraft et al. 2007).

2.3.1 Kinins

The kinin family includes bradykinin (BK), Lys-BK, and their des-Arg metabolites (des-Arg^9-BK, and Lys-des-Arg^9-BK), which are produced by cleavage of the C-terminal arginine of bradykinin (BK) and Lys-BK respectively by carboxypeptidases (Moreau, Garbacki et al.

2005), (Huang and Player 2010). Bradykinin and Lys-BK are short lived agonists of the B2 receptor and their metabolites des-Arg^9-BK, and Lys-des-Arg^9-BK mediate their activity through the B1 receptor (Bhoola, Figueroa et al. 1992). Bradykinin, has a major role in the kallikrein system (Margolius 1995), it is a vasoactive peptide that influences many processes including vascular permeability, blood pressure, vasodilatation, smooth muscle contraction, pain, release of nitric oxide from the endothelium and stimulation of the synthesis of cytokines (IL-1, TNF-α) by monocytes (Bhoola, Elson et al. 1992). Bradykinin is generated systemically and locally within the vascular wall (Nolly, Carretero et al. 1993).

2.3.2 Kinin receptors

There are two types of bradykinin receptors, known as B1 and B2, which are found in humans and other mammals, including the mouse. The human B1R gene (BDKRB1) is located on chromosome 14q32.1 – q32.2 and the three-exon structure of the human BDKRB2 gene is also located on chromosome 14q32 (Menke, Borkowski et al. 1994; Moreau, Garbacki et al. 2005). Bradykinin and Lys-Bk act at the B2 receptor, which is constitutively expressed. The carboxypeptidase metabolites of B2 (des-Arg^9-BK and Lys-des-Arg^9-BK) act at the B1 receptor, which is induced by pro-inflammatory cytokines and peptides, such as angiotensin II (AII), linked to AAA (Ceravolo, Fernandes et al. 2007).

Both kinin receptors are G protein receptors that can couple to the Gαq and Gαi families of proteins to stimulate increased intracellular Ca^{2+} and NO production (Leeb-Lundberg, Marceau et al. 2005) and activation of the MAP kinase cascade (Couture, Harrisson et al. 2001). The B2 receptor is expressed constitutively and has been detected in many tissue including on endothelial cells, smooth muscle cells, fibroblasts, mesangial cells and neutrophils (Couture, Harrisson et al. 2001) (Table 2.3). The B1 receptor is barely detectable under normal physiological conditions (Bourdet, Pecher et al. 2010) and expression is up-regulated in injured

tissue and after exposure to inflammatory cytokines, including IL-1 and TNFα (Zhou, Polgar et al. 1998) (Figure 2.8). This cytokine induced expression is mediated through specific MAP-kinase pathways and Nuclear Factor-κB (Medeiros, Cabrini et al. 2004). Evidence suggests that the kinin B1 receptors can amplify or substitute for the kinin B2 receptor, especially in chronic inflammation (Ahluwalia and Perretti 1999; Phagoo, Poole et al. 1999; Bockmann and Paegelow 2000). Mice with a targeted deletion of the gene for the B1 receptor are healthy, fertile, normotensive, and show a blunted hypotensive response to bacterial LPS and a reduced accumulation of PMN leucocytes in inflamed tissue (Bhoola, Elson et al. 1992). The B2 receptors are also directly activated by serine proteases, such as MPO. (Hecquet, Tan et al. 2000)

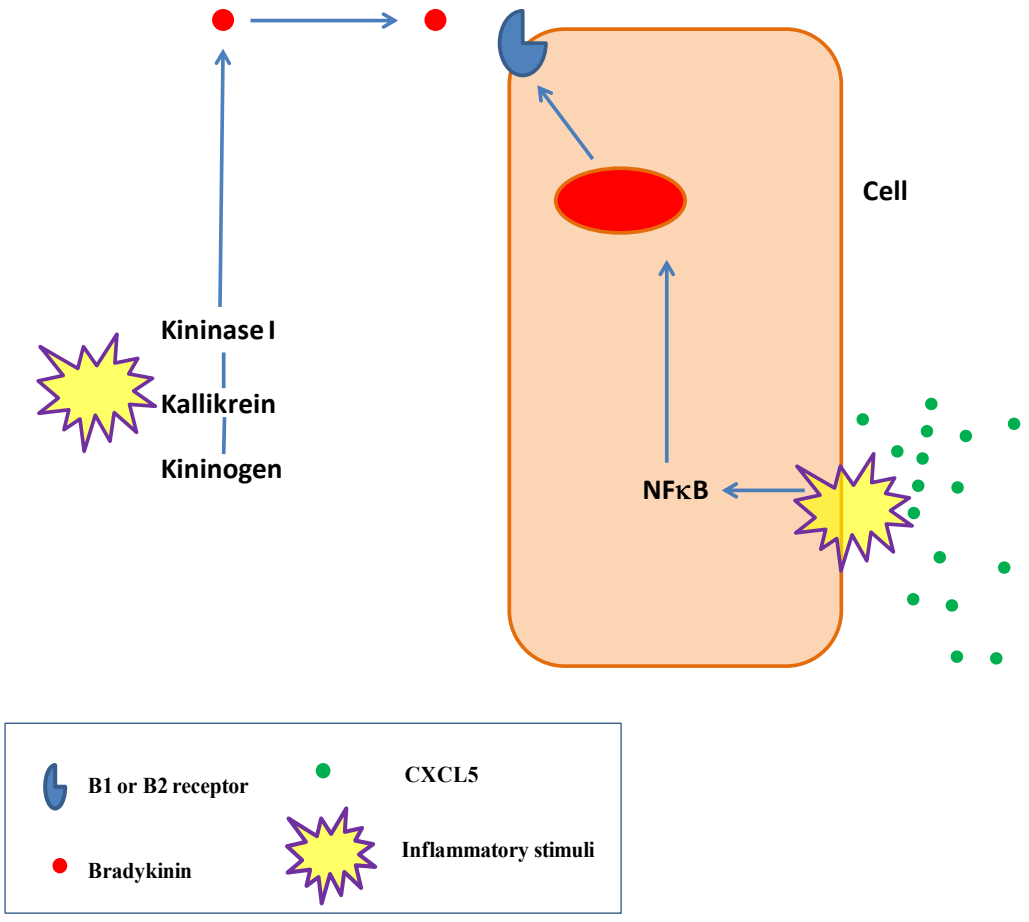


Figure 2.8 Schematic diagram demonstrating the proposed mechanisms of cytokine activation within a cell. Cytokines activate the transcriptional factor, NFκB, involved in the up-regulation of the B1 receptor. (Couture, Harrisson et al. 2001).

Kinin/receptor interactions can lead to receptor internalisation, potentially inducing a critical protective effect by reducing further activation (Pankaj, Philip et al. 2010). Studies have revealed important difference between the two receptor types in that the kinin B2 receptor is internalised rapidly and desensitized, whereas the kinin B1 receptor, once induced, is not (Proud and Kaplan 1988). Observation that kinin B1 receptor-induced responses are more persistent supports the lack of the internalization of the receptor-ligand complex (Levesque, Harvey et al. 1995). Interestingly, kallikreins may play a direct role in the modulation of cellular responses and in the regulation of blood pressure via B2 receptor activation. Data from various animal models support a role for B2 receptors in the acute phase of the inflammatory and pain response and a role for the B1 receptors in the chronic phase of such responses (Couture, Harrisson et al. 2001).

Table 2.3: Cellular distribution of kinin receptors

Receptor	Cell type/ tissue	Reference
B2r	Neutrophils, Endothelial cell, smooth muscle cells, fibroblasts, mesangial cells, most tissue, Macrophages	(Couture, Harrisson et al. 2001)
B1r	T Cells	(Shirasaki, Kanaizumi et al. 2009)
B1r	Neutrophil	(Koch, Haines et al. 1990)
B1r	Macrophages, fibroblasts, epithelial cells, smooth muscle cells and endothelial cells	(Gustafson, Schmaier et al. 1989)
B1r, B2r	Macrophages, Monocytes	(Bertram, Baltic et al. 2007)
B1r	Macrophages	(Bhoola, Elson et al. 1992)
B1r, B2r	Eosinophils	(Lamontagne, Nadeau et al. 1995)
B1r	Vascular endothelial, perivascular inflammatory cells, T lymphocytes CD3q cells, Human brain endothelial cells	(Bertram, Baltic et al. 2007)
B1r, B2r	Immature human monocyte derived dendritic cells	(Raidoo, Ramsaroop et al. 1997)
B2r	Dendritic cells, Smooth muscle cells	(Bertram, Baltic et al. 2007)
B1r	Endothelial cells, foamy macrophages, fibroblasts and smooth muscle cells	(Kakoki, Sullivan et al. 2010)
B1r, B2r	Vascular endothelium, primary sensory afferent neurons, vascular and nonvascular smooth muscle, epithelial cells, and some types of leukocytes.	(Moreau, Garbacki et al. 2005)
B1r	Macrophages, fibroblasts, neutrophil, endothelial cells	(Couture, Harrisson et al. 2001)
B1r	Macrophages, fibroblasts, epithelial cells, smooth muscle cells and endothelial cells, Neutrophils	(Duchene and Ahluwalia 2009)

2.3.3 Kinin receptor signalling

Various signal transduction mechanisms have been associated with kinins depending on the cellular type (Couture, Harrisson et al. 2001). These includes the activation of phospholipases A2, C and D with the subsequent release of prostaglandins, nitric oxide, inositol phosphates and diacylglycerol from membrane inositol phospholipids, leading to the mobilisation of intracellular calcium and activation of several isoforms of protein kinase C. Besides these classical pathways, the B2 receptor is also linked to the activation of protein tyrosine kinases and phosphatases as well as MAP kinase (Figure 2.9). Conversely, the B1 receptor is primarily linked to the activation of phospholipase C and the phosphoinositide pathway (Marceau, Hess et al. 1998; Couture, Harrisson et al. 2001).

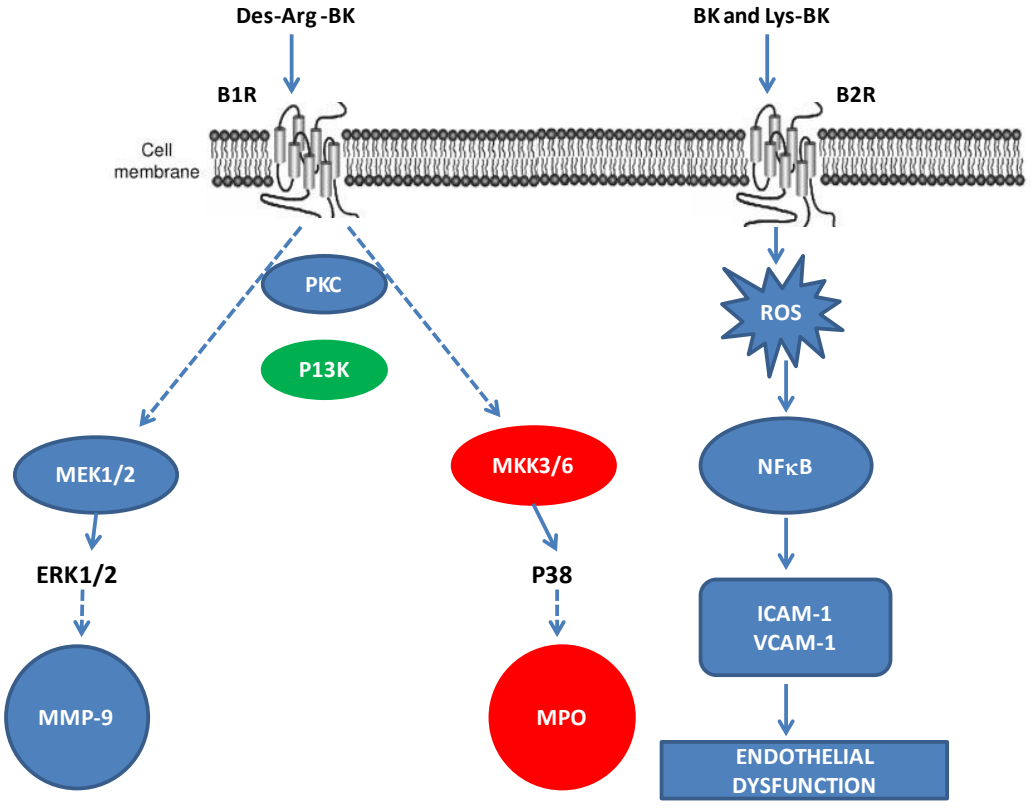


Figure 2.9 Schematic representation of B1 and B2 receptors and their proposed signally pathways. The B1 receptor activation induces neutrophil degranulation and the B2 receptor activation can lead to endothelial dysfunction (Higuchi, Ohtsu et al. 2007; Ehrenfeld, Matus et al. 2009).

2.4 KININ RECEPTORS AND DISEASE STATES

Substantial literature exists documenting roles for the B1 receptor in sepsis, asthma, allergy and diabetes (Marceau, Hess et al. 1998). Transgenic and knock out murine models of inflammation as well as kinin receptor antagonists and agonists have all indicated that the kinin receptors play a contributory role in many diseases (Table 2.2). Overproduction of kinins under pathophysiological conditions is implicated in a number of disorders, including pain, inflammation, hypotension, cardiovascular disease, asthma, colitis, pancreatitis, rhinitis, sepsis, and rheumatoid arthritis (Figure 2.10). (Marceau and Regoli 2004). The B2 receptor has been implicated in the early phases or acute state of inflammatory pain, whereas the B1 receptor participates prominently in the establishment and maintenance of chronic pain (Ahluwalia and Perretti 1999).

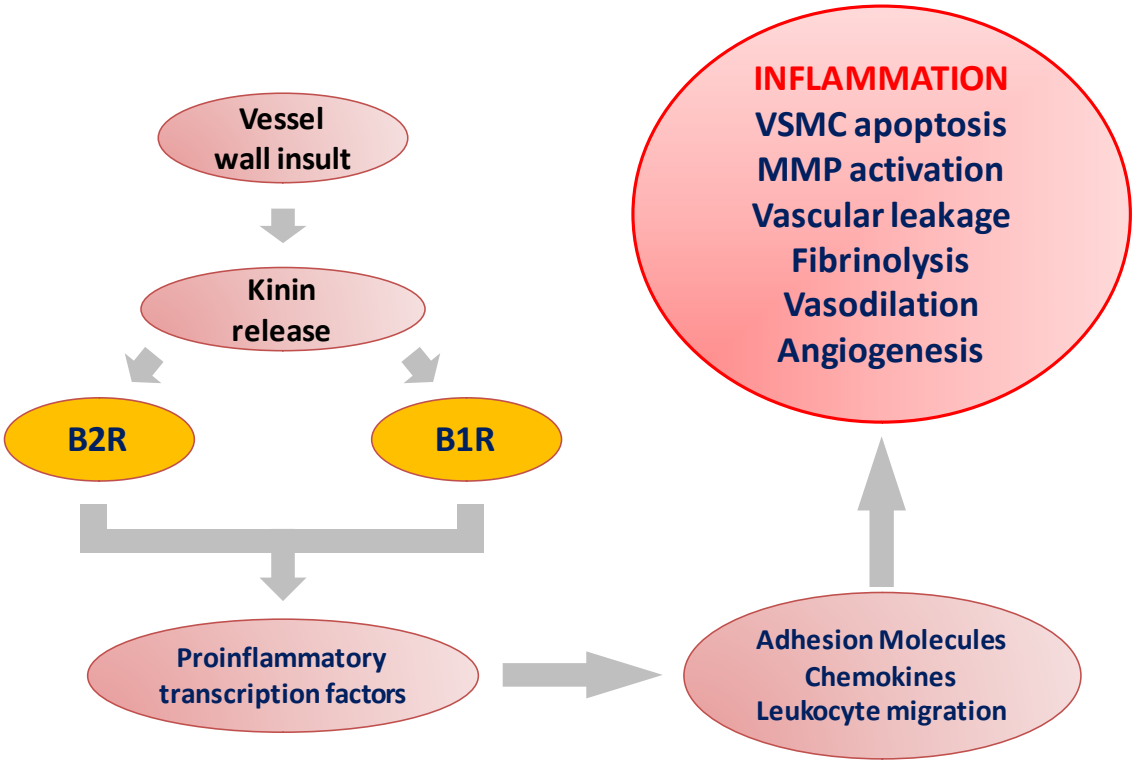


Figure 2.10 A schematic diagram demonstrating the result of kinin release and receptor activation, leading to inflammation, via leukocyte migration.

Table 2.4: Kinin receptors associated with disease

Receptor	Disease	Reference
B1r, B2r	Myocardial infarction	(Huang and Player 2010)
B1r	Atherosclerosis, renal ischaemia-reperfusion	(Duchene and Ahluwalia 2009)
B1r	Pain, diabetes, asthma, rheumatoid arthritis and multiple sclerosis, epilepsy, chronic inflammatory demyelinating polyneuritis, and systemic lupus erythematosus	(Couture, Harrisson et al. 2001)
B1r, B2r	Coronary heart disease, congestive heart failure and peripheral vascular and renal diseases	(Cassim, Shaw et al. 2009)
B1r	Insulin-dependent diabetes (type I diabetes), multiple sclerosis, inflammatory bowel disease, arteriosclerosis	(Moreau, Garbacki et al. 2005)
B2r	Asthma, chronic inflammatory pain, edema	(Moreau, Garbacki et al. 2005)
B1r, B2r	Multiple sclerosis,	(Bertram, Baltic et al. 2007)
B2r	Anti-GBM antibody-induced glomerulonephritis, systemic lupus erythematosus (SLE), vasulitis, Asthma	(Akbary, Wirth et al. 1996)
B2r	Insulin-dependent diabetes (type I diabetes)	(Constantinescu, Ventura et al. 1995)
B1r, B2r	Rheumatoid arthritis	(Cassim, Mody et al. 2002)
B1r, B2r	Cardiovascular disease, atherosclerosis, Inflammatory pain-acute, Chronic pain	(Ahluwalia and Perretti 1999)
B1r, B2r	Atherogenesis, Myocardial infarction	(Gabra and Sirois 2003)
B2r	Diabetic nephropathy	(Liu, Li et al. 2009)
B1r	Renal fibrosis, diabetic cardiomyopathy	(Bhoola, Figueroa et al. 1992; Sharma 2006)
B1r	Sepsis, asthma, allergy and diabetes	(Sharma 2006)
B1r, B2r	Pain, inflammation, hypotension, asthma, colitis, pancreatitis, rhinitis, sepsis, and rheumatoid arthritis	(Emanueli, Maestri et al. 1999)

Studies in B1 receptor knockout mice using induced peritoneal inflammation suggest that the B1 receptor may play an important role in the pathogenesis of chronic inflammation diseases (Marceau, Hess et al. 1998). The B1 receptor is likely to exert a strategic role, particularly in inflammatory diseases with an immune etiology such as diabetes, asthma, rheumatoid arthritis and multiple sclerosis (Couture, Harrisson et al. 2001). The protective effect of kinins in renal disease has been extended to the B1 receptor, as various aspects of the chronic inflammatory response seem to be limited by endogenous ligands of this receptor subtypes in animal models (Moreau, Garbacki et al. 2005).

Under chronic inflammatory conditions, such as renal fibrosis, B1 receptor KO mice have significantly lower expression of proinflammatory molecules, such as TGF-β, CCL2/MCP-1, and IL-6. B1 receptor KO mice also show an attenuation of diabetic cardiomyopathy, with improved systolic and diastolic function in comparison with diabetic control mice, suggesting that the B1 receptor is detrimental in diabetic cardiomyopathy, as it may mediate inflammatory and fibrotic processes.(Huang and Player 2010).

Targeted deletion of the B2 receptor in mice protects against the development of diabetic nephropathy (DN), providing additional evidence to support a relationship between the activity of the kallikrein-kinin system and renal impairment. Acute treatment of hyperfiltering diabetic rats with aprotinin, a kallikrein inhibitor, or with a B2-kinin receptor antagonist, increases the renal vascular resistance (Tan, Keum et al. 2007).

2.4.1 Kinin receptors in cardiovascular disease

A number of observations focus on kinins as potential mediators in endogenous cardiovascular protective mechanisms. This is, in part, due to the fact that components of the kallikrein-kinin system are localized in the heart and vascular tissues (Sharma 2006). For example, increased plasma levels of kallikrein, kininogen and BK have been demonstrated after myocardial

infarction (Lamontagne, Nadeau et al. 1995). The importance of kinins in the regulation of cardiovascular physiology has been documented in B2 receptor knockout mice, which develop enhanced levels of cardiac failure and hypertension (Emanueli, Maestri et al. 1999). B1 receptor expression has been localised to the endothelial cells, foamy macrophages, fibroblasts and smooth muscle cells, which are all prominent cell types involved in atherogenesis (Raidoo, Ramsaroop et al. 1997). B1 receptor antagonism or genetic inactivation in mice leads to a reduction in the levels of many markers of inflammation in tissues and improved outcome in models of atherosclerosis (Ahluwalia and Perretti 1999).

2.4.2 Therapeutic modulation of kinin receptors

i) Receptor agonists

The B1 receptor is specialised across species to respond to kinin metabolites generated by the arginine carboxypeptidases (either des-Arg9-BK, Lys-des-Arg9-BK or des-Arg10-KD) (Marceau, Hess et al. 1998; Moreau, Garbacki et al. 2005).

The B2 receptor has a high affinity for the "native" kinins, BK and Lys-BK, generated by either plasma or tissue kallikreins in all mammalian species, although none of the metabolites of BK retain a significant affinity for this receptor. B-9972 is a high potency B2 peptide agonist, that can alleviate pulmonary hypertension (Bawolak, Gera et al. 2007). The stimulation of the bradykinin receptors may have beneficial effects (Fincham, Bressan et al. 2009). The B2 receptor agonists may be beneficial in the treatment of cardiovascular disorders, such as congestive heart failure, hypertension, and ischemic heart disease (Huang and Player 2010). Various animal models have been shown that the stimulation of B2 receptors is not only implicated in the pathogenesis of inflammation, and tissue injury but also in powerful cardioprotective mechanisms, (Heitsch 2003). For example B2 receptor knockout mice exhibit salt-sensitive hypertension (Huang and Player 2010).

ii) Receptor antagonists

The use of kinin antagonists for targeting either receptor supports their involvement in conditions such as inflammatory hyperalgesia, fever and, perhaps, hepatic regulation of acute phase reactants (Marceau, Hess et al. 1998). The first series of compounds capable of antagonizing BK and des-Arg9-BK with specificity for B1 receptor included the prototype [Leu8] des-Arg9-BK. The first generation of B2 receptor antagonists was based on [D-Phe7]-BK (Moreau, Garbacki et al. 2005). These first prototype antagonists were developed with sepsis and pain as the disease targets, although the development of effective drugs against these targets has proved difficult (Leeb-Lundberg, Marceau et al. 2005). However, recent exciting discoveries in the cardiovascular field indicate that B1 receptor antagonists may have utility in the treatment of diseases such as atherosclerosis and renal disease (Duchene and Ahluwalia 2009). Icatibant (HOE 140), a widely studied peptide B2 receptor antagonist, has been found to significantly improve the ventilatory function in humans with asthma when administered in an aerosol form (Akbary, Wirth et al. 1996) and this reached the market in 2008 for the treatment of hereditary angioedema (HAE), it works by reducing inflammation (Huang and Player 2010).

The ‘‘third-generation’’ single-chain B1 and B2 receptor antagonists (B-9224, B-9430, 9330 and B-9668) were introduced with the new amino acid, a-2-indanyl-glycine at positions (L-isomer) and 7 (D-isomer) of the HOE-140 structure (Cassim, Mody et al. 2002). The understanding of the critical role the kinin receptors plays in health and disease has been greatly enhanced by the development of these antagonists (Bhoola, Elson et al. 1992). The development of novel non-peptidic (phosphonium) B1 receptor antagonists has stimulated renewed interest in this receptor as a target for the treatment of inflammatory diseases, particularly by demonstrating oral efficacy in inhibition of leukocyte recruitment in different inflammatory models (Ahluwalia and Perretti 1999).

As a large number of disease states such as chronic inflammatory pain, oedema, asthma, and sepsis have their basis in the inflammatory response, the development of novel antagonist drugs targeted at the B1 receptor and B2 receptor can provide therapeutic opportunities (Moreau, Garbacki et al. 2005). The clinical development of such drugs is at an early stage, with the few human clinical studies reported mainly based on peptide compounds (Proud, Bathon et al. 1995).

2.5 THE KALLIKREIN-KININ SYSTEM, NEUTROPHILS, AND AAA

Neutrophils are immune cells and instigators of an inflammatory response at sites of injury or disease. Active neutrophils are present within the adventitia and mural thrombus of AAA, providing evidence for their involvement in AAA (Cohen, Keegan et al. 1991). Circulating neutrophils are an important component of experimental AAA progression, as reducing the number of circulating neutrophils in aneurysm mouse models improved animal survival (Eliason, Hannawa et al. 2005). This suggests that the neutrophils play a major pathogenic role in AAA development. Further evidence indicates that aortic tissue from humans or mice with AAA contain much higher levels of proteases produced by neutrophils compared to healthy controls (Liu, Sukhova et al. 2006). Neutrophils routinely circulate in the body in a non-active state and in this normal state they do not attach efficiently to arterial endothelium. However, during the initial stages of AAA pathogenesis, activation of endothelial cells during an inflammatory event, such as tissue trauma or infection, leads to secretion of leukocyte chemotactic and activating factors such as IL-1, TNF-α, IL-8, CCL2/MCP-1 and IFN-γ, and expression of adhesion molecules at the endothelial cell surface increase neutrophil adhesiveness and transmigration through the endothelium (Davis, Pearce et al. 1993; Hannawa, Eliason et al. 2005; Houard, Touat et al. 2009). Neutrophils themselves produce multiple

neutrophil chemotactic factors, including IL-8, which can lead to their own recruitment (Houard, Touat et al. 2009).

Neutrophils contain the complete system for the synthesis and release of the kallikrein-kinin cascade, including the membrane-bound kinin-forming substrates (kininogens) They also express both the B1 and B2 receptors (Figure 2.11) (Proud and Kaplan 1988; Bhoola, Elson et al. 1992; Henderson, Figueroa et al. 1994). As such, it is postulated that establishment of a local neutrophil-derived kallikrein-kinin system perpetuates *in situ* neutrophil activation, endothelium permeability to plasma proteins and immune-inflammatory cells (monocyte/macrophages, lymphocytes), tissue damage and inflammatory response and within the artery wall (Bhoola, Elson et al. 1992) (Figure 2.12 and 2.13).

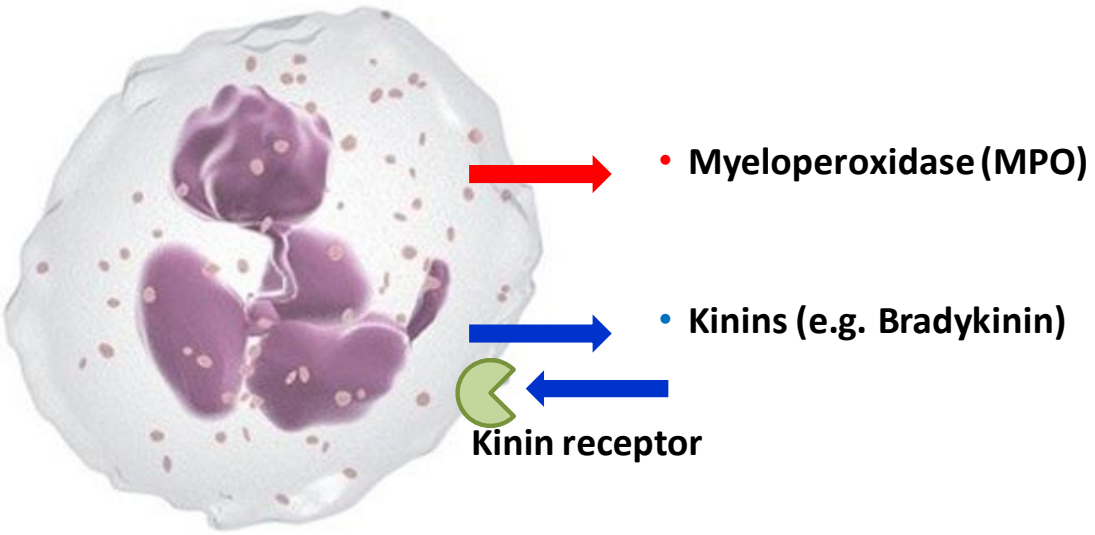


Figure 2.11. Schematic diagram illustrating the role of kinin receptors and neutrophils in AAA formation and predictive mechanisms involved, such as MPO production.

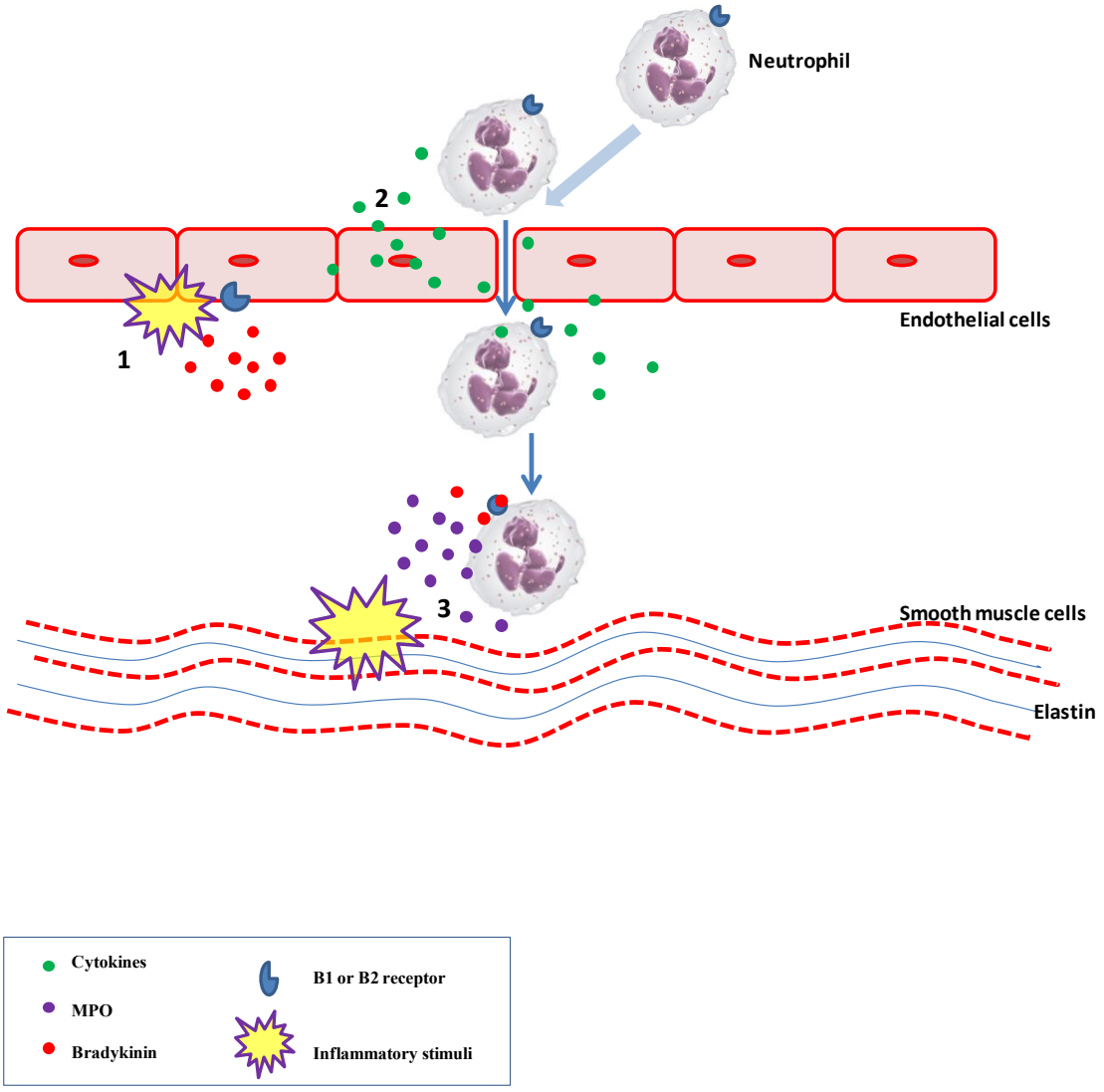


Figure 2.12 Proposed mechanisms of action for neutrophil recruitment driven by the B1 and B2 receptors. (1) Endothelial cells express the B1 or B2 receptor during inflammation. **(2)** Once activated by BK the endothelial cytokines are released, leading to migration of neutrophils. **(3)** Neutrophils expressing B1 or B2 receptors on their surface are driven to sites of inflammation by BK, leading to neutrophil degranulation and tissue damage by MPO.

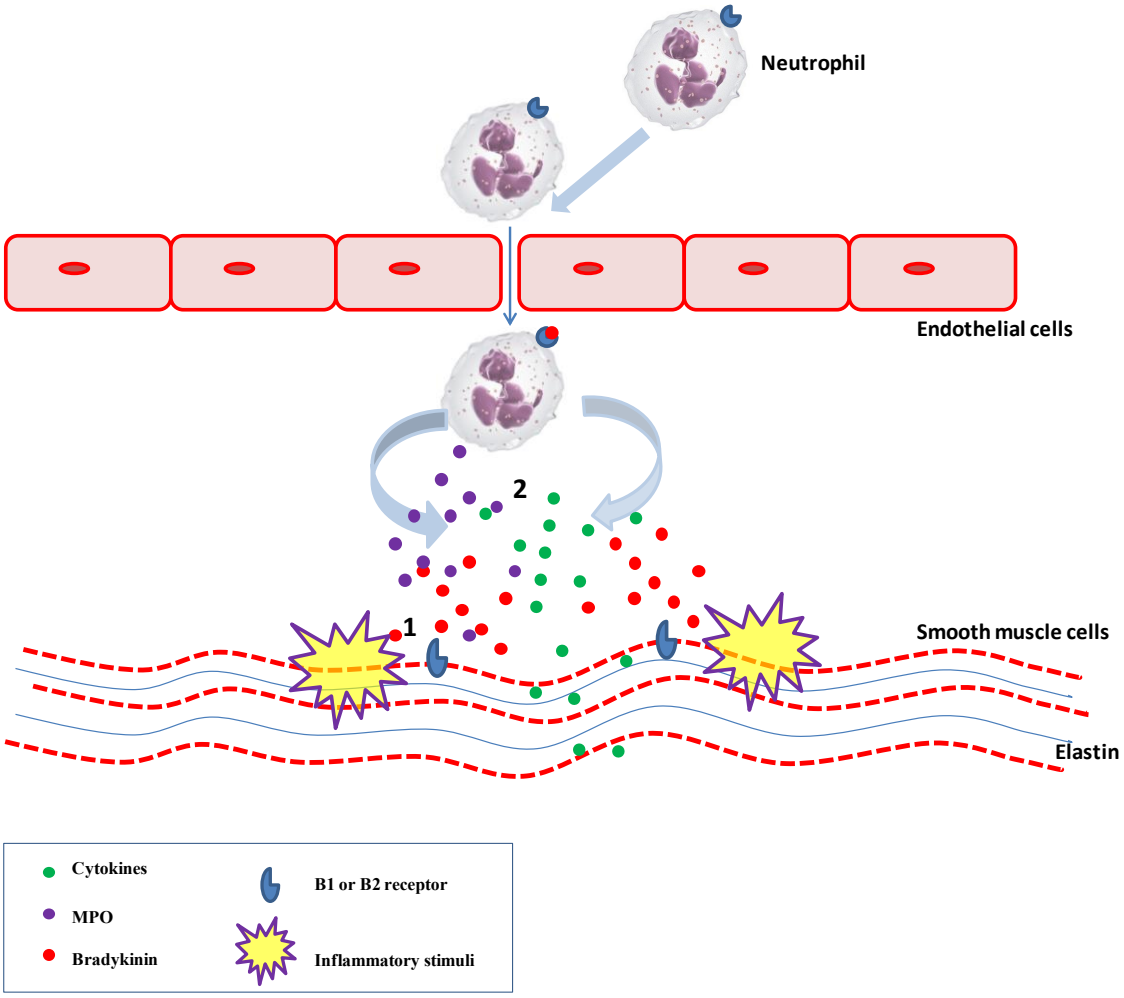


Figure 2.13 Proposed mechanism of action of neutrophil recruitment. Activation of neutrophils by BK following inflammation and transmigration to the vascular compartment, leads to cytokine release, further neutrophil recruitment, leading to degranulation and MPO release, resulting in tissue damage.

2.6 SUMMARY

AAA is an important cause of mortality in the elderly through aortic rupture. AAA is responsible for 2.1 % of deaths in men aged over 65, around half of the deaths occur if rupture of the aneurysm take place before the patient reaches hospital (Thompson, Ashton et al. 2009). The improvement of diagnostic tools has also lead to an increase in the discovery of AAA. A limited management approach is currently taken with diagnosed patients who are monitored over progressive years by repeated imaging and clinical review without clear treatment instigated. Surgical intervention is performed only where the risk of rupture is considered high. This *'wait-and-see'* approach results in physical and psychological burden that impacts significantly on patient well-being and quality of life. Development of a medical therapy aimed at limiting AAA formation and/or progression is urgently required. To date there is no non-surgical option available. It is of importance that the pathogenesis of AAA is understood. This study aims to further understand the pathogenesis of AAA, with a focus on treatment.

Sufficient evidence exists to propose a role for the kallikrein–kinin system in AAA. The capacity of kinins to induce the release of secondary mediators, such as cytokines is crucial in the maintenance of inflammation and the regulation of the cellular response (Bhoola, Elson et al. 1992). Kinins induce this release of cytokines, and cytokines have been shown to augment the effects of kinins, interplays that can enhance the inflammatory process leading to the formation and progression of AAA.

Neutrophils play a role in the kallikrein-kinin system, they express the B1 and B2 receptors on their surface, they can also synthesize and release other components of the KKS, including membrane bound kinin-forming substrates (kininogens) (Henderson, Figueroa et al. 1994), they are activated by plasma kallikrein, and are also producers of tissue kallikrein, underpinning a link between kinins and neutrophils in inflammation (Bhoola, Elson et al. 1992). Neutrophils are present within the adventitia and mural thrombus of human AAA (Cohen, Keegan et al.

1991) and studies in animal models suggest that depleting or blocking neutrophil function inhibits experimental AAA (Eliason, Hannawa et al. 2005).

The current study was undertaken to examine the potential link between kinins and neutrophils in the pathogenesis of AAA. The use of an animal model of AAA, and also mice deficient in the B1 and B2 receptors, helped direct this research. It also accessed the involvement of neutrophils in AAA progression, and investigated the affects of kinins on neutrophil activation.

Newly developed kinin receptor agonists, as well as antagonists with improved pharmacological properties in terms of their half-life and receptor selectivity, have demonstrated enhanced therapeutic potential in experimental models (Schulze-Topphoff, Prat et al. 2008). In addition to giving new insight into the pathogenesis aortic aneurysm, demonstration of a key role for kinin receptor-mediated neutrophil activation in promoting AAA will provide mechanistic evidence upon which novel therapeutic strategies can be based.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 ANGIOTENSION II INFUSED MOUSE MODEL OF AAA

3.1.1 *Animal studies.*

Approval for animal studies was obtained from the local ethics committee and experimental work performed in accordance with the institutional and ethical guidelines of James Cook University, prior to the commencement of study (# A1455, Appendix 2). Mice were kept in a temperature-controlled room on a 12-hour light/dark cycle, 60% humidity, and maintained on normal laboratory chow and water *ad libitum* for the duration of the experiments (Table 3.1).

Table 3.1 Mouse strains used in this research work.

Mouse Strains	Description	Source
C57BL/6	Background strain	JCU Animal facility
B6.B1rB2r$^{-/-}$	B6 background with B1r B2r KO	Masao Kakoki et al
B6.B2r$^{-/-}$	B6 background with B2r KO	Jackson laboratory, USA
ApoE$^{-/-}$	B6.129P2-Apoe ^{tm1Unc}/Arc (N10)	Animal Resources Centre, Canning Vale, WA
B1rB2r$^{-/-}$ApoE$^{-/-}$	B6.B1rB2r$^{-/-}$ crossed with ApoE$^{-/-}$	Generated this study
B2r$^{-/-}$ApoE$^{-/-}$	B6.B2r$^{-/-}$ crossed with ApoE$^{-/-}$	Generated this study

3.1.2 *Generation of kinin receptor-deficient ApoE$^{-/-}$ mice.*

C57BL/6 mice lacking both the bradykinin (kinin) B1 and B2 receptor genes (B1rB2r-null, Bdkrb1$^{-/-}$/Bdkrb2$^{-/-}$), and the kinin B2 receptor gene only (B2r-null, Bdkrb2$^{-/-}$), were generated by deleting the genomic region encoding the two receptors as previously described (Figure 3.1) (Kakoki, McGarrah et al. 2007). These B6.B1rB2r$^{-/-}$ mice were kindly provided by Masao Kakoki, M.D. Ph.D, University of North Carolina at Chapel Hill, USA, and the B6.B2r$^{-/-}$ (B6;129S7-*Bdkrb2*^{tm1Jfh}/J) mice were obtained from the Jackson laboratory, Maine, USA.

Kinin receptor-deficient C57BL/6 ApoE$^{-/-}$ mice (B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ApoE$^{-/-}$) were generated at the small animal handling facility of James Cook University by mating the respective null homozygote's with ApoE-deficient mice (B6.129P2-Apoe ^{tm1Unc}/Arc (N10)) sourced from the Animal Resources Centre, Canning Vale, Western Australia. Resultant double-heterozygous progeny were then intercrossed and the subsequent generations typed to identify and establish homozygous double deficient mouse lines, by Roby Jose (JCU). These homozygous double deficient mice were intercrossed to generate the knockout lines used for experiments. The control mice used were the ApoE-deficient mice (B6.129P2-Apoe ^{tm1Unc}/Arc (N10)) as above.

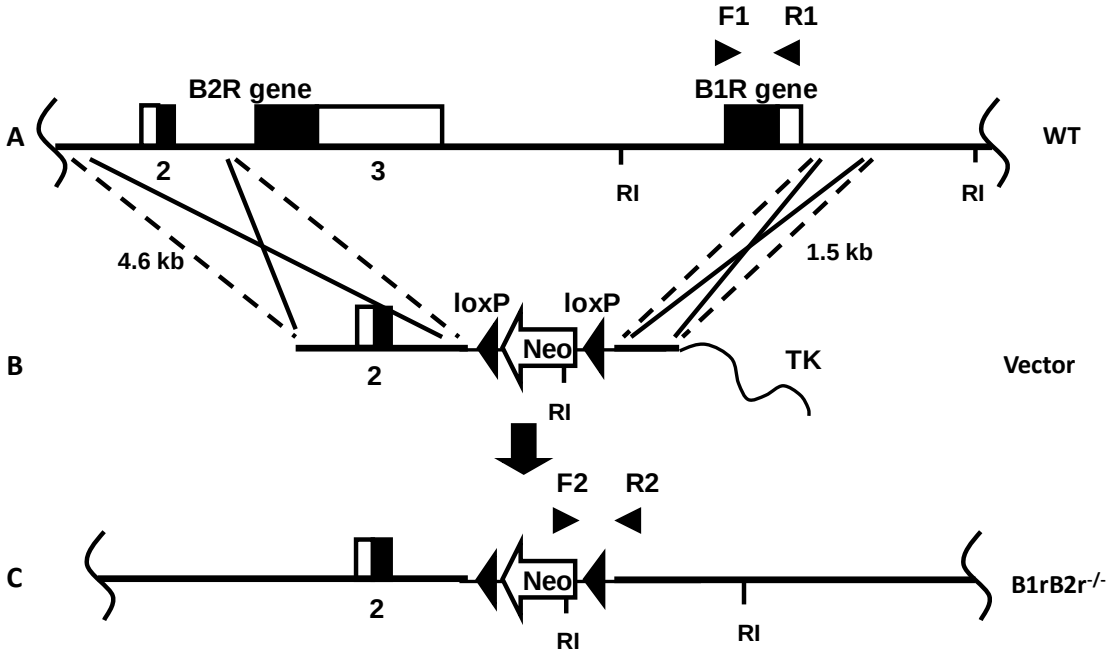


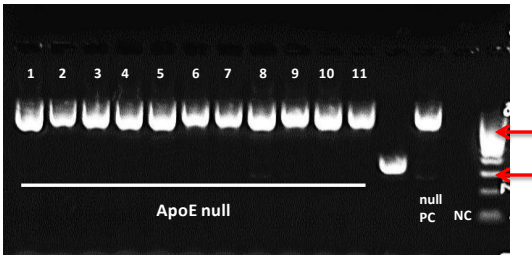
Figure 3.1 Generation of B1rB2r$^{-/-}$ mice lacking both the B1 receptor and B2 receptor. (**A**) the target locus in ES cells from C57BL/6J mice (R1, EcoR1 site). (**B**) the targeting vector, including the neomycin phosphotransferase gene (Neo) and thymidine kinase (TK) genes. (**C**) the resulting locus after homologous recombination. The location of the primers used to identify recombinants are shown (F1 and R1) to indicate the presence of B1 receptor gene (Wildtype) with primers (WT F1 and R1) and (F2 and R2) to indicate the absence of B1 receptor gene (B1rB2r$^{-/-}$) with primers (B1KO F2 and R2). Adapted from (Kakoki, McGarrah et al. 2007).

For genotyping, DNA extracted from ear clippings were amplified on an Applied Biosystems Veriti thermal cycler using primers 5'-TTC TAA CCA AAG CCA GCA GG-3'and 5'-ATG CTG ATG AAC AGG TTG GC-3' to identify the wild type (WT F1 and R1) allele and primers 5' ACG CGT CAC CTT AAT ATG CG-3'and 5'-GTC TTG TGA CAC TCT CTT GG-3'to detect the B1rB2r-null (B1KO F2 and R2) allele (both primer pairs amplifying at 94°C 30 sec, 59°C 30 sec and 72°C 30 sec). Primers 5'- TGT CCT CAG CGT GTT CTT CC-3' and 5'- GGT CCT GAA CAC CAA CAT GG-3' that identified the wild type (WT F3 and R3) allele and primers 5'- CTT GGG TGG AGA GGC TAT TC-3' and 5'- AGG TGA GAT GAC AGG AGA TC-3'that detected the B2r-null (B2KO F4 and R4) allele (both pairs amplifying at 94°C 30 sec, 64°C 30 sec and 72°C 30 sec) were used to type the B2r-deficient mice. ApoE null allele was identified using the primer pair 5'-CGA AGC CAG CTT GAG TTA CAG AA-3'and 5'-AGA GCC GGA GGT GAC AGA TCA G-3' (ApoE F5 and R5) (amplifying at 96°C 60 sec, 60°C 60 sec and 72°C 3 min), the primer details (Table 3.2) were sourced from Masao Kakoki. 5µl of the PCR products were loaded on a 1% agarose gel pre-stained with Gel-Red nucleic acid gel stain (Biotium Inc, Hayward, CA, USA) and electrophoresis performed for 45min at 100V. PCR products were visualised by ultraviolet transillumination (Figure 3.2).

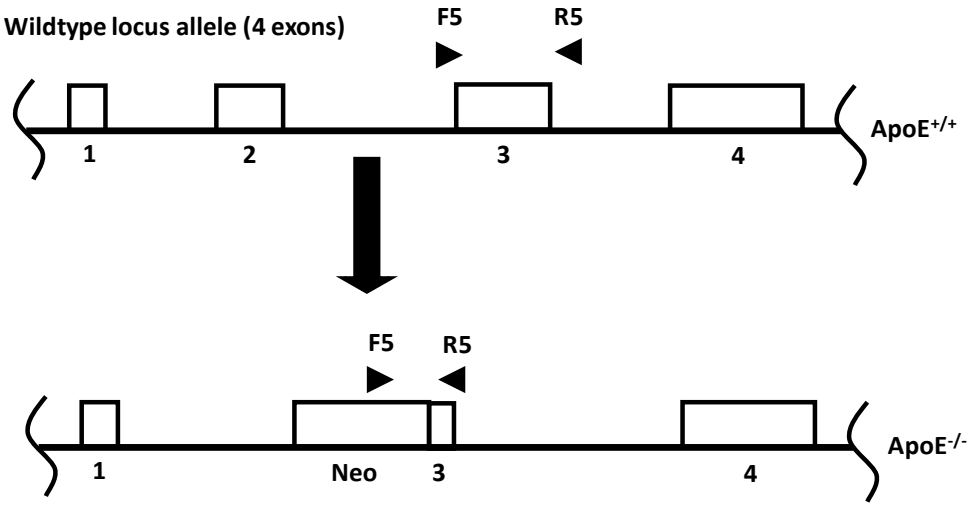
Table 3.2 Primer details

Primer name	Sequence	Description
WT F1	5'-TTC TAA CCA AAG CCA GCA GG-3'	Forward B1rB2r$^{+/+}$
WT R1	5'-ATG CTG ATG AAC AGG TTG GC-3'	Reverse B1rB2r$^{+/+}$
B1KO F2	5' ACG CGT CAC CTT AAT ATG CG-3'	Forward B1rB2r$^{-/-}$
B1KO R2	5'-GTC TTG TGA CAC TCT CTT GG-3'	Reverse B1rB2r$^{-/-}$
WT F3	5'- TGT CCT CAG CGT GTT CTT CC-3'	Forward B2r$^{+/+}$
WT R3	5'- GGT CCT GAA CAC CAA CAT GG-3'	Reverse B2r$^{+/+}$
B2 KO F4	5'- CTT GGG TGG AGA GGC TAT TC-3'	Forward B2r$^{-/-}$
B2 KO R4	5'- AGG TGA GAT GAC AGG AGA TC-3'	Reverse B2r$^{-/-}$
ApoE F5	5'-CGA AGC CAG CTT GAG TTA CAG AA-3'	Forward ApoE$^{-/-}$
ApoE R5	5'-AGA GCC GGA GGT GAC AGA TCA G-3'	Reverse ApoE$^{-/-}$

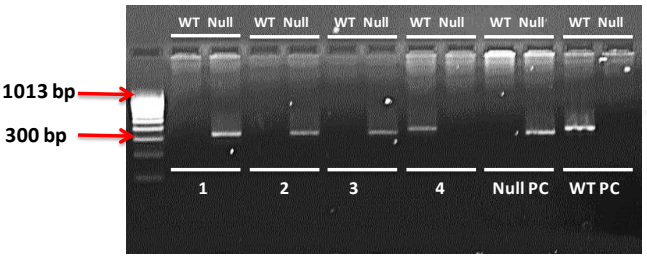
A



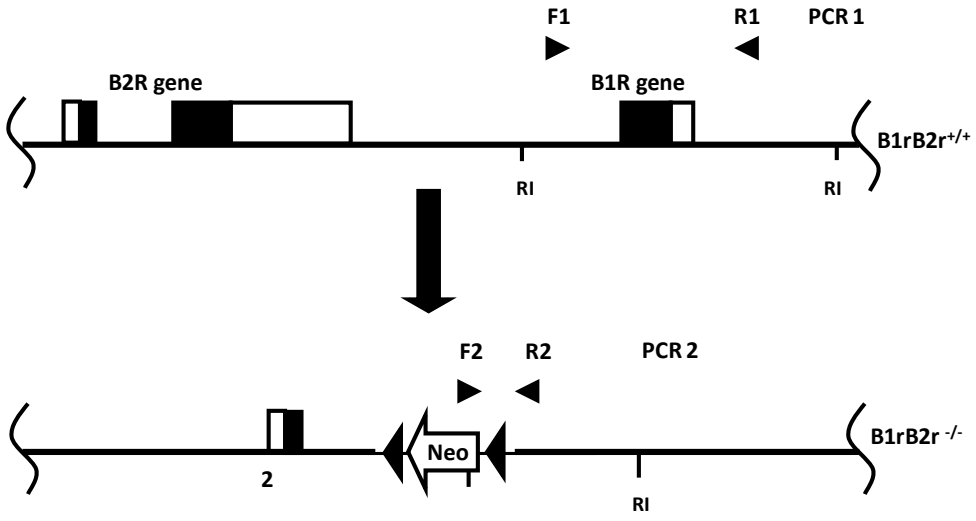
	ApoE(+/+)	ApoE(+/-)	ApoE(-/-)
WT allele	+ (300bp)	+ (300bp)	-



B



	B1RB2R(+/+)	B1RB2R(+/-)	B1RB2R(-/-)
WT allele	+ (363bp)	+ (363bp)	-
B1RB2R null allele	-	+ (337bp)	+ (337bp)



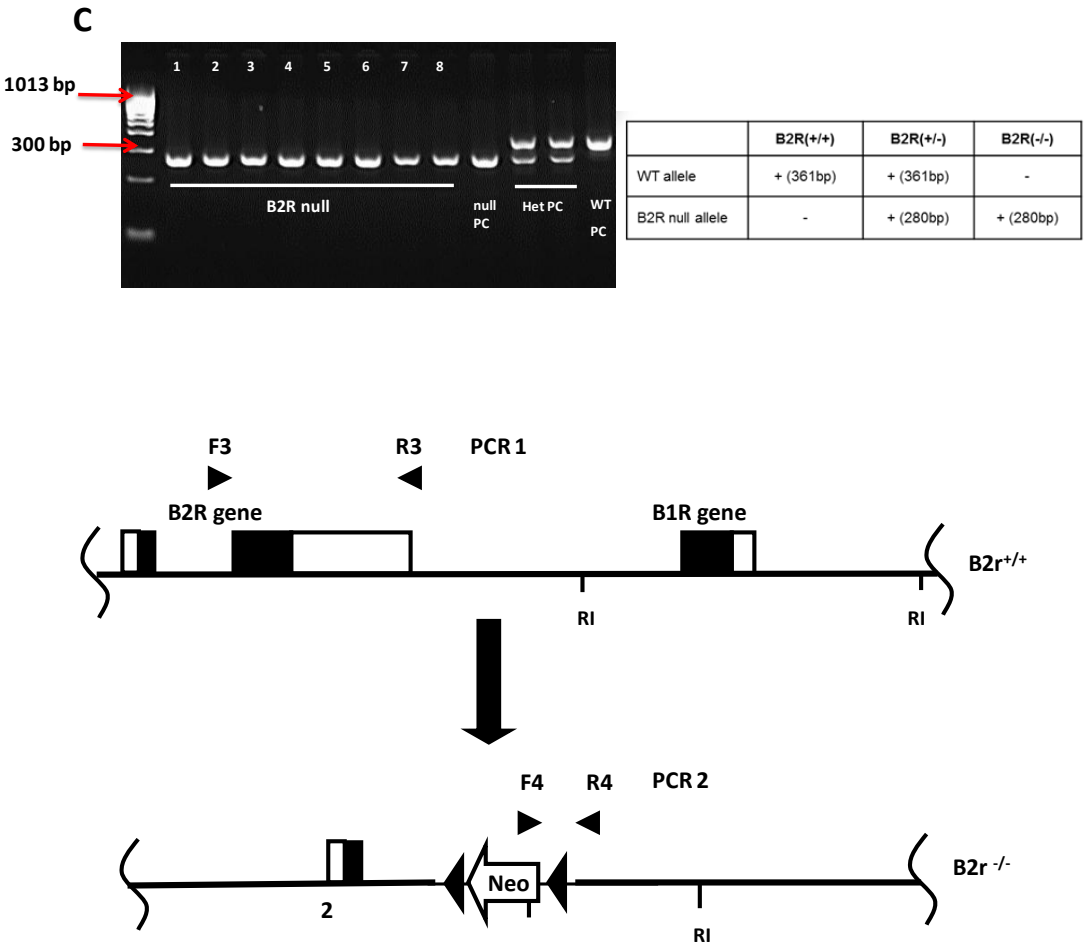


Figure 3.2. Homozygous null mutations in both the kinin B1 and B2 receptors (B1rB2r$^{-/-}$) or the B2 receptor alone (B2r$^{-/-}$) generated in Apolipoprotein E-deficient C57Bl/6 (ApoE$^{-/-}$) mice. Representative agarose gel electrophoretic profiles of PCR-amplified products and primer positions (line diagrams show wild type versus null) of **(A)** ApoE PCR, to identify ApoE$^{-/-}$ from wild type ApoE$^{+/+}$ (C57Bl/6), **(B)** B1rB2r PCR, to identify B1rB2r$^{-/-}$ from wild type B1rB2r$^{+/+}$ (ApoE$^{-/-}$) and **(C)** B2r PCR, to identify B2r$^{-/-}$ from wild type B2r$^{+/+}$ (ApoE$^{-/-}$).
WT, wild-type (DNA from mice of known genotype); PC, positive control (DNA from mice of known genotype); NC, negative control (No template controls).

3.1.3 *Angiotensin II ApoE mouse model*

Six-month old male mice on an ApoE$^{-/-}$ background were maintained on a normal diet and surgically implanted with osmotic minipumps (Model 1004, Alzet, Durect Corporation) that delivered angiotensin II (Sigma- Aldrich, Castle Hill, Australia) dissolved in sterile water at a dosage rate of 1.0μg/kg/min over 28 days. Briefly, the osmotic pumps were filled under sterile conditions, and weighed before and after filling to ensure the correct volume was added to the pump. The pumps were placed subcutaneously in the dorsal midline (interscapular space) via a small lateral incision, under ketamine (150mg/kg) and xylazine (10mg/kg) anaesthesia administered by intra-peritoneal injection. The incision was sutured closed and a topical antiseptic (Betadine, Purdue Products LP, USA) was applied to the wound. The mice were monitored twice daily for the duration of the experiment (day 28) or death (Figure 3.3). At experiment end (day 28) or death (before day 28) the mice were dissected and the aortas measured. Mice were classified as reaching; end of project (day 28), found dead (rupture) or found dead (non-rupture). All data was included in analyses.

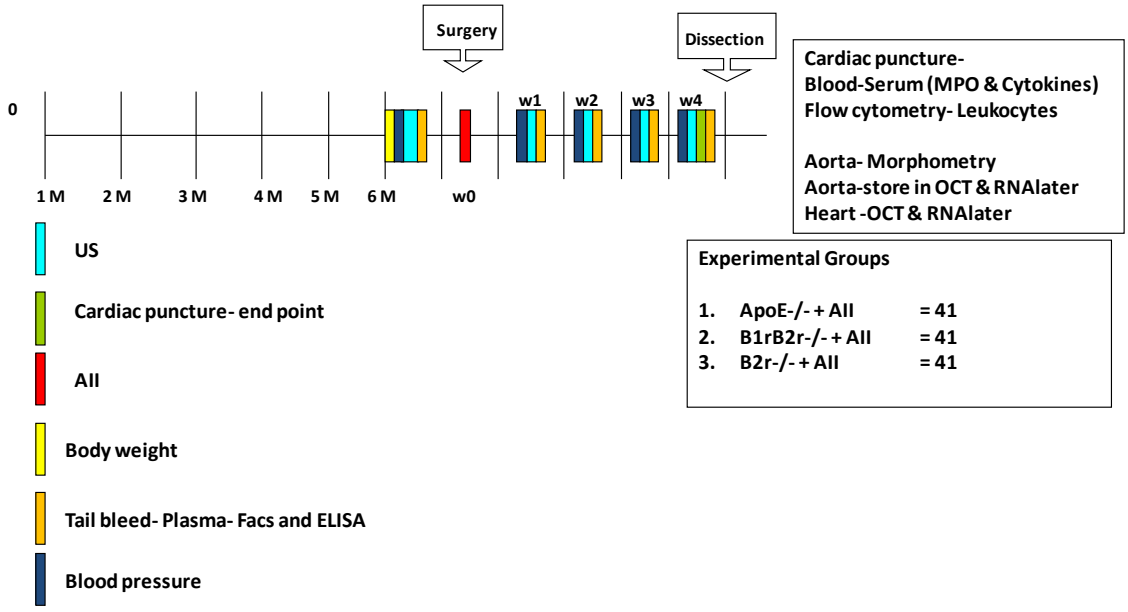


Figure 3.3 Study design for the AII – infused mouse model of AAA. Prior to pump surgery the baseline experiments were carried out. After pump insertion weekly experiments were carried out until study end, to assess AAA development.
US; Ultrasound, AII; angiotensin II, M; month, W; week.

3.1.4 *Measurement of supra-renal aortic diameter in vivo using ultrasound.*

The aortic diameter was monitored in all mice by ultrasound assessment prior to AII infusion (baseline), and at seven day intervals post insertion, on days 7, 14, 21 and 27 of the experimental period. The ultrasound measurements were performed on sedated mice (i.p., 40 mg/kg ketamine, 4 mg/kg xylazine) held in a dorsal recumbency using a MyLab^{TM} 30 VET XV GOLD platform (Esaote S.p.A, Italy) with a 40 mm linear transducer at an operating frequency of 10 MHz (LA435; Esaote, Italy) to provide a sagittal image of the SRA (Figure 3.4) as previously reported (Moran, Jose et al. 2013); (Golledge, Cullen et al. 2010). Maximum outer SRA diameter was measured at peak systole using the caliper measurement feature. All measurements were collected by two observers blinded to the treatment groups. It was established these measurements could be repeated with good intra-observer reproducibility (n=12 mice (reading 1 versus reading 2); coefficient of repeatability 0.902, 95% confidence intervals 0.671-0.973, $P < 0.0001$) (Figure 3.5) and inter-observer reproducibility (n=34 mice (observer 1 versus observer 2); coefficient of repeatability 0.947, 95% confidence intervals 0.894-0.974, $P < 0.0001$) (Figure 3.6).

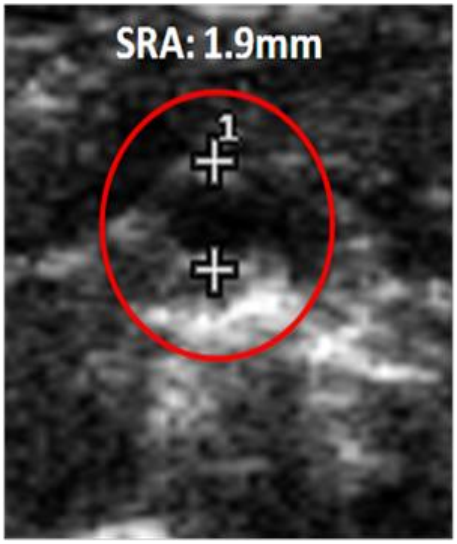


Figure 3.4. Representation of ultrasound image showing the mouse suprarenal aorta. Mice infused with AII that has a developing aneurysm. Electronic callipers (white crosses) show the maximal diameter of the outer aorta

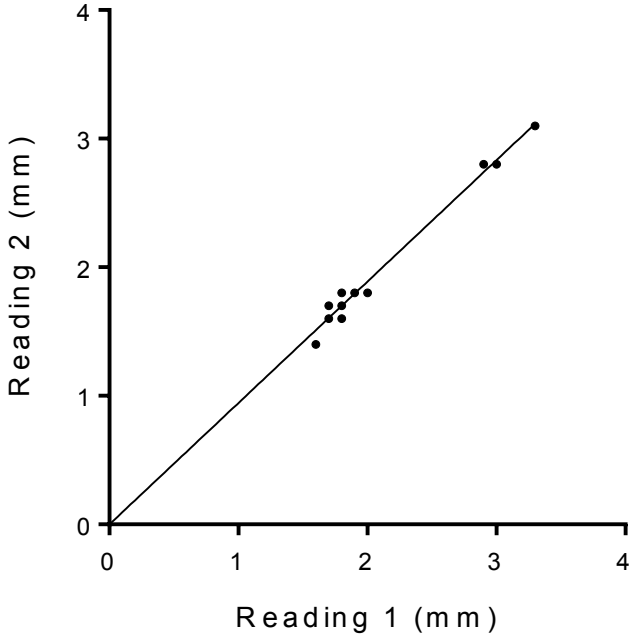


Figure 3.5. Assessment of reproducibility of SRA ultrasound readings as evaluated by the coefficient of variance between two SRA ultrasound readings by the same observer (intra-observer).

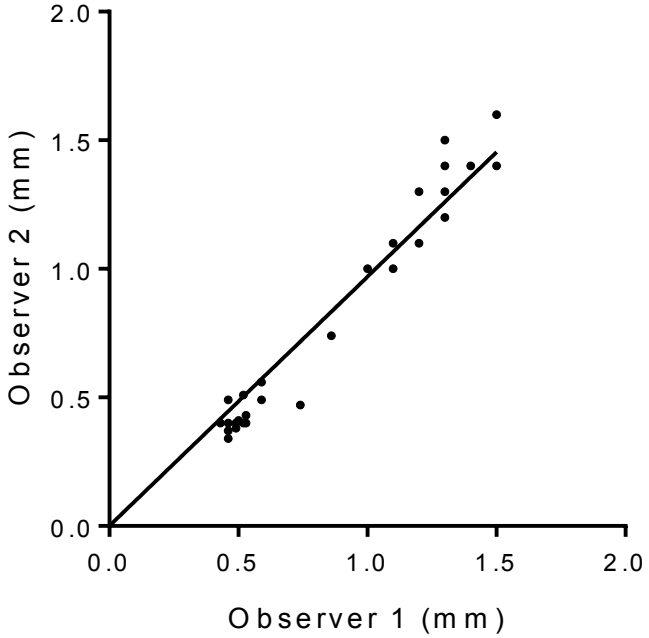


Figure 3.6. Assessment of reproducibility of SRA ultrasound readings as evaluated by the coefficient of variance between two SRA ultrasound readings by different observers (inter-observer).

3.1.5 *Blood collection*

Peripheral blood was collected from the lateral tail vein for flow cytometrical analysis of leukocytes (Chapter 5 and 6) and MPO ELISA (Chapter 6) into heparin coated tubes (BD Bioscience, San Jose, CA, USA). Briefly, the mouse was restrained in a tube restrainer, on a heating pad. A local anaesthetic cream (Emla^{TM}, AstraZeneca, Australia) was placed on the tail, and left for 15 minutes. The lateral tail vein in the distal one-third of the tail was visualised, then using a scalpel blade a swift incise across the tail vein was carried out. Drops of blood were collected into the heparin tubes, about 150μl in total and stored on ice. Pressure was applied to the wound using sterile gauze for 30 seconds to ensure haemostasis. Bloods were centrifuged for 10 min at approximately 2000 x g to separate plasma, the plasma was collected and aliquoted into 1.5 ml microfuge tubes and stored at -80^{o}C until required. Repeated thawing and freezing of samples was avoided.

Cardiac punctures were carried out on mice at termination of experiment (day 28), prior to PBS perfusion. Briefly, mice were euthanised by CO_2 asphyxiation and placed in a dorsal recumbent position. A needle (20- to 22-G) was placed in the left ventricle of the heart and advanced slowly until blood flows into the syringe. The blood was transferred into a heparin coated tube, and placed on ice. Plasma was prepared as above, in the tail bleed protocol, and stored at -80^{o}C until required. The blood obtained by cardiac puncture was used for neutrophil isolation experiments (Chapter 6).

3.1.6 *Aorta morphometry- ex vivo*

At study completion (day 28) after AII infusion mice were euthanised by CO_2 asphyxiation, the aorta was perfused with phosphate buffered saline (pH7.4) via a cannula placed in the left ventricle and atrium. The aorta was dissected form the aortic root (left ventricle) to the aortoiliac bifurcation. The adventitial fat was removed and the renal arteries preserved to

identify the suprarenal aortic region. The aortas were placed on a gradated template and digitally photographed (Coolpix 4500, Nikon). The aorta was segmented into four sections, arch (originating as the ascending aorta from the left ventricle and ending below the left subclavian artery), thoracic (below the left subclavian artery to the diaphragm), suprarenal (below the diaphragm to above the renal arteries) and infrarenal (below the renal arteries to the bifurcation into the right and left common iliac arteries) (Figure 3.7). The aortas, as well as the heart were stored in OCT (TissueTEK™, ProScitech, Townsville, Australia), or RNAlater™ (Ambion, Australia) and snap frozen in liquid nitrogen, and stored at -80°C for later analysis.

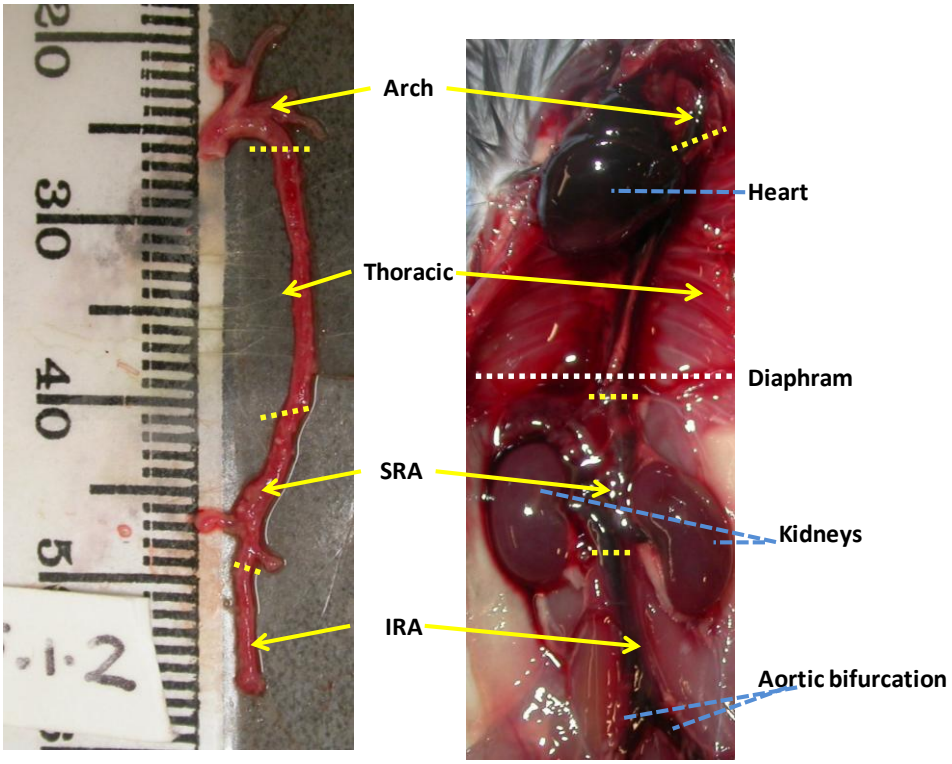


Figure 3.7 Representation of a dissected mouse aorta demonstrating the different regions measured in morphometric analysis and the graduated template (mm) for photography and measurement.

Maximum diameters of the arch, thoracic, suprarenal and infrarenal aorta were determined from digital images using computer-aided analysis (Adobe®Photoshop®CS5 Extended version 12, Adobe Systems Incorporated). It has been previously established that these measurements could be repeated with good intra-observer reproducibility (Moran, Jose et al. 2013); (Golledge, Cullen et al. 2010); (Moran, Cullen et al. 2009); (Rush, Nyara et al. 2009); (Krishna, Seto et al. 2012); (Golledge, Cullen et al. 2010). In this study it was also established these measurements could be repeated with good intra-observer reproducibility for example SRA (n=12 mice; coefficient of repeatability 0.944, 95% confidence intervals 0.801-0.985, $P < 0.0001$) (Figure 3.8) and inter-observer reproducibility, SRA (n=13 mice; coefficient of repeatability 0.967, 95% confidence intervals 0.887-0.990, $P < 0.0001$) (Figure 3.9). Both regional (arch, thoracic, suprarenal, infrarenal) and total aortic mean maximum diameter were determined for each mouse. The *mean maximum diameter* was used to describe the overall diameter of the aorta and was calculated from the sum of the maximum diameter of the individual aortic regions (arch, TA, SRA, IRA) divided by four.

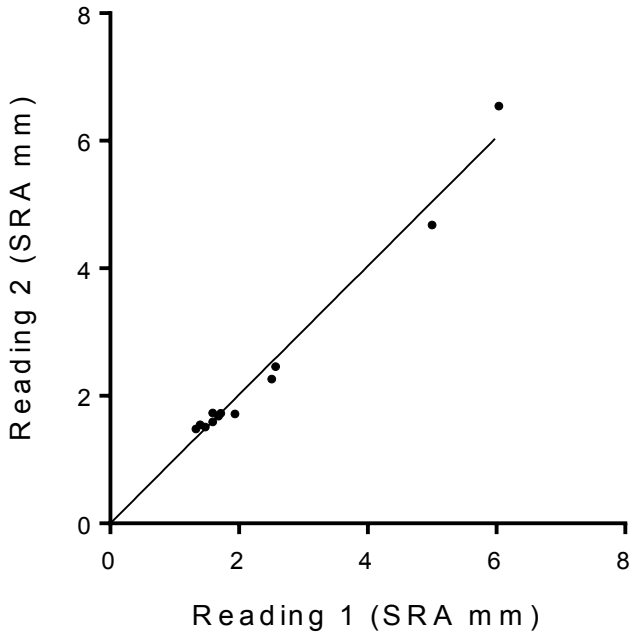


Figure 3.8. Assessment of reproducibility of SRA morphometry readings as evaluated by the coefficient of variance between two SRA morphometry readings by the same observer (intra-observer).

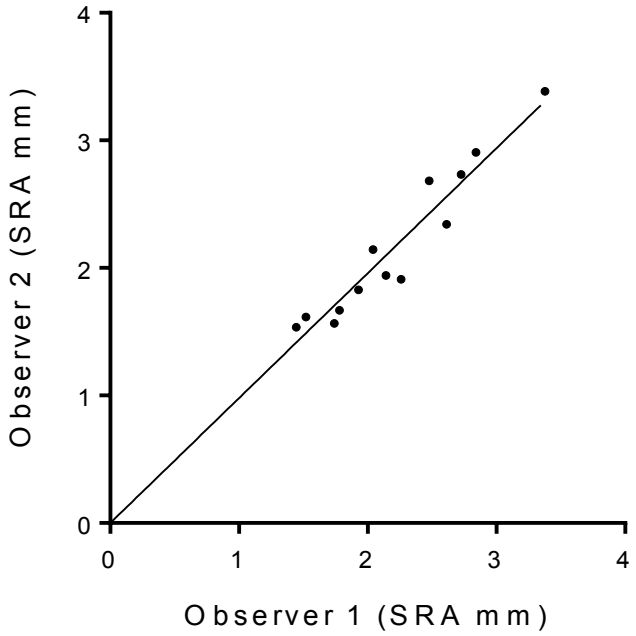


Figure 3.9. Assessment of reproducibility of SRA morphometry readings as evaluated by the coefficient of variance between two SRA morphometry readings by different observers (inter-observer).

3.1.7 *RNA extraction for B1 B2 receptor expression in mouse tissue and neutrophils*

Total RNA was extracted from mouse organs previously collected at dissection (heart and aorta; Chapter 5) and also from neutrophils isolated from whole blood (dextran/ficoll method) collected by cardiac puncture (Chapter 6). Total RNAs was isolated from mouse aorta, heart, and neutrophils with the Qiazol reagent Qiagen, Hilden, Germany) according to the manufacturer's protocol. The organs were cut into 5mm x 5mm pieces in a plate and placed in a tube. The samples were then treated with 500 μl of Qiazol reagent. The samples were then homogenised using a clean pestle and drill, and an additional 500μl of Qiazol reagent was added. The samples were left for 5 minutes at room temperature and 200 μl of chloroform (Sigma-Aldrich) was added, the samples were then shaken (~20 seconds), but not vortexed and left for 3 minutes, shaking at room temperature. The samples were centrifuged for 15 minutes at 10,000 g at 4 ^{o}C, making sure the samples were treated with care not to disturb the phases, care

was taken to remove the top aqueous phase. The samples were then treated with 500µl of 2-propanol. The samples were precipitated at -20 ^{o}C for 45 minutes.

RNA was purified using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, briefly, an equal volume of 70% ethanol was added, mixed and transferred to spin columns, then centrifuged (10,000 g, 30 seconds), and washed with RW1 buffer twice. The column was washed again with ethanol (100%) and the DNAse digestion step was carried out for 30 minutes at 37^{0}C, the columns were washed again with RW1 and RPE buffer. The RNA was eluted into ultra clean RNA tubes, in 20 µl (twice) of RNAse free water. The RNA was quantified using the Nanodrop spectrophotometer (Thermoscientific, Australia) at 260nm and 280nm to assess purity. RNA with an OD of 1.8-2.0 at 260/280 was used for further experiments.

3.1.8 *Reverse transcription PCR for B1rB2r expression*

Quantitative real-time qPCR was performed on a Corbett Life Science Rotor Gene 6000 (Qiagen Pty Ltd, Doncaster, Australia). Reactions were prepared on ice in 0.1ml RNase-DNase free PCR tubes in duplicate. The final reaction volume was 10µl, containing 15ng of RNA, 5µl SYBR green master mix (Qiagen) (containing, Taq DNA polymerase, dNTP mix and UNG treatment) 0.1µl of RT mix (Qiagen) and 0.2µl of appropriate primer made up to the final volume with RNase free water. The housekeeping gene control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was prepared and run simultaneously.

A three step melt thermal cycling program was used: 50^{o}C for 30 minutes for cDNA synthesis, 95^{o}C for 15 minutes (enzyme activation), 50 cycles of 94^{o}C, 15 seconds (denaturation), 55^{o}C for 30 seconds (annealing), 72^{0}C for 30 seconds (extension). Quantitative real time PCR was performed for B1r, B2r and the housekeeping gene GAPDH (Figure 3.10) using the one step

QuantiTect SYBR Green qPCR assay (Qiagen, Australia). The B1r primers and B2r primers were RT^2 qPCR primers commercially available from Qiagen (Australia), the GAPDH primers (F- 5'CATTTCCTGGTATGAGAAT3' and R- 5'GGTTTCTTACTCCTTGGA3') were also commercially obtained from Qiagen, Australia. The relative expression of the genes of interest was calculated by using the concentration Ct- standard curve method and normalised by the average expression of GAPDH (Formula below). The qPCR experiments had a minimum of five mice per group, the results were exported to Microsoft excel. The data was calculated using the Corbett Rotor gene run- analysis software and expressed as concentration.

Relative concentration = Gene of interest (Calculated concentration)/ Housekeeping gene (Calculated concentration)

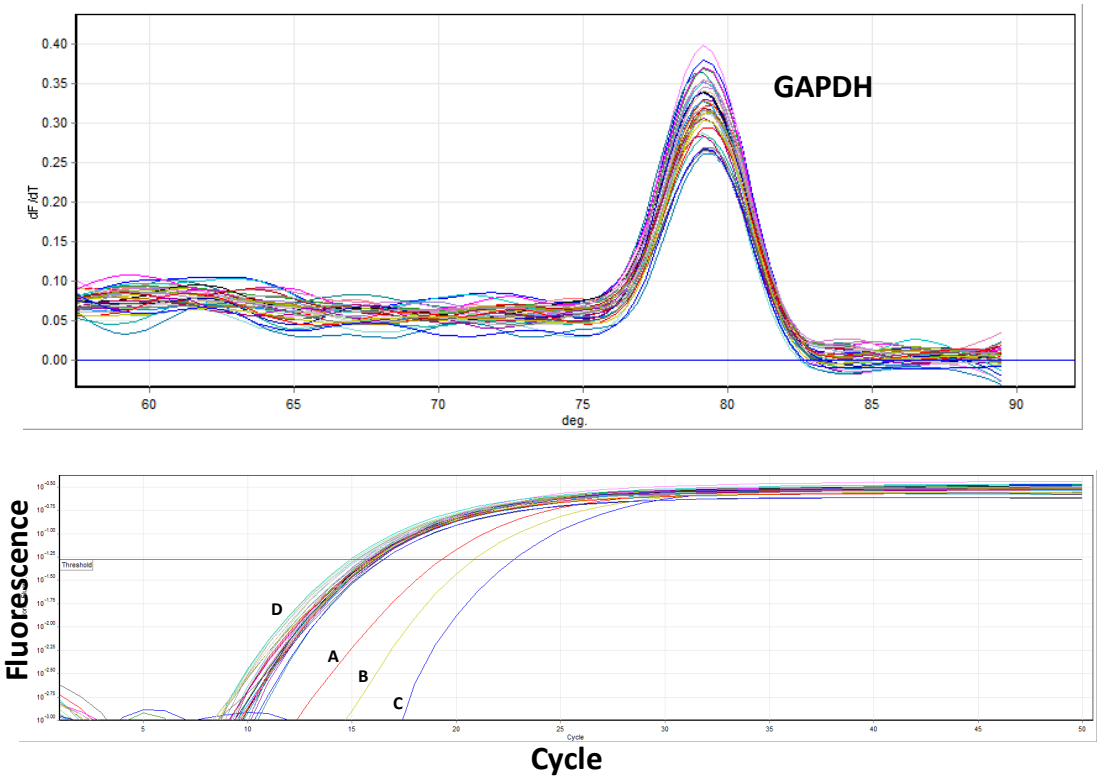


Figure 3.10. Quantitative RT-PCR for B1B2 receptor expression in heart tissue. (**A**) Melt curve showing GAPDH primers on a three step qPCR. (**B**) Fluorescence versus cycle number indicating amplified peaks for GAPDH, showing standards 1/20 (A), 1/50 (B) 1/160 (C) and samples (D).

3.2 IN VITRO STUDIES

3.2.1 *Preparation of murine neutrophils.*

Blood was collected from euthanised C57Bl/6 mice via cardiac puncture (20- to 22-G needle) and transferred to heparin-coated tubes. The blood was mixed with an equal volume of dextran saline solution (3% dextran T-500 in 0.9% NaCl) (Sigma-Aldrich), and incubated at room temperature for 20 minutes. The leukocyte-rich plasma (upper layer) was collected and centrifuged for 10 minutes at 1000 g at 5^{o}C to pellet cells. The supernatant was discarded and the pellet resuspended in a volume of 0.9% NaCl (equal to the starting volume of blood). The cell suspension was layered over 10ml of Ficoll-hypaque 1077 (Sigma-Aldrich) care was taken to maintain the sharp interface between the Ficoll and the cell suspension (Figure 3.11). The sample was centrifuged for 40 minutes at 1400 g, at 20^{o}C, with no brake. The top layer (saline) as well as the Ficoll-Hypaque layer was aspirated, leaving the neutrophil/ red blood cell pellet. The residual red blood cells were subjected to hypotonic lysis using red cell lysis buffer (Biolegend, San Diego, CA, USA) for 5 minutes at room temperature. Cells were centrifuged and washed twice with Hanks Buffered Saline Solution (Invitrogen, Camarillo, CA, USA) then resuspended in HBSS and immediately used for experiments.

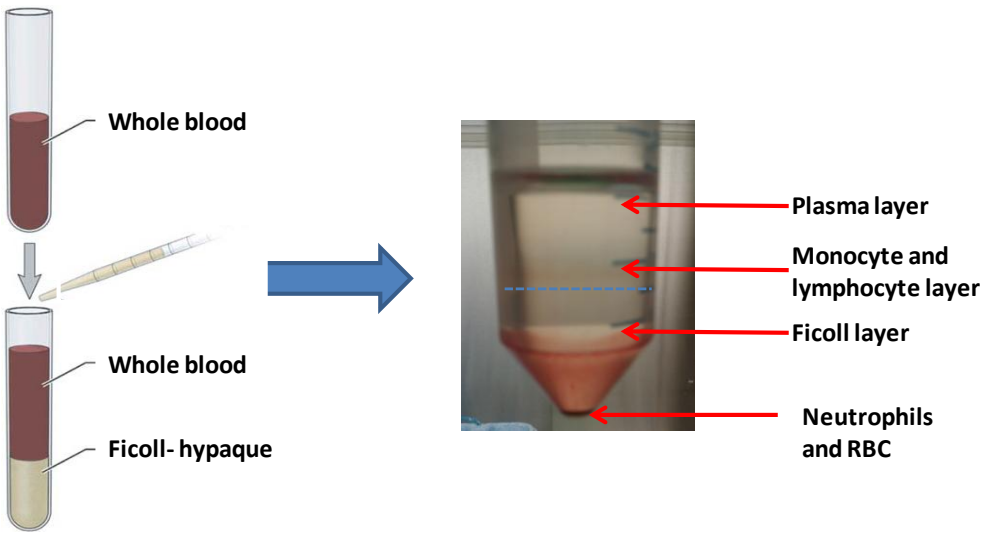


Figure 3.11 Isolation of neutrophils from mouse blood using Ficoll histopaque gradient method.

3.2.2 *Trypan blue test for neutrophil viability*

Trypan blue exclusion was used to assess the viability of isolated neutrophils. This is based on the principle that live cells possess intact membranes that exclude certain dyes, such as trypan blue, where as dead cells do not. The cell suspension is simply mixed with the trypan blue dye and then visually examined using a microscope to determine whether cells take up or exclude the dye (Strober 2001). A viable cell will have a clear cytoplasm and a nonviable cell will have a blue cytoplasm.

The neutrophil cell suspension (10μl) was mixed with an equal volume of 0.4% trypan blue and placed in a haemocytometer chamber (Hausser Scientific, Horsham, PA, USA), then placed under a microscope and counted to assess the dye uptake.

The total cell count was calculated:

Cell count per ml: = average count per haemocytometer square x dilution factor x 10^4 (count 10 squares)

3.2.3 *Kinin peptides.*

Bradykinin analogs B9430 (a non-selective B1/B2 receptor antagonist; DArg-Arg-Pro-Hyp-Gly-Igl-Ser-DIgl-Oic-Arg, Hyp: *trans*-4-hydroxyproline, Igl: α-(2-indanyl)glycine, Oic: octahydroindole-2-carboxylic acid**)**, B9330 (specific B2 receptor antagonist**;** DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DTic-Nig-Arg, Thi: β-(2-thienyl)alanine, Tic: tetrahydroisoquinoline-3-carboxylic acid, Nig: N-(2-indanyl)glycine**)**, and B9972 (specific B2 receptor agonist; DArg-Arg-Pro-Hyp-Gly-Igl-Ser-Oic-Igl-Arg**)**, were obtained from Dr Lajos Gera (University of Colorado, North Carolina, USA). These were synthesised as previously described (Stewart

1984.); (Bawolak, Gera et al. 2007); (Bironaite, Gera et al. 2004). Peptides were purified by high-pressure liquid chromatography (HPLC) and characterised by thin-layer chromatography and laser-desorption mass spectroscopy (Bironaite, Gera et al. 2004). In all *in vitro* studies the peptide concentration used was determined by dose response experiment. In all animal studies, relevant peptides were administered via intraperitonal (i.p.) injection (2mg/kg/dose) one day prior to the commencement of AII infusion and then every other day over the administration period. Control animals received 100 µl sterile saline i.p. at the same frequency.

3.2.4 *Flow cytometry to identify and enumerate neutrophils and monocytes*

For flow cytometry peripheral blood (50µl) was collected from tails of experimental animals into heparin tubes and stored on ice until use. The blood was then placed in FACs tubes and the red blood cells were lysed using 1x RBC lysis buffer (Biolegend, San Diego, CA, USA) for 5 minutes at room temperature, to eliminate red blood cells. Cells were washed with FACs buffer (2%FCS/PBS/0.05% sodium azide) and centrifuged at 450g for 5 minutes. To prevent any non-specific binding of the primary antibody to the cell surface, the FcR blocking reagent (1/50 of BD Pharmigen CD16/32) was added to the cells, diluted in FACs buffer, and incubated for 10 minutes at room temperature. The cells were centrifuged at 450g for 5 minutes. The primary antibodies, CD45-PerCP (1:200), CD11b-FITC (1:200), and Ly6G-PE (1:400) (BD Biosciences) were diluted in FACs buffer and added to the cells and incubated for 30 minutes at 4^{o}C, in the dark. As well as the stained cells and the control isotypes (mouse IgG APC, BD Bioscience), there were unstained cells and single stained cells, which were stained with one antibody only for example, only CD45-APC. These unstained and single stained cells were used to compensate during FACs. The cells were washed and centrifuged, 450g for 5 minutes and the pellet resuspended in 300µl FACs buffer. In order to quantify the cell population of interest, 10µl of AccuCheck counting beads (containing 10,000 beads) (Invitrogen, Camarillo, CA,

USA) were added to the samples. The cells were immediately acquired on a FACS Calibur flow cytometer (BD Biosciences, USA) and analysed with Cell Quest analysis software (BD Biosciences, USA).

3.2.4.1 *Flow cytometry gating strategy*

Cells were gated based on size (forward scatter, FSC) and granularity (side scatter, SSC). The dead cells in the extreme left of the plot were excluded. Doublets were excluded using FSC-H/ FSC-A plots and finally unstained cells were stained with propidium iodine (PI) (eBiosciences, Australia) to check the viability of the cells, the PI is taken up by the dead cells, which will be fluorescently labelled. The CD45 (APC) positive cells were gated next, and these were further gated into CD11b (FITC) and Ly6G (PE) positive cells, indicating activated neutrophils (Figure 3.12).

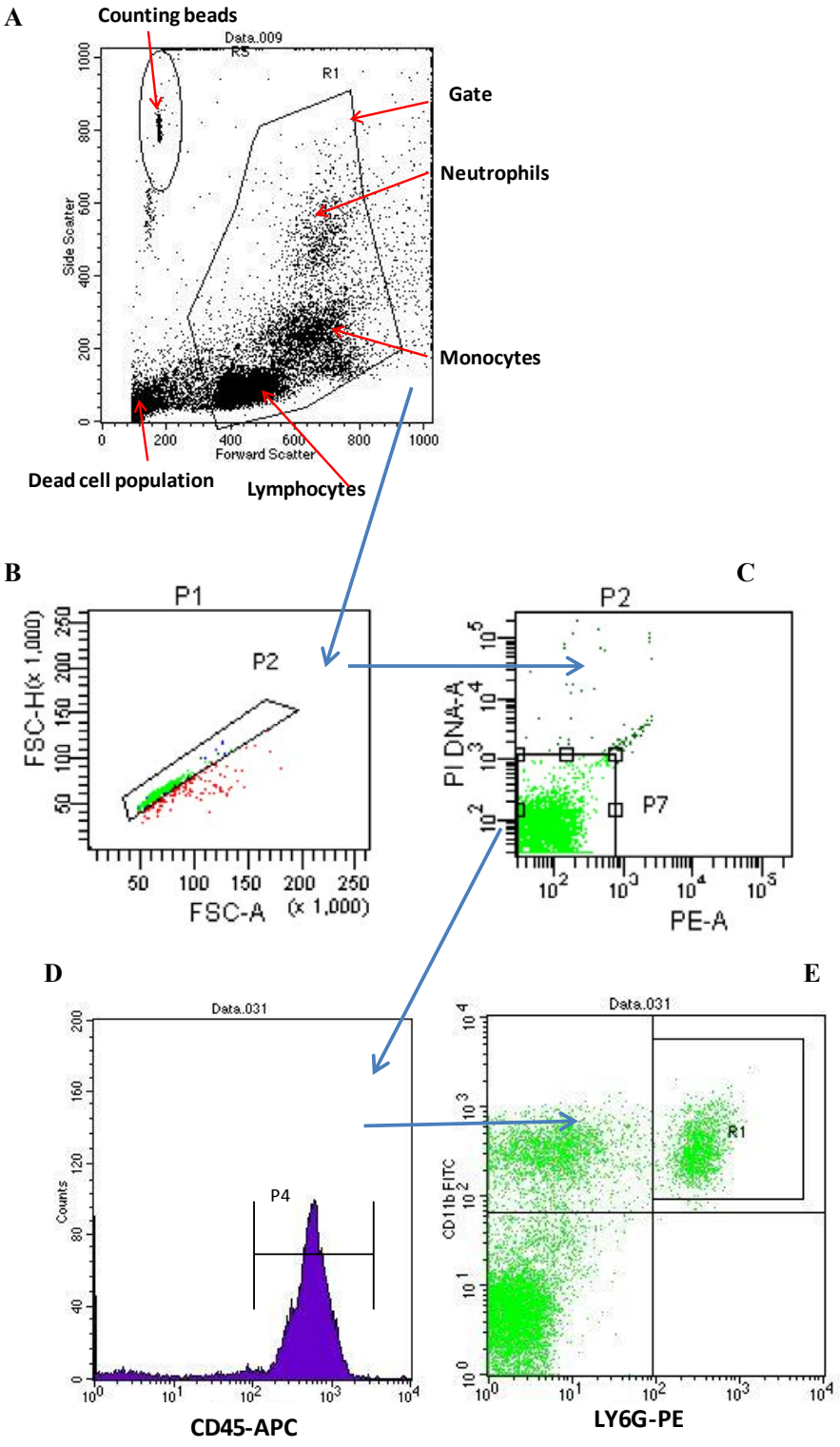


Figure 3.12 Flow cytometry gating stratagy (**A**) showing size and granularity of different cells, the populations include lymphocytes, monocytes and neutrophils to determine the gate, and the exclusion of the dead cells on the left. The counting beads can be seen, top left. **(B)** the P2 gate for singlets by removal of the doublets. **(C)** propidium iodine (PI) staining to gate viable cells (gate P3 indicates viable cells), nonviable cells take up PI and stain positive. (**D**) CD45 positive selected cells gated in P4 and (**E**) the P4 gated cells are further gated for CD11b and Ly6G positive selection (R1).

3.2.4.2 *Compensation controls*

An unstained sample (without any fluorescent dye) was analysed prior to testing the other samples. These readings are applied for standardising the instrument settings for the fluorescent compensation in the experiments. The single colour stains were also run to perform the compensation. The settings on the FACs Calibur were saved from the compensation for the running of the samples (Figure 3.13). Once the unstained cells and the single colour stains had been run and the compensation carried out the samples were run. A total number of 1000 events (of the counting beads) were acquired, which were gated on figure 3.12.

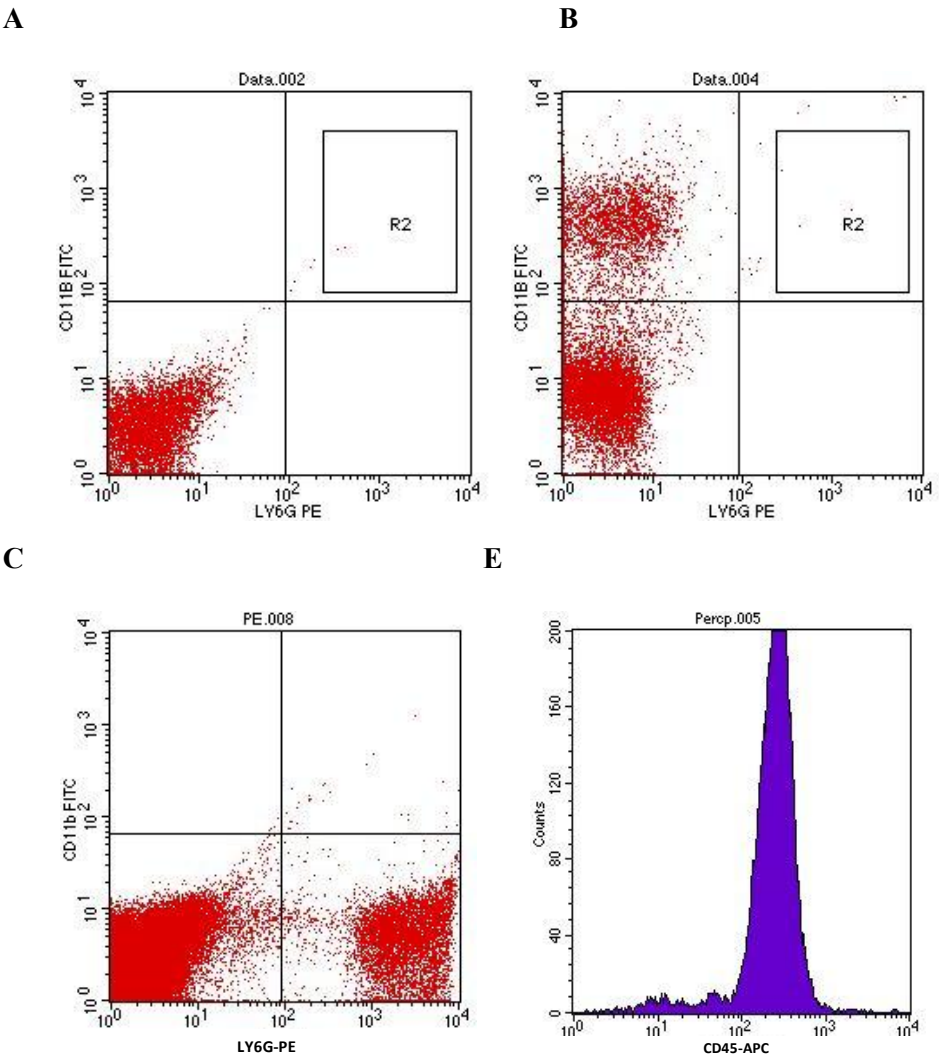


Figure 3.13 FACS plots showing (A) dot plot of unstained cells, (B) dot plot of FITC single stain colour, (C) dot plot of PE single colour stain, (D) histogram of single colour stain.

3.3 STATISTICS

Data were analysed using GraphPad Prism (version 6) and S Plus (version 8). The *in vitro* experiments were performed at least three times, and within these experiments the appropriate number of replicates carried out. Parametric or non-parametric tests were applied appropriate to distribution of data following analysis for normal distribution by the D'Agostino-Pearson omnibus normality test. Accordingly, end-point data were compared using unpaired t test or Mann-Whitney *U* test and expressed as mean ± SEM or median or box plots (min max).
End-point data, such as the mean maximum diameter (MMD) data and effect of kinin receptor deficiency on aortic arch atheroma severity was analysed using One-way ANOVA, and Tukey's post-hoc test or Kruskal-Wallis test (non-parametric ANOVA) and Dunn's post-hoc test was used to compare the differences between the groups. The data was expressed as mean ± SD (dot plots) or median and interquartile range (box plots), respectively.

Data presented over time (several time points) and between several treatment groups was analysed using Repeated measure Two-way ANOVA to determine the overall effect of treatment and with Tukey's Post-hoc test, to analyse effect of treatments between groups. SRA ultrasound data over time was presented as line graphs, with mean ± SEM and data analysed using Repeated measure ANOVA.

Contingency data were compared using Fisher's exact tests. Kaplan-Meier survival curves were analysed using log-rank (Mantel-Cox) test. In all cases P values less than 0.05 were considered significant.

Mann Whitney U test was performed to analyse the data from the qPCR. In all cases P values less than 0.05 were considered significant.

CHAPTER 4

THE EFFECT OF KININ RECEPTOR DEFICIENCY ON CARDIAC HYPERTROPHY IN THE APOE$^{-/-}$ MOUSE

Chapter 4

THE EFFECT OF KININ RECEPTOR DEFICIENCY ON CARDIAC HYPERTROPHY IN THE APOE$^{-/-}$ MOUSE

4.1 INTRODUCTION

The kallikrein-kinin system not only plays a major role in inflammation (Duchene and Ahluwalia 2009), but it is up regulated during vasodilatation, smooth muscle contraction (Tschope, Heringer-Walther et al. 2000), and plays a central role in the modulation of cardiovascular function (Cayla, Todiras et al. 2007). The use of kinin receptor agonists and antagonists and mouse models deficient in kinin receptors have made the study of these mechanisms more assessable (Cayla, Todiras et al. 2007), (McGiff and Quilley 1980), (Duka, Duka et al. 2006), (Madeddu, Parpaglia et al. 1995).

Expression of the Bradykinin receptor 2 (B2r) is constitutive in many tissues and has been identified in endothelial cells, smooth muscle cells, fibroblasts, mesangial cells and neutrophils (Couture, Harrisson et al. 2001). The B1 receptor is barely detectable under normal physiological conditions (Bourdet, Pecher et al. 2010) and it's expression is up-regulated in injured tissue and after exposure to inflammatory cytokines, including IL-1 and TNFα (Zhou, Polgar et al. 1998). Evidence suggests that the kinin B1 receptors can amplify or substitute for the kinin B2 receptor, especially in chronic inflammation (Ahluwalia and Perretti 1999; Phagoo, Poole et al. 1999; Bockmann and Paegelow 2000). Mice with a targeted deletion of the gene for the B1 receptor are healthy, fertile, normotensive, and show a blunted hypotensive response to bacterial LPS and a reduced accumulation of neutrophils in inflamed tissue (Bhoola, Elson et al. 1992). Kinin receptor deficient mice have been used to elucidate the function of these receptors in cardiovascular function (Cayla, Todiras et al. 2007), arthritis, renal disease and pain (Leeb-Lundberg, Marceau et al. 2005).

Kinins are formed by the enzymatic action of kallikreins, which are themselves potent molecules that mediate constriction of venules and dilation of arterioles (Bhoola, Elson et al. 1992). Kinins are believed to play a significant role in the regulation of blood pressure. This is because the kinin system is responsible for vasodilatation, reduction in total peripheral resistance, natriuresis, diuresis, increasing renal blood flow and releasing vasodilatation agents (Hecquet, Tan et al. 2000), (McGiff and Quilley 1980), (Sharma, Uma et al. 1996). It is suggested that both kinin receptors need to be blocked to lead to an increase in normal blood pressure in Wistar rats, indicating both receptors are involved (Duka, Duka et al. 2006). Increased blood pressure on the arterial wall has been proposed to be a proinflammatory stimulus, and there is thought to be an association between wall stress and the production of reactive oxygen species (Weiss, Kools et al. 2001). Kinins are not however the only factor influencing blood pressure.

The peptide angiotensin II (AII) also plays a role in regulating blood pressure, including impacting on vasoconstriction. The infusion of AII into hyperlipidemic apolipoptotein E-deficient ($ApoE^{-/-}$) mice elevates blood pressure, (Cassis, Gupte et al. 2009) which is increased within a day of infusion and can remain elevated for 16 days, although heart rate and pulse pressures are not obviously different in such mice (Weber, Rocic et al. 2005). The kallikrein-kinin system is linked to the rennin-angiotensin system, via several shared components. The B2 receptor interacts with angiotensin converting enzyme, linking both systems (Sharma 2006). How these important vascular pathways interact to alter cardiac parameters and furthermore how this impacts on AAA is unclear.

There are many limitations using human samples alone to investigate disease progression and mechanism, especially in diseases like AAA, as aneurysmal tissue is extremely difficult to obtain, and is restricted to the analysis of end stage disease from AAA samples (Daugherty and Cassis 2004). These limitations are overcome by the use of animal models, mainly rodents. The

use of these animal models to determine the mechanisms of disease progression are vital in AAA research.

In this Chapter ApoE$^{-/-}$ mice, and ApoE$^{-/-}$ mice deficient in both kinin receptors (B1rB2r$^{-/-}$ApoE$^{-/-}$) or the B2 receptor alone (B2r$^{-/-}$ApoE$^{-/-}$) were infused with AII. The influence of kinin receptors on cardiac related parameters, such as heart rate, blood pressure and hypertrophy were investigated. Normal blood pressure and heart rate were measured prior to AII infusion to provide a baseline value and then blood pressure and heart rate were monitored over a period of time to determine if there was any relationship between the expression of kinin receptors, AII and cardiac hypertrophy.

The expression of kinin receptors was also assessed to determine a relationship in these mice between AII, kinin receptor expression, and cardiac hypertrophy, heart rate, and blood pressure.

The following hypothesis was tested:

Kinin receptor deficiency promotes cardiac hypertrophy in an AII mouse model.

Specifically, the aim of this chapter was:

1. To assess the effect of kinin receptor deficiency on cardiac hypertrophy in an AII mouse model.

2. To assess the role of kinin receptors in the regulation of blood pressure and heart rate.

3. To assess the relationship between B1 and B2 kinin receptor expression in Apo E$^{-/-}$ mice hearts and whether up regulation of B1 receptor expression compensates for lack of B2 receptor expression in ApoE mice.

4.2 EXPERIMENTAL METHODS

4.2.1 *Animals and body weight*

Six month old male $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ or $B2r^{-/-}ApoE^{-/-}$ mice (n= 41 per group) were weighed before commencement of the study. The mice were infused with AII over 28 days during which blood pressure and heart rate was monitored. Heart weight and cardiac myofibre width were examined at necropsy at 28 days (Chapter 3, section 3.1.3).The mice were kept in a temperature-controlled room on a 12-hour light/dark cycle, 60% humidity, and maintained on normal laboratory chow and water *ad libitum*.

4.2.2 *Heart weight*

At termination of study mice were euthanised by CO_2 asphyxiation. Hearts were perfused with PBS via a 20-ml syringe with a 20-gauge needle placed in the right ventricle prior to heart excision. Whole hearts were taken, blotted dry and weighed before being bisected through the coronal plane. The hearts were placed in tissue moulds (Tissue Tek, ProSci Tech) face down and embedded in optimal cutting temperature (OCT) compound (Tissue Tek, ProSci Tech), snap frozen in liquid nitrogen and stored at -80^{o}C.

4.2.3 *Analysis of cardiac myofibre width*

Five-micrometer serial frozen tissue sections were cut using a cryostat (Shandon), air-dried onto poly-l-lysine-coated microscope slides, fixed in acetone (-20°C, for 10 minutes), and stained using standard Mayer's haematoxylin and eosin (H&E) method. Sections were placed PBS for 5 minutes. Rehydration was facilitated by a 2 minute wash in running tap water prior to staining in Mayer's Haematoxylin (ProSci Tech) for 30 dips. The slides were washed extensively (5-10 minutes) in running water, then stained then stained in eosin (6 dips) before being transferred

directly (dehydrated) to 50%, 75%, 80% and 100% ethanol (1 minute each). Two changes of xylene (2 x 2 minutes) cleared the sections, which were then mounted with coverslips and DePeX cement (ProSci Tech) in preparation for examination by light microscopy. Histological observation of cardiac myofibre width was performed with light microscopy and digitally photographed (Figure 4.1). The cardiac myofibre width was calculated from the images using computer-aided analysis (Adobe®Photoshop®CS5 Extended version 12, Adobe Systems Incorporated) using the ruler tool and the length calculated using a scale bar (0.01 mm). Five fibres were selected at random, and averaged, per heart section. It was established these measurements could be repeated with good inter-observer reproducibility (n=10 mice (observer 1 versus observer 2); coefficient of repeatability 0.900, 95% confidence intervals 0.624-0.977, P=0.0004)

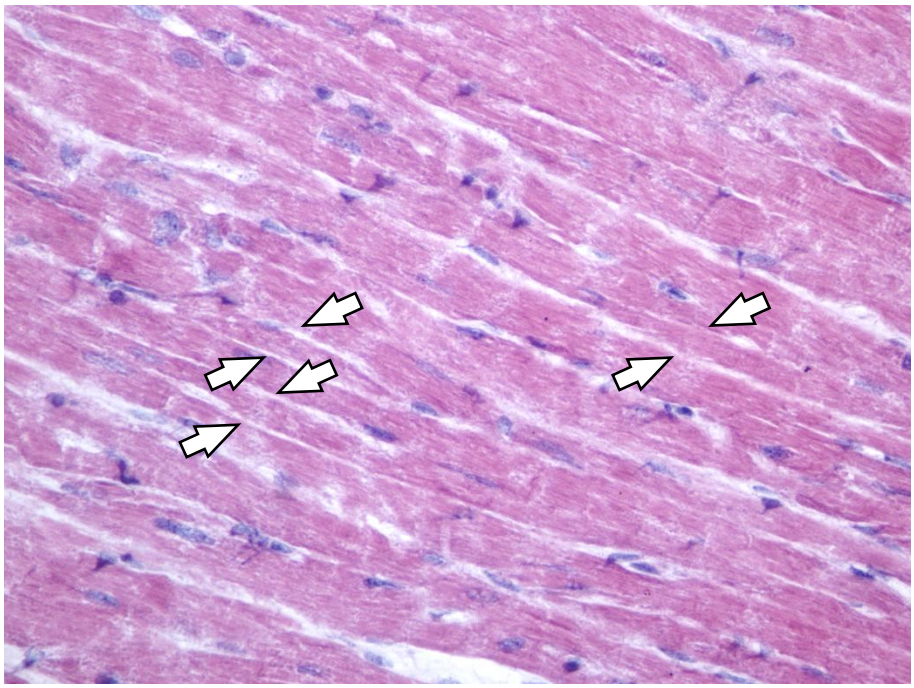


Figure 4.1 H& E stained heart section showing cardiac myofibre width (between arrows).

4.2.4 *Measurement of blood pressure and heart rate*

Systolic blood pressure (SBP) and heart rate (HR) were measured by volume pressure recording of the tail using the CODA non-invasive blood pressure system (Kent Scientific, Torrington, CT), at weekly intervals in conscious mice. Mice were acclimatized to the apparatus before use. To carry out the procedure, mice (in a restrainer/ holder) were placed onto the pre-warmed thermopad (38 °C). The tail was placed in the occlusion cuff and distension caused by arterial blood pulses was detected by the blood pressure machine. Pressure in the cuff was increased until the pulse was lost. Actual blood pressure was measured as the pressure at which a pulse was detected during cuff depressurization. Five readings were taken for each animal at each time point.

4.2.5 *Receptor expression of B1B2 receptors in hearts*

C57Bl/6, $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ or just $B2r^{-/-}ApoE^{-/-}$ mice (n= 5) per group without AII infusion were used for receptor expression experiments. Hearts were removed and stored in RNA later prior to RNA extraction. Total RNA was extracted from mouse hearts using RNeasy mini kit (Qiagen, Australia) according to manufacturer's instructions (Chapter 3, section 3.1.7), from C57Bl/6, $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice. SYBR Green PCR primers Bdkrb1 and Bdkrb2 (RT^2 qPCR primers) were commercially available from Qiagen and the GAPDH primers were also commercially obtained as described in Chapter 3, section 3.1.8). Quantitative real time PCR (qPCR) was performed for Bdkrb1 and Bdkrb 2 genes as described in Chapter 3, section 3.1.8) using a three step melt thermal cycling program.

4.2.6 *Statistics*

Data was analysed using GraphPad Prism (version 6) and S Plus (version 8). Parametric or non-parametric tests were applied as appropriate to distribution of data.

The body, heart weight and cardiac myofibre width (end-point data) was analysed using One-way ANOVA (parametric), and Tukey's post-hoc test or Kruskal-Wallis test (non-parametric ANOVA) and Dunn's post-hoc test to compare the differences between the groups. The data was expressed as mean ± SD or median and interquartile range, respectively.

Data presented over time (several time points) and between several treatment groups was analysed using Repeated measure Two-way ANOVA to determine the overall effect of treatment and with Tukey's Post-hoc test, to analyse effect of treatments between groups. This was used for blood pressure data (Section 4.3.3.1) and heart rate (Section 4.3.3.2) over time, and presented as a line graph (mean±SEM).

Receptor expression of B1r and B2r on hearts was analysed using Mann Whitney U tests. Statistical significance was taken as *P<0.05.

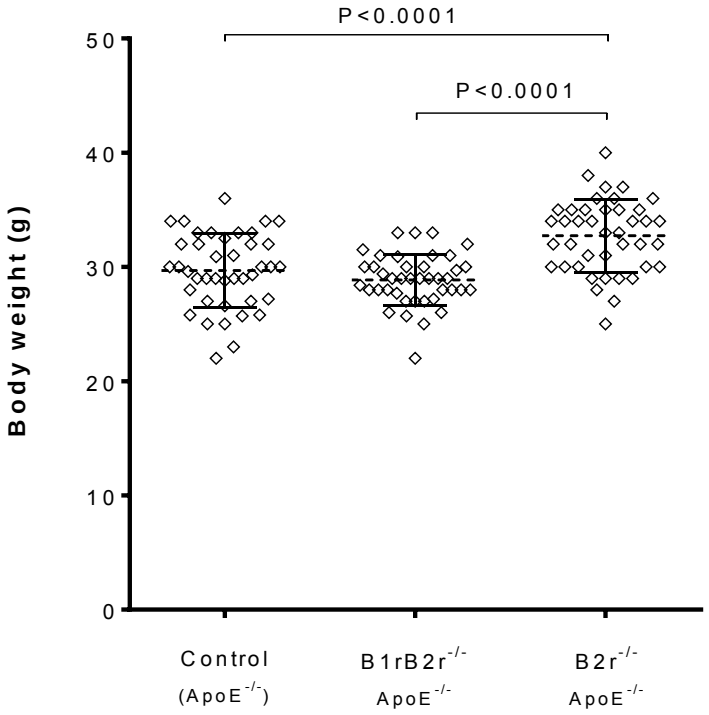
4.3 RESULTS

4.3.1 *Body and cardiac weight and kinin receptor deficiency in the ApoE$^{-/-}$mouse*

When body weights of ApoE$^{-/-}$, B1rB2r$^{-/-}$ ApoE$^{-/-}$ and B2r $^{-/-}$ ApoE$^{-/-}$ were compared at study commencement it was observed that there was a difference in the body mass of groups and when analysed with One-way ANOVA, a significant difference between the weight of the mice

at the P<0.05 level for the three groups was demonstrated (P<0.0001). Tukey's multiple comparisons test was carried out to compare each mouse group with all other mouse groups ($ApoE^{-/-}$ controls, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$) (Figure 4.2A and B).

A



B

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Actual P Value
$ApoE^{-/-}$ vs. $B1rB2r^{-/-}$	0.8220	-0.7146 to 2.359	0.4152
$ApoE^{-/-}$ vs. $B2r^{-/-}$	-3.044	-4.580 to -1.507	< 0.0001
$B1rB2r^{-/-}$ vs. $B2r^{-/-}$	-3.866	-5.402 to -2.329	< 0.0001

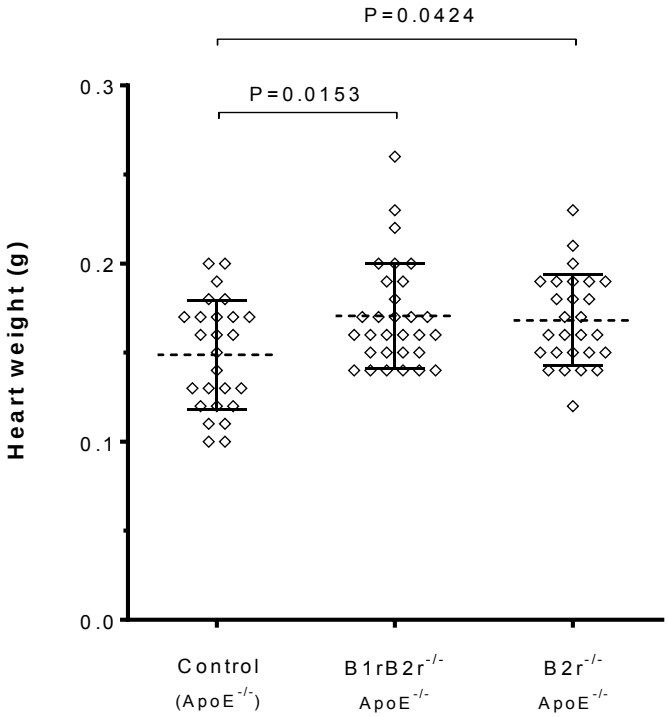
Figure 4.2 Comparison of body weight of kinin deficient and control mice. Baseline weight of mice prior to AII infusion. $ApoE^{-/-}$ (n=41), $B1rB2r^{-/-}ApoE^{-/-}$ (n=41), and $B2r^{-/-}ApoE^{-/-}$ (n=41) (**A**) Data presented as dot plots (mean ± SD), body weight (g). (**B**) Data was analysed by One way ANOVA with Tukeys multiple comparisons test (Table 4.1).

The $B2r^{-/-}ApoE^{-/-}$ mice were significantly larger in weight compared to the $ApoE^{-/-}$ control mice (32.73g±3.19 vs 29.69g±3.24; P<0.0001) and the $B1rB2r^{-/-}ApoE^{-/-}$ (32.73g±3.19 vs 28.87g±2.24; P<0.0001) (Figure 4.2A and B).

During the examination of the dissected aortas described in Chapter 3, section 3.13, at study completion, it was observed that the hearts from the kinin receptor deficient mice ($B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$) appeared hypertrophic compared to the $ApoE^{-/-}$ control mice. Subsequently, it was shown that there was a difference in the weight of the hearts between mice groups overall, and when analysed with One-way ANOVA, a significant difference (P<0.05) between the weight of the hearts for the three groups was demonstrated (P=0.0118). Tukey's multiple comparisons test was carried out to compare each mouse group with all other mouse groups ($ApoE^{-/-}$ controls, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$) (Figure 4.3A and B).

Hearts from the double-knockout $B1rB2r^{-/-}ApoE^{-/-}$ mice were significantly increased in weight compared to the $ApoE^{-/-}$ controls (0.17g±0.03 vs 0.14g±0.03; P=0.0153). Furthermore, hearts from the $B2r^{-/-}ApoE^{-/-}$ mice were significantly increased in weight compared to those from the $ApoE^{-/-}$ control mice (0.16g±0.02 vs 0.14g±0.03; P=0.0424) (Figure 4.3.A&B). The shape of the hearts looked different in the kinin deficient mice compared to the control group. This observation is important, changes to the size and shape of the heart or the chambers can indicate abnormalities, such as cardiomyopathy or hypertrophy.

A



B

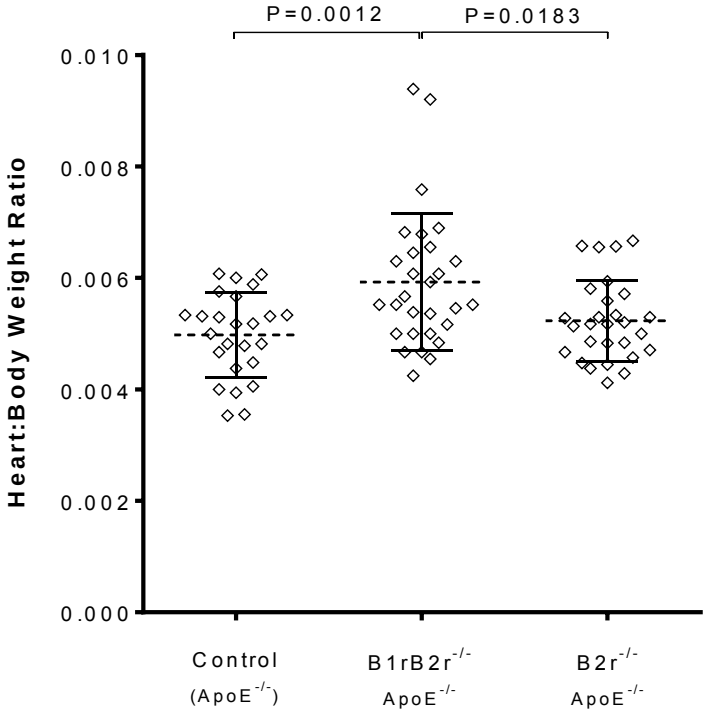
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Actual P Value
$ApoE^{-/-}$ vs. $B1rB2r^{-/-}$	-0.02182	-0.04012 to -0.003517	0.0153
$ApoE^{-/-}$ vs. $B2r^{-/-}$	-0.01930	-0.03807 to -0.0005320	0.0424
$B1rB2r^{-/-}$ vs. $B2r^{-/-}$	0.00251	-0.01560 to 0.02064	0.9411

Figure 4.3 Comparison of heart weight of kinin receptor deficient and control mice. (**A)** $ApoE^{-/-}$ (n=41), $B1rB2r^{-/-}ApoE^{-/-}$ (n=41), and $B2r^{-/-}ApoE^{-/-}$ (n=41) Data presented as dot plots (mean ± SD), heart weight (g). (**B**) Data was analysed by One way ANOVA with Tukeys multiple comparisons test (Table 4.2)

To determine the relative weight of the heart the overall weight of the mouse should be taken into consideration. For example larger animals could have larger hearts, so to compare the true cardiac weight based on the animal size the heart to body ratio was determined. With a difference in the heart-to-body ratio observed in mice groups overall, a significant difference ($P<0.05$) between the heart-to-body ratio for the three groups was demonstrated upon One-way ANOVA ($P=0.0011$). Tukey's multiple comparisons test was carried out to compare each mouse group with all other mouse groups ($ApoE^{-/-}$ controls, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$) (Figure 4.4A and B).

Through this analysis the heart-to-body ratio of the $B1rB2r^{-/-}ApoE^{-/-}$ mice were found to be significantly greater compared to the $ApoE^{-/-}$ control mice (0.0059 ± 0.001 vs 0.00494 ± 0.0007; $P=0.0012$) and similarly, the $B2r^{-/-}ApoE^{-/-}$ mice heart-to-body ratio tended to be larger compared to the $ApoE^{-/-}$ control mice (0.0052 ± 0.0007 vs 0.00494 ± 0.0007; $P=0.5921$) although not to a statistically significant level. The heart-to-body ratio of the $B1rB2r^{-/-}ApoE^{-/-}$ mice were also significantly larger than those of the $B2r^{-/-}ApoE^{-/-}$ mice (0.0059 ± 0.001 vs 0.0052 ± 0.0007; $P=0.0183$) (Figure 4.4A and B).

A



B

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Actual P Value
$ApoE^{-/-}$ vs. $B1rB2r^{-/-}$	-0.0009513	-0.001567 to -0.0003351	0.0012
$ApoE^{-/-}$ vs. $B2r^{-/-}$	-0.0002547	-0.0008758 to 0.0003665	0.5921
$B1rB2r^{-/-}$ vs. $B2r^{-/-}$	0.0006966	9.848e-005 to 0.001295	0.0183

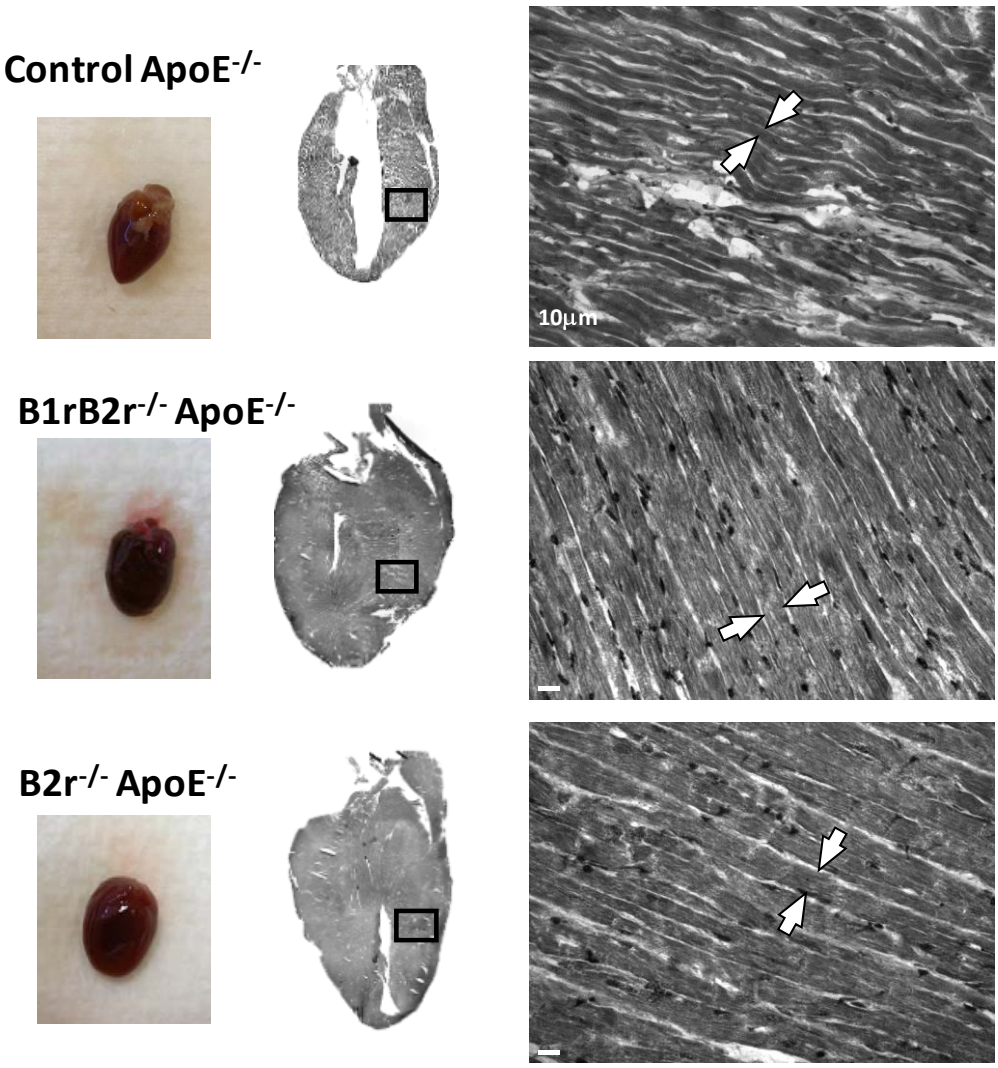
Figure 4.4 Comparison of heart: body ratio of kinin receptor deficient and control mice. $ApoE^{-/-}$ (n=25), $B1rB2r^{-/-}ApoE^{-/-}$ (n=29), and $B2r^{-/-}ApoE^{-/-}$ (n=28) (**A**) Data presented as dot plots (mean ± SD), heart: body ratio. (**B**) Data was analysed by One way ANOVA with Tukeys multiple comparisons test (Table 4.3).

4.3.2 *Cardiac hypertrophy*

Histological examination of hearts was performed to confirm the observation of cardiac hypertrophy in kinin receptor deficient mice. The hearts where bisected through the coronal plane, sectioned and stained with H & E to assess the cardiac myofibre width. A significant increase in cardiac myofibre width was observed in the B1rB2r$^{-/-}$ApoE$^{-/-}$ and more so in the B2r$^{-/-}$ApoE$^{-/-}$ compared to the ApoE$^{-/-}$ control group. It was observed that there was a difference in the cardiac myofibre width in mice groups overall, when analysed with One-way ANOVA, there was a significant difference ($P<0.05$) between the heart to body ratio for the three groups ($P<0.0001$). Tukey's multiple comparisons test was carried out to compare each mouse group with all other mouse groups (ApoE$^{-/-}$ controls, B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ApoE$^{-/-}$) (Figure 4.5A and B).

Through this analysis the cardiac myofibre widths of the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice were found to be significantly larger in size compared to the ApoE$^{-/-}$ control mice (0.01223±0.001 vs 0.00878 ±0.0008; $P<0.0001$) and the B2r$^{-/-}$ApoE$^{-/-}$ mice cardiac myofibre widths were significantly larger in size compared to the ApoE$^{-/-}$ control mice (0.01432±0.001 vs 0.00878±0.0008; $P<0.0001$). The cardiac myofibre widths of the B2r$^{-/-}$ApoE$^{-/-}$ mice were also significantly larger than those of the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice (0.01432±0.001 vs 0.01223±0.001; $P=0.007$) (Figure 4.5A and B).

A



B

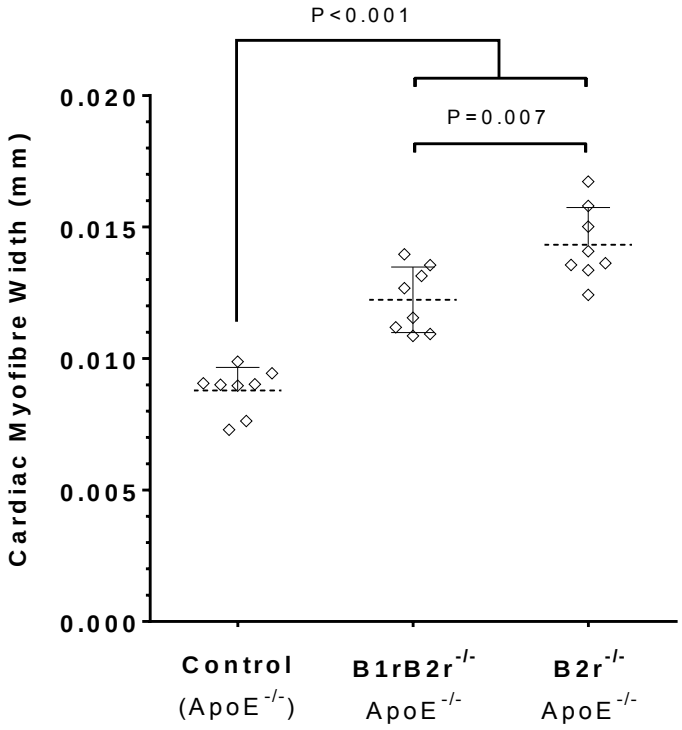


Figure 4.5 Comparison of cardiac myofibre width of kinin receptor deficient and control mice. $ApoE^{-/-}$ (n=8), $B1rB2r^{-/-}ApoE^{-/-}$ (n=8), and $B2r^{-/-}ApoE^{-/-}$ (n=8) (**A**) Representative images of the hearts, pictures indicate $ApoE^{-/-}$ $B1rB2r^{-/-}ApoE^{-/-}$, $B2r^{-/-}ApoE^{-/-}$ and H&E stain showing cardiac myofibre widths (white arrows) (**B**) Data presented as dot plots (mean ± SD), cardiac myofibre width (mm). Data was analysed by One way ANOVA with Tukeys multiple comparisons test.

4.3.3 *Cardiovascular parameters are influenced by kinin receptor deficiency in mice.*

4.3.3.1 *Infusion of AII leads to an increase in blood pressure*

The indirect (tail-cuff) method was used to assess systolic blood pressure (SBP) and heart rate. Baseline blood pressure was measured prior to commencement of AII infusion and every seven days thereafter. The effect of kinin receptor deficiency on blood pressure over time in AII infused $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice were analysed using Repeated Measure

Two way ANOVA. There was a significant effect of kinin receptor deficiency on blood pressure over time at the $P<0.05$ level for the three groups ($P<0.0001$) overall (Table 4.4).

Tukey's multiple comparisons test was used to compare each group with all other groups ($ApoE^{-/-}$ controls, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$). Mice deficient in both $B1rB2r^{-/-}ApoE^{-/-}$ kinin receptors had an increased SBP compared to control $ApoE^{-/-}$ mice at baseline (Week 0) (Figure 4.6). There was no significant difference in $B2r^{-/-}ApoE^{-/-}$ deficient mice compared to $ApoE^{-/-}$ mice at baseline. $B1rB2r^{-/-}ApoE^{-/-}$ mice also had a higher blood pressure compared to the $B2r^{-/-}ApoE^{-/-}$ mice. At seven days (week 1) post AII infusion the SBP was higher in all the mice groups, compared to baseline and stayed at a relatively constant level from weeks one to four. The SBP was significantly higher in the $B1rB2r^{-/-}ApoE^{-/-}$ mice compared to the $ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice (Figure 4.6) at all time points following AII infusion.

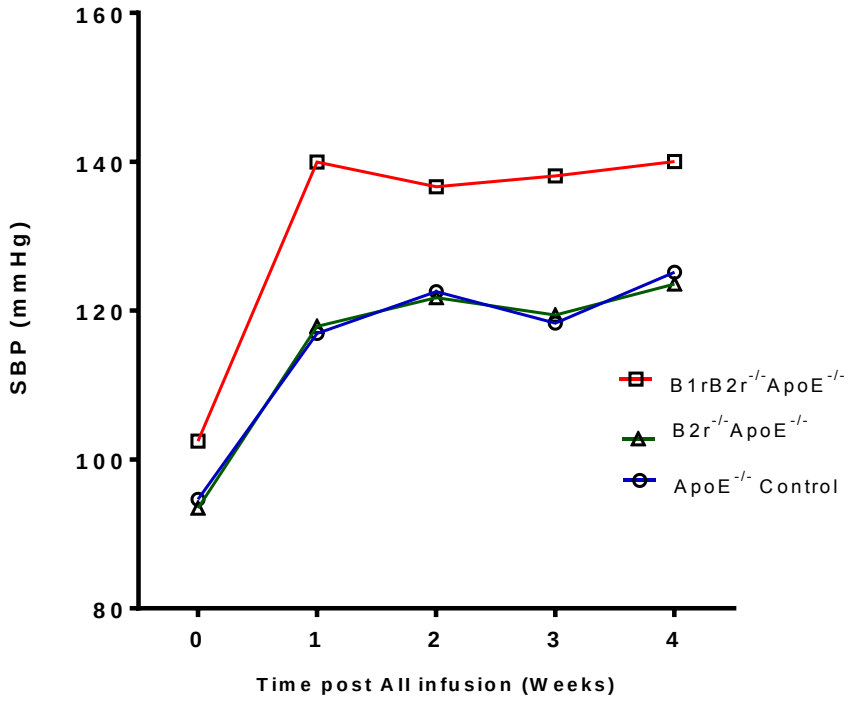


Figure 4.6 Comparison of change in blood pressure in AII-infused $ApoE^{-/-}$ (n=41), $B1rB2r^{-/-}$ $ApoE^{-/-}$ (n=41), and $B2r^{-/-}ApoE^{-/-}$ (n=41) mice over 28 days, as measured at one week intervals. Data is presented as Mean±SEM (graph) showing increasing blood pressure, determined prior to AII infusion and thereafter.

Table 4.4 Repeated Measure Two-Way ANOVA analysing the effect of kinin receptor deficiency on blood pressure, over time for ApoE$^{-/-}$ controls, B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ ApoE$^{-/-}$

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2972	8	371.6	F (8, 476) = 1.212	P = 0.2897
Time (Row)	82342	4	20586	F (4, 476) = 67.16	P < 0.0001
Group (Column)	27858	2	13929	F (2, 476) = 45.44	P < 0.0001
Residual	145910	476	306.5		

(SS, Sum of squares; DF, Degrees of Freedom; MS, Mean squares; F, F ration)

Table 4.5 Analysed by Repeated measures two-way ANOVA with Tukeys HSD Post Hoc tests

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Actual P value
Baseline (0)			
Control vs. B1rB2r$^{-/-}$	-7.813	-16.90 to 1.278	ns
Control vs. B2r$^{-/-}$	1.204	-7.887 to 10.30	ns
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	9.017	-0.07423 to 18.11	ns
Week 1			
Control vs. B1rB2r$^{-/-}$	-23.05	-32.89 to -13.21	P< 0.0001
Control vs. B2r$^{-/-}$	-0.9471	-10.93 to 9.036	ns
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	22.10	12.26 to 31.95	P< 0.0001
Week 2			
Control vs. B1rB2r$^{-/-}$	-14.09	-26.39 to -1.790	P=0.0200
Control vs. B2r$^{-/-}$	0.8121	-10.90 to 12.52	ns
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	14.90	3.629 to 26.17	P=0.0057
Week 3			
Control vs. B1rB2r$^{-/-}$	-19.78	-29.70 to -9.861	P< 0.0001
Control vs. B2r$^{-/-}$	-1.082	-11.56 to 9.395	ns
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	18.70	8.428 to 28.97	P< 0.0001
Week 4			
Control vs. B1rB2r$^{-/-}$	-14.87	-24.85 to -4.878	P=0.0015
Control vs. B2r$^{-/-}$	1.577	-9.604 to 12.76	ns
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	16.44	5.393 to 27.49	P=0.0015

Significance *P<0.05; ns, not significant

Because the baseline blood pressures varied between groups, blood pressure change over time was used to compare groups. As shown in Figure 4.7 AII infusion in B1rB2r$^{-/-}$ApoE$^{-/-}$ mice resulted in a mean increase in blood pressure of about 20% over the 28-day infusion period compared to ApoE$^{-/-}$ controls. The mean percentage relative increase in blood pressure was

markedly less in B2r$^{-/-}$ApoE$^{-/-}$ mice after 1 week AII infusion compared to the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice and this parameter was maintained at weeks 3 and 4.

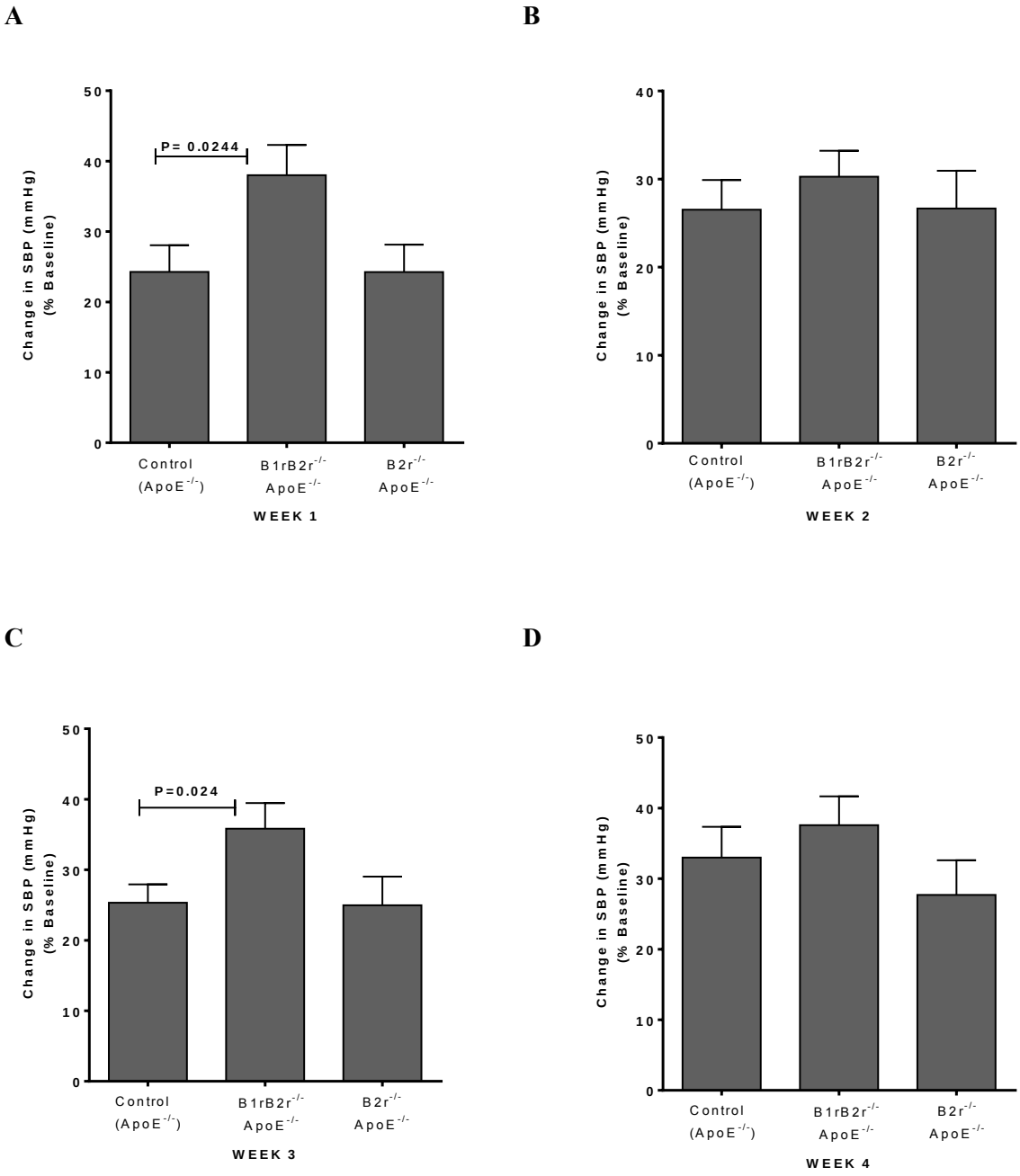


Figure 4.7 Change in SBP in AII-infused ApoE$^{-/-}$ (n=41), B1rB2r$^{-/-}$ApoE$^{-/-}$ (n=41), and B2r$^{-/-}$ ApoE$^{-/-}$ (n=41) mice over 28 days, as measured at one week intervals. (**A**) Percent change from baseline to week 1, (**B)** Week 2, (**C**) Week 3, (D) Week 4. Data is presented as Mean±SEM percent of blood pressure, determined prior to AII infusion**.** Analysed with Mann Whitney U test. **Significance compared to control *P<0.05.**

4.3.3.2 *Heart rate is not affected by AII infusion in ApoE$^{-/-}$, B1rB2$^{-/-}$ and B2$^{-/-}$ mice*

Heart rate was measured by the indirect tail cuff method. Baseline heart rate was measured prior to commencement of AII infusion and every week thereafter. The effect of kinin receptor deficiency on heart rate over time was analysed using repeated measure two way ANOVA. There was a significant ($P<0.05$) effect of kinin receptor deficiency on heart rate for the three groups ($P <0.0001$) overall (Table 4.6).

B2r$^{-/-}$ApoE$^{-/-}$ deficient mice had a significantly lower heart rate (BPM) compared to the ApoE$^{-/-}$ and the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice at baseline. In all groups the heart rate remained relatively stable overtime even after AII infusion, indicating little influence of AII on this parameter (Figure 4.8).

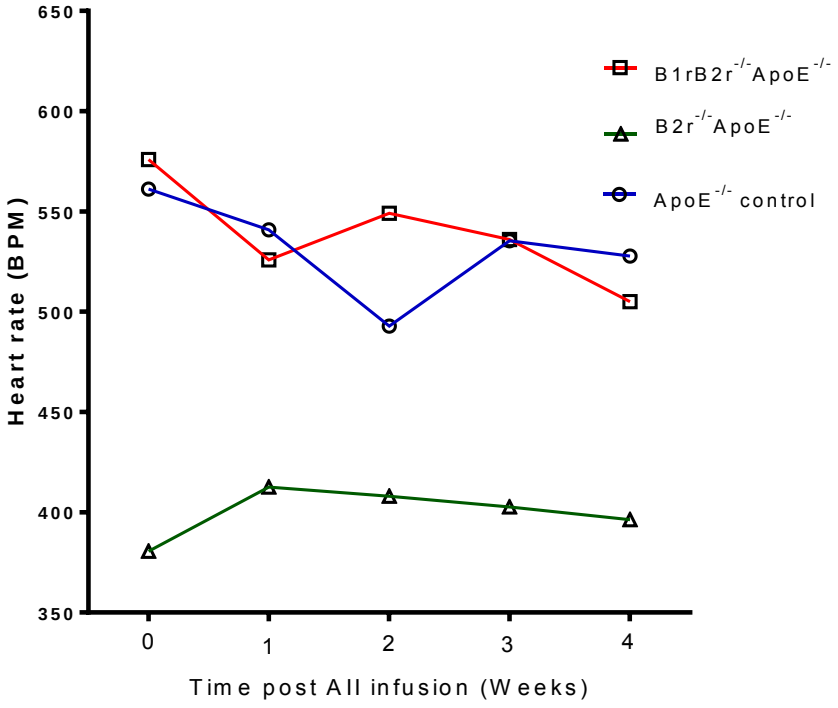


Figure 4.8 Comparison of change in heart rate (BPM) in AII-infused ApoE$^{-/-}$ (n=41), B1rB2r$^{-/-}$ ApoE$^{-/-}$ (n=41), and B2r$^{-/-}$ApoE$^{-/-}$ (n=41) mice over 28 days, as measured at 7-day (Week) intervals. Data is presented as Mean±SEM (graph) showing increasing heart rate, determined prior to AII infusion, and after.

Table 4.6 Repeated Measure Two-Way ANOVA analysing the effect of kinin receptor deficiency on heart rate, over time for ApoE$^{-/-}$ controls, B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ApoE$^{-/-}$

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	129061	8	16133	F (8, 429) = 2.387	P = 0.0159
Time (Row)	45677	4	11419	F (4, 429) = 1.690	P = 0.1513
Group (Column)	1.762e+006	2	880846	F (2, 429) = 130.3	P < 0.0001
Residual	2.899e+006	429	6758		

SS, Sum of squares; DF, Degrees of Freedom; MS, Mean squares; F, F ration

Table 4.7 Analysed by Repeated measures two-way ANOVA with Tukeys HSD Post Hoc tests

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	P Value
Baseline 0			
Control vs. B1rB2r$^{-/-}$	-14.72	-57.95 to 28.51	ns
Control vs. B2r$^{-/-}$	180.6	137.1 to 224.1	P< 0.0001
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	195.3	151.8 to 238.8	P< 0.0001
Week 1			
Control vs. B1rB2r$^{-/-}$	14.91	-33.87 to 63.68	ns
Control vs. B2r$^{-/-}$	128.3	79.86 to 176.7	P< 0.0001
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	113.4	66.14 to 160.6	P< 0.0001
Week 2			
Control vs. B1rB2r$^{-/-}$	-56.38	-119.5 to 6.701	ns
Control vs. B2r$^{-/-}$	84.82	26.13 to 143.5	P=0.0021
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	141.2	86.19 to 196.2	P< 0.0001
Week 3			
Control vs. B1rB2r$^{-/-}$	-0.6544	-52.20 to 50.89	ns
Control vs. B2r$^{-/-}$	132.7	79.33 to 186.0	P< 0.0001
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	133.3	84.47 to 182.2	P< 0.0001
Week 4			
Control vs. B1rB2r$^{-/-}$	22.71	-31.53 to 76.95	ns
Control vs. B2r$^{-/-}$	131.5	74.40 to 188.5	P< 0.0001
B1rB2r-/- vs. B2r$^{-/-}$	108.8	53.23 to 164.3	P< 0.0001

Significance *P<0.05; ns, not significant

4.3.4 *Receptor expression of B1 and B2 receptors in mouse hearts prior to AII infusion*

Relative gene expression levels of the B1 and B2 receptors in hearts were compared between the different mouse groups, C57Bl/6, $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice, by quantitative real time PCR, after normalising to GAPDH (Chapter 3, section 3.1.7). The relative gene expression of the B1 receptor was significantly greater in C57Bl/6 compared to $ApoE^{-/-}$ mice (P=0.0317) (Figure 4.10). There was no B1 receptor expression in the $B1rB2r^{-/-}ApoE^{-/-}$ as expected. Interestingly, the B1 receptor expression in the $B2r^{-/-}ApoE^{-/-}$ was significantly greater than in the $ApoE^{-/-}$ mice (P= 0.0159). The relative gene expression of the B2 receptor was greater in C57Bl/6 compared to $ApoE^{-/-}$ mice. There was no B2 receptor expression in the $B1rB2r^{-/-}ApoE^{-/-}$ or $B2r^{-/-}ApoE^{-/-}$ mice as expected.

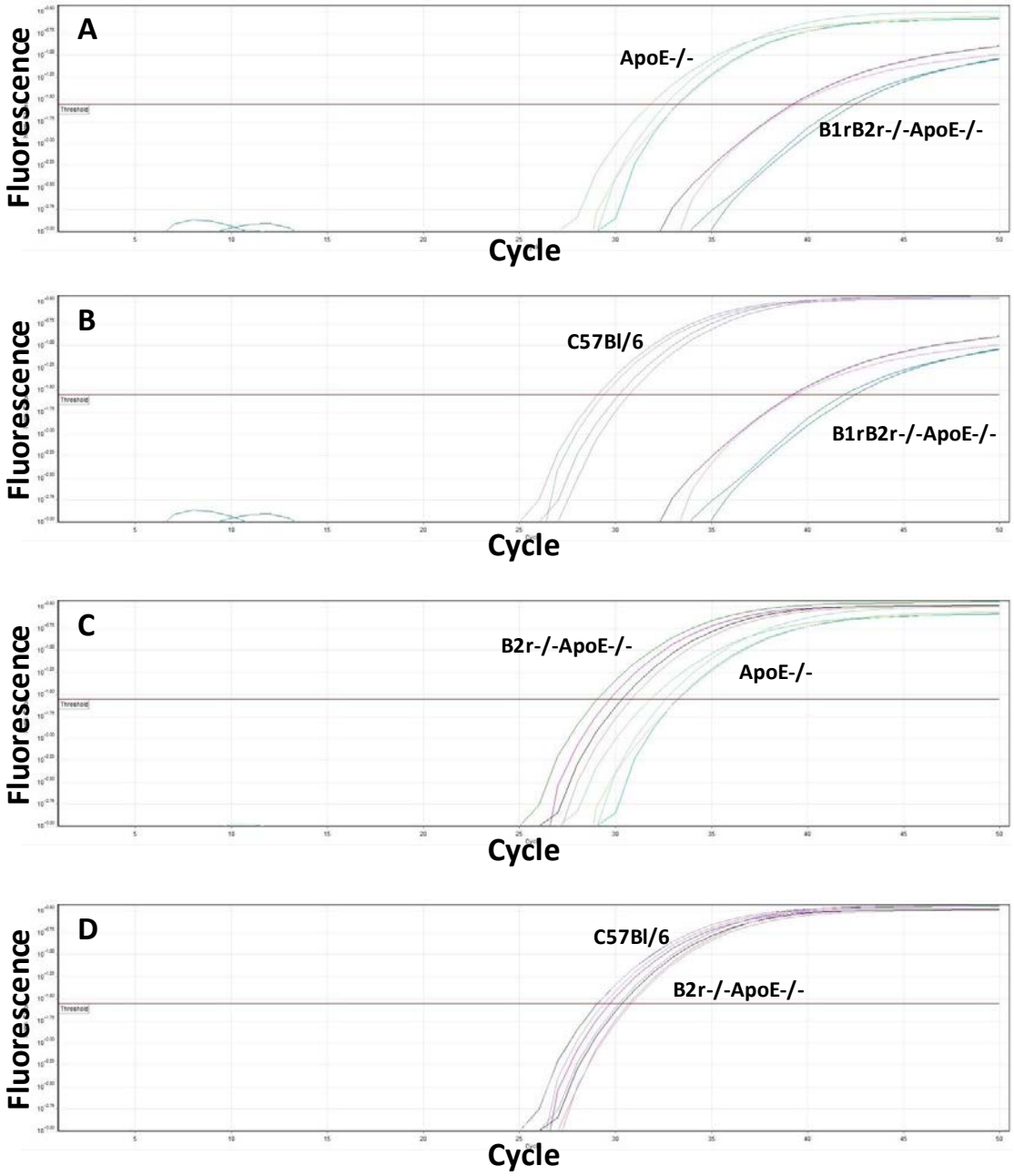


Figure 4.9 Amplification plot of heart RNA fromC57Bl/6, ApoE$^{-/-}$, B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ ApoE$^{-/-}$ mice using Bdkrb1 primers. (**A**) ApoE$^{-/-}$ versus B1rB2r$^{-/-}$ApoE$^{-/-}$ peak. (**B**) C57Bl/6 versus B1rB2r$^{-/-}$ApoE$^{-/-}$ (**C**) ApoE$^{-/-}$ versus B2r$^{-/-}$ApoE$^{-/-}$. (**D**) C57Bl/6 versus B2r$^{-/-}$ApoE$^{-/-}$. Replicates (n=5).

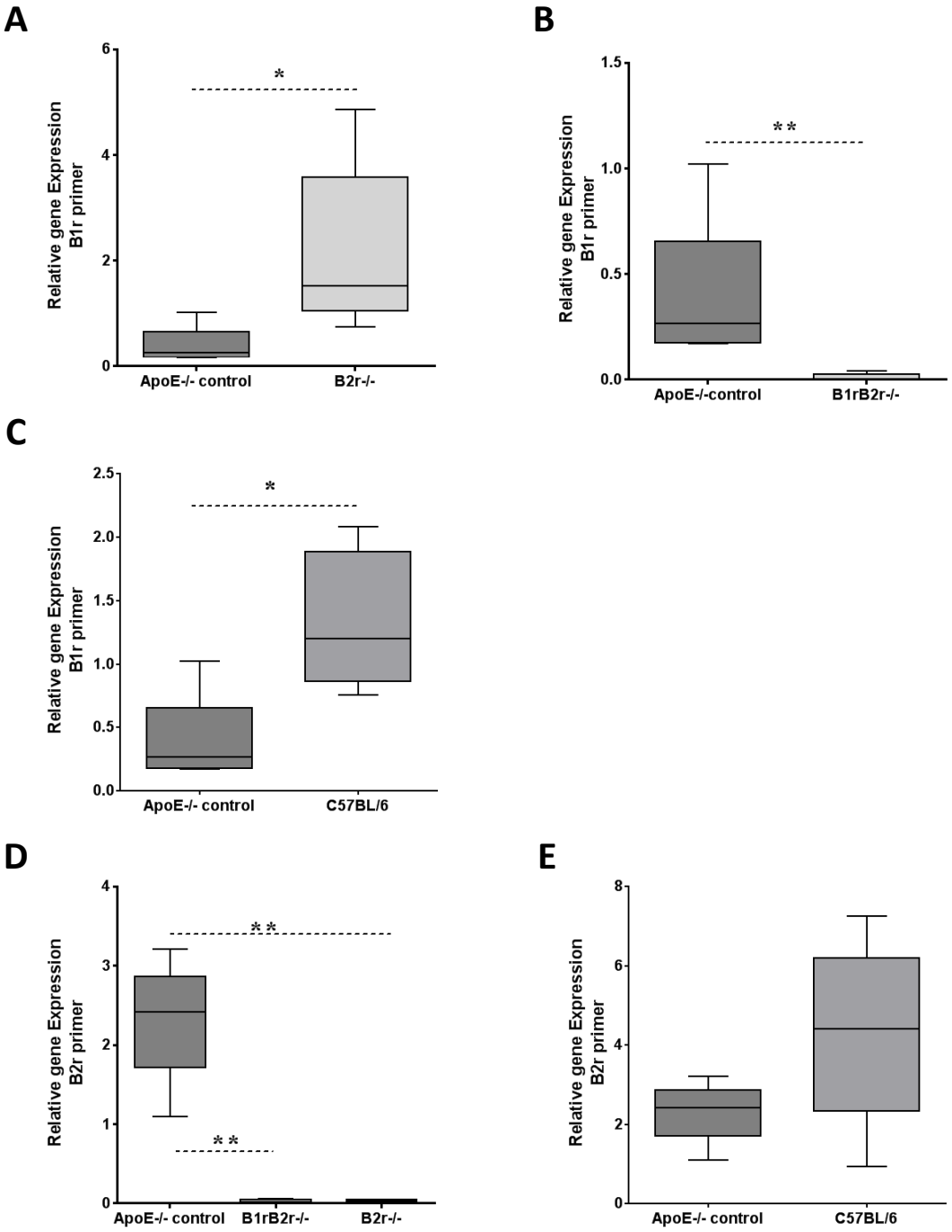


Figure 4.10 Relative gene expression calculated using concentration-Ct-standard curve and normalised by average expression of GAPDH. Data presented as box plots (min max), Analysed with Mann Whitney ***P<0.05. (A)** B1r expression in $ApoE^{-/-}$ versus $B2r^{-/-}ApoE^{-/-}$ P= 0.0159 (**B**) B1r expression in $ApoE^{-/-}$ versus $B1rB2r^{-/-}ApoE^{-/-}$ P= 0.0079 (**C**) B1r expression in C57Bl/6 versus $ApoE^{-/-}$ P=0.0317 (**D**) B2r expression in $ApoE^{-/-}$ versus $B1rB2r^{-/-}ApoE^{-/-}$ P= 0.0079 and $ApoE^{-/-}$ versus $B2r^{-/-}ApoE^{-/-}$ P= 0.0079 (**E**) B2r expression in C57Bl/6 versus $ApoE^{-/-}$ Heart sample (n=5) per mouse group.

4.4 DISSCUSSION

The most significant finding of this study was that cardiac hypertrophy was significantly greater in B1rB2r$^{-/-}$ApoE$^{-/-}$ mice, and B2r$^{-/-}$ApoE$^{-/-}$ mice compared to ApoE$^{-/-}$ mice. This data supports that B2r deficiency promotes cardiac hypertrophy in response to AII infusion. This increased effect in the B2r$^{-/-}$ApoE$^{-/-}$ mice could be due to up regulation of the B1r leading to even further hypertrophy. In the absence of the B2r, B1r up regulation appears to promote cardiac hypertrophy in response to AII. Other studies have reported similar findings. Madeddu *et al* (1995), noted that rats treated with a B2r antagonist peptide, Hoe 140, during the pre and postnatal phases of life showed greater systolic pressures, heart rates, and body weights than controls (Madeddu, Parpaglia et al. 1995), supporting the findings in this chapter.

A further aim of this chapter was to assess if kinin receptor deficiency affected blood pressure and heart rate. Data in this study demonstrated the effect of kinin receptor deficiency on blood pressure, and the hypertensive effect of AII in the AAA mouse model. It was noted that combined deficiency in B1rB2r mice led to an increased baseline in blood pressure prior to AII infusion compared to B2r$^{-/-}$ApoE$^{-/-}$ mice or the ApoE$^{-/-}$ control mice. This result suggests that in the absence of the B2r the B1r can compensate to normalise the blood pressure. When infused with AII, all mice groups experience a rise in blood pressure, again this effect was notably greater in the kinin deficient B1rB2r$^{-/-}$ApoE$^{-/-}$ mice. The response to AII was similar in the B2r$^{-/-}$ ApoE$^{-/-}$ mice and ApoE$^{-/-}$ control mice. Duka *et al* (2006) suggested that both kinin receptors had to be blocked to increase blood pressure in Wistar rats, supporting the concept that the receptors can compensate for each other. This group also noted that lack of blockade of B2r leads to increased expression of B1r suggesting an inter-relationship between the two receptors. In this respect, B1r can takes on the hemodynamic properties of B2r, indicating that both participate in maintenance of normal vasoregulation. Also the patterns of development of hypertension indicate that both B1r and B2r contribute to the maintenance of normal blood pressure, but one can compensate for inhibition of the other (Duka, Duka et al. 2006).

The findings of the current study suggest that there is limited direct relationship between blood pressure and cardiac hypertrophy, as the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice had high blood pressure and increased cardiac hypertrophy, yet the B2r$^{-/-}$ApoE$^{-/-}$ mice, which had lower blood pressure and further increased cardiac hypertrophy compared to the control mice. Surprisingly these responses appear independent of the changes in blood pressure during AII infusion which was most marked in the B1rB2r deficient mice.

A further interesting result was that B1 and B2 receptor expression was different in the mouse strains analysed. The B2 receptor deficiency was confirmed in the B1rB2r$^{-/-}$ApoE$^{-/-}$and B2r$^{-/-}$ ApoE$^{-/-}$ mice as expected, in the heart. The B1 and B2 receptor was significantly greater in C57Bl/6 compared to ApoE$^{-/-}$ mice. Published data suggests that the kinin B1 receptors can amplify or substitute for the kinin B2 receptor, especially in chronic inflammation (Ahluwalia and Perretti 1999; Phagoo, Poole et al. 1999; Bockmann and Paegelow 2000). Most interestingly, the B1 receptor expression in the B2r$^{-/-}$ApoE$^{-/-}$ mice was dramatically increased compared to both the C57Bl/6 and the ApoE$^{-/-}$ mice, in the heart. This finding is novel for this mouse strain. The data presented in this chapter supports the hypothesis that B1 kinin receptor expression is unregulated in the absence of the B2 kinin receptor. There are however important differences between the two receptor types suggesting that not all functions of B2r are compensated by B1r (Proud and Kaplan 1988). (Levesque, Harvey et al. 1995). Other important differences include that the B2r interacts with ACE.

Other studies have demonstrated a similar finding in different animal models. Duka *et al* noted that blockade of B2r leads to increased expression of B1r suggesting a relationship between the two receptors in rats. In this respect, B1r can takes on the hemodynamic properties of B2r, indicating that both participate in maintenance of normal vasoregulation. Also the patterns of development of hypertension indicate that both B1r and B2r contribute to the maintenance of normal BP, but one can compensate for inhibition of the other (Duka, Duka et al. 2006).

Further studies with kinin receptor knockout mice perhaps in the area of cardiac hypertrophy and further analysis into the signally pathways that lead to these mechanisms would be of benefit to determine the relationship between kinin receptors and hypertrophy.

To conclude, data from this chapter confirms:

1. Kinin receptor deficiency leads to cardiac hypertrophy in mice.

2. There is no relationship between blood pressure and cardiac hypertrophy.

3. B1 kinin receptor expression is up-regulated in the absence of the B2 kinin receptor.

This observation links the importance of the kinin receptors not only to cardiac hypertrophy, but also to blood pressure.

CHAPTER 5

THE EFFECTS OF KININ RECEPTOR DEFICIENCY ON ANGIOTENSIN II INDUCED AORTIC ANEURYSM FORMATION IN THE APOE$^{-/-}$ MOUSE

Chapter 5

THE EFFECTS OF KININ RECEPTOR DEFICIENCY ON ANGIOSTENSIN II INDUCED AORTIC ANEURYSM FORMATION IN THE APOE$^{-/-}$ MOUSE

5.1 INTRODUCTION

Although AAA is an important cause of morbidity and mortality in Western countries (Golledge, Muller et al. 2006) treatment is currently limited to endovascular or open surgical repair with no effective drug therapies available (Arko, Lee et al. 2002). Family history is an important risk factor (Norman and Powell 2007) and two genetic linkage studies have been linked to a region on chromosome 19 (19q13.4) with familial AAA (Shibamura H, et al. 2004), (Van Vlijmen-Van Keulen CJ, et al. 2005). This region, which has also been associated with intracranial aneurysm, harbours genes encoding members of the human tissue kallikrein family (van der Voet M et al. 2004), (Yousef GM, et al. 2000) including Kallikrein 1 (*KLK1*), previously known as tissue kallikrein (Yousef GM, et al. 2000). A single nucleotide polymorphism in *KLK1* has been associated with AAA (Biros, Norman et al. 2011). Plasma and tissue kallikrein are responsible for the generation of the kinins bradykinin and lys-bradykinin through cleavage of high or low molecular weight kininogen. These kinins stimulate one of the two bradykinin receptors (B1 and B2) in humans and other mammals including mice (Moreau, Garbacki et al. 2005). The B2 receptor (B2r) is constitutively expressed in many cells including polymorphonuclear neutrophils (Couture, Harrisson et al. 2001) but the B1 receptor (B1r) is barely detectable under normal physiological conditions (Bourdet, Pecher et al. 2010) and its expression is up-regulated in injured or activated tissue (Zhou, Polgar et al. 1998). Bradykinin, influences many physiological and pathophysiologicol processes including vascular permeability, blood pressure, vasodilatation, smooth muscle contraction, release of nitric oxide from the endothelium, inflammation, and stimulation of the synthesis of cytokines (IL-1, TNF-

α) by monocytes (Bhoola, Elson et al. 1992). In the previous chapter (Chapter 4, section 4.3.4) it was demonstrated that the kinin B1r is up regulated in the absence of the B2r in heart tissue, suggesting that cross regulation of signally through kinin receptors is complex and resulting in diverse effects.

AAA is often associated with atherosclerosis. The angiotensin II (AII) mouse model has been used extensively by many groups to study the contribution of AII-induced hypertension to several vascular pathologies, such as atherosclerosis and the formation of AAAs (Cassis, Gupte et al. 2009). AII stimulation via the AT receptors results in the formation of reactive oxygen species, leading to vascular inflammation in atherosclerosis and other vascular pathologies (Ross 1999). Similarly the kinin system has been linked to diverse cardiovascular diseases, and the complex link between kinin receptors, AAA, atheroma and RAS is yet to be clarified.

In a previous study within the Vascular Biology Unit, JCU (Rush, Nyara et al. 2009), utilising the $ApoE^{-/-}$ mouse model of AAA, microarrays were used to compare the gene expression of SRA segments of $ApoE^{-/-}$ mice to identify genes associated with AAA. A number of genes were up-regulated in non aneurysm forming areas indicating a potential protective role (including genes associated with the KKS). It was further demonstrated that kinin B2 receptors are up regulated in human AAA biopsies (Unpublished data, Rush et al). In a mouse model of AAA, aortic aneurysm and rupture was promoted by a B2r agonist while inhibited by kinin receptor antagonist. Moreover, progression of established AAA was inhibited by a B2r antagonist (Unpublished data, Moran et al). Furthermore, neutrophil depletion abrogated the ability of the B2r agonist to promote AAA within a mouse model (Unpublished data, Moran et al) (Appendix 1) suggesting that a link exists between B2r, AAA and neutrophils. Neutrophils have been linked to AAA in many studies (Cohen, Keegan et al. 1991; Hannawa, Eliason et al. 2005).

In this chapter, the relationship between bradykinin receptor expression and AII induced aneurysm formation was assessed using gene knockout mice lacking bradykinin receptors,

B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ApoE$^{-/-}$. The primary experimental outcomes for this chapter were analysed by measuring the aortic diameter of AII infused mice using ultrasound throughout AII infusion period and aortic morphometry at necropsy. The secondary outcomes included: (I) assessment of the arch of the aorta for atheroma severity; (II) kinin receptor expression in the aorta; (III) measurement of sera MPO associated with PMN activation; (IV) measurement of serum cytokines as indication of systemic inflammation; (V) flow cytometric analysis of circulating PMN and monocytes involved in the pathogenesis of AAA and atheroma severity. This chapter will determine if there was any relationship between the expression of kinin receptors, AII and AAA and atheroma.

It was hypothesised that:

1. Bradykinin receptor deficiency in the ApoE$^{-/-}$ mouse limits AII induced AAA and atherosclerosis.

2. Inflammatory leukocytes contribute to AAA and atheroma.

Specifically this Chapter aims to:

1. Determine if kinin receptor deficiency inhibits aneurysm development and atheroma severity in the AII-infused ApoE-/- mouse model

2. Assess the mechanisms involved in AAA and atheroma, by measuring markers of inflammation

3. Assess aortic expression of kinin B1r and B2r in Apo E$^{-/-}$ mice

5.2 EXPERIMENTAL METHODS

5.2.1 *Mouse model of AAA.*

The AII-infused ApoE$^{-/-}$ mouse model was used as described in Chapter 3. This model closely mimics many of the inflammatory characteristics associated with human AAA, including the enhanced propensity for the development of AAAs in male mice, with the incidence being approximately twice that of females (Daugherty and Cassis 2004). In addition, some of the genetic pathways believed to underpin human AAA are also relevant to aneurysm formation in this mouse model (Rush, Nyara et al. 2009). In the AII model an osmotic micro-pump (ALZET Model 1004, Durect Corporation, USA) containing AII (Sigma-Aldrich) is inserted into the subcutaneous space left of the dorsal midline under anaesthesia (i.p.; 150 mg/kg ketamine, 10 mg/kg xylazine) to administer AII at a rate of 1.0 μg/kg/min over 28 days (Chapter 3, section 3.13).

5.2.2 *Kinin receptor deficiency and aneurysm formation*.

To investigate the potential link between kinin receptor signalling and AAA, the effect of kinin receptor gene deletion on AII-induced AAA in an ApoE$^{-/-}$ mouse model was examined. ApoE-deficient mice and ApoE$^{-/-}$ mice that were homozygous for null mutations in both the kinin B1 and B2 receptors (B1rB2r$^{-/-}$ApoE$^{-/-}$) or the B2 receptor alone (B2r$^{-/-}$ApoE$^{-/-}$) were generated (Chapter 3, section 3.1.2). Groups of six month old male ApoE$^{-/-}$ (controls; n=41), B1rB2r$^{-/-}$ ApoE$^{-/-}$ (n=41), and B2r$^{-/-}$ApoE$^{-/-}$ (n=41) mice were infused with AII (Chapter 3, section 3.1.3) over 28 days. The diameter of the suprarenal aorta (SRA) was monitored by ultrasound (Chapter 3, section 3.1.4) at 7-day intervals, while regional maximum diameters of the aortic arch, thoracic aorta (TA), SRA, and infrarenal aorta (IRA) were determined at study end (Figure 5.1), or after death due to rupture by morphometric analysis (Chapter 3, section 3.1.6).

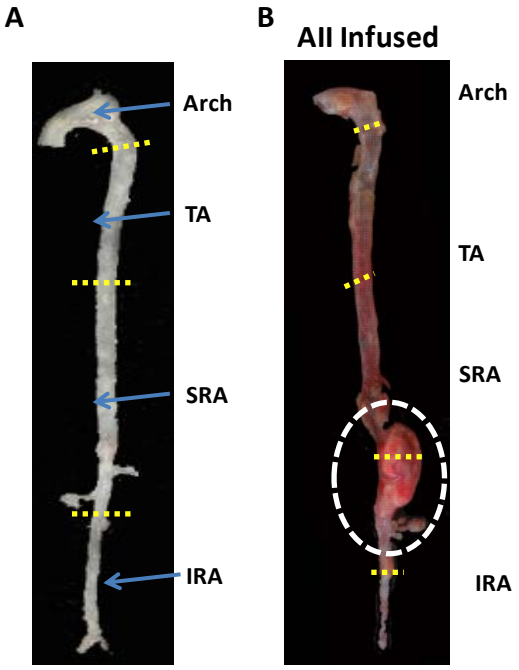


Figure 5.1 Representation of morphometry image of the mouse aorta demonstrating (**A**) aortic regions: Arch, TA, SRA and IRA (**B**) mouse aorta infused with AII, indicating the different regions measured by morphometry (yellow lines) (Arch, TA, SRA, IRA) the white circle indicates the dilation in the SRA region.

5.2.3 *Expression of kinin B1 and B2 receptors in the mouse aorta*

To determine whether B1r and B2r were expressed and to compare the expression in different mouse strains, aortas were harvested from n=5 C57Bl/6, ApoE$^{-/-}$, B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ ApoE$^{-/-}$ mice without AII infusion and stored in RNA later prior to isolation of total RNA. Total RNA was extracted from aorta using RNeasy mini kit (Qiagen, Australia) according to manufacturer's instructions (Chapter 3, section 3.1.7). SYBR Green PCR primers Bdkrb1 and Bdkrb2 (RT^2 qPCR primers) were commercially available from Qiagen and the GAPDH primers were also commercially obtained as described in Chapter 3, section 3.1.8). Quantitative real time PCR (qPCR) was performed for Bdkrb1 and Bdkrb 2 genes as described in Chapter 3, section 3.1.8) using a three step melt thermal cycling program.

5.2.4 *Quantification of atheroma in the aortic arch by sudan IV staining*

The aortic arch tissues collected at dissection and stored in OCT were thawed at room temperature, and washed in PBS to remove excess OCT. The arch segments were opened longitudinally with a scalpel and pinned to a waxed membrane in a petri dish. The segments were washed in 70% ethanol for 5 minutes. Segments were then stained with Sudan IV (0.1% Sudan (Sigma) in an equal volume of 100% acetone and 70% ethanol), for 60 minutes, with gentle agitation. The segments were washed twice in 80% ethanol for 5 minutes to decolourise the tissue and then washed in running tap water. The segments were placed on a gradated template and digitally photographed (Coolpix 4500, Nikon). Lipid areas were indicated by the red stain. The amount of stain was calculated from the images using computer-aided analysis (Adobe®Photoshop®CS5 Extended version 12, Adobe Systems Incorporated) by drawing around the stained areas and the total tissue area using the draw tool (Figure 5.2) and % atheroma was calculated using the formula: red stain area/ total x 100. An example of atheroma analysis is shown in Figure 5.2.

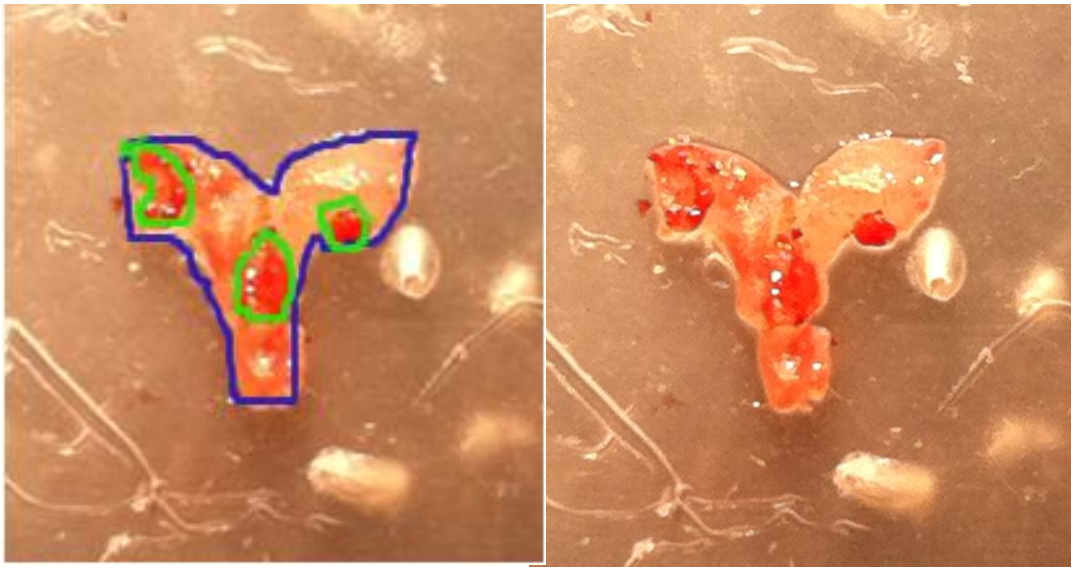


Figure 5.2 Representation of an ApoE$^{-/-}$ mouse aortic arch stained with Sudan IV. Red areas (outlined in green) indicate areas of lipid deposition and the blue outline is the total area measured.

5.2.5 *Flow cytometry analysis of circulating neutrophils and monocytes in AII infused mice*

The activation status of circulating neutrophils and monocytes was determined by flow cytometric analysis (Chapter 3, section 3.2.4) of blood samples obtained via tail bleed (Chapter 3, section 3.1.5) 5 days prior to the commencement of AII infusion and at 7 day intervals after AII infusion. Heparinised blood samples (50 µl) were depleted of red cells using an erythrocyte lysis buffer (eBioscience), and surface staining was done using antibodies to CD45- PerCP (1:200) (leukocyte marker), CD11b-FITC (1:200) (myeloid marker), and Ly6G-PE (1:400) (PMN marker)(BD Biosciences) diluted in PBS containing 2mM EDTA (Amresco) and 0.5% (w/v) BSA (ICN Biomedicals). Neutrophils were $CD45^+$, $CD11b^+$, $Ly6G^+$ whilst monocytes were $CD45^+$, $CD11b^+$, $Ly6G^-$. Samples were preincubated for 30 minutes with anti CD16/32 (1:50) to prevent FcR binding before addition of surface staining antibody cocktails. Counting beads (Invitrogen) were added. Viable cells were identified by forward/side scatter profile and in some cases by propidium iodide (PI) exclusion (Figure 5.3). Comparing forward scatter-area to forward scatter-linear gate was used to exclude doublets from analysis. The stopping event was set at 1000 counting beads. Acquisition was performed on a FACs Calibur flow cytometer (BD, USA) and analyzed using Cell Quest Pro analysis software (BD Biosciences, USA).

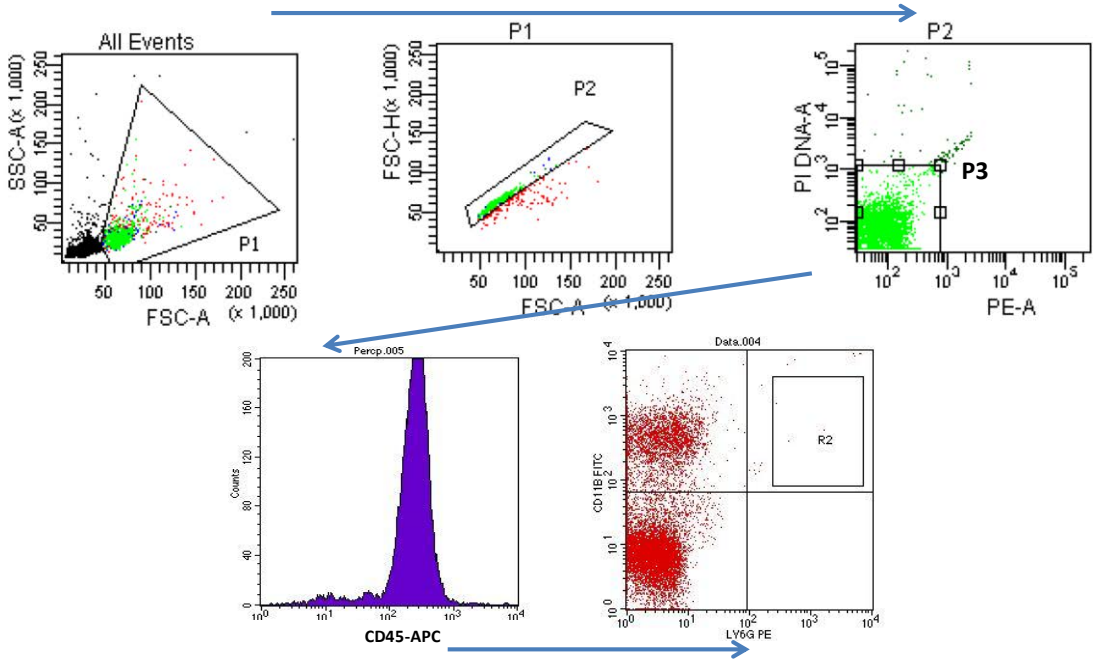


Figure 5.3 Flow cytometry gating strategy indicating forward/ side scatter; exclusion of doublets; plot demonstrating viable cells stained with propidium iodine (PI). P3 gate indicates viable cells, dead cells are excluded (PI positive). CD45 gating and CD11b and Ly6G gating.

5.2.6 *Measurement of circulating inflammatory cytokines by bead array*

Plasma was prepared from heparinised tail vein blood collected prior to AII infusion and at week two. Experimental groups were as described in 5.2.2. Plasma cytokine levels were determined using the BD CBA Mouse Inflammation kit (BD Biosciences) according to the manufacturer's instructions. Briefly, antibody coated beads were used to detect IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70. Serial dilutions of the provided cytokine standards (in duplicate) and samples were prepared and acquired using FACS Calibur. Standard curves were generated and cytokines quantified in samples using the FCAP Array software (v 3.0, BD, USA). (Appendix 4).

5.2.7 *Determination of circulating plasma MPO*.

The total concentration of MPO in plasma samples taken from kinin receptor-deficient (B1rB2r$^{-/-}$ ApoE$^{-/-}$and B2r$^{-/-}$ApoE$^{-/-}$ and ApoE$^{-/-}$) mice, was determined by ELISA (total MPO, Mouse, ELISA kit) following procedures recommended by the manufacturer (Hycult Biotech). In brief, plasma was obtained from blood samples collected by tail bleed (Chapter 3, section 3.1.5). Serial dilutions of the provided MPO standards were prepared (run in duplicates) and assayed as described by the manufacturer's instructions (Appendix 4). Data was expressed as the change in plasma MPO over baseline levels determined in plasma sampled prior to AII infusion. Mean intra-assay coefficient of variation for MPO determined from the standard curves of duplicate standards was 1.57%.

5.2.8 Statistics

Data was analysed using GraphPad Prism (version 6) and S Plus (version 8). Parametric or non-parametric tests were applied as appropriate to distribution of data. The percentage change in SRA diameter (ultrasound) (Section 5.3.1), and percentage change of baseline MPO measured in plasma (Section 5.3.8) collected prior to AII infusion of the kinin deficient mice and ApoE$^{-/-}$, was presented using mean±SEM, percent from baseline (baseline was determined prior to AII infusion), presented as one graph per week and analysed with Mann Whitney *U* test.

Data presented over time (several time points), between the kinin deficient mice and ApoE$^{-/-}$, was analysed using Repeated measure Two-way ANOVA, to determine the overall effect of treatment, and with Tukey's Post-hoc test, to analyse treatment between each group. This was used for, ultrasound data (Section 5.3.1), over time, and presented as a line graph (mean±SEM).

End-point data, such as the mean maximum diameter (MMD) data and effect of kinin receptor deficiency on aortic arch atheroma severity was analysed using One-way ANOVA, and Tukey's

post-hoc test or Kruskal-Wallis test (non-parametric ANOVA) and Dunn's post-hoc test was used to compare the differences between the groups. The data was expressed as mean ± SD (dot plots) or median and interquartile range (box plots), respectively.

Kaplan-Meier survival curves were analysed using log-rank (Mantel-Cox) test and contingency data (rupture) were compared using Fisher's exact tests. In all cases P values less than 0.05 were considered significant.

Receptor expression of B1 and B2 receptors in aortas was analysed using Mann Whitney *U* test, statistical significance was taken as *P<0.05. Flow cytometry data of plasma and inflammatory cytokine levels were analysed by a Mann–Whitney U test P<0.05 when compared to control mice, and data presented as Mean±SEM.

5.3 RESULTS

5.3.1 Kinin receptor deficiency inhibits AII-induced AAA in ApoE$^{-/-}$ mice

5.3.2.1 *Ultrasound of AII infused ApoE$^{-/-}$, B1rB2r$^{-/-}$ and B2r$^{-/-}$ mice*

The potential link between kinin receptors and AAA was investigated using kinin receptor deficient mice infused with AII. ApoE-deficient mice homozygous for null mutations in both the kinin B1 and B2 receptors (B1rB2r$^{-/-}$ApoE$^{-/-}$) or the B2 receptor alone (B2r$^{-/-}$ApoE$^{-/-}$) were generated (Chapter 3, section 3.1.2). Groups of ApoE$^{-/-}$, B2r$^{-/-}$ ApoE$^{-/-}$ and B1rB2r$^{-/-}$ ApoE$^{-/-}$ mice were infused with AII for 28 days (Section 5.2.4). Dilatation of the SRA in these mice was monitored at seven-day intervals using ultrasound (Figure 5.4), and regional and mean total maximum aortic diameter were determined morphometrically at completion of the study period.

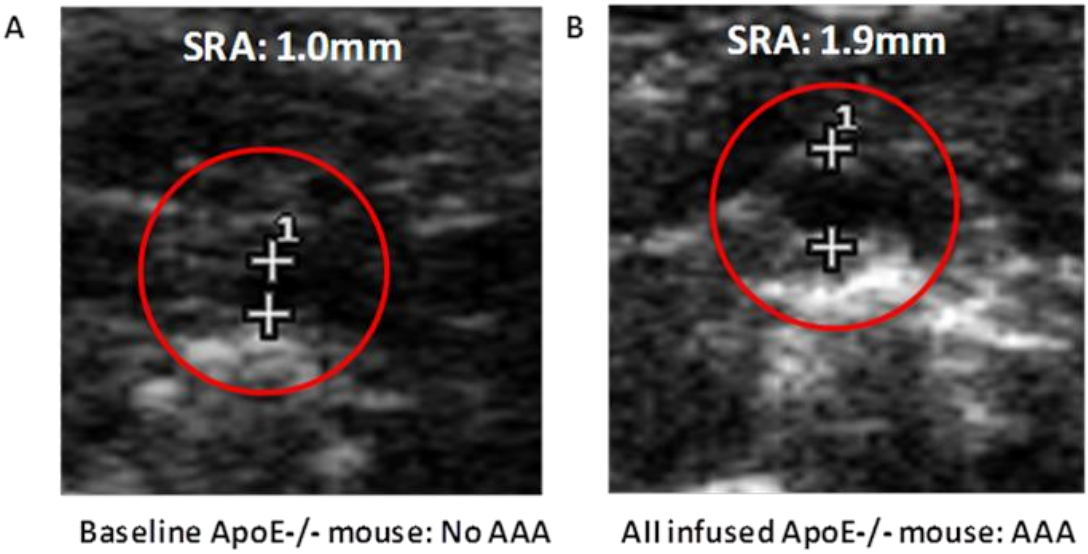


Figure 5.4 Representation of ultrasound image showing the mouse suprarenal aorta. (**A**) mice at baseline reading, prior to AII infusion (**B**) mice infused with AII that has a developing aneurysm. Electronic callipers (white crosses) show the maximal diameter of aorta.

Baseline SRA diameter was measured by ultrasound prior to commencement of AII infusion and every seven days after. The effect of kinin receptor deficiency on aortic diameter, over time by ultrasound in $ApoE^{-/-}$ controls, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ was analysed using Repeated Measure Two Way ANOVA. There was a significant effect of kinin receptor deficiency on aortic diameter at the $p<0.05$ level for the three groups ($P < 0.0001$) overall (Table 5.1).

The overall significance was tested by Repeated Measure Two-Way ANOVA, Tukey's multiple comparisons test was carried out to compare each mouse group with all other mouse groups ($ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$). At baseline (day 0), it was noted that there was no significant difference in SRA diameter between $ApoE^{-/-}$ controls and $B1B2r^{-/-}$ApoE, but there was between $ApoE^{-/-}$ controls and $B2r^{-/-}$ApoE, and $B1B2r^{-/-}$ApoE and $B2r^{-/-}$ApoE. There was no significant difference between the SRA diameter in any of the groups of mice in week 1. A significant difference in aortic diameter as measured by ultrasound was observed in weeks 3 and 4 between $ApoE^{-/-}$ controls and $B1B2r^{-/-}$ApoE and $ApoE^{-/-}$ controls and $B2r^{-/-}$ApoE in weeks 2, 3 and 4 (Figure 5.5).

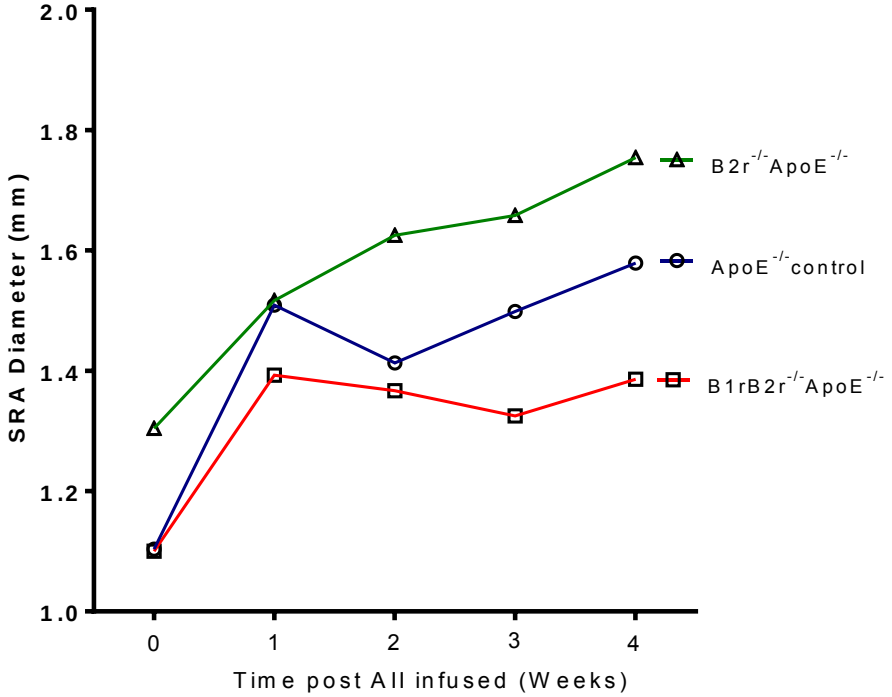


Figure 5.5 Comparison of change in maximum SRA diameter in AII-infused ApoE$^{-/-}$ (n=41), B1rB2r$^{-/-}$ApoE$^{-/-}$ (n=41), and B2r$^{-/-}$ApoE$^{-/-}$ (n=41) mice over 28 days, as measured by ultrasound at 7-day (Week) intervals. Data is presented as Mean±SEM showing increasing diameter suprarenal aortic diameter, determined prior to AII infusion, and after. Analysed by Repeated measures two-way ANOVA (Table 5.1) with Tukeys HSD Post Hoc tests significance *P<0.05 (Table 5.2)

Table 5.1 Repeated Measure Two-Way ANOVA analysing the effect of kinin receptor deficiency on aortic diameter, over time for ApoE$^{-/-}$ controls, B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ApoE$^{-/-}$

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.928	8	0.1160	F (8, 360) = 3.068	P = 0.0023
Time (Row)	8.637	4	2.159	F (4, 360) = 57.11	P < 0.0001
Group (Column)	4.779	2	2.390	F (2, 90) = 26.08	P < 0.0001
Subjects (matching)	8.245	90	0.09162	F (90, 360) = 2.423	P < 0.0001
Residual	13.61	360	0.03780		

SS, Sum of squares; DF, Degrees of Freedom; MS, Mean squares; F, F ration

Table 5.2 Tukeys HSD Post Hoc tests

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	P value
Baseline (Day 0)			
Control vs. $B1rB2r^{-/-}$	0.003333	-0.1216 to 0.1282	ns
Control vs. $B2r^{-/-}$	-0.2008	-0.3399 to -0.06181	P=0.0021
$B1rB2r^{-/-}$ vs. $B2r^{-/-}$	-0.2042	-0.3407 to -0.06760	P=0.0014
Week 1			
Control vs. $B1rB2r^{-/-}$	0.1166	-0.008300 to 0.2415	ns
Control vs. $B2r^{-/-}$	-0.007576	-0.1466 to 0.1314	ns
$B1rB2r^{-/-}$ vs. $B2r^{-/-}$	-0.1242	-0.2607 to 0.01240	ns
Week 2			
Control vs. $B1rB2r^{-/-}$	0.04636	-0.07853 to 0.1713	ns
Control vs. $B2r^{-/-}$	-0.2120	-0.3510 to -0.07295	P=0.0011
$B1rB2r^{-/-}$ vs. $B2r^{-/-}$	-0.2583	-0.3949 to -0.1218	P< 0.0001
Week 3			
Control vs. $B1rB2r^{-/-}$	0.1735	0.04859 to 0.2984	P=0.0034
Control vs. $B2r^{-/-}$	-0.1598	-0.2989 to -0.02082	P=0.0195
$B1rB2r^{-/-}$ vs. $B2r^{-/-}$	-0.3333	-0.4699 to -0.1968	P< 0.0001
Week 4			
Control vs. $B1rB2r^{-/-}$	0.1927	0.06779 to 0.3176	P=0.0009
Control vs. $B2r^{-/-}$	-0.1754	-0.3144 to -0.03635	P=0.0089
$B1rB2r^{-/-}$ vs. $B2r^{-/-}$	-0.3681	-0.5046 to -0.2315	P< 0.0001

Significance *P<0.05; ns, not significant

As baseline (Day 0) diameters varied between groups (Fig 5.5 and Table 5.2) the change in maximum SRA diameter was also calculated as a percentage of the baseline measurement to facilitate between group comparisons. As shown in Figure 5.6, AII infusion in $ApoE^{-/-}$ control mice resulted in a mean increase in SRA diameter of 40-50% over the 28-day infusion period. In contrast, the mean % increase in SRA diameter was markedly less in $B2r^{-/-}ApoE^{-/-}$ mice after 7 days (Week1) AII infusion (~20% above baseline) and this protection against aortic dilatation was maintained at weeks 3 and 4 with mean SRA dilatation of 25% and 31% above baseline, respectively (Figure 5.6). The mean % increase in SRA dilatation in mice deficient in both B1 and B2 receptors tended to be lower than that of $ApoE^{-/-}$ controls during the first 14 days (2 Weeks) of AII infusion, although statistical significance could not be demonstrated. However a

maintained mean SRA dilatation at 24% and 31% above baseline demonstrated significantly reduced SRA dilatation at weeks 3 and 4, respectively compared to $ApoE^{-/-}$ controls (Figure 5.6).

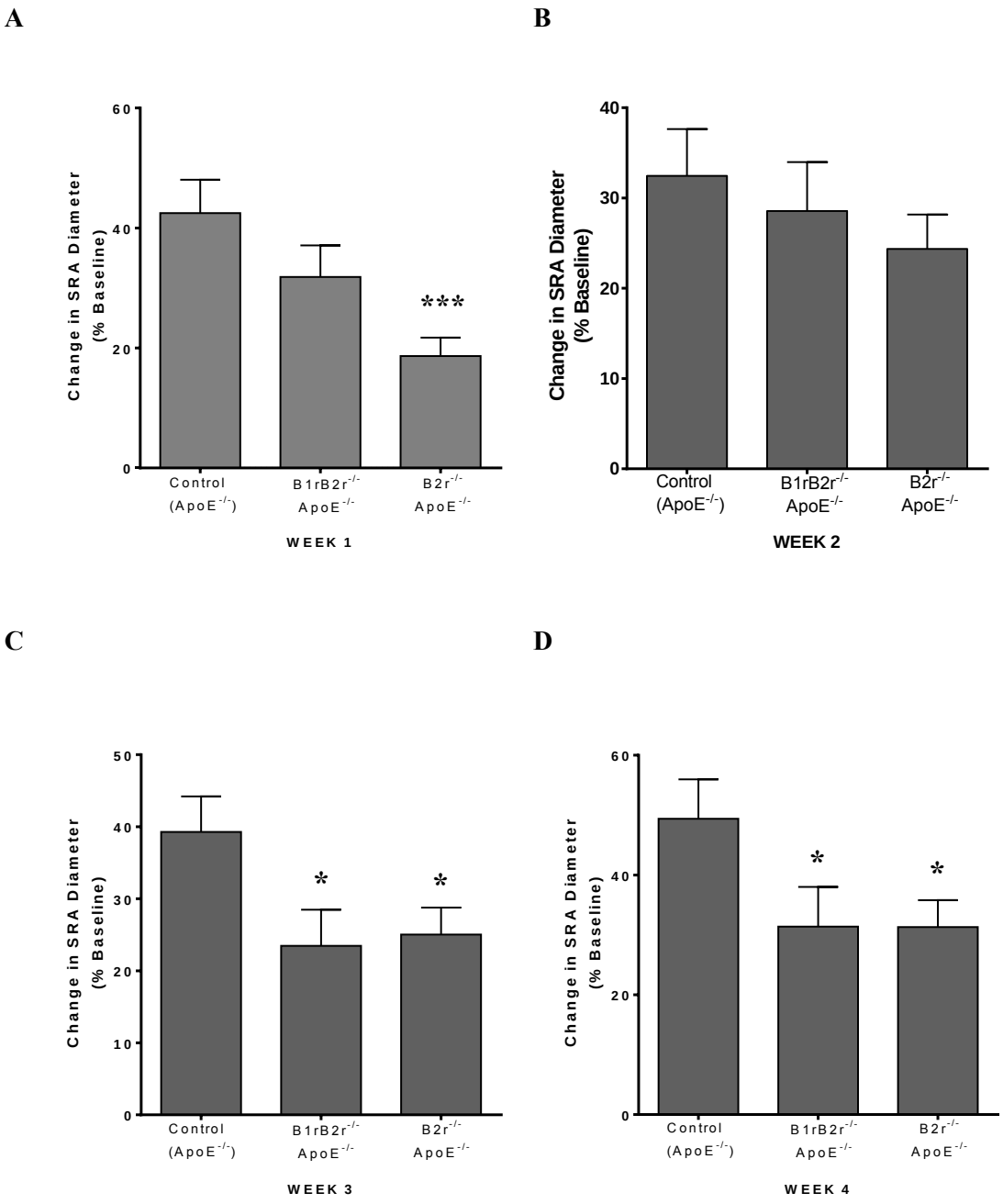


Figure 5.6 Change in maximum SRA diameter in AII-infused $ApoE^{-/-}$ (n=41), $B1rB2r^{-/-}ApoE^{-/-}$ (n=41), and $B2r^{-/-}ApoE^{-/-}$ (n=41) mice over 28 days, as measured by ultrasound at 7-day intervals. (**A**) Percent change from baseline to week 1, (**B)** Week 2, (**C**) Week 3, (D) Week 4. Data is presented as Mean±SEM percent of SRA baseline diameter, determined prior to AII infusion**.** Analysed with Mann Whitney U test***P<0.05.**

5.3.2.2 *Aortic morphometry of AII infused ApoE$^{-/-}$, B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ApoE$^{-/-}$ mice*

The observation that kinin receptor deficiency abrogated AII-induced dilatation of the SRA in this mouse model was reflected by morphometric data obtained from harvested aortas at the end of the study (Figure 5.7). We observed an overall reduction in mean total maximum aortic diameter in B1rB2r$^{-/-}$ApoE$^{-/-}$, B2r$^{-/-}$ApoE$^{-/-}$ mice compared to the ApoE$^{-/-}$control when analysed with Kruskal-Wallis test (non-parametric ANOVA), there was a significant effect of kinin receptor deficiency on aortic diameter at the $P<0.05$ level for the three groups ($P = 0.0035$). Dunn post-hoc multiple comparisons test was carried out to compare each mouse group with all other mouse groups (ApoE$^{-/-}$ controls, B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ApoE$^{-/-}$) (Figure 5.8).

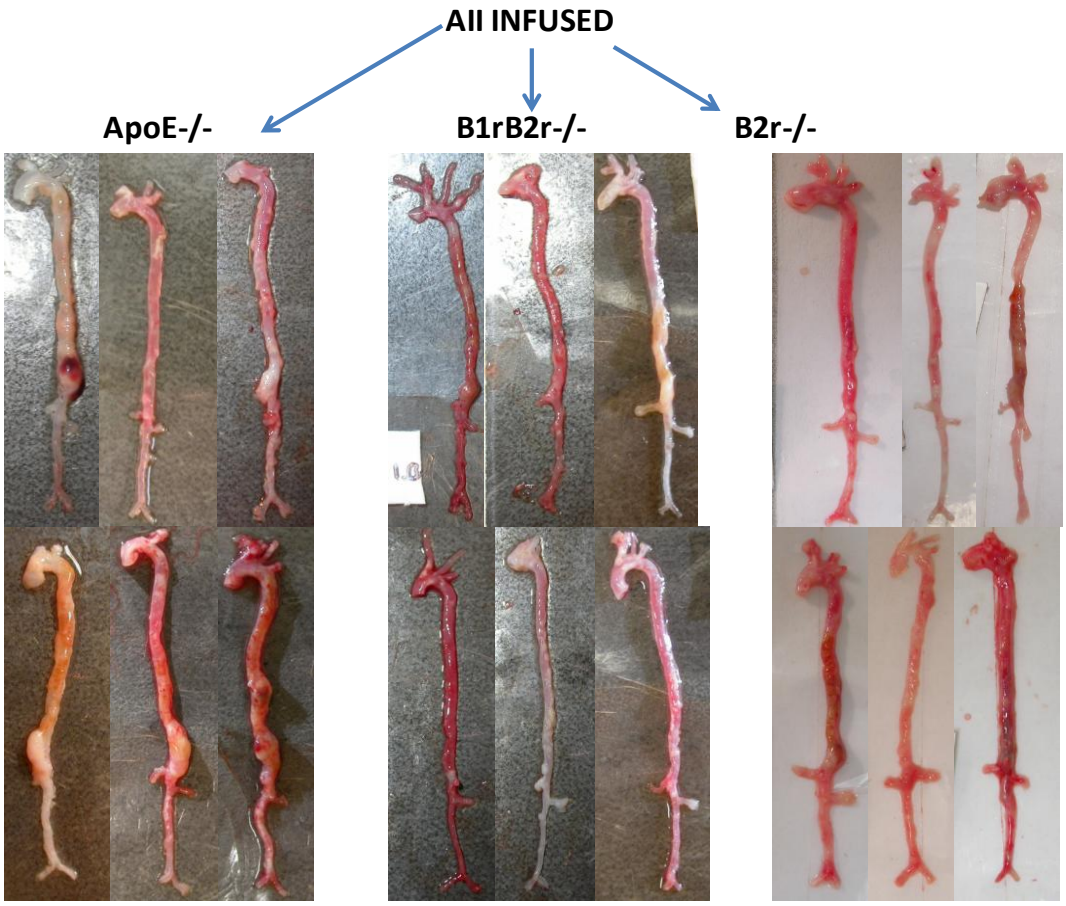


Figure 5.7 The influence of kinin receptor deficiency on aneurysm formation in ApoE$^{-/-}$ mice. Examples of aortas harvested from ApoE$^{-/-}$ (control) mice and ApoE$^{-/-}$ mice deficient in kinin B1/B2 receptors (B1rB2r$^{-/-}$ApoE$^{-/-}$) and B2 receptor alone (B2r$^{-/-}$ApoE$^{-/-}$) after infusion with AII over a 28-day experimental period. Determination of maximum diameters in these vessels by morphometric analysis was performed using digital images taken with the aortas remaining *in situ*. Representative images shown. All images are shown in Appendix 5.

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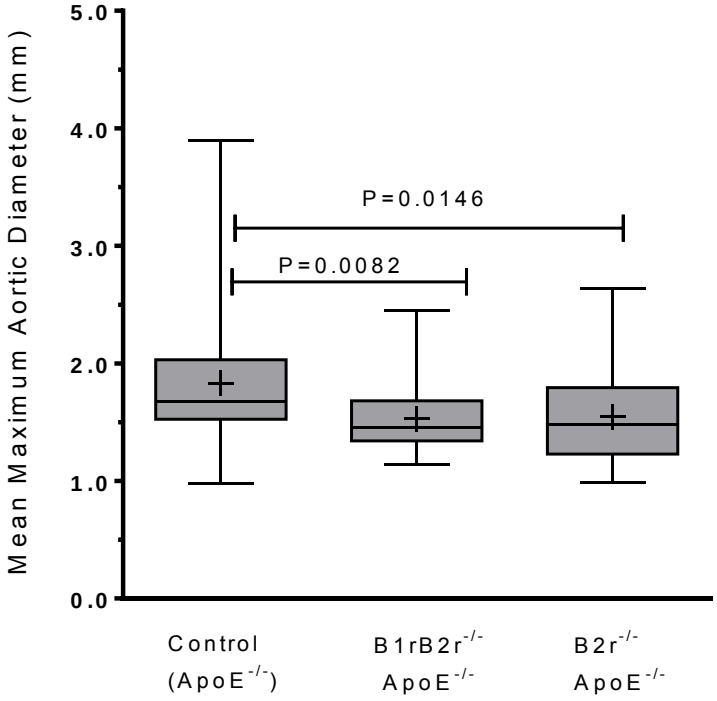


Figure 5.8 Total maximum aortic diameter in AII-infused $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$, and $B2r^{-/-}$ $ApoE^{-/-}$ mice after 28 days, calculated by averaging morphometrically determined regional (Arch, TA, SRA, IRA) maximum diameters. (**A**) Data presented as box plots (min max), maximum aortic diameter (mm). Data was analysed by Kruskal-Wallis test with Dunn post-hoc multiple comparisons test AII, angiotensin II; TA, thoracic aorta; SRA, suprarenal aorta; IRA, infrarenal aorta. + indicates mean.

In AII-infused $ApoE^{-/-}$ mice deficient in both kinin receptors (B1r $B2r^{-/-}ApoE^{-/-}$), mean maximum aortic diameter was significantly lower compared to $ApoE^{-/-}$ mice (1.53±0.29 vs 1.83±0.55) and $B2r^{-/-}ApoE^{-/-}$ mice mean maximum aortic diameter was significantly lower compared to $ApoE^{-/-}$ mice (1.54±0.37 vs 1.83±0.55). An analysis of each of the different regions of the aortas (arch, thoracic, suprarenal and infrarenal) was carried out and is presented in Table 5.4.

Table 5.3 Maximum aortic diameter following AII infusion

Group	ApoE$^{-/-}$ control	B1rB2r$^{-/-}$ ApoE$^{-/-}$	B2r$^{-/-}$ApoE$^{-/-}$	Overall Comparison 1-Way ANOVA
Number	41	41	41	
Aortic arch	2.01 (0.46)	1.87 (0.36)	2.01 (0.56)	NS
TA	1.76 (0.41)	1.49 (0.22)**	1.47 (0.42)**	P= 0.0006
SRA	2.27 (0.97)	1.78 (0.56)**	1.80 (0.58)*	P= 0.0034
IRA	1.29 (0.76)	0.98 (0.38)*	0.89 (0.26)**	P= 0.0017
Average maximum diameter	1.83 (0.54)	1.53 (0.29)**	1.55 (0.37)**	P= 0.0018

Diameters are means (standard deviation) in mm. Average maximum aortic diameters were calculated by averaging maximum diameters from all 4 segments in each mouse. Analysis was carried out by One-way ANOVA (overall comparison), with Tukeys post hoc test *significant of aortic dilatation compared between each group and control, P<0.05. TA, thoracic aorta; SRA, suprarenal aorta; IRA, infrarenal aorta.

5.3.2.3 *Kinin receptor deficiency influences survival in AII infused ApoE mice*

Deficiency in both kinin receptors (B1rB2r$^{-/-}$ApoE$^{-/-}$ mice) apparently improved survival following AngII-infusion but statistical significance could not be demonstrated when compared with ApoE-/- mice (*P*= 0.1252) (Figure 5.9). AII-induced aortic rupture was reduced to ~50% in B1rB2r$^{-/-}$ApoE$^{-/-}$ mice compared to ApoE$^{-/-}$ mice, but again statistical significance could not be demonstrated (Figure 5.10). The frequency of aortic rupture was similar in groups of B2r$^{-/-}$ ApoE$^{-/-}$ compared to ApoE$^{-/-}$ mice.

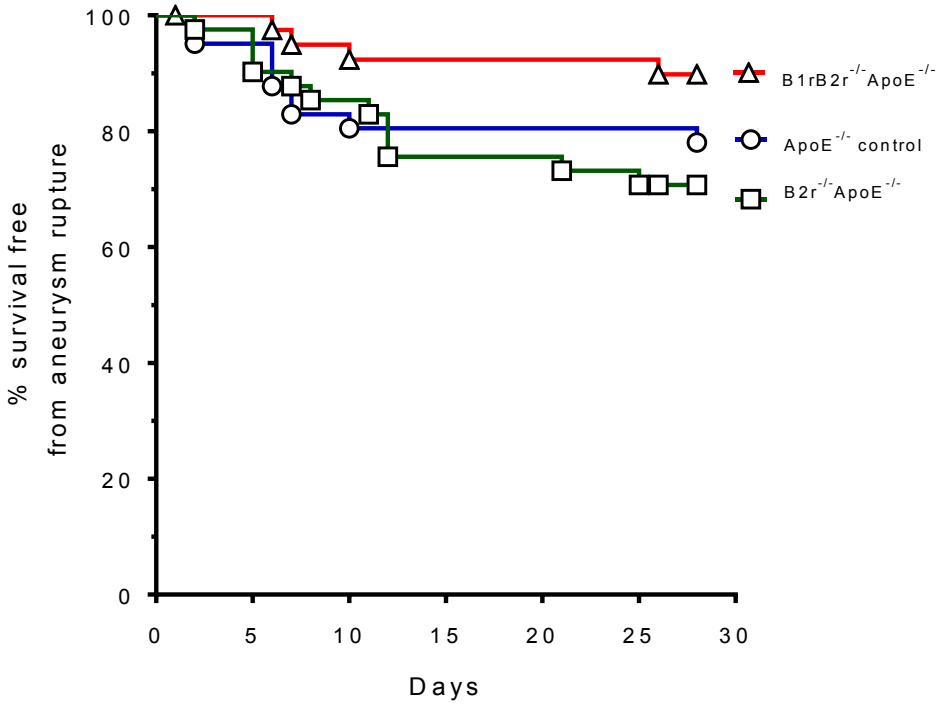


Figure 5.9 Kaplan-Meier curves of survival free from aneurysm rupture in $ApoE^{-/-}$ mice deficient in $ApoE^{-/-}$ (○; n=41), $B1rB2r^{-/-}ApoE^{-/-}$, (Δ; n=41), and $B2r^{-/-}ApoE^{-/-}$ (□; n=41). P=0.1252, and P=0.5023 versus $ApoE^{-/-}$ control respectively.

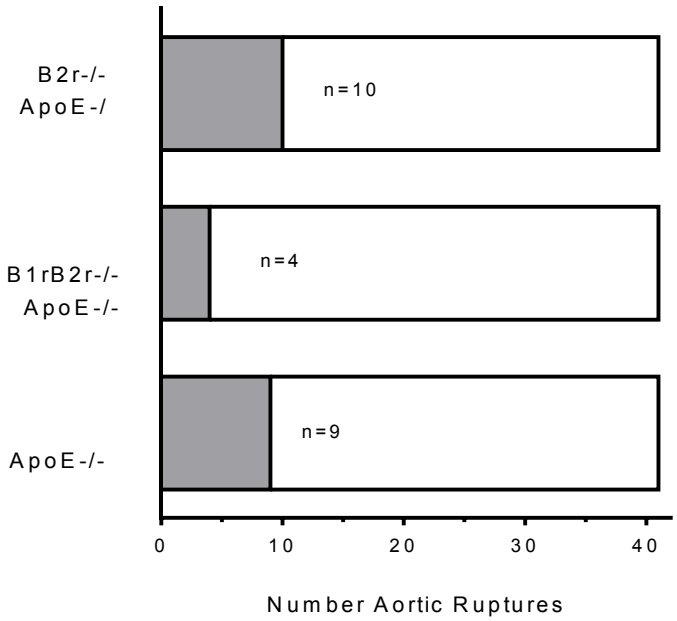


Figure 5.10 Number of aortic ruptures in AII infused mice where n=41 per group. Number of ruptures are indicated (grey). P=0.225 and P=1.000 $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ versus $ApoE^{-/-}$.

5.3.2 *Expression of kinin B1 and B2 receptors in mouse aortas prior to AII infusion*

Relative gene expression levels of the kinin B1 and B2 receptors were compared between the different mouse groups, C57Bl/6, $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice n=5 per group, by quantitative real time PCR, using Bdkrb1 and Bdkrb 2 primer sets (Chapter 3, section 3.1.7). After normalising to GAPDH, the relative expression of both B1 and B2 receptors, were assessed. Previously receptor expression was determined using heart tissue, and results showed differential expression in mouse strains (Chapter 4, section 4.3.4). Receptor expression was determined in aortic tissue (Figure 5.11). There was no B1 receptor expression in the $B1rB2r^{-/-}ApoE^{-/-}$ aorta as expected. Interestingly, the B1 receptor expression in the $B2r^{-/-}ApoE^{-/-}$ was greater than in the $ApoE^{-/-}$ mice aorta as shown previously for heart tissue (Chapter 4). The relative gene expression of the B2 receptor was greater in C57Bl/6 compared to $ApoE^{-/-}$ mice, although not significant. There was no B2 receptor expression in the $B1rB2r^{-/-}ApoE^{-/-}$ or $B2r^{-/-}ApoE^{-/-}$ mice as expected.

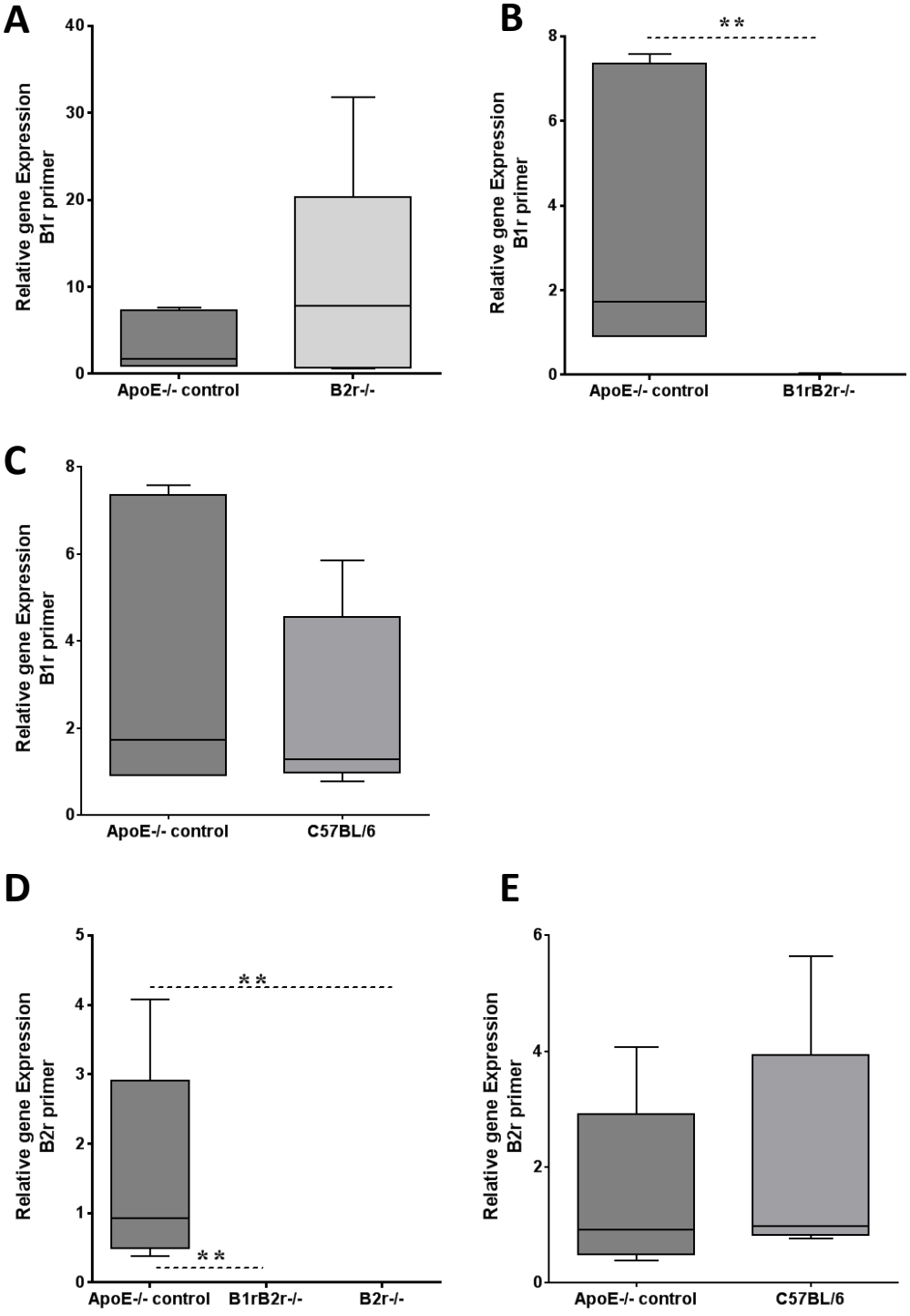


Figure 5.11 Relative gene expression calculated using concentration-Ct-standard curve and normalised by average expression of GAPDH. Data presented as box plots (min max), Analysed with Mann Whitney ***P<0.05. (A)** B1r expression in $ApoE^{-/-}$ versus $B2r^{-/-}ApoE^{-/-}$ (**B**) B1r expression in $ApoE^{-/-}$ versus $B1rB2r^{-/-}ApoE^{-/-}$ P= 0.0079 (**C**) B1r expression in C57Bl/6 versus $ApoE^{-/-}$ (**D**) B2r expression in $ApoE^{-/-}$ versus $B1rB2r^{-/-}ApoE^{-/-}$ P= 0.0079 and $ApoE^{-/-}$ versus $B2r^{-/-}ApoE^{-/-}$ P= 0.0079. (**E**) B2r expression in C57Bl/6 versus $ApoE^{-/-}$. Aorta sample (n=5) per mouse group.

5.3.3 *Aortic arch atheroma*

The effect of kinin receptor deficiency on aortic arch atheroma severity was assessed by Sudan IV staining. The percentage staining area in the $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice infused with AII was compared (Figure 5.12 and 5.13). We observed an overall reduction in the percentage area stained with Sudan red in $B1rB2r^{-/-}ApoE^{-/-}$, $B2r^{-/-}ApoE^{-/-}$ mice compared to the $ApoE^{-/-}$ when analysed with One-way ANOVA, there was a significant ($P<0.05$) effect of kinin receptor deficiency on aortic arch atheroma for the three groups ($P <0.0001$). Tukey's multiple comparisons test was carried out to compare each mouse group with all other mouse groups ($ApoE^{-/-}$ vs $B1rB2r^{-/-}ApoE^{-/-}$ vs $B2r^{-/-}ApoE^{-/-}$) (Figure 5.13).

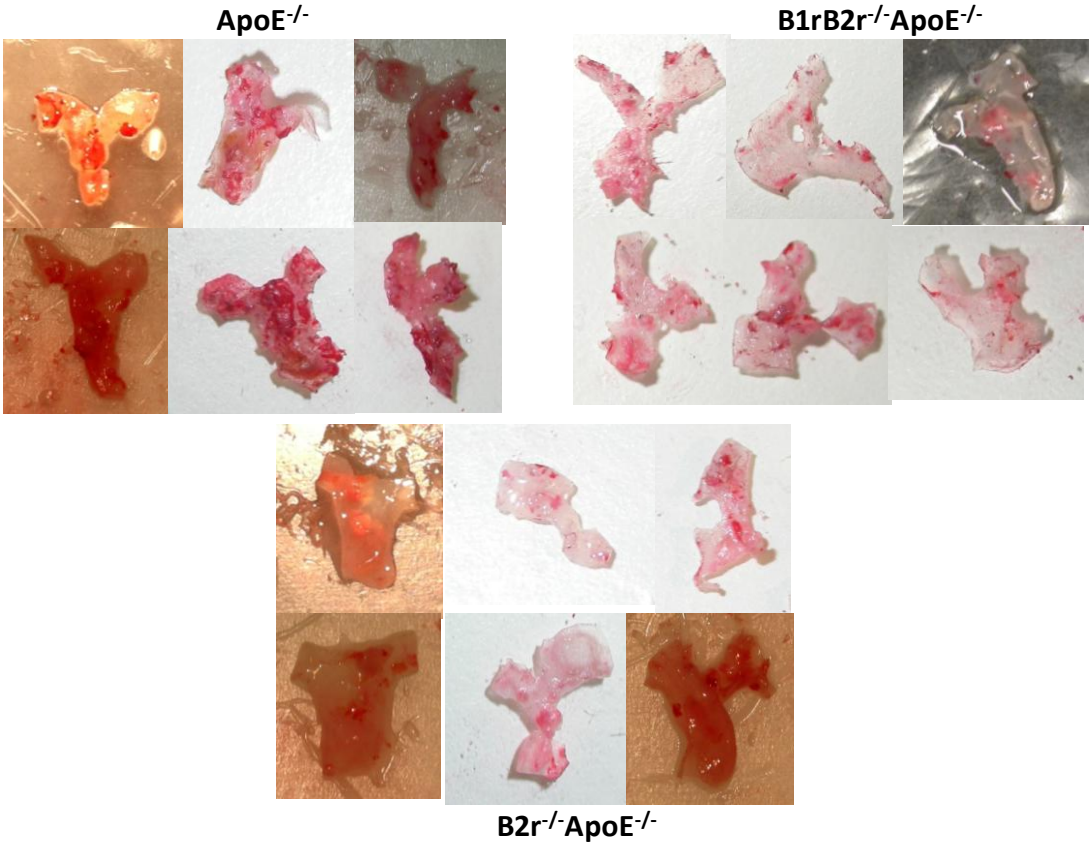
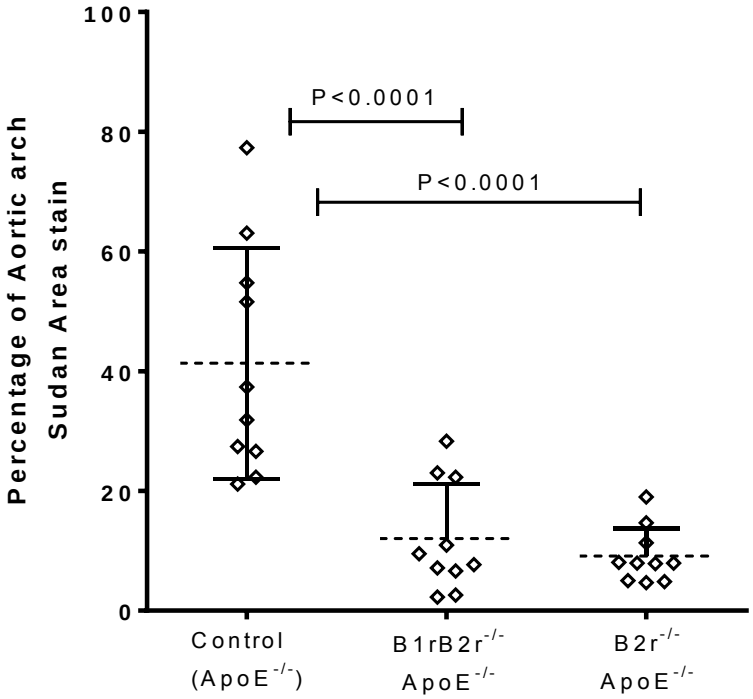


Figure 5.12 Representative aortic arch samples stained with Sudan IV. Areas of red indicate lipid deposition.

A



B

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Actual P Value
ApoE$^{-/-}$ control vs. B1rB2r$^{-/-}$	29.31	15.34 to 43.28	< 0.0001
ApoE$^{-/-}$ control vs. B2r$^{-/-}$	32.22	18.25 to 46.19	<0.0001
B1rB2r$^{-/-}$ vs. B2r$^{-/-}$	2.907	-11.06 to 16.88	0.8642

Figure 5.13 Assessment of atheroma severity in the aortic arch by Sudan IV staining area in AII-infused ApoE$^{-/-}$, B1rB2r$^{-/-}$ApoE$^{-/-}$, and B2r$^{-/-}$ApoE$^{-/-}$ mice after 28 days, calculated by stained red area divided by total area (x100). (**A**) Data presented as dot plots (mean ± SD), maximum aortic diameter (mm). (**B**) Data was analysed by One way ANOVA with Tukeys multiple comparisons test (Table 5.4) AII, angiotensin II; TA, thoracic aorta; SRA, suprarenal aorta; IRA, infrarenal aorta.

5.3.4 *Analysis of circulating neutrophils and monocytes within mice following AII infusion*

The difference in proportion of circulating neutrophils and monocytes populations in kinin receptor-deficient mice compared to the ApoE$^{-/-}$ mice was evaluated using flow cytometry. CD11b^{+}, Ly6G^{+} are neutrophils whereas CD11b^{-}, Ly6G^{-} are monocytes (Figure 5.14 A and B). At baseline, prior to AII infusion the CD11b+, Ly6G+ neutrophil population was lower in all mice groups compared to post AII infusion. Compared with the baseline (n = 25), the percentage of gated leukocytes that were neutrophils was greater in the post AII infused mice in all mouse groups (ApoE$^{-/-}$ mice; 10.98±0.733% vs. 14.36 ±1.588%, B1rB2r$^{-/-}$ApoE$^{-/-}$ mice; 12.08±1.053% vs. 20.23 ±3.658%, and B2r$^{-/-}$ApoE$^{-/-}$ mice; 14.60 ±1.177% vs 21.75 ±2.234%) (Figure 5.14 A and B*)*. There were significantly more neutrophils at baseline in the B2r$^{-/-}$ApoE$^{-/-}$ mice (P=0.0078) compared to the control group (Figure 5.15). There were also significantly more B2r$^{-/-}$ApoE$^{-/-}$ neutrophils at week one compared to the ApoE$^{-/-}$ mice (P=0.0359). During week four the neutrophil population returned to just above baseline level, with the percentage higher in the kinin knockout mice compared to the ApoE$^{-/-}$ mice (Figure 5.15).

At baseline, prior to AII infusion the CD11b+, Ly6G^{-} population was lower in all mice groups compared to post AII infusion. Compared with the baseline (n = 25), the percentage of positive monocytes was greater in the post AII infused mice in all mouse groups (ApoE$^{-/-}$ mice; 13.02±0.756% vs. 16.46 ±1.651%, B1rB2r$^{-/-}$ApoE$^{-/-}$ mice; 12.10±1.034% vs. 13.62 ±1.253%, and B2r$^{-/-}$ApoE$^{-/-}$ mice; 10.32 ±0.662% vs 18.13 ±1.543%) (Figure 5.14 A and B*)*. There was a significantly lower percentage of B2r$^{-/-}$ApoE$^{-/-}$ mice monocytes at baseline compared to the control group (P=0.013) and the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice (P=0.01) (Figure 5.16). The B2r$^{-/-}$ApoE$^{-/-}$ monocyte number decreased in week two (P=0.0051) and four (P=0.008), compared to the control group and the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice (Wk2 P<0.0001) and (Wk4 P=0.002). Indicating less monocytes in circulation of the B2r$^{-/-}$ApoE$^{-/-}$ mice compared to the ApoE$^{-/-}$ mice and the double receptor knockouts (Figure 5.16).

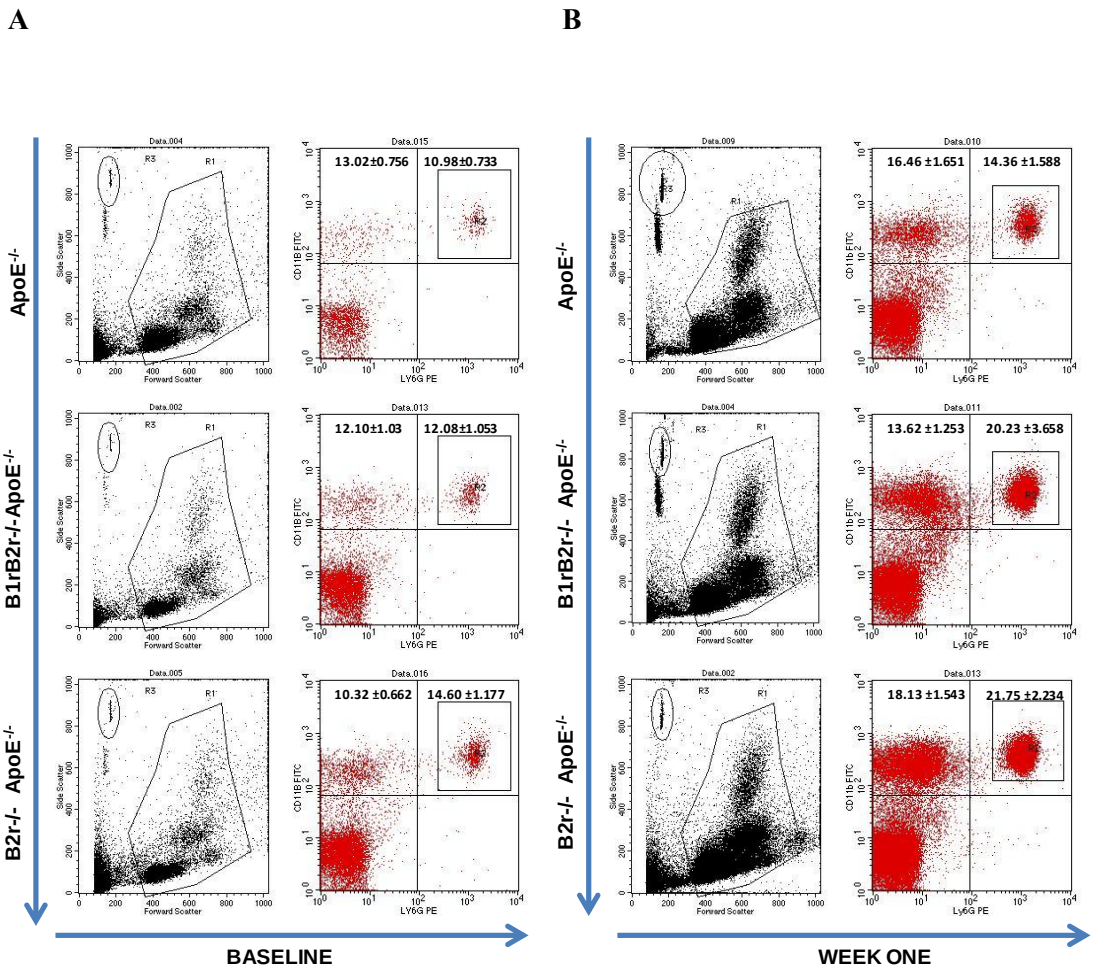


Figure 5.14. Flow cytometry detection of extracellular expression of CD11b and Ly6G on cells in the circulation of AII infused mice. Comparison between (**A**) baseline prior to AII infusion and (**B**) week one, post AII infusion. The scattergrams indicate the forward and side scatter plot for cell size and granularity, first row of (**A**) and (**B**) and the percentage population of the CD11b and Ly6G stained cells, for each of the mice groups; control ($ApoE^{-/-}$ mice), the $B1rB2r^{-/-}$ $ApoE^{-/-}$ mice and the $B2r^{-/-}$ mice $ApoE^{-/-}$.

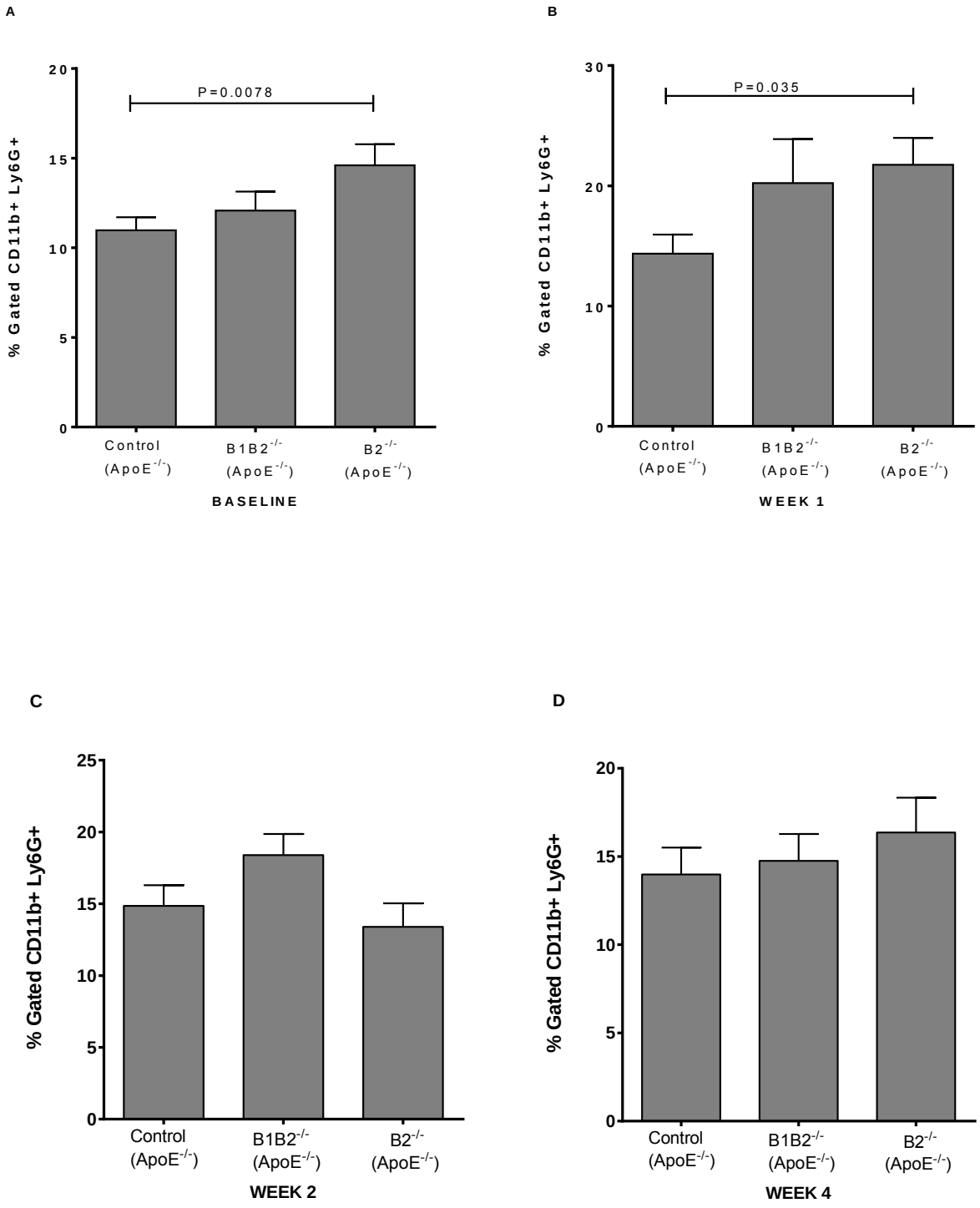


Figure 5.15 Flow cytometry analysis of $CD11b^+$ and $Ly6G^+$ cells (neutrophils) in circulation of AII infused mice. The percentage of $CD11b^+$ $Ly6G^+$ neutrophils within the leukocyte population were monitored prior to AII infusion and post AII infusion over a four week period using flow cytometry on blood from tail bleeds of $ApoE^{-/-}$ mice deficient in kinin B2 receptor compared with relevant $ApoE^{-/-}$ control, and kinin B1/B2 receptor-deficient $ApoE^{-/-}$ mice. Data is presented as Mean±SEM, statistical significance was determined by a Mann–Whitney U test $P<0.005$

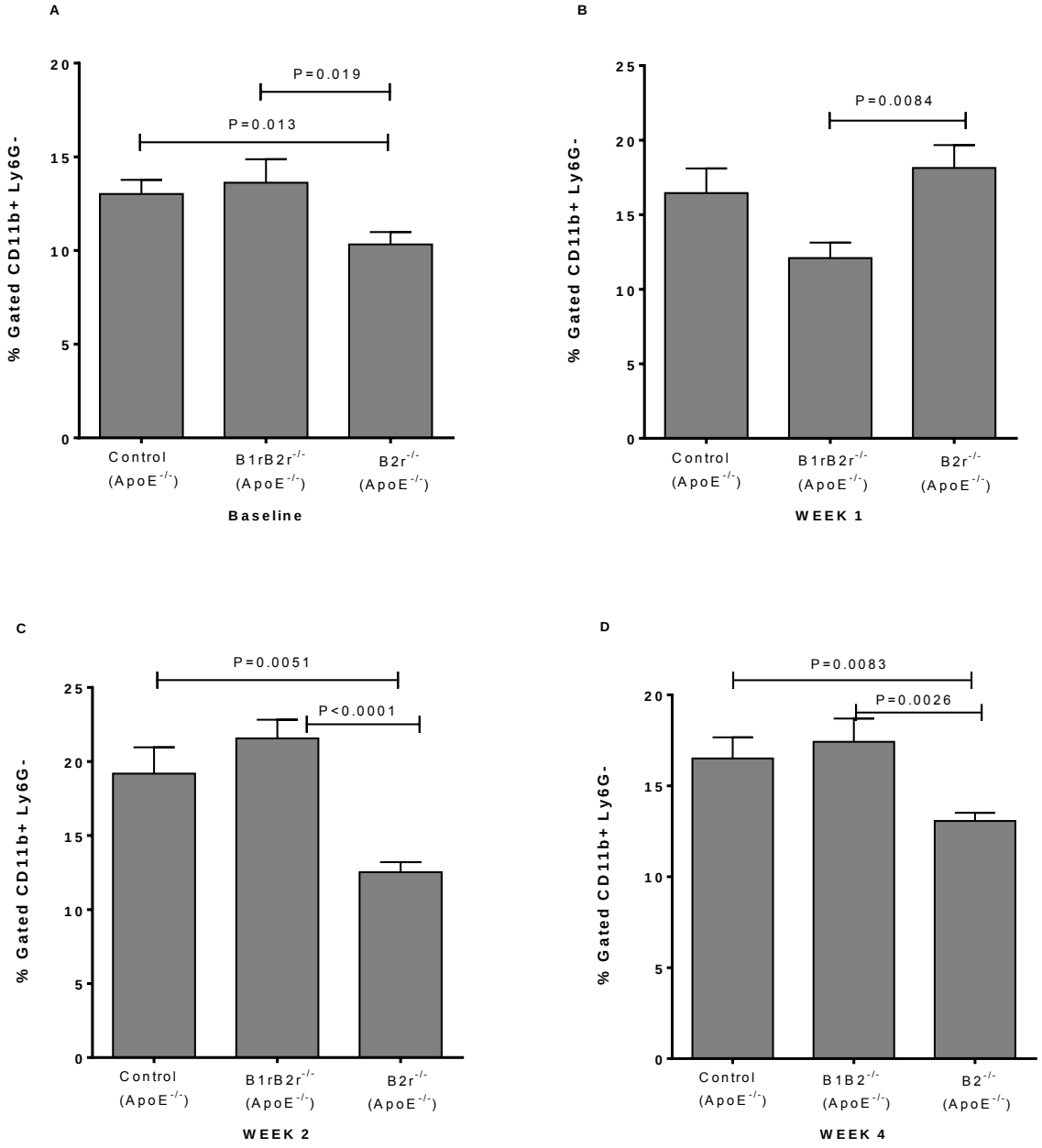


Figure 5.16 Flow cytometry analysis of $CD11b^-$ and $Ly6G^-$ cells (monocytes) in circulation of AII infused mice. The percentage of $CD11b^-$ $Ly6G^-$ neutrophils within the leukocyte population were monitored prior to AII infusion and post AII infusion over a four week period using flow cytometry on blood from tail bleeds of $ApoE^{-/-}$ mice deficient in kinin B2 receptor compared with relevant $ApoE^{-/-}$ control, and kinin B1/B2 receptor-deficient $ApoE^{-/-}$ mice. Data is presented as Mean±SEM, statistical significance was determined by a Mann–Whitney U test $P<0.005$

5.3.5 *Analysis of inflammatory cytokines in plasma of AII infused mice*

The mouse plasma samples from the kinin receptor-deficiency study (section 5.2.2) were next analysed to determine the levels of circulating inflammatory cytokines, using a bead array (Table 5.6). Again, plasma was collected prior to the commencement of AII infusion (baseline) and again 14 days later, by tail bleed. The level of six different inflammatory cytokines (TNF-α, IFN-γ, MCP-1, IL-6, IL-10 and IL-12p70) present in plasma samples taken at baseline and week 2 were assessed.

Consistently higher levels of TNF-α were detected in the plasma from B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ ApoE$^{-/-}$ mice compared to the ApoE$^{-/-}$ control mice. In each of the mouse groups the level of IFN-γ was also lower at baseline compared to week 2, indicating that AII infusion lead to an increase in these cytokine levels. Blood from the control mice harboured lower levels of IFN-γ compared to B2r$^{-/-}$ApoE$^{-/-}$ and the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice, with the latter exhibiting the highest level. In contrast, MCP-1 levels were higher in all the baseline samples of each mouse group compared to week 2, with the highest levels present in the B2r$^{-/-}$ApoE$^{-/-}$ mice. The IL-6 cytokine levels were similar between the control mice and the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice, with lower baseline levels and higher week 2 levels. There was no IL-10 present in any of the samples. The IL-12 levels were highest in the B1rB2r$^{-/-}$ApoE$^{-/-}$ mice, with similar levels in the control and B2r$^{-/-}$ ApoE$^{-/-}$ mice, all had higher week 2 levels compared to the baseline (Table 5.5). These cytokines indicate inflammation.

Table 5.5: Plasma level of inflammatory cytokines in control and Bradykinin receptor-deficient ApoE$^{-/-}$ mice infused with AII over 2 weeks

Cytokine	Baseline			Week 2		
Pg/ml	**ApoE$^{-/-}$**	**B1rB2r$^{-/-}$ ApoE$^{-/-}$**	**B2r$^{-/-}$ ApoE$^{-/-}$**	**ApoE$^{-/-}$**	**B1rB2r$^{-/-}$ ApoE$^{-/-}$**	**B2r$^{-/-}$ ApoE$^{-/-}$**
IFN-γ	*0.07±0.07*	*0.055±0.27*	*0.22±0.16*	*0.33±0.22*	*0.81±0.32*	*0.41±0.18*
IL-6	*3.16±1.68*	*2.12±0.99*	*6.54±2.56*	*21.79±11.70*	*27.47±23.82*	*2.66±1.12*
MCP-1	*15.86±2.8*	*9.62±1.96*	*36.58±1.78*†	*10.01±3.06*	*8.91±1.48*	*14.17±1.78*
TNFα	*0.0±0.0*	*0.49±0.49*	*0.0±0.0*	*0.0±0.0*	*1.34±0.96*	*1.93±1.1*
IL-12	*3.39±1.55*	*9.70±2.06**	*2.05±0.87*†	*4.41±1.76*	*10.68±4.30*	*3.34±1.68*
IL10	*0.0±0.0*	*0.0±0.0*	*0.0±0.0*	*0.0±0.0*	*0.0±0.0*	*0.0±0.0*

*AII, angiotensin II; Serum level of inflammatory cytokines; * P<0.05 compared to baseline control; † P<0.05 compared to baseline B1rB2r$^{-/-}$ ApoE$^{-/-}$ ‡ P<0.05 compared day 14 control; Δ P<0.05 compared to week 2 B1rB2r$^{-/-}$ ApoE$^{-/-}$, Data is presented as Mean±SEM, statistical significance was determined by a Mann–Whitney U test .*

5.3.6 *Determination of plasma MPO levels in AII infused mice*

Seeking to further support the hypothesis that neutrophil activation via the kinin B2 receptor plays a role in the evolution of AAA, mouse plasma samples obtained from kinin receptor-deficient and ApoE$^{-/-}$ mice were assayed for MPO (Section 5.2.2) study. Plasma MPO is a marker of neutrophil activation. Plasma was collected prior to commencement of AII infusion (baseline), and then a second time 2 weeks later, after infusion by tail bleed. The level of MPO present in plasma samples taken at week 2 was calculated as a percentage of baseline for comparative assessment between groups.

The median increase in circulating MPO in AII-infused ApoE$^{-/-}$ mice was 174% over 14 days (Figure 5.17). Plasma from mice lacking expression of both the B1 and B2 receptors exhibited a lower median increase in circulating MPO of 100%, although the lower percentage change was not statistically significant when compared to that of the ApoE$^{-/-}$ controls (P=0.163). A median increase in circulating MPO of only 73% above baseline over 14 days was observed in the plasma of AII-infused ApoE$^{-/-}$ mice deficient only in the B2 receptor, reflecting a

significant (P=0.0006) reduction in neutrophil activation in these animals compared to control mice (Figure 5.17). These findings further support the link between B2 receptors, neutrophil activation and AAA.

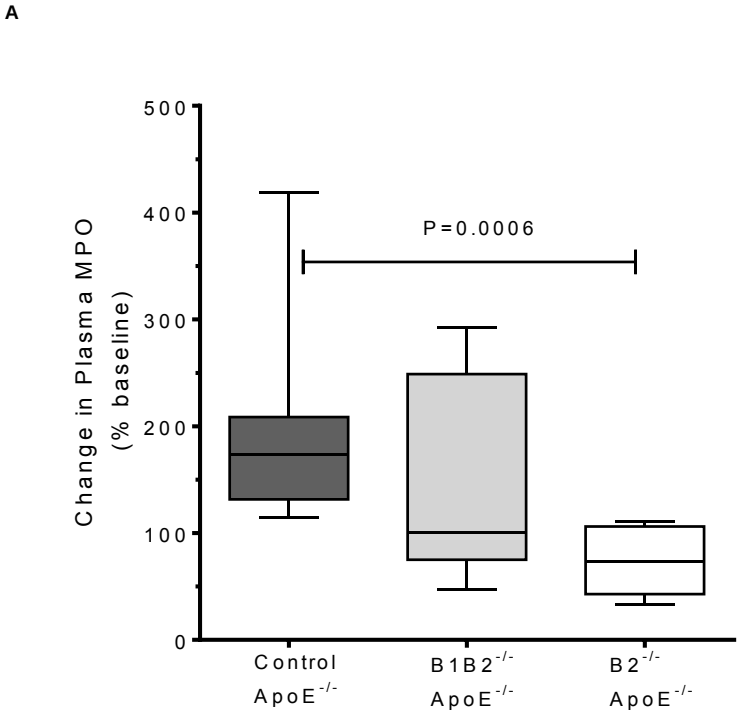


Figure 5.17 Circulating levels of MPO are significantly reduced following 14 days AII-infusion in (**A**) ApoE$^{-/-}$ mice deficient in kinin B2 receptor compared with relevant control (dark grey box). Light-grey box represents kinin B1/B2 receptor-deficient ApoE$^{-/-}$ mice. Data presented as box plots (min max) for percentage change of baseline MPO measured in plasma collected prior to AII infusion. Data was analysed with Mann Whitney U test ***P=0.0006. MPO, myeloperoxidase

5.4 DISCUSSION

Data presented in this chapter shows that kinin receptor deficiency, in a mouse model influences AAA and atherosclerosis. Preliminary microarray data from the AII infused ApoE$^{-/-}$ mouse model identified many genes relevant to aneurysm development or protection, those up-regulated in non aneurysm forming areas indicating a potential protective role (Rush, Nyara et al. 2009). A single nucleotide polymorphism in *KLK1* has been associated with AAA (Biros, Norman et al. 2011). Further preliminary data demonstrates the expression of the B1 and B2 kinin receptors within human AAA biopsies, however B2r predominated and was significantly up regulated in AAA tissue compared to paired control tissue (Unpublished data, Rush et al).

Previously unpublished data from Rush and Moran (VBU, JCU, Townsville) suggested that a kinin receptor agonist clearly promoted AAA development and rupture while a kinin antagonist inhibited AAA development and rupture providing the basis for the subsequent studies presented in this chapter. Initially, the influence of a peptide agonist (B9972, selective to the B2 receptor) and antagonist (B9430, blocks at both B1 and B2 receptors) on AAA development and rupture within the AII mouse model was examined. It was also noted that blockade of the kinin receptor via administration of the kinin receptor antagonist attenuated the ability of AII to induce aortic dilatation, underlining the observed protective effect against aortic rupture. This finding was somewhat surprising as a previous study employing a similar mouse model reported that kinin B1r deficiency promoted the development of AAA (Merino, Todiras et al. 2009).

Neutrophils contain the complete system for the synthesis and release of the kallikrein-kinin cascade, and express both the B1r and B2r (Proud and Kaplan 1988; Bhoola, Elson et al. 1992; Henderson, Figueroa et al. 1994). Active neutrophils are also present within the adventitia and mural thrombus of AAA, providing evidence for their involvement in AAA (Cohen, Keegan et al. 1991). The effect of neutrophil depletion on the ability of B2r agonism to exacerbate AII

induced AAA in a mouse model has been demonstrated in a preliminary study (unpublished data, Moran et al). Depletion of neutrophils in $ApoE^{-/-}$ mice inhibited the ability of the B2r agonist B9972 to promote AAA development and rupture. This data suggesting an important link between neutrophils and AAA is supported by other studies in rodent models demonstrating a role for neutrophils in promoting AAA development. The mechanisms driving their aortic recruitment and role in AAA pathogenesis is poorly understood (Eliason, Hannawa et al. 2005; Hannawa, Eliason et al. 2005; Kurihara, Shimizu-Hirota et al. 2012; Watanabe, Fujioka et al. 2012).

In this chapter, the role of kinin receptors in AAA was investigated using a kinin receptor deficient AII infused mouse model. It was hypothesised that kinin receptor deficiency would inhibit AII-induced aortic aneurysm formation in the $ApoE^{-/-}$ mouse, and studies were conducted employing $ApoE^{-/-}$ mice deficient in either the B2 receptor alone ($B2r^{-/-}ApoE^{-/-}$), or combined deficiency of both kinin receptors ($B1rB2r^{-/-}ApoE^{-/-}$). Study outcomes demonstrated that both B2r and B1rB2r deficiency decreased aortic dilatation, although the effect was most marked in the $B1rB2r^{-/-}ApoE^{-/-}$ genotype. This was initially demonstrated using ultrasound and confirmed at study end upon aortic morphometric analysis, this was particularly noted in the SRA region of the aorta. Deficiency in both B1 and B2 receptors promoted model survival, with $B1rB2r^{-/-}ApoE^{-/-}$ mice exhibiting a decreased aortic rupture rate compared control $ApoE^{-/-}$ mice, although not significant. In contrast, aortic rupture and survival rate remained comparable between $B2r^{-/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ controls. An explanation for this could be receptor compensation as described in the previous chapter (Chapter 4, section 4.3.4), where the expression of the B1r was shown to be significantly increased in $B2r^{-/-}ApoE^{-/-}$ mice compared to $ApoE^{-/-}$ control mice. Other studies support this finding demonstrating that expression of each of the two kinin receptors is mutually linked and modulation of one can result in the up regulation or compensatory expression of the other (Xu, Carretero et al. 2005). The kinin receptors have a role in inflammation, not only in leukocyte recruitment but also cytokine stimulation.

The effect of kinin receptor deficiency on atheroma severity was also investigated. The AII mouse model has been used by many groups to study vascular pathologies such as atherosclerosis and abdominal aortic aneurysms (Cassis, Gupte et al. 2009). In the current study it was noted that $ApoE^{-/-}$ control mice had significant development of atheroma in the aortic arch compared to either of the $B2r^{-/-}ApoE^{-/-}$ or $B1rB2r^{-/-}ApoE^{-/-}$ mice . It was also shown that $B1rB2r^{-/-}ApoE^{-/-}$ mice had markedly more aortic arch atheroma compared to the $B2r^{-/-}ApoE^{-/-}$ mice. It has been suggested that up-regulation of B1 receptor expression has been noted in atheromatous plaques. Kinins are potent vasodilators that are involved in the regulation of vascular tone. The activation of the kinin receptors results in the release of nitric oxide, which can lead to damage to vascular smooth muscle cells, further leading to the development of atherosclerosis (McLean, Perretti et al. 2000) .

It was demonstrated in unpublished preliminary data (Moran et al) (Appendix 1), and in other studies, in the AII animal model of AAA that depleting or blocking neutrophil function inhibits the development of aneurysms (Eliason, Hannawa et al. 2005). Inflammation also has a major role in the development of AAA. Given the links between the kallikrein-kinin system, neutrophils, and AAA pathogenesis, the role of kinin receptor-mediated neutrophil activation in experimental aortic aneurysm was examined. Circulating level of myeloperoxidase (MPO) is often used as a diagnostic marker for neutrophil activation in inflammatory diseases such as vasculitis. It is not only a useful biomarker for disease but is also pathogenic. The release of the serine protease MPO directly leads to cell destruction and the additional recruitment of inflammatory cells. High levels of MPO can lead to vessel damage and this process is clearly linked to the activation of neutrophils (Lau et al, 2004). Plasma concentration of MPO was markedly lower in mice that were deficient in kinin B2 receptors compared to control mice, suggesting that the inhibition of aortic dilatation in response to AII in these mice was potentially associated with a reduced state of neutrophil activation within the vessel wall.

In support of this, flow cytometry analysis of blood indicated that the population of activated CD11b and Ly6G positive neutrophils increased in the circulation of mice after AII infusion compared to the baseline. However, the relative number of these circulating cells was higher in the kinin receptor deficient compared to the control group. The percentage population of these cells was also higher at weeks one and two compared to week four indicating neutrophil circulation peaked during the early period. It is thought that the kinin receptors play a role in promoting neutrophil transmigration through blood vessel walls via endothelial cell junctions. This may in part explain why the neutrophil numbers were higher in the plasma of the kinin deficient compared to the control mice (Correa *et al*, 2005; Calixto *et al,* 2004). In addition, circulating numbers of CD11b positive monocytes were shown to be reduced in $B2r^{-/-}ApoE^{-/-}$ mice compared to both $B1rB2r^{-/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ controls. This is particularly relevant in light of the role of macrophages in the development of atherosclerosis (Fukumoto, Libby et al. 2001; Kolodgie, Gold et al. 2003) and the fact that aortic arch atheroma was correspondingly less severe in $B2r^{-/-}ApoE^{-/-}$ mice.

Higher levels of inflammatory cytokines were found within the plasma of AII compared to control mice. In contrast, levels of the anti-inflammatory cytokine IL-10 were relatively decreased. The increase in MCP-1, IL-6 and IL-12 levels is perhaps as expected in the AAA model, where these cytokines potentially contribute to the pathogenesis of vascular disease and injury (Sprague *et al,* 2009). Why they are increased in the kinin receptor deficient mice remains unclear at this point and requires further investigation.

The current mouse model studies demonstrated that both B2r deficiency alone and combined B1rB2r deficiency inhibited AII-induced aortic dilatation, although this effect was most marked in $B1rB2r^{-/-}ApoE^{-/-}$ mice when taking into account aortic rupture and model survival data. There seems to be no relationship between blood pressure (Chapter 4) and aneurysm formation, yet combined deficiency in B1r and B2r leads to an increase in blood pressure, indicating both

receptors are required to maintain normal haemodynamic pressures. Atheroma severity is decreased by kinin receptor deficiency, suggesting there is a relationship between the receptors and atheroma.

In conclusion, this data illustrates that kinin receptor deficiency or blockade and neutrophils depletion limits AAA development, progression and rupture within a mouse model. Further investigation into the role of neutrophils and kinins receptors in AAA, and the mechanisms involved are investigated in the following chapter.

CHAPTER 6

THE ACTION OF KININ RECEPTOR ACTIVATORS AND BLOCKERS ON MURINE NEUTROPHILS IN VITRO

Chapter 6

THE ACTION OF KININ RECEPTOR ACTIVATORS AND BLOCKERS ON MURINE NEUTROPHILS IN VITRO

6.1 INTRODUCTION

Neutrophils can synthesize, store and release the components of the active kallikrein-kinin system (Gustafson, Schmaier et al. 1989; Henderson, Figueroa et al. 1994; Saxena, Shaw et al. 2011). Neutrophils are activated by plasma kallikrein and are also producers of tissue kallikrein, underpinning a potential link between kinins and neutrophils in inflammation (Bhoola, Elson et al. 1992). Both kinin receptors are expressed on neutrophils (Bhoola, Elson et al. 1992; Bertram, Misso et al. 2007). Activation of the B1 receptor induces chemotaxis of neutrophils (Gustafson, Schmaier et al. 1989). Neutrophils are present within the adventitia and mural thrombus of human AAA (Cohen, Keegan et al. 1991), where they have the potential to release serine proteases which can damage the vessel wall. This neutrophil activation triggers the release of neutrophil granules, which damage the aortic tissue, particularly in the destruction of the collagen matrix (Wright, Moots et al. 2010). The recruitment and activation of neutrophils is a hallmark of vascular inflammation. The release of serine proteases such as myeloperoxidase (MPO) during the respiratory burst is a good marker of neutrophil activation (Lau, Mollnau et al. 2005). Significantly, it was demonstrated in unpublished preliminary data (Moran et al) (Appendix 1), and in other studies, in the AII animal model of AAA that depleting or blocking neutrophil function inhibits the development of aneurysms (Eliason, Hannawa et al. 2005). Neutrophil depletion abrogated the ability of the B2 receptor agonist to promote AAA within a mouse model of AAA (Moran, Unpublished data) (Appendix 1).

Data presented in the previous chapter (Chapter 5) demonstrated that kinin receptor deficiency in mice protected against experimental AAA in a mouse model. It was also demonstrated that the neutrophil activation (MPO levels) was decreased in kinin receptor deficient mice. In this

chapter, an *in vitro* investigation of the effect of kinin receptor activators and blockers on neutrophil activity, and the production of MPO was assessed, indicating the activation status. The use of AII and the kinin receptor activators and blockers on the neutrophils mimics the AII mouse model in the previous chapter. AII is a know activator of neutrophils (Paragh, Szabó et al. 2002) but the effect of the kinin receptor activators and blockers remains to be assessed.

Neutrophil activation via the B1r and B2r was assessed, using the production of MPO as a marker of activity. This was carried out to provide mechanistic insight into neutrophil degranulation, the release of serine proteases such as MPO are thought to lead to vessel damage in vivo (Wright, Moots et al. 2010). This vessel damage may contribute to the pathology of AAA.

It is hypothesised that:

> Kinin receptor activation of neutrophils stimulates the release of the potent serine protease myeloperoxidase

Specifically this Chapter aims to:

1. Determine the pattern of B1 and B2 kinin receptor expression in Apo $E^{-/-}$ mice neutrophils

2. Assess the action of kinin receptor-agonists, and antagonists on MPO production, *in vitro*

6.2 EXPERIMENTAL METHODS

6.2.1 *Receptor expression of kinin B1 and B2 receptors on neutrophils*

Total RNA was extracted from purified neutrophils (Chapter 3, section 3.2.1) (1.2×10^6/ml) obtained from C57Bl/6, $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice using RNeasy mini kit (Qiagen, Australia) according to manufacturer's instructions (Chapter 3, section 3.1.7). SYBR Green PCR primers Bdkrb1 and Bdkrb2 (RT^2 qPCR primers) were commercially available from Qiagen and the GAPDH primers were also commercially obtained as described in Chapter 3, section 3.1.8). Quantitative real time PCR (qPCR) was performed for Bdkrb1 and Bdkrb 2 genes as described in Chapter 3, section 3.1.8) using a three step melt thermal cycling program.

6.2.2 *Dose response for AII and kinin peptide neutrophil activation*

The concentration of AII and the kinin peptides to use for *in vitro* neutrophil stimulation assays was determined experimentally by dose response assays. Human plasma physiological level of AII is 3fmol/ml (10.40 pg/ml) and BK is 0.2– 7.1 pmol/ml (25.5 ng/ml). Purified neutrophils (Chapter 3, section 3.2.1) (1.2×10^6/ml) from C57Bl/6 mice were resuspended in HBSS (Invitrogen) and seeded into a 96 well plate and incubated for 30 minutes at 37°C, 5% CO_2 in the presence of either HBSS alone (negative control), phorbol 12-myristate 13-acetate (PMA, Sigma; 50 ng/ml, positive control), AII alone 10 ng/ml, 1 ng/ml**,** 0.5 ng/ml, 0.10 ng/ml and 0.05 ng/ml, or Kinin B2 receptor agonist (B9972) 0.1nM, 1nM and 10nM, the doses were selected from previous studies (Koyama, Sato et al. 2000; Vega, El Bekay et al. 2010). Plates were then centrifuged (650g; 5 min) and the supernatant taken for assessment of MPO activity using a commercially available fluorescence activity assay (EnzChek® Myeloperoxidase (MPO) Activity Assay Kit, Invitrogen). Briefly, the activity assay determines the peroxidation activities of MPO in cell lysates. Peroxidation is detected using a nonfluorescent Amplex UltraRed

reagent, which is oxidized by the H_2O_2 generated redox intermediates MPO-I and MPO-II to form a fluorescent product. The supernatant standards and samples were run in duplicate.

A BMG PolarStar plate reader with 485 nm excitation and 530 nm emission was used to read plates. The MPO activity of each sample was expressed as relative fluorescence units (RFU) and was calculated by subtracting the fluorescence measured from the negative control wells (HBSS only) from the fluorescence of each test well. Serial dilutions of the provided MPO standards were prepared and assayed as described in the manufacturer's instructions (Appendix 4). Mean intra-assay coefficient of variation for MPO determined from the standard curves of duplicate standards was 0.64%. The mean inter-assay coefficient of variation for MPO determined from the standard curves of two separate assays was 4.07%.

6.2.3 *Immunofluorescence assays of activated neutrophils.*

In order to perform immunofluorescence assays to confirm/ visualise neutrophil activation (6.2.1), purified neutrophils (Chapter 3, section 3.2.1) (1.2×10^6/ml) were resuspended in HBSS (Invitrogen) and seeded onto glass coverslips treated with 0.001% polylysine (Sigma), and treated with PMA, (Sigma; 50 ng/ml), AII (Sigma;10 ng/ml) alone or left unstimulated (HBSS) and incubated for 30 minutes at 37°C, 5% CO_2. Non-adherent cells were discarded, by washing with PBS. Adherent cells were fixed with 100% ethanol at 4°C for 10 minutes. Subsequently, the slides were blocked with in PBS and 1% bovine serum albumin for 1 hour at RT. Incubation with primary antibody, goat anti-mouse MPO (1:50) (Santa Cruz), was performed for 30 minutes at RT. Secondary antibodies was Alexa Fluor 488conjugated rabbit anti- goat IgG (1:1000) (Abcam). Nuclei were stained with DAPI (1 μg/ml) (Sigma). Images were acquired on a Zeiss ApoTome Imager.Z1 fluorescence microscope using Axio Vision 4.8 software (Carl Zeiss, Germany).

6.2.4 *Assessment of neutrophil activation by kinin receptor agonists and antagonists.*

Purified neutrophils (Chapter 3, section 3.2.1) (1.2×10^6/ml) obtained from C57Bl/6 mice by cardiac puncture were resuspended in HBSS (Invitrogen) and seeded onto a 96 well plate and incubated for 30 minutes at 37°C, 5% CO_2 in the presence of either HBSS alone (negative control), phorbol 12-myristate 13-acetate (PMA, Sigma; 50 ng/ml, positive control), AII (10 ng/ml) alone, AII + kinin B2 receptor agonist B9972 (10 nM), AII + kinin B1/B2 receptor antagonist B9430 (10 nM), or AII + kinin B2 receptor antagonist B9330 (10 nM). Plates were then centrifuged (650 g; 5 min) and the supernatant taken for assessment of MPO activity using a commercially available fluorescence activity assay (EnzChek® Myeloperoxidase (MPO) Activity Assay Kit, Invitrogen) as described above.

6.2.5 *Statistics*

Data was analysed using GraphPad Prism (version 6) and S Plus (version 8). Parametric or non-parametric tests were applied as appropriate to distribution of data.

End-point data, such as the neutrophil activation data (Section 6.2.3 and 6.2.4) was analysed using One-way ANOVA (parametric), and Tukey's post-hoc test or Kruskal-Wallis test (non-parametric ANOVA) and Dunn's post-hoc test was used to compare the differences between the groups. The data was expressed as mean ± SD (dot plots) or median and interquartile range (box plots), respectively.

Receptor expression of B1B2 receptors on neutrophils was analysed using Mann Whitney U test, statistical significance was taken as *$P<0.05$, and data is presented as Mean±SEM.

6.3 RESULTS

6.3.1 *Kinin B1 and B2 receptor expression in murine ApoE$^{-/-}$ neutrophils*

Relative gene expression levels of the B1 and B2 receptors on purified neutrophils from C57Bl/6, ApoE$^{-/-}$, B1rB2r$^{-/-}$ApoE$^{-/-}$ and B2r$^{-/-}$ApoE$^{-/-}$ mice was compared, by quantitative real time PCR, using Bdkrb1 primers and Bdkrb 2 (Chapter 3, section 3.1.7). After normalising to GAPDH, the relative expression of both B1 and B2 receptors, were assessed (Figure 6.1). There was no B1 receptor expression in the B1rB2r$^{-/-}$ApoE$^{-/-}$ as expected. Again, as in Chapters 4 and 5, the B1 receptor expression in the B2r$^{-/-}$ApoE$^{-/-}$ was greater than in the ApoE$^{-/-}$ mice, indicating up regulation. There was no B2 receptor expression in the B1rB2r$^{-/-}$ApoE$^{-/-}$ or B2r$^{-/-}$ApoE$^{-/-}$ mice as expected in the neutrophils.

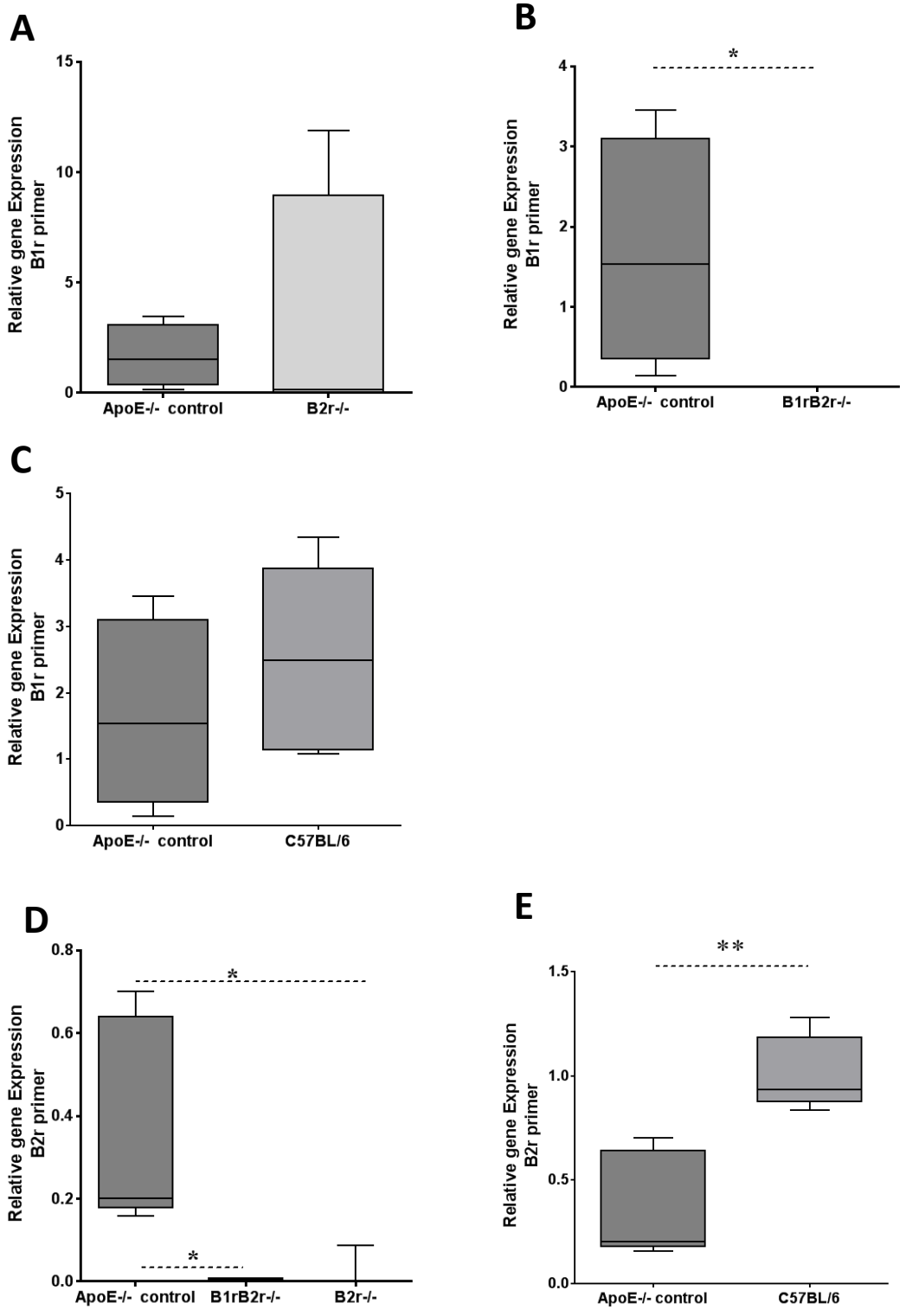


Figure 6.1 Relative gene expression calculated using concentration-Ct-standard curve and normalised by average expression of GAPDH. Data presented as box plots (min max), Analysed with Mann Whitney ***P<0.05. (A)** B1r expression in $ApoE^{-/-}$ versus $B2r^{-/-}ApoE^{-/-}$ (**B**) B1r expression in $ApoE^{-/-}$ versus $B1rB2r^{-/-}ApoE^{-/-}$ P= 0.0286 (**C**) B1r expression in C57Bl/6 versus $ApoE^{-/-}$ (**D**) B2r expression in $ApoE^{-/-}$ versus $B1rB2r^{-/-}ApoE^{-/-}$ P= 0.0159 and $ApoE^{-/-}$ versus $B2r^{-/-}ApoE^{-/-}$ P= 0.0179. (**E**) B2r expression in C57Bl/6 versus $ApoE^{-/-}$ P=0.0079. Neutrophil sample (n=5) per mouse group.

6.3.2 *Dose response for AII and kinin peptide neutrophil activation*

The measurement of the respiratory burst (using MPO activity) of stimulated neutrophils under the influence of agonists and antagonists, such as a kinin receptor agonist in combination with AII, may provide insights into the mechanisms of activation by assessing whether agonists enhance and antagonists block MPO activity in neutrophils. Initially a dose response assay was carried out to determine the optimal concentration of AII or the B2r agonist required to activate the neutrophils. In this assay neutrophil activation is measured by determination of MPO activity. The observation that neutrophil activation resulted in increased MPO activity and the optimal concentration for this activation was assessed. The results analysed using Kruskal-Wallis test (non-parametric ANOVA) (Figure 6.2 & 6.3). The concentration of AII selected for future experiments was 10ng/ml. The concentration of the B2r agonist B9972 used for further experiments was 10nM.

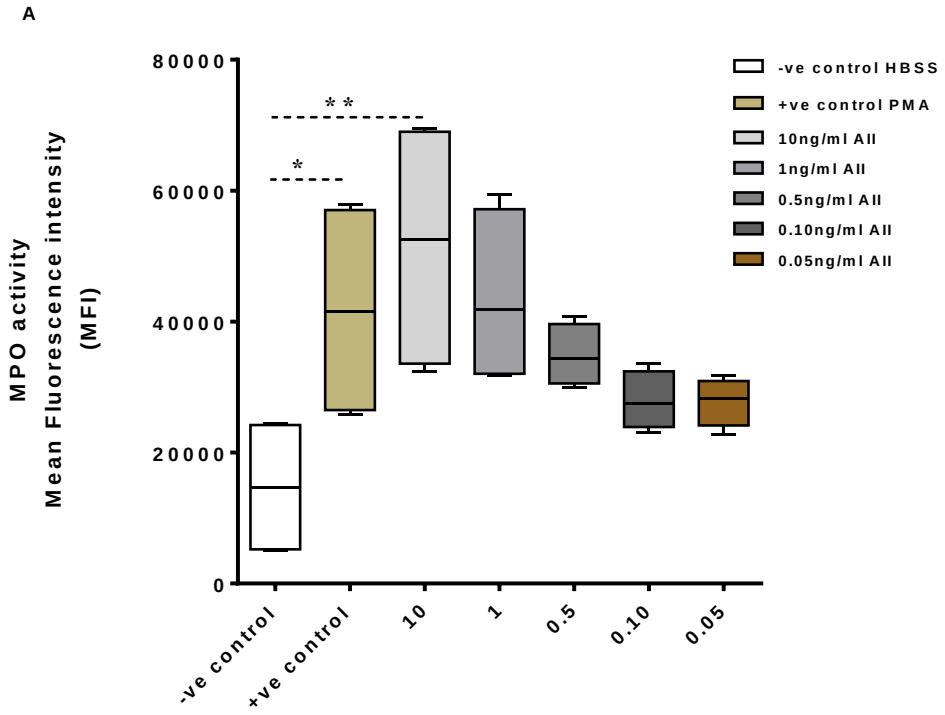


Figure 6.2 Activation of purified murine neutrophils (1.2×10^6/ml) *in vitro* monitored using AII dose response assays. Data represents neutrophils suspended in HBSS alone (negative control), PMA (50 ng/ml, positive control) in both panels. Panel **(A)** AII 10 ng/ml, 1 ng/ml**,** 0.5 ng/ml, 0.10 ng/ml and 0.05 ng/ml**.** (**A**) Data was analysed by Kruskal-Wallis test with Dunn post-hoc multiple comparisons test.* P=0.0369 and **P=0.0046.

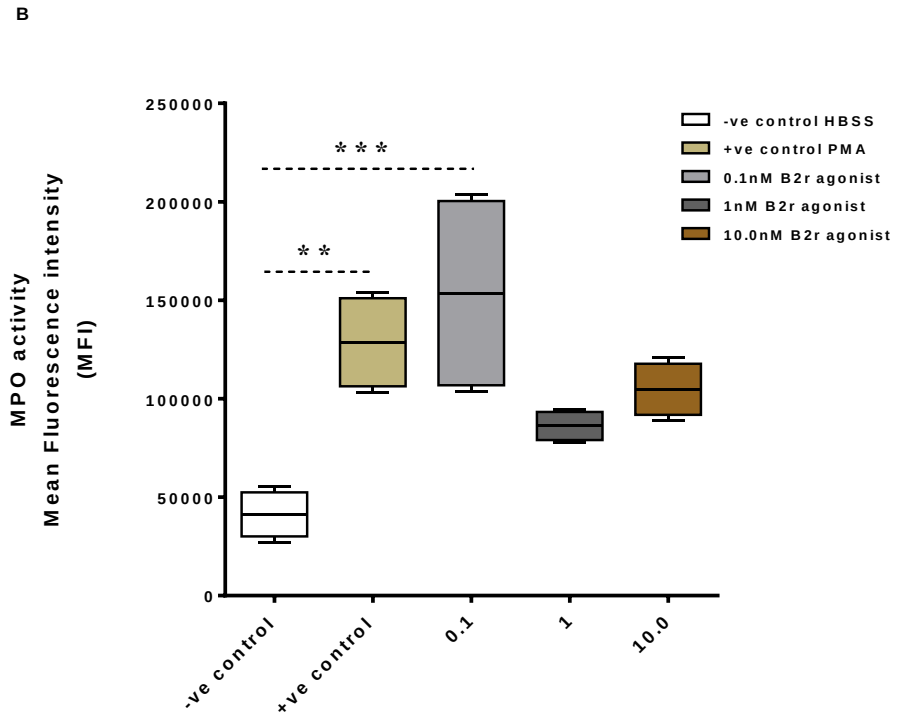


Figure 6.3 Activation of purified murine neutrophils (1.2×10^6/ml) *in vitro* monitored using dose response assays. Data represents neutrophils suspended in HBSS alone (negative control), PMA (50 ng/ml, positive control) in both panels. **(B)** Kinin B2 receptor agonist 0.1nM, 1nM and 10nM. Data presented as box plots (min max). Data was analysed by Kruskal-Wallis test with Dunn post-hoc multiple comparisons test. (**B**) **P=0.0024 and ***P=0.0002

6.2.3 *Neutrophil activation in vitro*

The activation of neutrophils at the site of AAA formation is likely to be an important step in disease development and progression. Activation is associated with a respiratory burst and the release of serine proteases such as MPO. Additionally extracellular DNA can be found as a component of so called neutrophil extracellular traps (NETs). NETs are extracellular web-like structures harbouring DNA, and granular proteins that are triggered by cell lysis associated with reactive oxygen species produced by NADPH oxidase in activated neutrophils (Brinkmann, Reichard et al. 2004). The contents of the neutrophil granules also spill into the surrounding area and this process may lead to tissue damage in the body, via the vessels. To monitor the activation of neutrophils in vitro, enriched murine neutrophils were incubated with HBSS alone (negative control), PMA (50 ng/ml, positive control), or AII (10 ng/ml) alone and the cells were

analysed using immunofluorescence microscopy to monitor and visualise activation markers and signs of cell destruction. The PMA- and AII-stimulated but not HBSS control neutrophils were observed to lose the distinct granule pattern seen in control cells and in some cases the cell contents were apparently being extruded from the cell (Figure 6.4). Thus, these observations indicated that the neutrophils were being activated following treatment with PMA or AII.

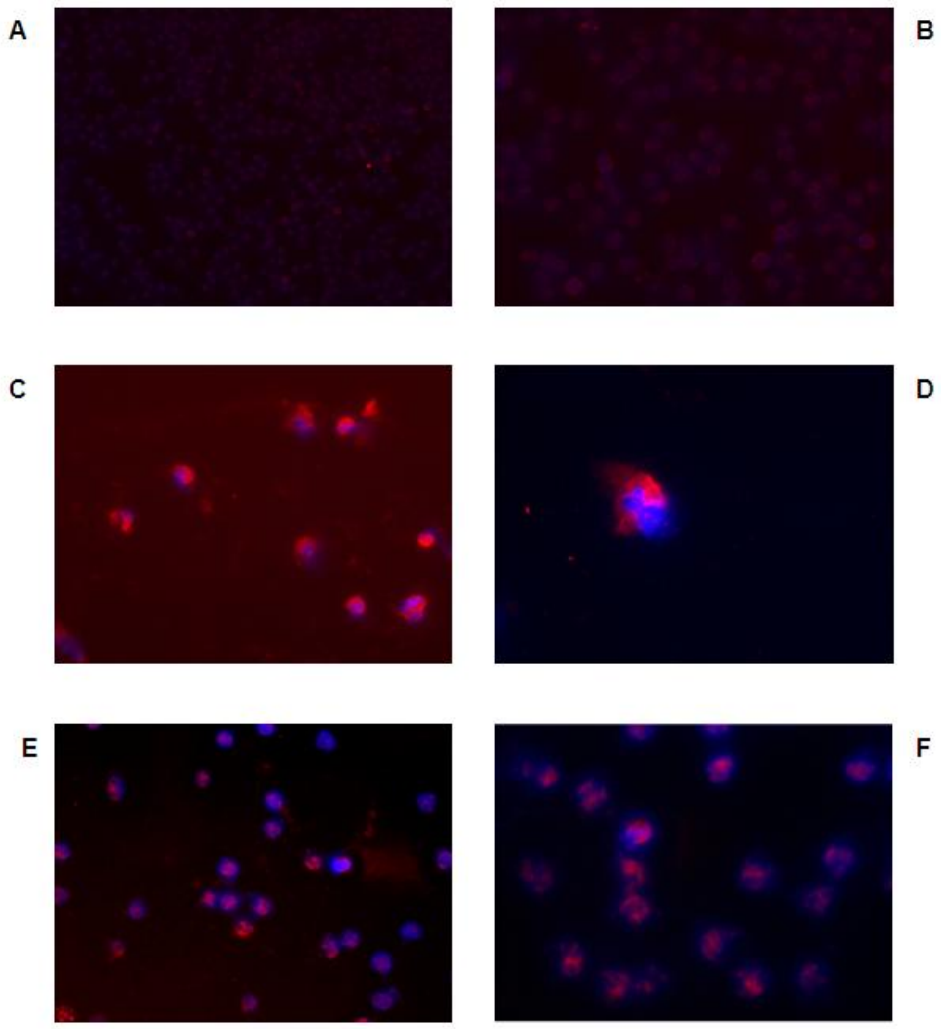


Figure 6.4 Immunofluorescence microscopy of neutrophil activation and MPO production. (**A**) Murine neutrophils incubated with HBSS alone (magnification x 20). (**B**) HBSS alone (magnification x 40). (**C**) Neutrophils were activated with PMA (50 ng/ml) (magnification x 40). (**D**) PMA (50 ng/ml) (magnification x 63). (**E**) Neutrophils were activated with AII (10 ng/ml) (magnification x 40). (**F**) AII (10 ng/ml) (magnification x 63). All neutrophils were immunostained for MPO and DNA. AII, angiotensin II; MPO, myeloperoxidase; HBSS, Hanks Buffered Salt Solution.

6.2.4 *Augmentation of AII-induced neutrophil activation by kinin B2 receptor agonist and inhibition by B1 and B2 receptor antagonism in vitro.*

Neutrophils are considered important in the pathogenesis of AAA (Eliason, Hannawa et al. 2005), (Rizas, Ippagunta et al. 2009), (Lindeman, Abdul-Hussien et al. 2009), (Pagano, Zhou et al. 2009). Thus modulation of kinin B1 and B2 receptor expression in neutrophils could be an important cellular mechanism under pinning the *in vivo* effects of kinin receptor activation ($ApoE^{-/-}$ mice) and deficiency ($B1rB2r^{-/-}$ and $B2r^{-/-}$) on AAA observed in the AII mouse model (Chapter 5, section 5.2.2). Thus, the action of B2 receptor agonist and B1r and B2r antagonists on neutrophil activation was investigated *in vitro*. To this end, murine neutrophils obtained from C57Bl/6 mice were suspended in Hank's Balanced Salt Solution (HBSS) alone (baseline for cell activation), or exposed to AII in the presence or absence of either B2 receptor agonist or B1r and B2r antagonists. Culture supernatants were then assayed for activity of MPO, a hallmark indicator of neutrophil respiratory burst, as a measure of neutrophil activation. The observation that neutrophil activation resulted in increased MPO activity ($P<0.05$) was demonstrated using Kruskal-Wallis test, there was an overall significant effect of MPO activity for the treatment groups combined P=0.0007. Dunn post-hoc multiple comparisons test was carried out to compare each treatment group with all other treatment groups (AII, B2r agonist, B1rB2r antagonist and B2r antagonist) (Figure 6.5A and Table 6.1).

As expected, incubation of neutrophils in the presence of PMA resulted in significant cell activation with a median MPO activity in culture supernatants from 1.5- 12 RFU above unstimulated neutrophils (P=0.0114) (Figure 6.5A). Exposure of neutrophils to AII alone produced a similar yet less dramatic effect on neutrophil activation with MPO activity increased 1.5- 9 RFU above baseline (Figure 6.5A). Addition of the kinin B2 receptor agonist to AII-stimulated neutrophils resulted in significantly increased neutrophil activation compared to cells exposed to AII alone and compared to unstimulated cells (P=0.0006). Remarkably, MPO

activity in the supernatant of these cells was in fact 1.5-fold higher than that of PMA-treated cells (positive control) (Figure 6.5). In contrast, culture of AII-stimulated neutrophils in the presence of B2r or B1/B2 receptor antagonists suppressed neutrophil activation with MPO activity similar to base line levels and was lower than that measured in cells exposed to AII alone (Figure 6.5A). The major contribution of the B2r to neutrophil activation was demonstrated by the specific B2 agonist and AII induced MPO activity, with a change from 9 - 17 RFU. Whereas the dual antagonist had no significant difference compared to the B2r antagonist, suggesting a lesser role for the B1r in signalling. Furthermore the B2 agonist exacerbates AII effects on neutrophil activity. The blocking of the B2r abrogates AII mediated neutrophil activation in vivo. Why this occurred is unclear, although an explanation may be derived from emerging evidence of cross-talk and inter-regulation between receptors of the angiotensin and kinin systems (Rodrigues ES 2009), (Xu, Carretero et al. 2013).

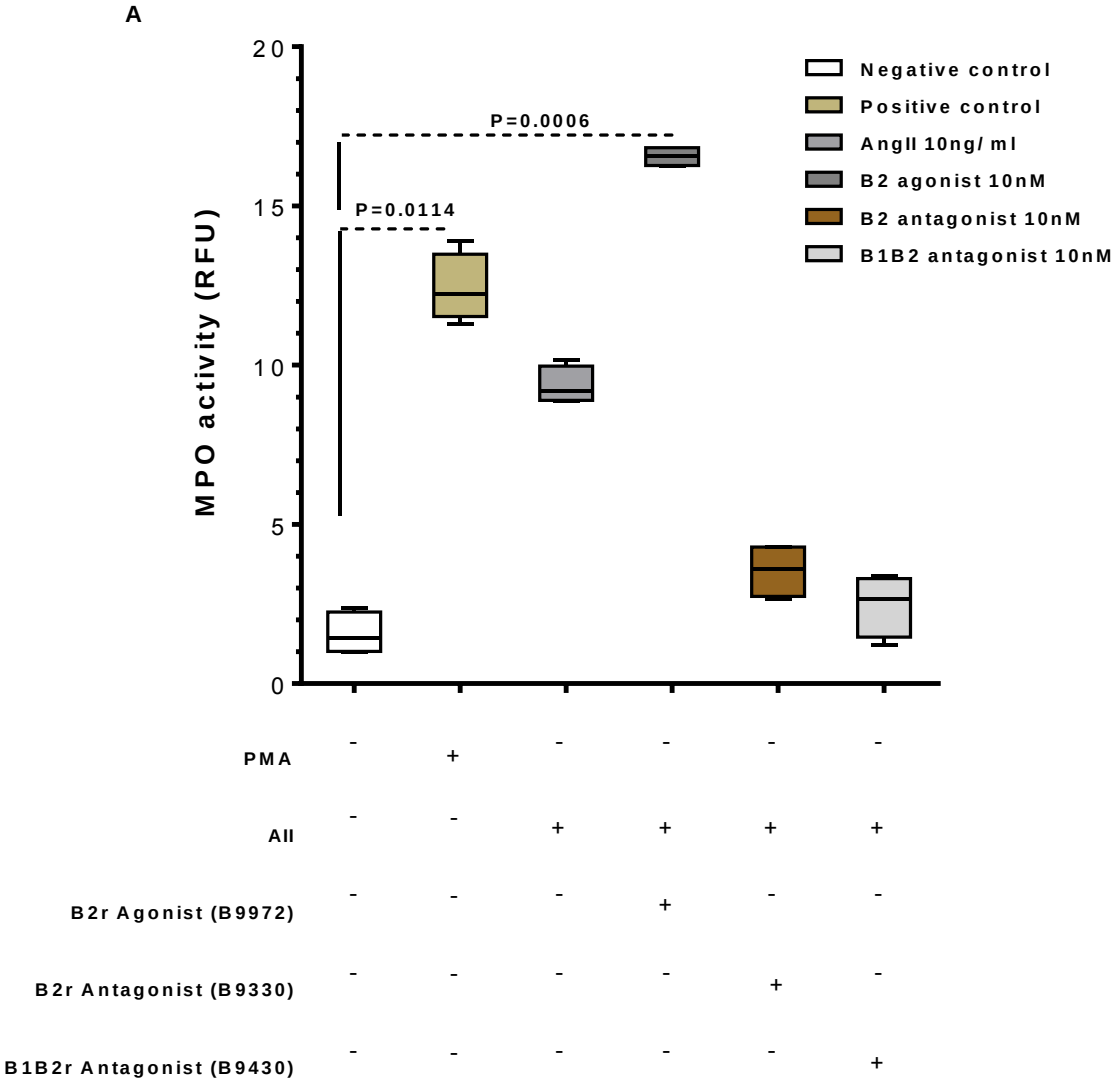


Figure 6.5 AII activation of murine neutrophils *in vitro* is augmented by kinin B2 receptor agonist. **(A)** Purified neutrophils (1.2×10^6/ml) were suspended in HBSS alone (negative control), PMA (50 ng/ml, positive control), AII (10 ng/ml) alone, AII + kinin B2 receptor (B2r) agonist (10 nM), AII + kinin B1/B2 receptor antagonist (10 nM), or AII + kinin B2 receptor antagonist (10 nM). The B2 receptor agonist (B2r agonist) significantly enhanced AII-stimulated MPO activity (i.e. neutrophil activation). AII-mediated neutrophil activation was significantly inhibited by B2 (B2r antagonist) and combined B1/B2 (B1B2r antagonist) receptor blockade. Data presented as box plots (min max) for relative fluorescence units (RFU) of n=4 repeat experiments.

Table 6.1 Data was analysed by Kruskal-Wallis test with Dunn post-hoc multiple comparisons test

Dunn's multiple comparisons test	Mean rank diff.	Summary	P Value
Negative control vs. Positive control	-15.25	*	0.0114
Negative control vs. AII 10ng/ ml	-11.25	ns	0.1222
Negative control vs. B2 agonist 10nM	-19.25	***	0.0006
Negative control vs. B2 antagonist 10nM	-6.250	ns	> 0.9999

AII, angiotensin II; MPO, myeloperoxidase; PMA, phorbol 12-myristate 13-acetate.

6.4 DISCUSSION

Published work and data presented earlier in this thesis supports the concept that the kallikrein-kinin system and neutrophils play a role in the pathogenesis of AAA. It was demonstrated that kinin receptor deficiency in mice protected against experimental AAA compared to ApoE controls in an AII mouse model. It was also demonstrated that the neutrophil activation (MPO levels) was decreased in kinin receptor deficient mice. Examination of end-stage human AAA biopsies demonstrates a transmural infiltrate of inflammatory cells including cells of the innate immune system such as neutrophils (Abdul-Hussien, Hanemaaijer et al. 2009; Houard, Touat et al. 2009; Abdul-Hussien, Hanemaaijer et al. 2010; Folkesson, Silveira et al. 2011). A number of studies in rodent models have demonstrated that neutrophils promote AAA development however the mechanism driving their aortic recruitment is incompletely understood (Eliason, Hannawa et al. 2005; Hannawa, Eliason et al. 2005; Kurihara, Shimizu-Hirota et al. 2012; Watanabe, Fujioka et al. 2012). The kallikrein-kinin system has long been recognized for its role in driving innate immune responses and neutrophils have been identified to contain the full complement of molecules required to activate the kallikrein-kinin cascade (Proud and Kaplan 1988; Bhoola, Elson et al. 1992; Henderson, Figueroa et al. 1994).

Consequently, here, the role of kinin receptor agonists and antagonists in neutrophil activation and the production of MPO were examined. In Chapter 5 the levels of MPO, a marker of neutrophil activation, were found to be markedly reduced in mice that were deficient in kinin B2 receptors compared to similar AII treated control mice. The levels of MPO are often used as a diagnostic marker in inflammatory diseases, including vasculitis. High levels of MPO can lead to vessel damage and this process is clearly linked to the activation of neutrophils (Lau et al, 2004). Indeed, the evidence for neutrophil activation in this chapter further supports a role for MPO production and release via kinin receptor activation. The release of this MPO could have an effect on surrounding vessels in vivo and perhaps even lead to AAA associated vessel damage although this was not confirmed in this Chapter.

In vitro evidence in this Chapter indicates that the kinin B2 receptor agonist (B9972) can promote neutrophil activation, this neutrophil activity was even higher compared to activation with AII or the control PMA alone, in contrast the kinin B1B2/ B2 receptor antagonists (B9330 and B9430), which actually inhibited neutrophil activation. This evidence further links the kinin receptors to neutrophil activation, and the production of MPO. This data further confirms that the kinin receptors are involved in neutrophil activation and MPO production. This production of MPO is believed to play a role in vessel damage (Lau et al, 2004), leading to inflammation and further recruitment of leukocytes. The role of kinins, kinin receptors and neutrophils has been investigated in other studies, but the role in AAA pathogenesis via neutrophil activation and MPO production is limited and the mechanisms need further clarifying. Bockmann et al noted that kinins are responsible for the release of inflammatory mediators, such as cytokines on leukocytes, particularly neutrophils, this was demonstrated using kinin peptides. The kallikrein system and neutrophils were investigated in relation to proteinase-activated receptor (PAR-4) mediated inflammation (Houle et al, 2005), particularly looking at granulocyte recruitment via the release of tissue kallikrein on neutrophils, and the activation of PAR on neutrophils, this affected vascular permeability leading to the production of active kinins and recruitment of further neutrophils. The chemotactic effects of kinin B1 receptor agonists on neutrophils were investigated by Ehrenfeld et al, in relation to inflammation in humans, in vitro experiments on human neutrophils indicated that kinin B1 and B2 receptor agonists induced migration of cells in chemotaxis chambers.

The use of the AII in the *in vitro* study was intended to mimic the animal study in the previous chapter. AII is a known activator of neutrophils (Paragh, Szabó et al. 2002), yet the kinin B2 receptor agonist (B9972) activated the neutrophils further, leading to more MPO activity. AII induces leukocyte recruitment to the vessel wall, and constitutes a hallmark of early stages of atherosclerosis, AT1 receptors for AII are found in circulating neutrophils (Vega et al, 2010).

It is thought that the kinin receptors play a role in promoting the transmigration of neutrophil into the endothelial cell junctions and the vessel wall. In the previous chapter it was noted that the numbers of neutrophils were higher in the plasma of the kinin deficient compared to the control mice (Calixto *et al,* 2004).

It was also confirmed that neutrophils express both the B1r and the B2r. The expression of the B1r was increased in the B2r deficient mice. Evidence suggests that activity at kinin B1 receptors can amplify or substitute for the kinin B2 receptor, especially in chronic inflammation (Ahluwalia and Perretti 1999), (Phagoo, Poole et al. 1999). In fact neutrophils contain the complete system for the synthesis and release of kinins.

Thus taken together our findings suggest that kinins promote the activation of neutrophils via B2 receptors. In summary, MPO not only serves as an index of neutrophil recruitment and activation, but it also displays traditional cytokine like properties that can serve to modulate the activation state of leukocytes in inflammatory vascular disease. Increased intraluminal levels of MPO is a hallmark of systemic inflammatory disease (Lau *et al*, 2004).

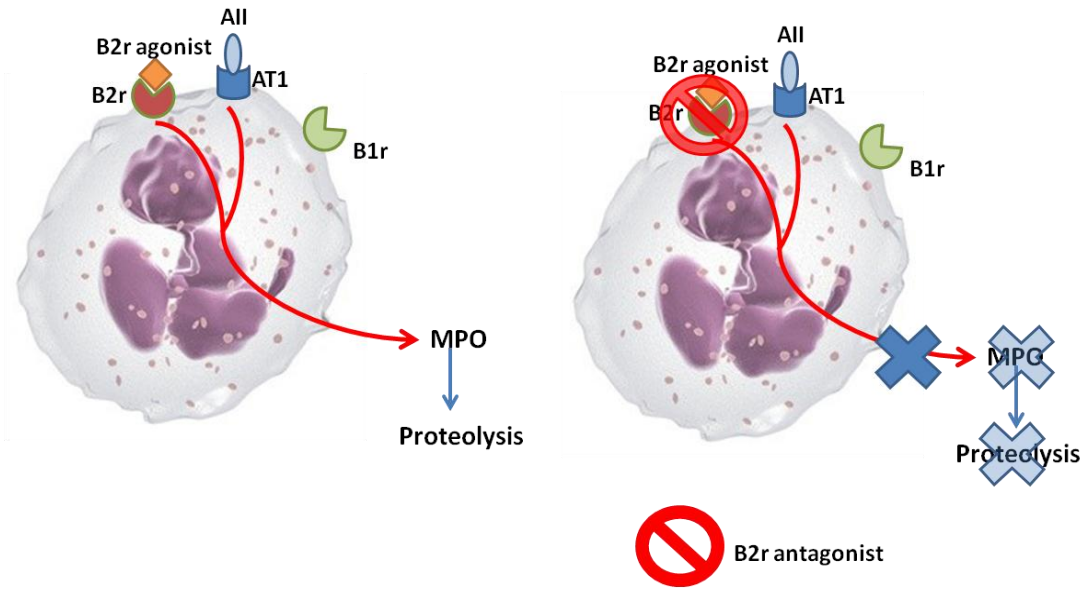


Figure 6.6 Demonstrating the role of kinin receptor agonists and antagonists on MPO production from the activated neutrophil.

CHAPTER 7

GENERAL DISCUSSION

GENERAL DISCUSSION

Abdominal aortic aneurysm (AAA) is a common, late age-at-onset disorder affecting ~1.6 to 7.2 % of the general population age 50 years and older (Conway, Malkawi et al. 2012; (Lenk, Tromp et al. 2007). AAA is asymptomatic and can remain undetected for long periods of time, leading to a potential increase in aneurysm size and potentially rupture. Open surgery is one treatment option for this condition, with a 5% peri-operative mortality rate in elective patients (Arko, Lee et al. 2002) although endovascular aneurysm repair (EVAR) is favoured. AAA is being increasingly identified at an early stage due to the availability of ultrasound screening programs and the large volume of abdominal imaging being undertaken for unrelated problems. Previous randomized trials have demonstrated that early elective surgery of AAAs measuring <55mm does not reduce mortality (2002; Lederle, Wilson et al. 2002; Ouriel, Clair et al. 2010; Cao, De Rango et al. 2011). Once identified therefore small AAAs are simply monitored by repeat imaging however up to 70% of these expand to a size that later requires surgery (2002; Lederle, Wilson et al. 2002; Ouriel, Clair et al. 2010; Cao, De Rango et al. 2011). There is currently no effective drug therapy to limit AAA expansion (Golledge and Norman 2011).

Tissue biopsies reveal hallmark pathology of AAA as a persistent proteolytic imbalance that results in excess matrix destruction and progressive weakening of the arterial wall (Abdul-Hussien, Soekhoe et al. 2007). Examination of the end-stage human AAA biopsies demonstrates a transmural infiltrate of inflammatory cells including cells of the innate immune system such as neutrophils (Abdul-Hussien, Hanemaaijer et al. 2009; Houard, Touat et al. 2009; Abdul-Hussien, Hanemaaijer et al. 2010; Folkesson, Silveira et al. 2011). In

several vascular conditions inflammatory cells can enter the aorta wall by migration leading to localised damage and this could be the case in AAA, although the primary process driving the progression is unresolved (Lindeman, Abdul-Hussien et al. 2009; Abdul-Hussien, Hanemaaijer et al. 2010). These migrating cell populations involve neutrophils, macrophages and T cells (Daugherty and Cassis 2004; Lindeman, Abdul-Hussien et al. 2009), that release a cascade of chemokines and cytokines, such as IL-6, which then activate factors such as proteases, leading to degradation of the vessel wall.

Daugherty *et al*, noted that an infusion of Angiotension II induced enhanced formation of abdominal aortic aneurysms in apolipoprotein E (ApoE) deficient mice, with males being more susceptible to AAA formation, just as in humans. AAA is often associated with atherosclerosis. The angiotensin II (AII) mouse model has been used extensively by many groups to study the contribution of AII-induced hypertension to several vascular pathologies, such as atherosclerosis and the formation of AAAs (Cassis, Gupte et al. 2009). The key mechanisms contributing to the progression of AAA in this mouse model include inflammation, ECM degradation, angiogenesis and VSMC loss (Daugherty and Cassis 2004). The use of animal models has proven to be particularly useful in the study of the early phase development of AAA, including the analysis of inflammation and cell recruitment. A number of studies in rodent models have demonstrated that neutrophils promote AAA development however the mechanism driving their aortic recruitment is incompletely understood (Eliason, Hannawa et al. 2005; Hannawa, Eliason et al. 2005; Biros, Norman et al. 2011; Kurihara, Shimizu-Hirota et al. 2012; Watanabe, Fujioka et al. 2012).

A role for the kallikrein system, a group of blood proteins that play a role in inflammation, has been implicated in AAA using the murine Angiotension II model (Daugherty and Cassis 2004). The kallikrein-kinin system has long been recognized for its role in driving innate immune responses and neutrophils have been identified to contain the full complement of molecules

required to activate the kallikrein-kinin cascade (Proud and Kaplan 1988; Bhoola, Elson et al. 1992; Henderson, Figueroa et al. 1994). Previous genetic studies have highlighted genetic regions within the kallikrein-kinin system to be associated with aneurysm development (Shibamura, Olson et al. 2004; Van Vlijmen-Van Keulen, Rauwerda et al. 2005) (Yousef, Chang et al. 2000; van der Voet, Olson et al. 2004) (Biros, Norman et al. 2011). Bradykinin, which is a principal effector of the kallikrein system (Margolius 1995), is a vasoactive peptide that influences many processes including vascular permeability and inflammation. There are two types of bradykinin receptors, known as B1 and B2, which are found in humans and other mammals, including the mouse (Moreau, Garbacki et al. 2005). The B1 receptor is barely detectable under normal physiological conditions (Bourdet, Pecher et al. 2010) and expression is up-regulated in injured tissue and after exposure to inflammatory cytokines, including IL-1 and TNFα (Zhou, Polgar et al. 1998). The B2 receptor is expressed constitutively and has been detected in many tissue including on endothelial cells, smooth muscle cells, fibroblasts, mesangial cells and polynuclear neutrophils (Couture, Harrisson et al. 2001).

Clinically, the current deficiency in the management of AAA is the availability of effective drugs to limit AAA progression, this thesis is aimed at assessing the role of the kinin receptors in AAA progression and the assessment of blockers of this pathway as potential therapeutic targets.

The primary aims of the study were:

1. To determine the role of kinin receptors in aortic aneurysm using the AII mouse model in which atherosclerosis and cardiac function were additionally assessed, and,

2. To demonstrate that action via the kinin receptors on neutrophils leads to neutrophil activation and production of MPO, a serine protease that stimulates inflammation and matrix degradation within the artery wall.

In this study it was shown that kinin receptor deficiency reduces experimental AAA and atherosclerosis. Data obtained from studies using mice deficient in B1 and/or B2 receptors (Chapter 5) provides some evidence for the contribution of kinin receptors in the pathogenesis of both AAA and aortic atheroma. These studies demonstrated that both B2r deficiency alone and combined B1r and B2r deficiency inhibited the induction of AAA. Deficiency of both the B1 and B2 receptors increased survival of the mice compared to the $ApoE^{-/-}$ control group, and also the $B1rB2r^{-/-}ApoE^{-/-}$ had a decreased rupture rate compared to the control group although this was not significant. This finding was surprising as a previous study employing the same mouse model reported that kinin B1 receptor deficiency promoted the development of AAA (Merino, Todiras et al. 2009) The expression of the two kinin receptors has previously been demonstrated to be linked and deficiency of one of the receptors shown to promote up regulation of the other (Xu, Carretero et al. 2005), this was confirmed in Chapter 4 significantly in the heart tissue, and in Chapter 5 and 6 in the aorta and neutrophils, although not significant, demonstrating that B2 receptor deficiency leads to increased expression of the B1 receptor.

Preliminary studies in the Vascular Biology Unit (James Cook University) demonstrated that kinin B2 receptors are up regulated in human AAA biopsies, and in a mouse model of AAA, a B2 receptor agonist promoted, while a kinin receptor antagonist, inhibited AAA development and rupture and a B2 receptor antagonist inhibited progression of established AAA (unpublished data, Rush et al). Further, neutrophil depletion abrogated the ability of the B2 receptor agonist to promote AAA

within a mouse model (unpublished data, Moran et al). Based on these observations together with the current data demonstrating kinin receptor deficiency limits aortic aneurysm and atherosclerosis (Chapter 5), it is hypothesised that the B2 receptor is the key kinin receptor involved in promoting AAA.

Inflammation within the artery wall is a characteristic feature of AAA and neutrophils, immune-inflammatory leukocytes, are believed to play an important role AAA pathogenesis, demonstrated by animal studies in which depletion or blockade of neutrophil function resulted in the inhibition of aortic dilatation and rupture (Eliason, Hannawa et al. 2005). Myeloperoxidase (MPO), a marker of neutrophil activation, is released from activated neutrophils may contribute to vessel wall damage (Lau et al, 2004). All components of the kallikrein-kinin signaling system and kinin production have been identified in neutrophils (Bhoola, Elson et al. 1992). Given that kinin receptor deficiency limited aortic dilatation in an *in vivo* model of AAA (Chapter 5), whether action via kinin receptors affected neutrophil activation was investigated (Chapter 6). Serum MPO was markedly reduced in mice that were deficient in kinin B2 receptors compared to the $ApoE^{-/-}$ control mice after AII infusion. *In vitro*, activation of purified neutrophils, indicated by the measurement of MPO activity in the assay supernatant, was stimulated or inhibited when challenged with kinin B2 receptor agonist or antagonist, respectively. Together, these findings provide evidence for kinin B2 receptor-mediated activation of neutrophils in aortic dilatation.

The population of circulating activated ($CD11b^{+}$ $Ly6G^{+}$) neutrophils increased in $ApoE^{-/-}$ mice in response to AII infusion. Interestingly, the number of these circulating cells was higher in kinin receptor deficient mice compared to ApoE-/- control mice. The percentage population of these cells was higher at weeks one and two compared to week four indicating neutrophil circulation peaked during this period. It is thought that kinin receptors mediate neutrophil transmigration through endothelial cell junctions into the artery wall (Correa *et al*, 2005;

Calixto *et al,* 2004), which may account for the increased numbers of circulating neutrophils observed in kinin deficient mice compared to ApoE-/- controls.

The kallikrein-kinin system not only plays a major role in inflammation (Duchene and Ahluwalia 2009), they are up regulated during vasodilatation and smooth muscle contraction (Tschope, Heringer-Walther et al. 2000), and also play a central role in the modulation of cardiovascular function (Cayla, Todiras et al. 2007). Kinins are believed to play a role in the regulation of blood pressure (Hecquet, Tan et al. 2000), (McGiff and Quilley 1980), (Sharma, Uma et al. 1996). The use of kinin agonists and antagonists, and also mice deficient in kinin receptors have made the study of these mechanisms more assessable (Cayla, Todiras et al. 2007), (McGiff and Quilley 1980), (Duka, Duka et al. 2006), (Madeddu, Parpaglia et al. 1995). The peptide angiotensin II also plays a role in the increase of blood pressure, including vasoconstriction. In the current study, blood pressure and heart rate prior to AII infusion, then change in blood pressure and heart rate in response to AII infusion over time, was evaluated in $ApoE^{-/-}$, $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice to assess whether the reduction in aortic dilatation and atherosclerosis observed in the kinin receptor deficient mice (Chapter 5) was associated with AII-induced changes in cardiac function. Data in Chapter 4 demonstrated that kinin deficiency for both receptors lead to an increase in blood pressure in non AII infused mice. This was not the case in the $B2r^{-/-}ApoE^{-/-}$ mice, which had a similar blood pressure to the control mice. When infused with AII, all mice groups experienced an elevation in blood pressure, again this effect was noticed greater in the kinin deficient $B1rB2r^{-/-}ApoE^{-/-}$ mice. Other groups have noted similar; Duka *et al* suggested that blockade of both kinin receptors is required for increased blood pressure in Wistar rats. In the same study, inhibition or absence of the B2r was associated with the up regulation of B1r, with B1r taking on the hemodynamic properties of B2r suggesting that both kinin receptors participate in vasoregulation and can compensate for the other (Duka, Duka et al. 2006). Madeddu *et al* noted that rats given Hoe 140 (B2r antagonist) during prenatal and postnatal phases of life showed greater systolic pressures, heart rates, and body weights than controls (Madeddu, Parpaglia et al. 1995). Benetos

et al also found that a kinin antagonist (B4146, a competitive antagonist) produced an increase in mean arterial pressure in Wistar rats, while heart rate remained unchanged (Benetos, Gavras et al. 1986). Other rodent studies have demonstrated no change in blood pressure with kinin receptor antagonism or deficiency (Cayla *et al*. 2007) (Bao, Gohlke et al. 1992). The difference in these studies by other groups and those carried out in this thesis include, different mice strains, different time points and even different animal types, all of which will alter the outcome and give different results.

A decreased heart rate was observed in $B2r^{-/-}ApoE^{-/-}$ mice compared to both $B1rB2r^{-/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ controls in non AII infused mice (Chapter 4). AII infusion had little effect on the heart rate of any mice group. Other studies have reported that heart rate of $B1rB2r^{-/-}$ mice is significantly lower compared to controls (Cayla, Todiras et al. 2007), while Yang *et al* did not observe any difference in blood pressure or heart rate between kinin-deficient mice or rats and their controls (Yang, Liu et al. 1997).

An interesting observation in Chapter 4 is that kinin deficient mice develop cardiac hypertrophy, most extensively in $B1rB2r^{-/-}ApoE^{-/-}$ mice compared to the control mice. Kinins are growth-inhibitory to cardiomyocytes. Knockout of kinin B2 receptor (B2R) signalling causes dilated and failing cardiomyopathy (Emanueli, Maestri et al. 1999; Maestri, Milia et al. 2003). Kinins improve cardiac function and act as cardioprotective agents via activation of signal transduction pathways that generate NO (Emanueli, Maestri et al. 1999). This links the importance of the kinin receptors not only to blood pressure and heart rate, but also to cardiac dysfunction, as data in this thesis and other published data demonstrates that kinins are protective and the blockade of kinins via the receptors leads to hypertrophy or dysfunction.

It has been noted that patients with AAA frequently have atherosclerosis, yet it remains unknown if this is due to similar risk factors or a casual relationship between diseases. It has been suggested that AAA is a pathological response to aortic atherosclerosis. Another theory is

arterial remodelling (Golledge and Norman 2010). The AII mouse model has been used extensively by many groups to study the contribution of AII-induced hypertension to several vascular pathologies, such as atherosclerosis and the formation of AAAs (Cassis, Gupte et al. 2009). The effect of kinin deficiency on atheroma severity was also investigated in this thesis (Chapter 5). It was shown that the $ApoE^{-/-}$ control mice had significantly increased atheroma in the aortic arch compared to the $B1rB2r^{-/-}ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$ mice after AII infusion. This result is consistent with other studies involving AII and atherosclerosis. AII stimulation *in vivo* results in the production of reactive oxygen species, leading to vascular inflammation in atherosclerosis (Ross 1999). It has been suggested that up-regulation of B1 receptor expression has been noted in atheromatous plaques (McLean, Perretti et al. 2000). The B1 receptor is believed to be involved in chronic inflammation, including expression in human atherosclerotic lesions, and Merino *et al* noted that deletion of the B1 receptor in $ApoE^{-/-}$ mice aggravates atherosclerosis. Results in this thesis noted that mice deficient in the B2 receptor ($B1rB2r^{-/-}$ $ApoE^{-/-}$ and $B2r^{-/-}ApoE^{-/-}$) were protected against atheroma, suggesting a role for the B2 receptor in the pathogenesis.

This thesis has linked the role of the kinin receptors to inflammation, AAA and atherosclerosis pathogenesis and also it was noted that mice deficient in the kinin receptors had cardiac hypertrophy and increased blood pressure, indicating a role also in modulation of cardiovascular function. Many studies in mice have demonstrated that AII infusion induces cardiac hypertrophy and AAA, and the Kallikrein –kinin system has been linked to both of these diseases.

Ultimately any therapy for AAA is aimed at preventing aortic rupture. For ethical reasons it is very difficult to assess mechanisms promoting AAA rupture in patients. In the current study use of kinin deficiency reduced the frequency of AAA development. The ability of the kinin agonist to promote neutrophil activation was also noted. These finding highlight the potential

importance of kinins receptors and neutrophils in AAA development and also provide a model to investigate other strategies which could limit the critical end stage of AAA.

Generalisation of the findings from this study should take into account the limitations of translating from animal models to humans. While we have thoroughly investigated the role of kinin receptors using a mouse model applying these findings to patients directly would require trials of kinin modifying agents in patients. It is well recognized that angiotensin converting enzyme inhibitors promote the half-life of kinins by slowing their degradation (Marceau, Hess et al. 1998; Moreau, Garbacki et al. 2005). It has been reported that angiotensin converting enzyme inhibitors promote human AAA progression in one study and it is possible that this result could be related to stimulation of the kinin cascade (Miyake and Morishita 2009). The effect of angiotensin converting enzyme inhibitors on AAA progression remains controversial and is currently being examined in a randomized controlled trial (Golledge and Norman 2011). True examination of the impact of kinins on human AAA would require trialing of agents which directly inhibit the kinin B2 receptor.

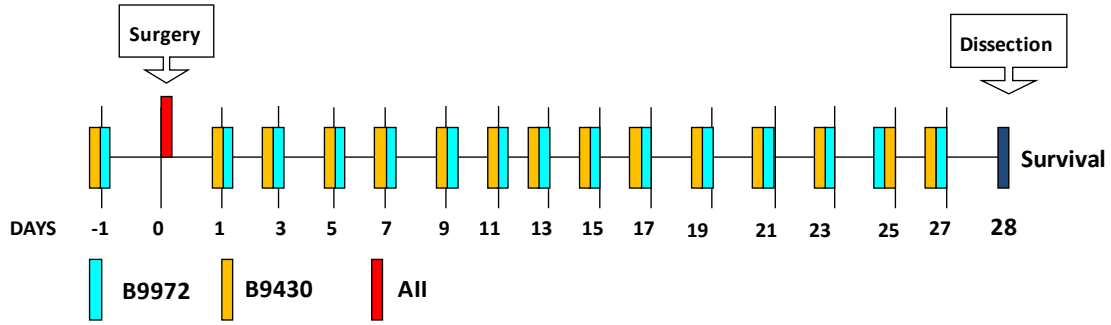
In conclusion, our data illustrates that blockade of the kinin pathway limits AAA development and progression within a mouse model. Neutrophils are activated by kinin agonists. These findings suggest that if safe and effective means of blocking the kinin pathway can be developed for use in patients this strategy could be an effective means of treatment for AAA. Both neutrophils and the kallikrein–kinin system appear to be key mediators in AAA pathogenesis.

APPENDIX 1

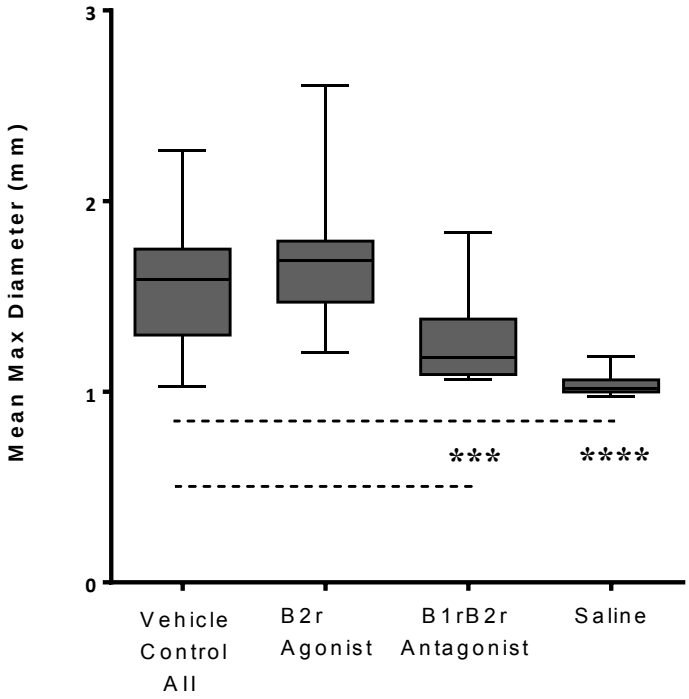
APPENDIX 1 PRELIMINARY DATA

Effect of kinin receptor agonist and antagonist on aneurysm formation.

This experiment was carried out by Dr Catherine Rush (JCU). Thirteen week old male ApoE$^{-/-}$ mice infused with AII were divided into groups receiving either the kinin receptor agonist (B9972, selective to the B2 receptor) (n=24), kinin receptor antagonist (B9430, blocks at both B1 and B2 receptors) (n=25), or vehicle control (n=27) over a 28-day AII infusion period, relevant peptides were administered via 100 µl intraperitonal (i.p.) injection (2mg/kg/dose) at commencement of AII infusion and then every second day over the administration period. A fourth group of mice (n=27) were infused with saline for 28 days as experimental controls. Maximum diameter of aortic arch, thoracic aorta (TA), suprarenal aorta (SRA), and infrarenal aorta (IRA) was determined by morphometric analysis at study end or after fatality during the study period as previously described (Moran, Cullen et al. 2009; Rush, Nyara et al. 2009; Golledge, Cullen et al. 2010; Golledge, Cullen et al. 2010; Krishna, Seto et al. 2012; Moran, Jose et al. 2013)



Study design for the AII – infused mouse model of AAA with peptide challenge. Prior to pump surgery the peptide injections were carried out using either B9972 or B9430. After pump insertion every second day further injections of either B9972 or B9430 were carried out until study end.
AII; angiotensin II.



AII-induced AAA in ApoE$^{-/-}$ mice. Mean maximum aortic diameters were compared between groups, ApoE$^{-/-}$ mice infused with saline (n=27), and ApoE$^{-/-}$ mice infused with AII and administered vehicle control (n=27), B2 receptor agonist (n=24), and B1/B2 receptor antagonist (n=25) (**A**) Data presented as box plots (min max), maximum aortic diameter (mm).

Maximum aortic diameter in relation to AII and kinin peptide administration.

Group	Saline infused control	AII & vehicle control	AII & B1/B2 antagonist	AII & B2 agonist
Number	27	27	25	24
Aortic arch	1.36 (0.11)*	1.85 (0.45)	1.51 (0.32)*	2.15 (0.42)
TA	1.12 (0.06)*	1.49 (0.42)	1.24 (0.18)	1.54 (0.35)
SRA	0.99 (0.08)*	2.10 (0.74)	1.54 (0.59) *	2.02 (0.70)
IRA	0.69 (0.06)*	0.80 (0.19)	0.79 (0.08)	1.06 (0.28)
Average maximum diameter	1.04 (0.05)*	1.56 (0.30)	1.17 (0.42) *	1.63 (0.46)

Diameters are means (standard deviation) in mm. Average maximum aortic diameters were calculated by averaging maximum diameters from all 4 segments in each mouse. Analysis was carried out by One-way ANOVA (overall comparison), with Tukeys post hoc test *significant inhibition of aortic dilatation compared to vehicle control, $P<0.05$

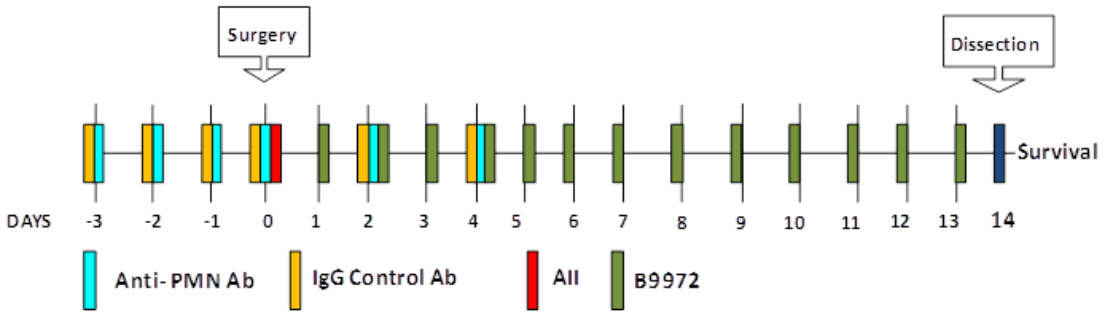
TA, thoracic aorta; SRA, suprarenal aorta; IRA, infrarenal aorta.

Effect of neutrophil depletion on aneurysm formation in the presence of kinin B2 receptor activation.

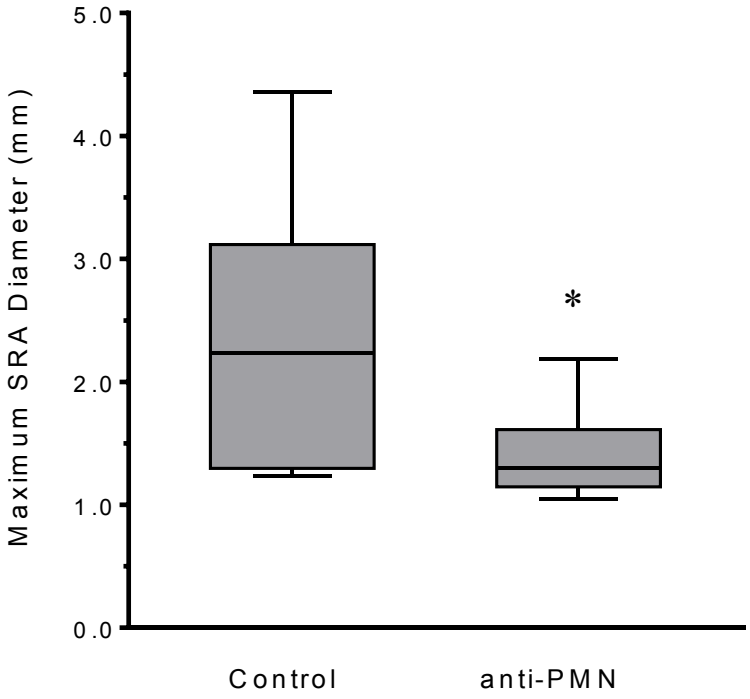
This experiment was carried out by Dr Corey Moran (JCU). Twenty-four thirteen week old male ApoE$^{-/-}$ mice were infused with AII (Chapter 3, section 3.1.3) and received the kinin B2 receptor agonist (B9972) over 14 days. The experiment was run for 14 days as neutrophils are short lived in an immune response, and are recruited to sites of inflammation within days of AII infusion. Neutropenia was induced in half the mice (n=12) prior to AII infusion and administration of the receptor agonist. Regional maximum aortic diameters were determined by morphometric analysis at study end or if death occurred during the experimental period (Chapter 3, section 3.1.6).

Mice received four 250 µl i.p. injections of rabbit anti-mouse PMN (AIAD31140; Accurate Chemical and Scientific) diluted 1:10 in saline immediately prior to (days -3, -2, -1) and at commencement of AII infusion (day 0), with additional injections at days 2 and 4 post commencement of AII infusion. Control mice were administered IgG antibody using an identical regimen (Figure 5.2). Depletion of circulating neutrophils was confirmed by flow cytometic analysis (Chapter 3, section 3.2.4) of 50 µl blood samples obtained via tail bleed (Chapter 3, section 3.1.5) 5 days post commencement of AII infusion. Heparinised blood samples were depleted of red cells by lysis buffer (BioLegend), and flow cytometry was performed using antibodies to CD45-APC (1:200), CD11b-FITC (1:200), and Ly6G-PE (1:400) (BD Biosciences) diluted in PBS containing 2mM EDTA (Amresco) and 0.5% (w/v) BSA (ICN Biomedicals). Samples were preincubated for 20 min with anti-CD16/32 to prevent FcR binding before addition of surface staining antibody cocktails. Viable cells were identified by forward/side scatter profile and in some cases by propidium iodide (PI) exclusion. A forward scatter-area against forward scatter-linear gate was used to exclude doublets from analysis. When applied, quadrants were set such that positive staining was >99% of that observed in the

presence of negative control antibody. Flow cytometry was performed on a FACs Calibur (BD Biosciences, USA) and analysed with Cellquest analysis software (BD Biosciences, USA). Analysis was performed with the operator blinded to the origin of experimental samples.



Study design for neutrophil depletion in the AII – infused mouse model of AAA with B2 receptor challenge. Prior to pump surgery the neutrophil depletion injections were carried out. After pump insertion over the 14 day experiment mice received injections of B9972 until study end. AII; angiotensin II.



Morphometric analysis demonstrating significantly smaller maximum SRA diameter in anti-PMN mice administered kinin B2 receptor agonist compared to agonist-administered IgG control mice. Data presented as box plots (min max), data expressed as maximum SRA diameter (mm). Analysed with Mann Whitney *P=0.009. AII, angiotensin II; PMN, polymorphonuclear leukocytes.

APPENDIX 2

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APPENDIX 3

APPENDIX 3

1 X PHOSPHATE BUFFERED SALINE (PBS) pH 7.4

For 1000 ml (stock 10X)

8gm of Nacl

0.2gm of KCl

1.44gm of Na_2HPO_4

0.24gm of KH_2PO_4

Adjust pH to 7.4. Adjust volume to 1L with additional distilled H_2O.Sterilize by autoclaving.

For 100 ml (working 1 X)

10 ml of stock solution

90 ml of distilled H_2O

2% FLOW CYTOMETRY BUFFER

100 ml of 1x PBS

2 % fetal calf serum (FCS)

0.05 gm Sodium Azide

Mix well. Adjust pH to 7.4. Sterilize by autoclaving. Store at 4°C

3% DEXRAN/ 0.9% NaCl

To 250ml distilled H_2O:

7.00gm Dextran

2.25gm NaCl

Mix well. Store at 4°C.

SUDAN IV SOLUTION

500ml Acetone

500ml 70% Ethanol

5gm Sudan IV

SUDAN DESTAIN

500ml 80% Ethanol

APPENDIX 4

Appendix 4

Cytokine bead array standard curves (Chapter 5, section 5.2.6)

Plex Components

		Analyte		
Name	Lot Number	Name	Model	2nd Reporter
Bead 2		Mouse TNF	Quantitative	No
Bead 3		Mouse IFN-γ	Quantitative	No
Bead 4		Mouse MCP-1	Quantitative	No
Bead 5		Mouse IL-10	Quantitative	No
Bead 6		Mouse IL-6	Quantitative	No
Bead 1		Mouse IL-12p70	Quantitative	No

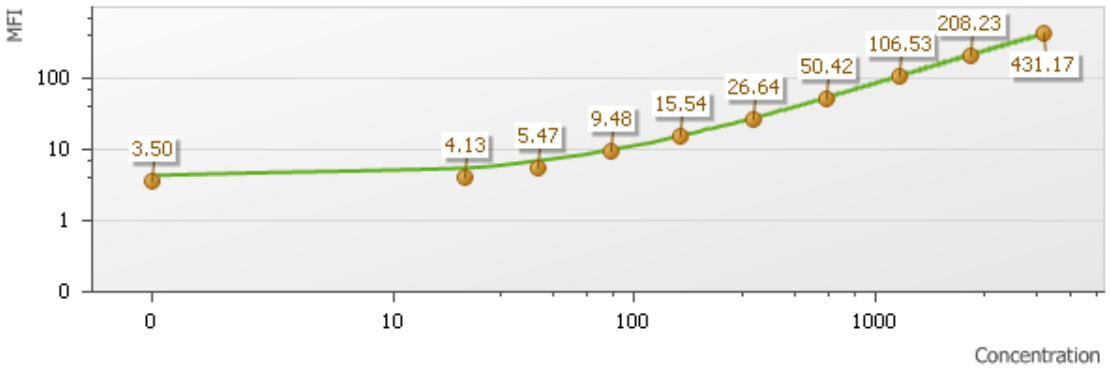
Standard Samples of Quantitative Analysis

Reporter Parameter 1	
Sample Name	Concentration
Std001	0.00
Std002	20.00
Std003	40.00
Std004	80.00
Std005	156.00
Std006	312.00
Std007	625.00
Std008	1,250.00
Std009	2,500.00
Std010	5,000.00

Bead 1-Mouse IL-12p70

R^2=99.90% A: 1.450 B: 3.467 C: 8.526 D: 10.657 E: 0.000

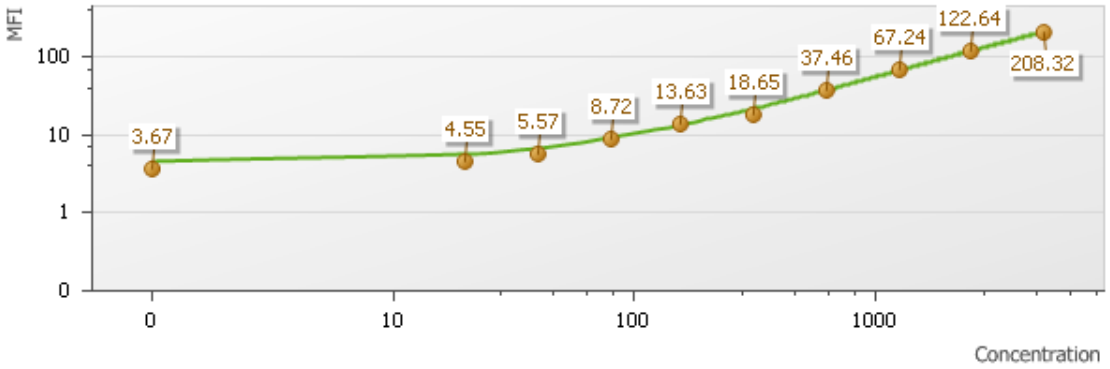
Fitting type: 5 Parameter Logistic



Bead 2-Mouse TNF

R^2=99.85% A: 1.527 B: 3.497 C: 8.442 D: 9.068 E: 0.000

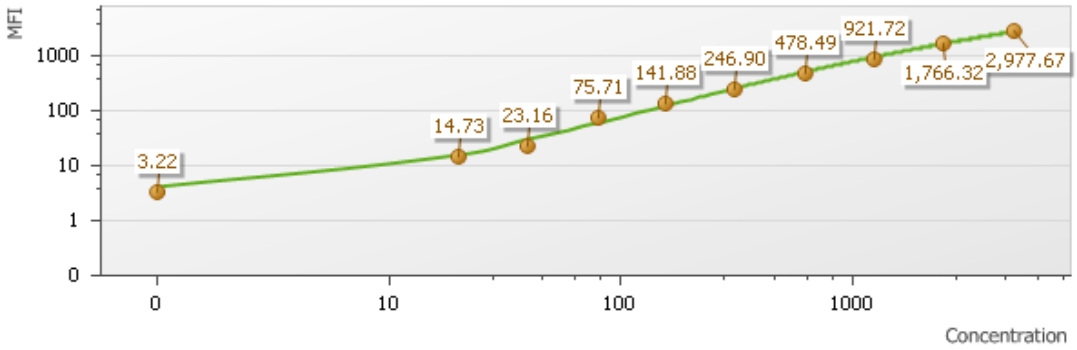
Fitting type: 5 Parameter Logistic



Bead 3-Mouse IFN-γ

R^2=99.70% A: 1.422 B: 2.375 C: 6.827 D: 11.817 E: 0.000

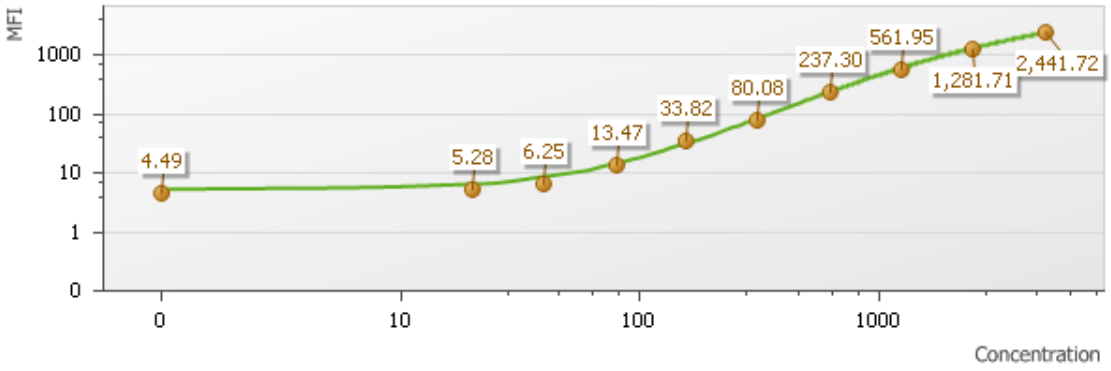
Fitting type: 5 Parameter Logistic



Bead 4-Mouse MCP-1

R^2=99.90% A: 1.634 B: 4.813 C: 6.489 D: 9.419 E: 0.000

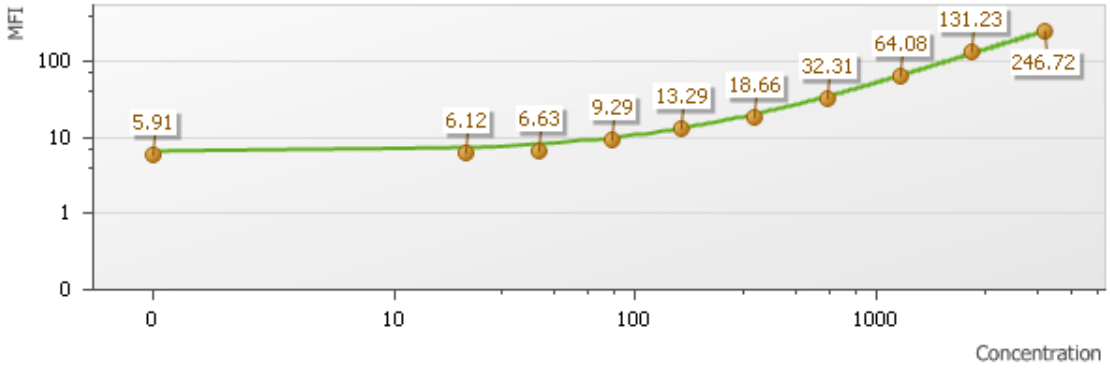
Fitting type: 5 Parameter Logistic



Bead 5-Mouse IL-10

R^2=99.89% A: 1.901 B: 4.227 C: 8.782 D: 9.654 E: 0.000

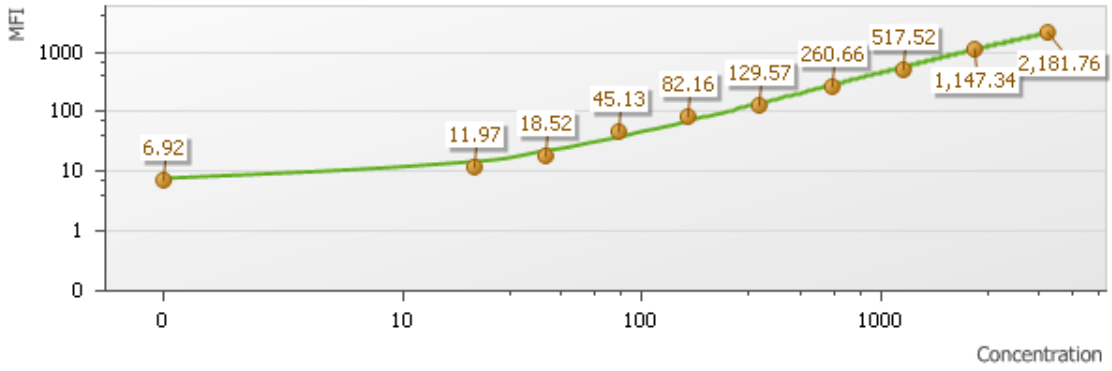
Fitting type: 5 Parameter Logistic



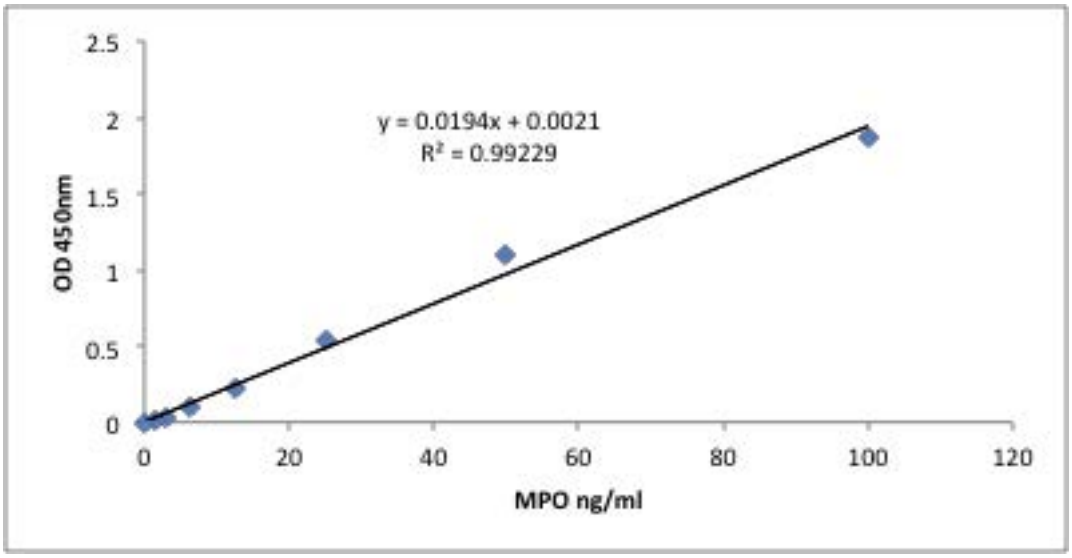
Bead 6-Mouse IL-6

R^2=99.64% A: 2.010 B: 2.686 C: 8.468 D: 13.205 E: 0.000

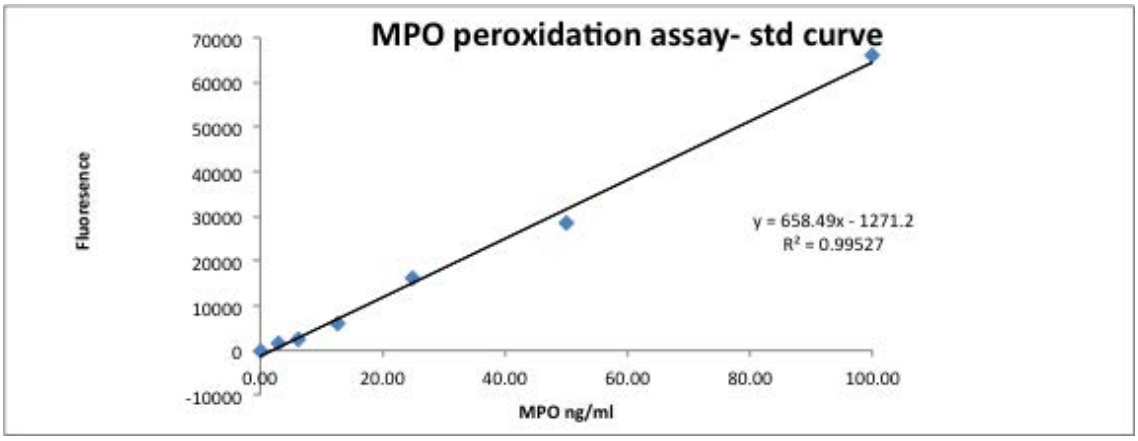
Fitting type: 5 Parameter Logistic

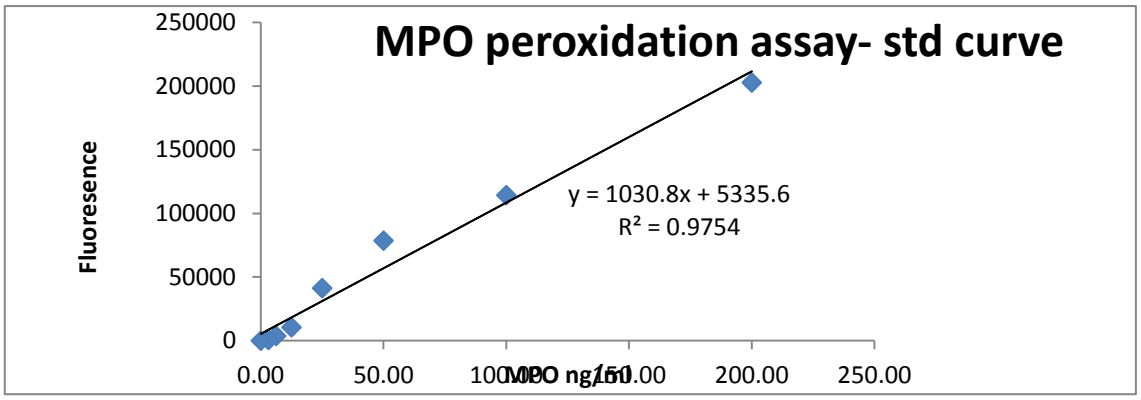


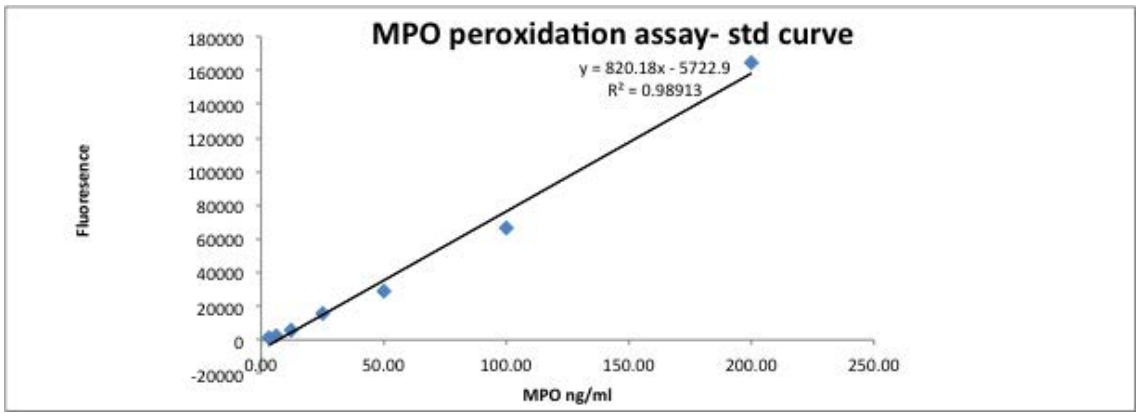
Standard curve MPO ELISA (Chapter 5, section 5.2.7)



Standard curve MPO activity assay (Chapter 6, section 6.2.2)




MPO peroxidation assay- std curve
Fluoresence
250000
200000
150000
100000
50000
0
y = 1030.8x + 5335.6
R² = 0.9754
0.00
50.00
100.00
150.00
200.00
250.00
MPO ng/ml


MPO peroxidation assay- std curve
Fluoresence
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
-20000
y = 820.18x - 5722.9
R² = 0.98913
0.00
50.00
100.00
150.00
200.00
250.00
MPO ng/ml

APPENDIX 5

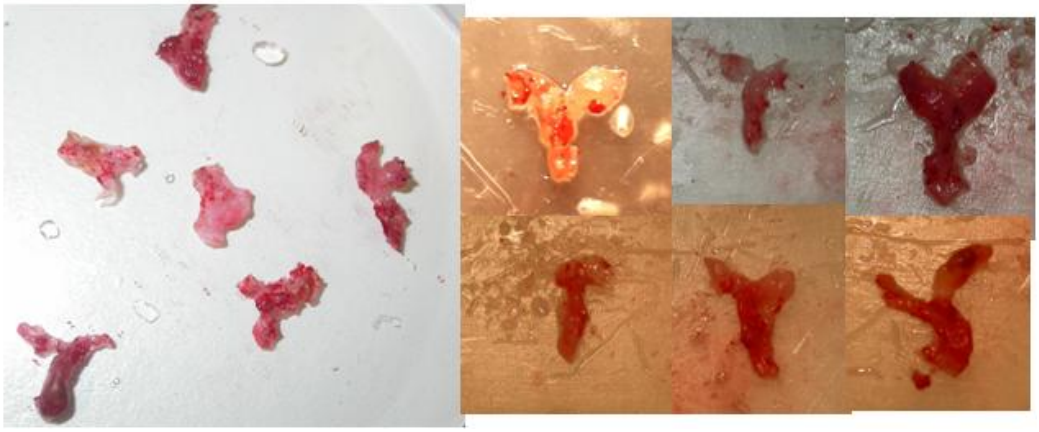
APPENDIX 5

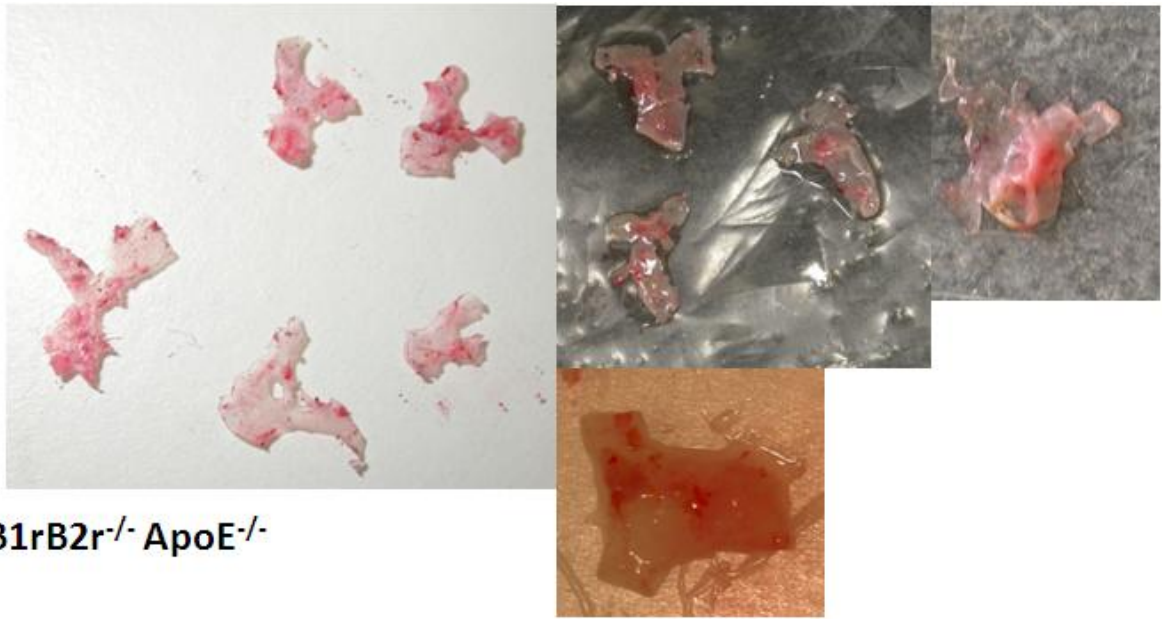
ApoE$^{-/-}$ control

B1rB2r$^{-/-}$ ApoE$^{-/-}$

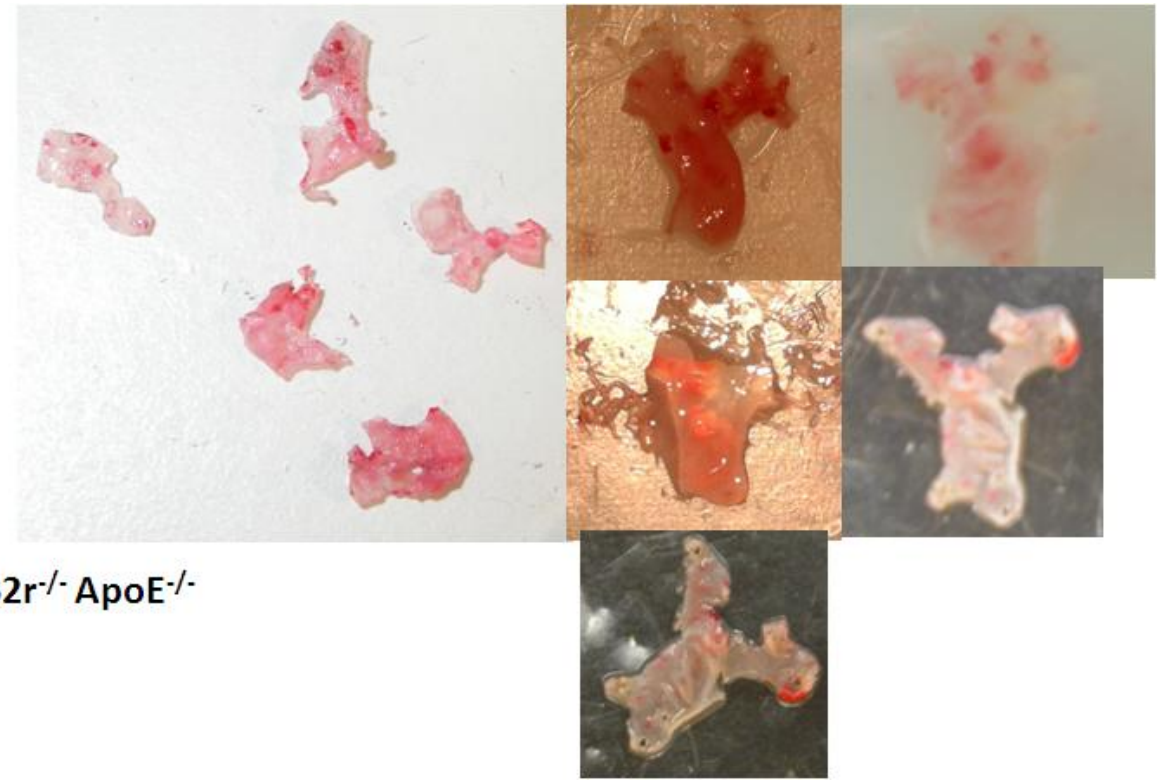
B2r$^{-/-}$ ApoE$^{-/-}$

ApoE$^{-/-}$





B1rB2r$^{-/-}$ ApoE$^{-/-}$



B2r$^{-/-}$ ApoE$^{-/-}$

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