Role of resistance vasculature in the development and maintenance of hypertension in chronic kidney disease

Ko Jin Quek, B.Sc. (Hons), M.Sc. (Hons)

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Supervisor:

Professor Jacqueline K. Phillips

Co-supervisor:

Professor Alberto Avolio

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MACQUARIE University
SYDNEY·AUSTRALIA

Declaration of originality

I hereby declare that the work presented in this thesis has not been submitted for a higher degree to any other university or institution. To the best of my knowledge this submission contains no material previously published or written by another person, except where due reference is stated otherwise. Any contribution made to the research by others is explicitly acknowledged.

Ko Jin Quek

Department of Biomedical Sciences
Faculty of Medicine and Health Science
Macquarie University

January 2016

This thesis is dedicated

to my beloved family

Declaration of contributions

Chapter 3

Candidate performed all experiments, analysed data and interpreted results. Candidate was the major contributor to the manuscript. Jacqueline Phillips, Tim Murphy and Alberto Avolio contributed to conception and design of experiments, data analysis, interpretation of results and editing, revision and final approval of manuscript. Rochelle Boyd assisted with data collection. Omar Ziad Ameer Al-adhami assisted with the preparation of histological sections, imaging and data analysis, and contributed to final approval of manuscript. Barbara Zangerl and Mark Butlin assisted with the preparation of pressure myography equipment, review and approval of final manuscript..

Chapter 4

Candidate performed all experiments, analysed data and interpreted results. Candidate was the major contributor to the manuscript. Jacqueline Phillips and Alberto Avolio contributed to conception and design of experiments, data analysis, interpretation of results and editing, revision and final approval of manuscript. Omar Ziad Ameer Al-adhami assisted with data collection and analysis, the preparation of histological sections and imaging, and contributed to final approval of manuscript. Tim Murphy provided constructive feedback on this chapter. Ibrahim Salman assisted with data collection.

Chapter 5

Candidate performed all experiments, analysed data and interpreted results. Candidate was the major contributor to the manuscript. Jacqueline Phillips, Tim Murphy and Alberto Avolio contributed to conception and design of experiments, data analysis, interpretation of results and editing, revision and final approval of manuscript. Omar Ziad Ameer Al-adhami, Qi Jian Sun, Rochelle Boyd, Manasha Saha, Cara Hildreth and Ibrahim Salman assisted with rat treatments and animal monitoring. Omar Ziad Ameer Al-adhami assisted with the preparation of histological sections. Rochelle Boyd, Omar Ziad Ameer Al-adhami and Ibrahim Salman assisted with data collection. Ian Wright provided constructive feedback on this chapter.

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Abstract

Hypertension is a significant complication among chronic kidney disease (CKD) patients, resulting in higher mortality rates in these patients than renal disease itself. The process of cardiovascular damage starts very early during progression in well-defined CKD, long before the renal treatment stage is reached (Vanholder et al., 2005), thus making kidney disease the most common "secondary" form of hypertension (Cohen and Townsend, 2009, Ross and Banerjee, 2013). Resistance arteries play a particularly important role in the maintenance of hypertension in these CKD patients (Li et al., 1997, Paisley et al., 2009), as they are the main contributors to total peripheral resistance (TPR) (Beevers et al., 2001, Mulvany et al., 1978, Mulvany and Halpern, 1977). Although it is well-established that alterations in resistance arteries are likely to contribute to increased cardiovascular risk and act as a substrate for end-organ damage (Schiffrin, 2012), resistance artery alterations are still poorly understood in the CKD-mediated hypertensive state, and hence were the focus of this thesis.

To investigate the effects of CKD on the resistance vasculature as part of the overall cardiovascular disease (CVD) state, we employed an established rat model of CKD-mediated hypertension, the Lewis polycystic kidney (LPK) rat. In a series of four studies, we sought to examine alterations in resistance artery structure, function and biomechanical properties, in association with disease progression. In addition, we investigated the effects of treatment (AT_1 receptor antagonism and calcium channel blockade) on the resistance vasculature.

In study 1, we explored temporal changes in LPK resistance artery structure, function and biomechanical properties, in association with hypertension and renal disease progression. We investigated three time-points: 6 weeks of age, where LPKs are hypertensive and have gross derangement of the kidney cortex and medulla; 12 weeks of age, where LPKs demonstrate signs of renal dysfunction in addition to hypertension and increased sympathetic nerve activity (SNA); and 18 weeks, where LPK have marked renal disease and still present with hypertension and elevated SNA (Phillips et al., 2007). These experiments revealed that alterations in the vasculature tended to emerge after the 6 week time-point in LPK, and that older age was associated with negative outcomes. Older LPK resistance arteries underwent structural alterations which were characterised by eutrophic and hypertrophic inward remodelling at established and severe renal disease time points, respectively, and these structural alterations were significantly associated with the hypertension. Stiffness was increased in 6 and 18 week LPK, and impaired endothelium-dependent relaxation was evident in 12 and 18 week LPK. These findings suggested that in CKD-mediated hypertension, multiple effects on resistance arteries are seen, which result in the activation of compensatory

mechanisms: structural mechanisms eventually become maladaptive, thus exacerbating the disease, while functional mechanisms involve endothelial dysfunction, impairing vasorelaxation. Thus, study 1 revealed that impairments in the LPK vasculature arise at as early as 6 weeks, and generally worsen as hypertension pursues and with progression to marked renal disease.

Study 2 aimed to ameliorate the deleterious alterations in the vasculature found in study 1, via treatment with an angiotensin type 1 (AT_1) receptor antagonist, valsartan, to block the effects of angiotensin II (Ang II). Chronic treatment performed from 4-18 weeks of age reduced hypertension, improved collagen-elastin ratios and structural vascular remodelling outcomes. In addition, LPK treated with valsartan exhibited vasoconstriction and vasorelaxation responses that were comparable to Lewis controls, and demonstrated improved stiffness and compliance properties. Thus, study 2's findings highlighted the important contribution of Ang II and AT_1 receptors to resistance artery structural, functional and biomechanical properties.

Similar to study 2, study 3 also aimed to ameliorate the deleterious alterations in vasculature found in study 1. However, in addition to this, study 3 aimed to further investigate whether valsartan's effectiveness was due to the specific blockade of Ang II's effects, or because of the resultant reduction of blood pressure. Hence, study 3 involved treatment of LPK rats with amlodipine to block the effects of voltage-dependent L-type Ca^{2+} channels (LTCCs), and therefore result in an antihypertensive effect without directly affecting Ang II signalling. Similar to valsartan outcomes, chronic treatment with amlodipine performed from 4-18 weeks of age reduced hypertension, improved collagen-elastin ratios, improved structural vascular remodelling outcomes, and normalised dysfunctional vasoconstriction and vasorelaxation responses in LPK. However, relative to valsartan, the effectiveness of amlodipine in normalising these parameters was to a lesser extent. In addition, amlodipine was unable to improve stiffness and compliance properties in LPK rats.

In study 4, we investigated myogenic tone and endothelial dysfunction, examining sensitivity to endothelial-derived constricting and relaxing factors. In addition, because endothelial dysfunction has been shown to be receptor-specific, we investigated the effect of different agonists on endothelial responses. Study 4 revealed increased myogenic tone and vasoconstriction to Ang II in LPK rats, along with endothelial dysfunction that correlated with the degree of renal impairment, regardless of the agonist used (bradykinin, BK; or aceylcholine, ACh).

Collectively, these studies showed that hypertension and renal impairment in a rat model of CKD are associated with alterations in the mesenteric resistance arteries, and that

pharmacological treatments are able to mitigate the majority of these observed changes. These modifications of structural, functional and biomechanical resistance artery properties serve to protect end-organs by mediating pressure changes; however, with the persistence of both hypertension and renal dysfunction, these changes can ultimately lead to further exacerbation of these disease states. Overall, these studies provide a better understanding of the pathological resistance vasculature consequences in the CVD and CKD states, and thus help to direct pharmacological targets for more effective therapeutic outcomes.

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σ	Stress
σ_0	Stress at baseline diameter
η	Viscosity
AA	Arachidonic acid
AASK	African American Study of Kidney disease
AC	Adenylate cyclase
ACEi	Angiotensin converting enzyme inhibitor
ACh	Acetylcholine
ADPKD	Autosomal dominant polycystic kidney disease
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of variance
AR	Alizarin red
ARB	Angiotensin receptor blocker
ARPKD	Autosomal recessive polycystic kidney disease
AT_1	Angiotensin type 1 receptor
AT_2	Angiotensin type 2 receptor
ATP	Adenosine triphosphate
B	Constant related to the rate of increase of the stress-strain curve
BF	Blood flow
BK	Bradykinin
BW	Body weight
Ca	Calcium
$(Ca^{2+})_i$	Intracellular calcium
cAMP	cyclic adenosine monophosphate
CCB	Calcium channel blocker
cGMP	Cyclic guanosine monophosphate
CKD	Chronic kidney disease
CO	Cardiac output
COX	Cyclooxygenase
CSA	Cross sectional area
CT	Collecting tubules

CVD	Cardiovascular disease
D	Diameter
D_e	External diameter
D_i	Internal diameter
D_0	Baseline diameter measured at 3 mmHg
DNA	Deoxyribonucleic acid
ε	Strain
EC_{50}	Effective concentration 50%
ECs	Endothelial cells
ECF	Extracellular fluid
ECM	Extracellular matrix
EDH	Endothelium-derived hyperpolarisation
eGFR	Estimated GFR
eNOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease
ET	Tangential elastic modulus
ET-1	Endothelin-1
FGF-23	Fibroblast growth factor 23
GFR	Glomerular filtration rate
GTP	Guanosine triphosphate
HI	Heart index
IDNT	Irbesartan in Diabetic Nephropathy Trial
IEL	Internal elastic lamina
IL-10	Interleukin 10
Indo	Indomethacin
iNOS	Inducible nitric oxide synthase
IP_3	Inositol phosphate 3
K^+ Ca^{2+}	Calcium-activated potassium channel
KCl	Potassium chloride
KDOQI	National Kidney Foundation Kidney Disease Outcomes Quality Initiative
KI	Kidney index
L	Length
L-Arg	L-Arginine
LF	Low frequency

L-NAME	Nω-nitro-L-arginine methyl ester
LPK	Lewis polycystic kidney
LTCC	L-type Ca^{2+} channel
LVH	Left ventricular hypertrophy
LVI	Left ventricle index
MAP	Mean arterial pressure
MCSA	Media cross sectional area
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MSB	Martius scarlet blue
MsPGN	Mesangioproliferative glomerulonephritis
NA	Noradrenaline
nd	Not detected
Nek-8	Never in mitosis gene a-related kinase 8
NIMA	Never in mitosis gene a
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NPHP	Nephronophthisis
NPHP9	Nephronophthisis 9
P	Pressure
P	Phosphate
PC-1	Polycystin-1
PE	Phenylephrine
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PKA	Protein kinase A
PKD	Polycystic kidney disease
PKHD1	Polycystic kidney and hepatic disease 1 gene
PTH	Parathyroid hormone
PWV	Pulse wave velocity
r	Radius
R	Resistance

RAS	Renin angiotensin system
R_{max}	Maximum response
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SHR	Spontaneously hypertensive rats
SNA	Sympathetic nerve activity
SNP	Sodium nitroprusside
SNS	Sympathetic nervous system
SO	Shikata's orcein
SR	Sarcoplasmic reticulum
TBI	Total-body irradiation
TNF-α	Tissue necrosis factor alpha
TPR	Total peripheral resistance
TxA_2	Thromboxane A_2
UPC	Urinary protein creatinine ratio
UUO	Unilateral ureteral obstruction
vCa^{2+}	Voltage-sensitive Ca^{2+} channels
VK	Von Kossa
VSMC	Vascular smooth muscle cell
WT	Wall thickness
WKY	Wistar-Kyoto

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Chapter 1

Literature review

Quek, K.J.

1.1 Hypertension and cardiovascular disease

High blood pressure, or *hypertension*, is the extended elevation of blood pressure (Behbahani, 2010, Foëx and Sear, 2004, AIHW, 2014b). Hypertension is common in Australia, amongst both males and females (AIHW, 2014b), and has been shown to be responsible for more deaths and disease than any other biomedical risk factors (Lopez et al., 2006, AIHW, 2014b). Hypertension results in increased morbidity and mortality because of its' negative effects on end-organs, which leads to several severe pathological outcomes such as kidney and cardiovascular disease (CVD): diseases which can subsequently result in events such as stroke and heart attack (Foëx and Sear, 2004, AIHW, 2014b).

Cardiovascular disease refers to all diseases and conditions of the heart and blood vessels (Access Economics and National Heart Foundation of Australia, 2005). In 2008, CVD was responsible for more deaths (49% of all deaths) than any other disease group in Australia, and still continues to dominate Australia's health profile today (AIHW, 2014b). Although mortality rates from acute events such as stroke and heart attack have been declining, CVD remains a high cause of mortality and is becoming more associated with periods of chronic disabling illness (AIHW, 2014b).

There is a positive and continuing relationship between blood pressure levels and CVD risk (Chobanian et al., 2003, AIHW, 2014b), where risk of CVD increases with every increase in blood pressure from the "ideal" <130 mmHg systolic blood pressure (SBP) level (Mancia et al., 2013). Hypertension accounts for 42% of the CVD burden, and hence is the biggest contributor to CVD (AIHW, 2014b). Thus, hypertension has occasionally also been considered a CVD in itself, seeing as the risk factors for hypertension (e.g. age, poor diet, obesity, lack of physical activities and increased alcohol consumption) are very similar to those for other forms of CVD (AIHW, 2014b). Hypertension can be classified into two major categories: primary hypertension and secondary hypertension.

1.1.1 Primary hypertension

Primary hypertension (also referred to as essential hypertension) is the chronic elevation of blood pressure without a known cause (Carretero and Oparil, 2000, Oparil et al., 2003). Primary hypertensive cases are the most common form of hypertension, comprising

approximately 90-95% of all hypertensive cases (Mulvany, 2008, Carretero and Oparil, 2000), and are characterised by an increase in sympathetic activation and total peripheral resistance (TPR), and play a large role in the development of left ventricular hypertrophy (LVH) (Feihl et al., 2008, Pries et al., 2001, Sonoyama et al., 2007). Primary hypertension has been estimated to have a heritability link of 30-50%, with at least 43 genetic variants believed to affect systolic and diastolic blood pressure (Ehret and Caulfield, 2013). These effects on SBP are of particular importance, because increased SBP variability is associated with increased organ damage (Leoncini et al., 2013). Note, however, that although a genetic predisposition to hypertension has been proposed, many other factors such as increased sympathetic activity or increased stress are also believed to contribute (Oparil et al., 2003), and that the underlying cause of primary hypertension remains unknown.

1.1.2 Secondary hypertension

Unlike primary hypertension, increased blood pressure in secondary hypertension (also referred to as non-essential hypertension) is linked to a known diagnosed condition (Chiong et al., 2008). Various conditions may result in the dysregulation of organ systems and thus lead to a dysregulation of blood pressure, with such conditions including but not limited to: pregnancy, endocrine dysfunction, neurological causes, aortic diseases, and chronic kidney disease (CKD) (Chiong et al., 2008). Of all the various conditions, kidney disease is the most common cause of secondary hypertension, with over 58% of patients with CKD being hypertensive (Hilleman and Lynch, 1999, Toto, 2005, US renal data system, 2014, Kidney Health Australia, 2014b). Hence, hypertension is considered an important co-morbid factor associated with CKD (Baumeister et al., 2009, Middleton and Pun, 2010).

Through its effects on kidney volume expansion and overall increased systemic vascular resistance, kidney disease has the potential to exacerbate uncontrolled hypertension (Buffet and Ricchetti, 2013). Though kidney disease can cause hypertension, hypertension likewise can result in kidney injury (Iino et al., 2003, Klag et al., 1996, Young et al., 2002, AIHW, 2014b, KDOQI, 2012). The presence of hypertension is a major determinant of the progression of kidney dysfunction as well as mortality from CVD (Fall and Prisant, 2005, Levin et al., 2001), because hypertension and kidney disease can act in a cyclic manner,

resulting in severe deterioration of both kidney and cardiovascular functioning (Iino et al., 2003, Buller et al., 2004, Yerram et al., 2007). Untreated hypertension has the potential to renal blood vessels by causing an increase in blood vessel wall thickness and internal vessel diameter narrowing, which thereby reduces blood supply and decreases kidney function (AIHW, 2014b). As a result of this damage, the kidney's ability to filter fluid and waste from the blood can become impaired, thus leading increased blood volume overall — and ultimately, an increase in blood pressure (Buffet and Ricchetti, 2013).

1.2 Chronic kidney disease

Chronic kidney disease is an increasing public health concern at a local and global level (Yerram et al., 2007, Jha et al., 2013), resulting in high premature mortality rates in Australia (ANZDATA registry, 2014), and affecting up to 7% of the world population (Couser et al., 2011). In fact, in Australia it has been estimated that approximately 1 in 3 people have an increased risk of developing CKD (Australian Bureau of Statistics, 2013), and 1 in 10 people over 18 years of age present with indicators of CKD, such as reduced renal function and/or the presence of albumin in urine (Wong and Vanhoutte, 2010, Australian Institute of Health and Welfare, 2011).

Chronic kidney disease is a broad term for heterogenous disorders affecting the structure and function of the kidney, and is defined as the progressive loss of kidney function occurring over a period of months or years (Yerram et al., 2007, AIHW, 2014b). The three most common causes of kidney disease in Australia are hypertension, diabetes and glomerulonephritis (Kidney Health Australia, 2014b). The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI) (2012) criteria for the definition of CKD include: (1) kidney damage for a minimum of 3 months, defined by structural or functional abnormalities of the kidney, with or without a low glomerular filtration rate (GFR); or (2) a GFR <60 mL/min/1.73 m^2 (approximately corresponding to a creatinine level >1.5 mg/dL in men or >1.3 mg/dL in women) for at least 3 months, with or without kidney damage (Chobanian et al., 2003). The former is indicated by pathological abnormalities or kidney damage markers, such as abnormal blood or urine composition, or abnormal imaging test results – for example, the presence of albuminuria (>300 mg/d or 200 mg/g creatinine)

(Chobanian et al., 2003). In contrast, for the latter, a GFR <60 mL/min/1.73 m^2 for more than 3 months represents a minimum of 50% reduction in kidney function relative to a normal adult level, and may be associated with a number of complications (KDOQI, 2012).

In the CKD state, although most renal impairment cases can be detected and diagnosed at the early stages via regular blood tests, symptoms may only begin to appear following up to a 90% loss of kidney functioning (AIHW, 2014a). In severe cases, the loss of kidney functioning may be so great that if left untreated, can lead to death because of the inability to sustain life (AIHW, 2014b). Disease of this severity is referred to as end-stage renal disease (ESRD), and requisites kidney replacement therapy in order to survive — this therapy may involve either a kidney transplant, or dialysis, which serves to remove wastes or excess fluids from the body (AIHW 2010d). Progression from the initiation of CKD to ESRD has been classified through 5 stages (KDOQI, 2012, Schiffrin et al., 2007, Rocco and Berns, 2012):

1. Kidney damage characterised by a normal or increased GFR (≥90 mL/min/1.73m^2), with an approximately 3.3% prevalence.
2. Kidney damage characterised by a mildly reduced GFR (60-89 mL/min/1.73m^2), with an approximately 3% prevalence. Stage 2 is accompanied by a 1.5 fold increase in CVD risk, relative to non-CKD patients. At this stage, treatments focus primarily on blood pressure control; however, treatment actions also include dietary modifications and renoprotection.
3. Moderately decreased kidney function characterised by a low GFR (30-59 mL/min/1.73m^2), with an approximately 4.3% prevalence. Stage 3 is accompanied by a 2-4 fold increase in CVD risk, relative to non-CKD patients. Treatment focus and actions are the same as stage 2.
4. Severely decreased kidney function characterised by a very low GFR (15-29 mL/min/1.73m^2), with an approximately 0.2% prevalence. Stage 4 is accompanied by a 4-10 fold increase in CVD risk, relative to non-CKD patients. For most patients at stage 4 and beyond, renal replacement therapy is required.
5. Kidney failure (ESRD) characterised by an extremely low GFR (<15 mL/min/1.73m^2), with an approximately 0.1% prevalence. Similar to stage 4, most patients require renal replacement therapy.

1.2.1 Markers of chronic kidney disease

Glomerular filtration provides a measure of the amount of ultrafiltrate of blood as it is filtered through the glomerular capillaries (Levey et al., 2014). The level of GFR, which is calculated from mathematical formulas (Dias et al., 2013) based on the number of nephrons and the determinants of single-nephron GFR (Levey et al., 2014), is considered to be one of the best measures of overall kidney function, for diagnostic and investigation purposes in disease states (KDOQI, 2012). Glomerular filtration rate is affected by various factors, such as age, ethnicity and obesity (Jafar et al., 2011, Levey et al., 2014). The markers of kidney damage are determined by the type of renal disease at present, and may include, for example, abnormalities in imaging tests, or abnormalities in the composition of blood or urine, and may or may not be accompanied with decreased GFR (KDOQI, 2012).

Proteinuria is a very sensitive marker of kidney damage that is increased early on in the disease state for many types of CKD (KDOQI, 2012), and is associated with risk of CVD as well as progression to ESRD. In addition, decreased proteinuria levels are associated with reduced risk of cardiovascular morbidity and mortality, and increased preservation of kidney function (Sarafidis et al., 2007).

Albuminuria is another commonly used indicator for glomerular damage, where its presence in the urine – irrespective of how minute ("microalbuminuria") – is the earliest manifestation of renal failure (KDOQI, 2012): urinary albumin excretion has been shown to possess both diagnostic and prognostic values that are comparable to decreased estimated GFR (eGFR) values (Chobanian et al., 2003). Albumin (molecular weight ~68,000 daltons) and other low molecular weight globulins are the most abundant urine proteins in most types of CKD (KDOQI, 2012), and many studies have produced supporting evidence that the ratio of albumin-to-creatinine values or total protein-to-creatinine values obtained from a spot urine sample is reflective of the excretion rates of albumin or total protein, respectively, as measured in timed urine samples (KDOQI, 2012). Albumin excretion is difficult to detect at small concentrations, as this requires specific and sensitive assays; however at increased concentrations, albumin excretion is easily detected in total urine protein investigations (KDOQI, 2012). As well as its significance as a marker of renal impairment, albuminuria also plays an important role in prognosis estimates for both the progression of renal impairment and development of CVD (KDOQI, 2012).

1.3 Chronic kidney disease and its association with cardiovascular disease

Although the treatment options of dialysis and kidney transplantation are available for patients at later stages of CKD, life expectancy for CKD patients is still considerably reduced because of a major contributing factor to mortality: CVD (Foley, 2010, Tonelli et al., 2006).

Although CVD was first recognised as a problem in patients receiving dialysis (i.e. those with CKD stage 5), CVD risk is also increased during the earlier stages of CKD, and is proportional to the degree of impaired kidney function (Go et al., 2004). The development of cardiovascular damage begins early on during the progression of CKD, even preceding the renal treatment stage (Vanholder et al., 2005), thus making kidney disease the most common "secondary" form of hypertension (Cohen and Townsend, 2009, Ross and Banerjee, 2013). In fact, patients of all stages of CKD are considered the highest risk group for development of CVD (KDOQI, 2012, ANZDATA registry, 2014), and patients with CKD are more likely to die from CVD before requiring dialysis, a kidney transplant, or progressing to ESRD (Keith et al., 2004, McCullough et al., 2008b, Gullion et al., 2006, Sarnak et al., 2003). At least 85% of patients with stage 3 CKD or greater have hypertension (Cohen and Townsend, 2009), and observational studies indicate that patients with stage 3 CKD are more likely to die from CVD than they are likely to die from kidney disease (Foley et al., 2005). Furthermore, patients who do survive kidney disease and subsequently require dialysis will generally have well-established cardiovascular complications (Foley et al., 1995). Hence, the management of CKD, and in particular at the early stages of disease, should be principally focused on the improvement and minifying of cardiovascular risk factors (Kidney Health Australia, 2014a).

The mechanisms underlying amplified risk of CVD among CKD patients are yet to be fully understood (AIHW, 2014b). Patients with CKD appear to have an increased risk for CVD for several reasons: (1) CKD is associated with an increase in prevalence of CVD risk factors; (2) CKD is an independent risk factor for CVD; (3) CVD is a risk factor for CKD; and (4) CVD risk factors promote the development and progression of CKD (Schiffrin et al., 2007, Go et al., 2004, AIHW, 2014b, Levin et al., 2001).

1.3.1 Contributors of increased cardiovascular disease risk in chronic kidney disease

Irrespective of whether CVD was a cause or complication of CKD, the amplified risk of CVD in individuals with CKD may be attributed to: (1) a greater prevalence of ‘‘traditional’’ risk factors for CVD; and (2) a higher prevalence of risk factors related to the haemodynamic and metabolic complications of CKD (also known as ‘‘CKD-related’’ or ‘‘non-traditional’’ CVD risk factors) (Figure 1.1) (KDOQI, 2012). It is believed that the pathophysiology underpinning the development of CVD in CKD involves complex interactions between traditional and non-traditional risk factors, as well as uraemia-related risk factors (Yerram et al., 2007), where non-traditional risk factors that are specifically linked with renal failure are likely to play a causative role (Vanholder et al., 2005). Risk for CVD rises progressively with the number of risk factors (Access Economics and National Heart Foundation of Australia, 2005).

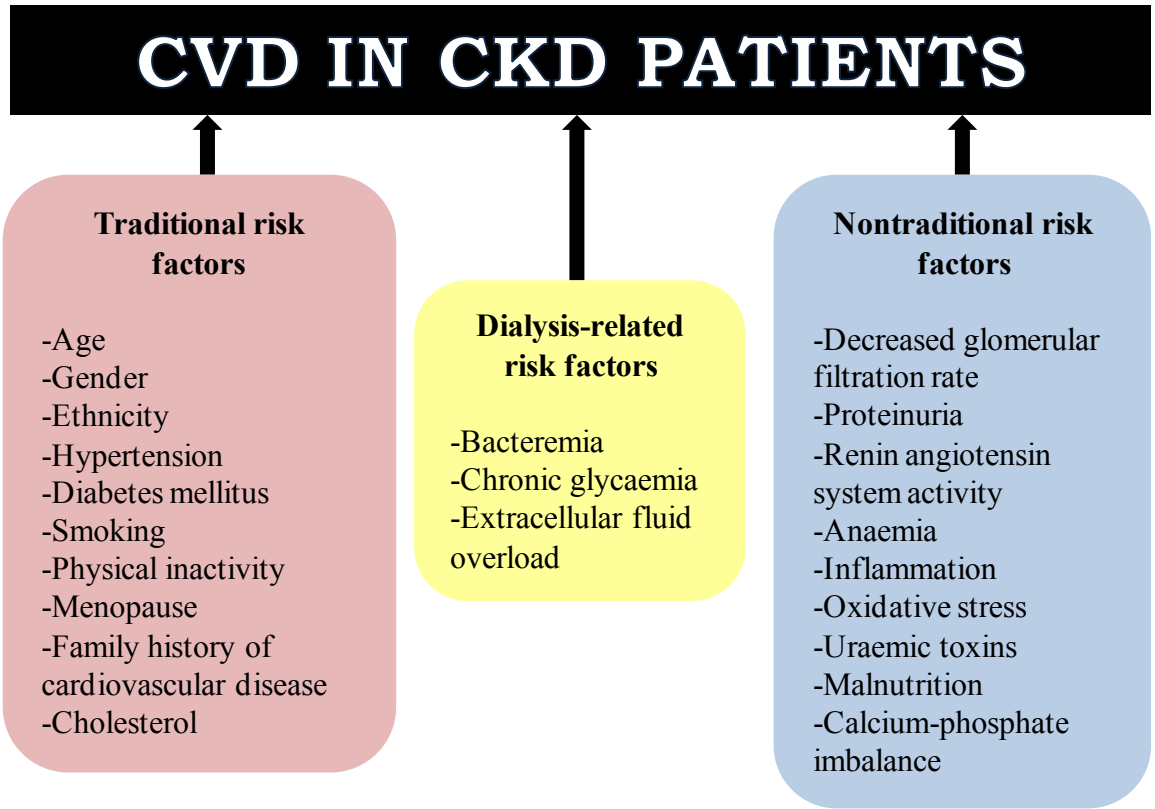

Figure 1.1. Schematic representation of contributors to cardiovascular disease (CVD) in chronic kidney disease (CKD) patients. Contributors can be broadly categorised into

traditional risk factors, dialysis-related risk factors, and non-traditional risk factors. Adapted and modified from (Rucker and Tonelli, 2009) and (KDOQI, 2012).

1.3.1.1 Traditional risk factors

Traditional risk factors are genetic, behavioural and biomedical conditions associated with a greater risk of CVD and CKD. Genetic factors such as older age, male gender and family history of CVD (Sarnak and Levey, 2000, AIHW, 2014b, WHO, 2003, Access Economics and National Heart Foundation of Australia, 2005), behavioural risk factors such as tobacco smoking, physical inactivity and poor nutrition (Sarnak and Levey, 2000, AIHW, 2014b, AIHW, 2008, NHMRC, 2001, WHO, 2003), and biomedical risk factors such as diabetes, high cholesterol, obesity and high blood pressure itself (AIHW, 2014b), are all correlated with increased prevalence of CVD and CKD. As mentioned previously (see section 1.1 Hypertension and cardiovascular disease, hypertension has also been considered a CVD in its own right, because the risk factors for hypertension are largely the same as those for other forms of CVD (AIHW, 2014b).

1.3.1.2 Non-traditional risk factors

In addition to traditional risk factors, non-traditional risk factors are believed to play important contributory roles to CVD and CKD. Non-traditional factors are uraemia-related risk factors – that is, other CVD risk factors – that increase in prevalence or severity as renal function declines (Sarnak and Levey, 2000). Uraemia-related risk factors are accumulations that occur in CKD patients due to their impaired renal clearance abilities, and hence increasingly significant and worsened as renal impairment continues to worsen (Yerram et al., 2007). These include, among others, proteinuria, increased uraemic toxin levels, oxidative stress in the vasculature, and chronic volume overload (Kao et al., 2010).

1.3.1.3 Dialysis risk factors

Finally, other risk factors include those related to dialysis. Patients receiving treatment with renal replacement therapy are at high mortality risk because of infection, which has been shown to contribute to approximately 10% of deaths (Bray et al., 2014).

1.4 Pathogenesis of hypertension in chronic kidney disease

The pathogenesis of hypertension in kidney disease patients has traditionally been attributed to a combination of both volume overload and sodium retention, along with an over-activation of the renin angiotensin system (RAS) (Phillips, 2005, Savoia and Schiffrin, 2006), however other factors contribute to or exacerbate hypertension in CKD, such as: endothelial dysfunction (Wang et al., 1999, Wang et al., 2000) (described later: see section 1.5.2.1) altered RAS functioning, vascular remodelling and calcification (London, 2003, London et al., 2003) (described later: see section 1.5.1.2.6) and sympathetic nervous system (SNS) hyperactivity (Cohen and Townsend, 2009, Schlaich et al., 2009). In the setting of CKD, these factors interact with each other in a complex manner to result in an increase in cardiovascular complications. These factors are explained in more detail below.

1.4.1 Renin angiotensin system

The RAS plays a major role in the development of hypertension and progression of kidney disease in patients with CKD (Cohen and Townsend, 2009), as evidenced by the effectiveness of RAS inhibitors in controlling blood pressure in renal disease (Martínez-Maldonado, 1998). The RAS is believed to be the most important endocrine system, with regards to its effects on blood pressure (Beevers et al., 2001, Foëx and Sear, 2004), and is well-established to play a large contributory role in the pathogenesis of renal impairment, via not only its vasoconstriction-inducing properties, but also its ability to disturb glomerular and tubular functions (Iino et al., 2003).

Though the RAS is located throughout the body, the majority of renin originates from the juxtaglomerular apparatus of the kidney (Foëx and Sear, 2004). The juxtaglomerular apparatus monitors renal perfusion pressure and sodium concentrations in the distal tubular

fluid (Foëx and Sear, 2004). Renin is synthesised and secreted from the juxtaglomerular apparatus of the kidney in response to glomerular under-perfusion (as a result of decreased blood pressure) or a reduced sodium load at the macula densa of the distal renal tubule (Beevers et al., 2001, Hilleman and Lynch, 1999). Renin is also released in response to stimulation of β1 adrenoceptors by noradrenaline (NA) released from the renal sympathetic nerve terminals (Beevers et al., 2001, Ecder and Schrier, 2009, Schweda and Kurtz, 2004, Foëx and Sear, 2004). The protease renin cleaves angiotensinogen to yield the inactive decapeptide angiotensin I (Ang I) (Foëx and Sear, 2004, Hilleman and Lynch, 1999), which is then converted into an active octapeptide, Angiotensin II (Ang II), by the angiotensin-converting enzyme (ACE) (Foëx and Sear, 2004, Criscione et al., 1993, Hilleman and Lynch, 1999, Morgado and Neves, 2012). Angiotensin I or Ang II can lead to the production of Ang-(1-7) (Santos, 2014) which is formed in the blood vessel endothelium (Santos et al., 1992), and has been shown to have vasodilatory effects (Silva et al., 2011), as well as antiproliferative effects in vascular smooth muscle cells (VSMCs) (Freeman et al., 1996). Angiotensin II acts directly on specific angiotensin type 1 (AT_1) and angiotensin type 2 (AT_2) receptors, and indirectly through the release of other agents acting in a paracrine or autocrine fashion, such as endothelin-1 (ET-1), growth factors or cytokines (Touyz and Schiffrin, 2000, Neves et al., 2003). Although the majority of actions of Ang II occur via the AT_1 receptors (Carey and Siragy, 2003), the role of AT_2 receptors are of high importance, as they negatively modulate AT_1 receptor function, and have vasodilatory effects in the resistance vasculature (Padia and Carey, 2013). High Ang II concentrations will suppress renin secretion via a negative feedback loop (Beevers et al., 2001, Hilleman and Lynch, 1999), and therefore regulate Ang II's resultant concentrations.

Angiotensin II's activation of the AT_1 receptor causes smooth muscle contraction, has a direct effect on vascular remodelling (see section 1.5.1.2.4) and has a key role in RAS effects on the SNS. Angiotensin II is known to mediate central sympathoexcitation (Sun et al., 2009, Fink, 2009), as well as alter sympathetic outflow to organs such as the heart and vasculature (Schlaich et al., 2009, Gao et al., 2008). In addition, it stimulates ET-1 production which leads to further increased vasoconstriction (Schiffrin, 2005). Angiotensin II stimulates the release of aldosterone from the adrenal gland (specifically, the zona glomerulosa), which consequently leads to a further increase in blood pressure which is related to sodium and water retention

(Beevers et al., 2001, Foëx and Sear, 2004, Neves et al., 2003), as well as the release of prostacyclin, and catecholamines. The local production of aldosterone and the binding to its receptor in VSMCs in turn, contributes to regulation of vascular tone and vascular remodelling in hypertension (Neves et al., 2003).

1.4.2 Sympathetic nervous system activity

The SNS has an important role in maintaining a normal blood pressure (Beevers et al., 2001, Foëx and Sear, 2004). Hypertension in patients with CKD and ESRD is paralleled by increases in SNS activity (Neumann et al., 2004, Vink et al., 2013, Blankestijn, 2007), as indicated by increased muscle sympathetic nerve activity (SNA) and circulating catecholamines (Zoccali et al., 2002, Klein et al., 2003). Altered SNS activity, as a consequence, augments the hypertensive state, via an increase in cardiac output (CO) and TPR. The enhanced SNA is believed to be driven by the gradually worsening renal impairments, because nephrectomy or denervation has been shown to correct blood pressure and SNS activity in human and animal studies (Phillips, 2005). Other evidence for the role of increased SNS activity in CKD has been demonstrated by Harrison et al. (2010), who found that ganglionic blockade significantly reduced mean blood pressure in a polycystic kidney disease (PKD) rat model (the Lewis polycystic kidney [LPK] rat) compared to Lewis control animals (52% vs. 4%), whereas plasma-renin values and Ang II were decreased in the worsened disease phase, suggesting a critical role for the SNS in the pathogenesis of the cardiovascular features of the disease (Phillips et al., 2007). In hypertensive patients, both increased release of, and enhanced peripheral sensitivity to, NA can be found (Foëx and Sear, 2004). However, it is unclear what stimulates the initial sustained elevations in sympathetic overactivity (Hilleman and Lynch, 1999).

The kidney has an important, dual role in the pathogenesis of increased SNS activity, as it is both a recipient and generator of sympathetic signals, through both efferent and afferent activity, respectively (Vink et al., 2013). There is an extensive network of sensory nerve fibres in the kidney, and many laboratory data indicate that sympathetic activation plays a role in hypertension in CKD, through not only direct vascular constriction, but also interactions with the RAS, via stimulation of renin release from the juxtaglomerular apparatus, and direct

stimulation of transtubular sodium reabsorption (Cohen and Townsend, 2009, Ritz et al., 1998, Beevers et al., 2001, Ecder and Schrier, 2009, Schweda and Kurtz, 2004, Foëx and Sear, 2004). Because the kidney is not only a target of sympathetic activity, but also a source of signals, it has the potential to directly modulate sympathetic drive and blood pressure (Phillips, 2005). In renal failure, the kidney drives an increase in efferent SNS activity, via afferent signals to central integrative autonomic nuclei and as a consequence, the increased SNS activity contributes to a further decline of renal function, and thus increases the risk of cardiovascular events in these renal disease patients (Phillips, 2005).

1.5 Effects of hypertension in chronic kidney disease on the resistance vasculature

Patients with hypertension may have an increase in CO and/or increase in TPR (Foëx and Sear, 2004); however, the haemodynamic hallmark of hypertension is increased TPR (Cooper and Heagerty, 1997). Although all blood vessels (diameters ranging from 3 mm lumen diameter in the rat aorta to about 7 μm in the capillaries) contribute to TPR to some extent, TPR is mostly determined by the small arteries (Beevers et al., 2001, Mulvany et al., 1978, Mulvany and Halpern, 1977). This is because small arteries are responsible for the major pressure drop that occurs between large conduit arteries and capillaries, by exerting their function through the resistance which they present to blood flow (Feihl et al., 2008, Hilleman and Lynch, 1999, Mulvany, 2002, Rizzoni et al., 2003, Short, 1966). Small arteries [arteries with lumen diameters between 100 and 300 μm (Steeds et al., 1999, Feihl et al., 2006, Luksha et al., 2011)], also known as resistance arteries, play an important role in hypertension (Li et al., 1997, Paisley et al., 2009). Although it is well-established that alterations in resistance arteries such as vascular remodelling are likely to contribute to increased cardiovascular risk and act as a substrate for end-organ damage (Schiffrin, 2012), resistance artery alterations are still poorly understood in hypertension and CKD, and hence are the focus of this thesis.

The aorta's distensibility under normal conditions ensures constant blood flow to target organs, and buffers increased pressures to protect end organs (Ng et al., 2011a). Hence, arteriosclerotic changes in the large conduit arteries which involve remodelling and an increase in calcium deposition (London et al., 2002, Wheeler, 2003, Ng et al., 2011b, Ameer et al., 2014b) can ultimately lead to target organ damage through their effects on the small

resistance arteries (Abdu et al., 2001). Increased stiffness, as measured by pulse-wave velocity (PWV), is a measure of arterial stiffness that has revealed associations with increased cardiovascular risk prediction in high-risk ESRD patients (Ng et al., 2011a). Initially, high mean arterial blood pressures may increase large artery stiffness through the loading of stiff components of the arterial wall at high blood pressure levels (Laurent et al., 2009). This increase in stiffness may in turn damage small resistance arteries because of transmission of pressure waves, resulting in consequent resistance vessel structural adaptations (James et al., 1995). The structural adaptation of small resistance arteries, which is characterised by an increased wall-lumen ratio, can then contribute to further increases in mean blood pressure (Park and Schiffrin, 2001), thus resulting in a vicious cycle that ultimately leads to the maintenance of high blood pressure.

Although small artery structural alterations of resistance arteries may be the first detectable manifestation of target organ damage in hypertension (Park and Schiffrin, 2001), functional alterations in the arteries such as endothelial dysfunction are also of high importance, as they can contribute to or exacerbate vascular remodelling in resistance arteries (described later: see section 1.5.2 Functional alterations). Endothelium function in hypertensive patients show a trend to decrease in the group with the highest blood pressure, and significantly decreases in those with the stiffest blood vessels (Park and Schiffrin, 2001). Endothelial cells appear to play a critical role in the development of vascular structural alterations, because removal of endothelium (a key element in mediating vascular response to shear stress) prevents vascular changes due to flow alteration (Langille and O'Donnell, 1986, Goto et al., 2000).

1.5.1 Structural alterations

Resistance arteries play a particularly important role in the maintenance of hypertension in CKD, because small alterations in the resistance artery lumen diameter can result in dramatic increases in TPR, and therefore hypertension (Intengan and Schiffrin, 2000). A framework proposed by Mulvany (2002) for understanding the relationship between blood pressure and resistance artery structure is shown in Figure 1.2. The cardiovascular system is believed to try and maintain blood pressure at an optimum level, as determined by the various functions of

the body (Julius, 1988). If blood pressure deviates from this "required" blood pressure, then fast (active) and slow (passive) processes are activated to compensate for this deviation.

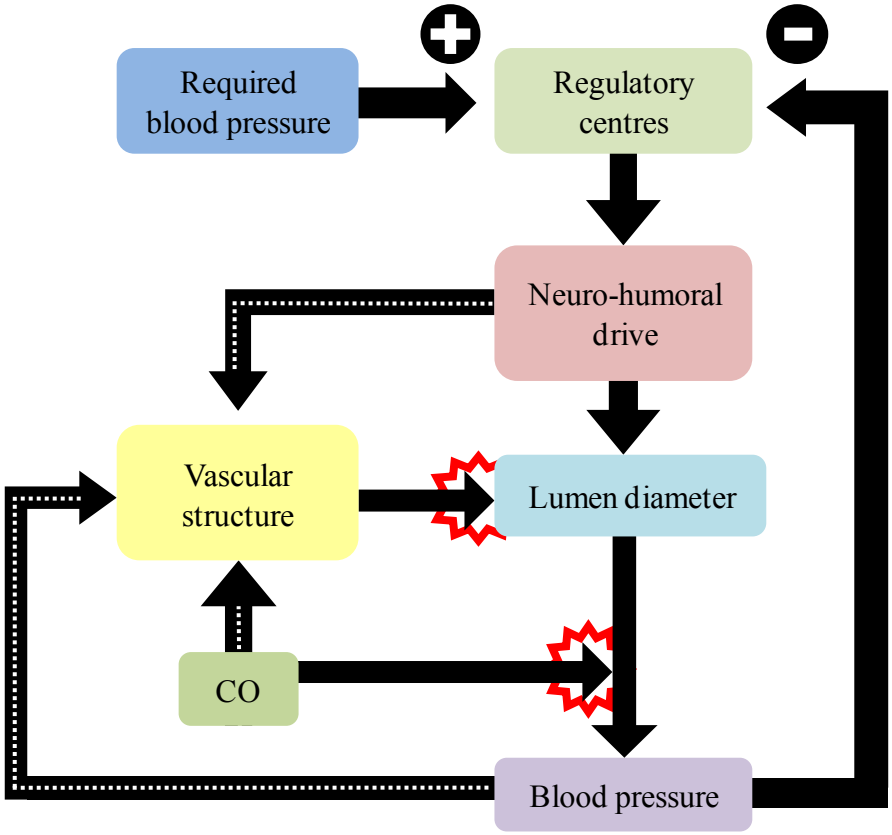

Figure 1.2. Diagram demonstrating the relationship between blood pressure, neurohumoral drive and resistance artery vasculature. Solid lines indicate fast processes, while dashed lines indicate slow processes. The star behind the arrow indicates amplification effects. The cardiovascular system tries to maintain blood pressure at a "required" blood pressure. Any deviation from the required pressure results in a feedback signal sent to regulatory centres, which will increase or decrease neurohumoral drive accordingly. Alterations in neurohumoral drive then affect resistance vessel structure and, together, these two stages will affect total peripheral resistance (TPR), and hence blood pressure. Vascular structure is affected by blood pressure, cardiac output (CO) and neuro-humoral drive, which act via slow processes to drive structural changes. In turn, vascular structure has amplifying effects on lumen diameter, further exacerbating structural changes. Similarly, CO also has amplifying effects, acting with

lumen diameter changes to further increase blood pressure. Adapted and modified from (Mulvany, 2002).

1.5.1.1 Fast processes and active mechanisms

Fast processes result in quick blood pressure alterations which are only temporary, whereas slow processes result in longer-lasting effects. Fast processes are a negative feedback mechanism that are inherently stable (Mulvany, 2002). For example, if the pressure is too low, baroreceptors detect these changes, resulting in fast processes which involve sending a signal to central autonomic regulatory centres to increase the neurohumoral drive (Mulvany, 2002). This increased neurohumoral drive then affects resistance vessel lumen diameter, with the ultimate effect of this drive being determined by the strength of neurohumoral drive signal, as well as resistance artery structure (Mulvany, 2002). The decreased lumen diameter then results in an increased TPR through its interactions with CO, thus resulting in an increased blood pressure (Mulvany, 2002). Fast processes altering lumen diameter primarily depend on the contractile activation and interaction of actin with myosin in VSMCs, which are known as active functional mechanisms of resistance arteries (Martinez-Lemus et al., 2009).

The active properties of a vessel are determined by the state of contraction of the individual VSMCs, their number, and their arrangement (Mulvany, 2002). The active mechanisms through which resistance arteries may increase systemic vessel resistance are a function of the activation level of the individual VSMCs; that is, their myogenic tone (Mulvany, 1999). Myogenic tone is VSMC's intrinsic ability to contract in response to an increase of transmural pressure, independent of any neural, metabolic or hormonal mediation (Davis and Hill, 1999, Davis et al., 2001, Feihl et al., 2006, Sonoyama et al., 2007). Myogenic tone becomes more vigorous as vessel size decreases (Davis, 1993), and therefore gains in importance with decreasing vessel calibre; resistance arteries in particular exhibit substantial luminal narrowing (or even closure) in reaction to an increase in transmural pressure (Davis, 1993, Uchida and Bohr, 1969).

Myogenic tone serves to protect the distal capillaries against deleterious local hypertension (Davis and Hill, 1999) and deliver blood to the capillaries in the correct quantity and at the

correct pressure (Mulvany, 2002). This short-term protection has an immediate result: augmented myogenic tone amplifies resistance to blood flow, which leads to a proximal increase in blood pressure (Feihl et al., 2006) so that flow resistance is concentrated on the arterial side of the system, and capillary pressure is therefore maintained at a low level (Pries et al., 1995, Pries et al., 2001). However, if the elevation of transmural pressure persists, short-term modulating myogenic constriction will initially be prolonged, but will eventually progressively give way to structural rearrangement of wall material around a smaller lumen, i.e. the passive adaptation of resistance vessels (Bakker et al., 2002, Martinez-Lemus et al., 2004, Mulvany, 2002, Sonoyama et al., 2007). Myogenic tone is only a short-term modulator, while structural passive adaptation of resistance vessels is a significant contributor to the maintenance of elevated blood pressure for any prolonged period (Mulvany, 2002).

1.5.1.2 Slow processes and passive mechanisms (vascular remodelling)

Following the activation of fast processes, slow passive processes involving alterations in resistance artery structure ensue (Mulvany, 2002). For example, if the required blood pressure were to increase such as in hypertension, slow processes would be activated to ensure adequate long-term blood flow to all blood vessels (Mulvany, 2002).

Passive mechanisms through which resistance arteries may result in an increase in TPR is via structural modifications. These alterations in vascular structure involve an increase in resistance vessel wall-lumen ratio, as a result of an increased neurohumoral drive and an increased blood pressure (Mulvany, 2002), and are grouped under the generic name of *vascular remodelling* (Mulvany, 2002, Feihl et al., 2008). Due to the increased resistance vessel wall-lumen ratio, the neurohumoral drive necessary to maintain increased blood pressure will be reduced, and any subsequent neurohumoral drive hereafter may be amplified due to altered resistance artery structure (Mulvany, 2002).

A variety of factors contribute to the pathogenesis of vascular remodelling in CKD-mediated hypertension (see Figure 1.3), some of which include but are not limited to: neurohumoral/hormonal drive (see section 1.5.1.2.5 Vascular remodelling and the sympathetic nervous system), RAS activation (see section 1.5.1.2.4 Vascular remodelling and

the renin angiotensin system), and functional alterations (see section 1.5.2 Functional alterations).

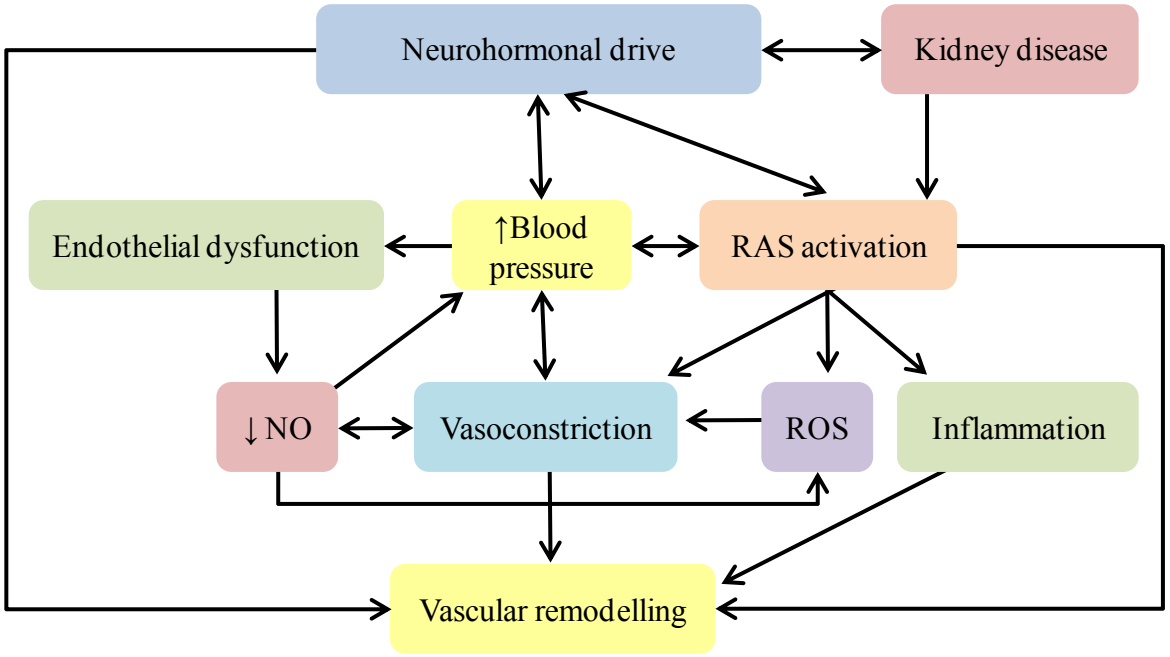

Figure 1.3. Simplified schematic illustration of the pathogenesis of vascular remodelling. Blood pressure interacts with a variety of factors to contribute to vascular remodelling, including neurohumoral/hormonal drive alterations, renin angiotensin system (RAS) alterations, vasoconstriction of the blood vessels, and endothelial dysfunction. Endothelial dysfunction results in a decrease in nitric oxide (NO), which consequently affects reactive oxygen species (ROS) levels as well as blood pressure and vasoconstriction (described later: see section 1.5.2). Complex interactions between neurohumoral/hormonal drive, kidney disease and RAS activation lead to vasoconstriction, increased ROS and inflammation; all of which lead to vascular remodelling. Adapted and modified from (Mulvany, 2002, Intengan and Schiffrin, 2001).

Passive remodelling results in altered pressure-diameter relationships of small resistance arteries (Neves et al., 2003). Structural alterations in the resistance arteries are known to play a significant role in the persistent increases in TPR (Intengan and Schiffrin, 2000, Laurant et

al., 1997, Pries et al., 2001), and thus the prediction of cardiovascular events in hypertensive patients (Mathiassen et al., 2007, Rizzoni et al., 2003, Intengan and Schiffrin, 2001).

In hypertension, the arterial system undergoes structural remodelling that is characterised by hypertrophy of the VSMCs with encroachment on the lumen (and therefore an increased wall-lumen ratio), as well as associated decreased arterial distensibility (Touyz, 2000, Laurant et al., 1997). Vascular remodelling of resistance vessels is hypothesised as being one of the first sites of target organ damage due to increased intraluminal pressures from hypertension (Schiffrin et al., 2000, Intengan and Schiffrin, 2001): though vascular remodelling may initially be adaptive, it eventually becomes maladaptive and compromises organ function, contributing to cardiovascular complications of hypertension (Intengan and Schiffrin, 2001).

1.5.1.2.1 Vascular remodelling parameters

Specifically, though the term "remodelling" in the context of hypertension generally refers to a decreased internal lumen diameter, increase in wall thickness and no net change in media cross sectional area (MCSA), there are in fact other types of remodelling that may occur. Definitions of the various types of remodelling rely on specific measurements of the artery: internal lumen diameter, media wall thickness, wall-lumen ratio and MCSA (Figure 1.4).

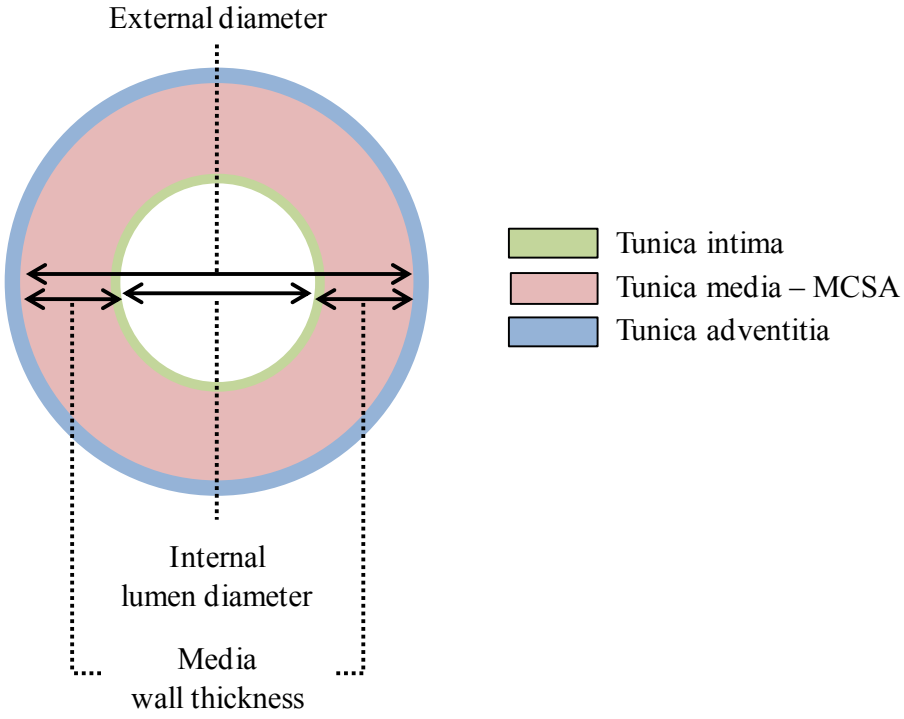

Figure 1.4. Schematic illustration of parameters measured from arteries to define remodelling. MCSA, media cross sectional area. Adapted and modified from (Short, 1966).

1.5.1.2.2 Subtypes of vascular remodelling

Arterial remodelling is a multidirectional process – as shown in Figure 1.5. For example, *outward* remodelling refers to an increase in lumen diameter whereas *inward* remodelling is a decrease in lumen diameter (Mulvany, 1999). Other terminology utilises the MCSA as the point of reference. Remodelling that involves an increase in the area occupied by the vessel media is called *hypertrophic* remodelling. In hypertrophic remodelling, the MCSA increases, indicating the presence of smooth muscle growth (Mulvany, 1996). This increase in vessel thickness may encroach on the lumen (Behbahani et al., 2010, Mulvany, 1996, Intengan and Schiffrin, 2000), resulting in a narrowed lumen diameter which is thus associated with an increased media-lumen ratio (also known as wall-lumen ratio) and MCSA (Intengan and Schiffrin, 2000, Mulvany et al., 1985). In contrast to hypertrophic remodelling, remodelling that yields a decrease in MCSA is termed *hypotrophic* remodelling (Mulvany, 1999). Finally, remodelling in which there is no change in MCSA is called *eutrophic* remodelling (Mulvany, 1999). In eutrophic remodelling, vascular wall material is rearranged around a narrowed

lumen without evidence of net growth (Behbahani et al., 2010, Mulvany, 2002, Intengan et al., 1999). In inward eutrophic remodelling, outer and inner lumen diameters are reduced, MCSA is unaltered, and wall-lumen ratio is increased, without an increase in vascular stiffness (Intengan and Schiffrin, 2001). The mechanisms leading to inward eutrophic remodelling are poorly understood, but could result from inward growth combined with peripheral apoptosis, or from vasoconstriction embedded in an expanded extracellular matrix (ECM) (Intengan and Schiffrin, 2001). A negative consequence of the inward eutrophic remodelling response is that increased systemic pressure results in increased TPR, because vascular diameters are decreased throughout the network (Pries et al., 2001). The type of remodelling greatly affects the resistance generated to flow (Mulvany, 1999). For instance, compared to a normal arteriole, a vessel with eutrophic remodelling generates more resistance to blood flow upon smooth muscle contraction.

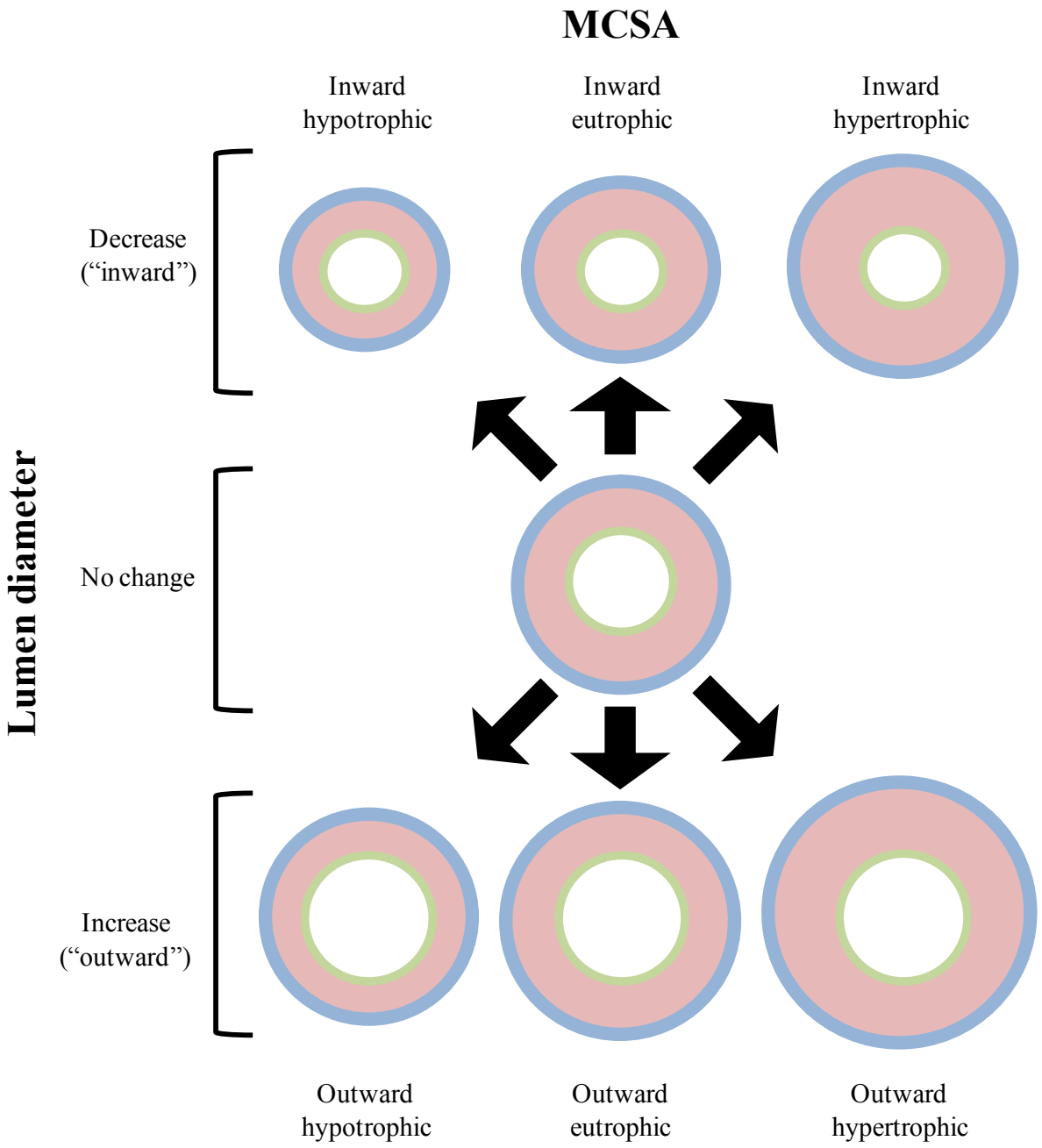

Figure 1.5. Diagram showing the various types of vascular remodelling. The starting point of the vessel is shown in the centre. Vascular remodelling can be classified according to structural changes in the lumen diameter and changes in the media cross sectional area (MCSA). Lumen diameter changes can be inward (decrease in lumen diameter) or outward (increase in lumen diameter), while MCSA changes may be hypotrophic (decrease in MCSA), eutrophic (unaltered MCSA), or hypertrophic (increase in MCSA). Adapted and modified from (Mulvany, 1999).

1.5.1.2.3 Role of vascular remodelling in total peripheral resistance

The important contributory role of resistance artery remodelling to TPR is best explained by 1) Laplace's and 2) Poiseuille's laws:

Laplace's law

Laplace's law is a tool used to understand the varying structure of different blood vessels in different conditions. For any tubular flow within an object of cylindrical geometry, Laplace's law states:

$$\boldsymbol{\sigma = \frac{P \times r}{WT}}$$

In this equation, σ is the intramural (average circumferential) stress, P is the transmural pressure (the difference between luminal and extraluminal pressures), r represents the lumen diameter ("radius"), and WT is the vessel wall thickness.

In large arteries, an elevation of transmural pressure causes a compensatory distension (an increase in lumen diameter), decrease in wall thickness, and therefore an increase in intramural stress. Because large arteries have very little myogenic response, in the presence of persistent elevation of transmural pressure, the only mechanism that allows regulation of intramural stress in the presence of distension is an increase in growth response. This growth response involves a constant increase in blood vessel wall thickness to a level that is sufficient enough to normalise intramural stress.

In contrast to large arteries, for the same degree of elevation of transmural pressure, small arteries exhibit a different coping mechanism. This is because resistance arteries have greater myogenic tone, as well as smaller lumen radii and wall thickness relative to large arteries. This means that resistance arteries are able to actively adjust their lumen size and wall thickness to increased pressure, and that their intramural stress values are much lower (Feihl et al., 2008). Therefore, when exposed to hypertension, these small arteries do not exhibit a significant change in intramural stress, and therefore do not exhibit hypertrophy (Feihl et al., 2008). Instead, resistance arteries typically undergo a process consisting of the rearrangement of VSMCs and ECM, whilst still maintaining a constant cell number and mass – "eutrophic inward remodelling".

Poiseuille's law

Because of their small size, resistance artery eutrophic inward remodelling changes play a significant role in the determination of blood vessel resistance. The large effect of changes in resistance vessel structure on TPR is demonstrated by Poiseuille's law:

$$R \propto \frac{\eta \times L}{r^4}$$

In this equation, R is the resistance, η is the viscosity of blood, L is the length of the resistance artery, and r represents the lumen diameter ("radius").

As a structural response to hypertension, resistance vessels decrease their lumen diameter and increase in vessel wall thickness. This decrease in lumen diameter, although it may be a minor reduction, consequently has major effects on TPR and blood pressure, because TPR is inversely proportional to the 4th power of the vessel radius (Cooper and Heagerty, 1997, Savoia and Schiffrin, 2006). Lumen diameter is a primary functional characteristic of resistance vessels, determining TPR and therefore blood pressure, blood flow (BF), and cardiac work (Faury et al., 1999). These hypertension-induced structural changes in resistance arteries serve to protect the smaller capillary vessels from elevated blood pressure. However, though the decreased lumen diameter and increased vessel wall thickness are adaptive mechanisms to compensate for the change in blood pressure, these structural alterations will become key players in the continued elevation of pressure (Cooper and Heagerty, 1997, Rizzoni et al., 2003, Mulvany, 2008).

1.5.1.2.4 Vascular remodelling and the renin angiotensin system

The RAS is an important contributor to vascular remodelling (Savoia et al., 2011, Min et al., 2005), with Ang II playing a particularly important role in VSMC hypertrophy and inflammation (Schiffrin, 2004, Zhang et al., 2005).

Beyond contraction of smooth muscle, Ang II has the ability to promote many processes in cardiovascular tissue, including cell growth, migration, differentiation, and apoptosis, as well as modulation of ECM composition and turnover (Feihl et al., 2008, Griffin et al., 1991). It has been extensively documented that Ang II can directly induce cell growth, in part via formation of ROS, and activate inflammatory mechanisms contributing to vascular

remodelling (Luft, 2001). Angiotensin II is greatly implicated in the development of the progression of structural alterations (vascular remodelling) in small resistance arteries (Touyz, 2005): it has been demonstrated that long-term infusions of non-pressor doses of Ang II are able to induce resistance vessel vascular remodelling, involving significantly increased media width, MCSA, and wall-lumen ratio (Griffin et al., 1991). This is via the AT_1 receptors through smooth muscle growth (Touyz et al., 1999, Gibbons et al., 1992) and collagen deposition (Benetos et al., 1997), as has been demonstrated in rat resistance arteries with primary hypertension (Aalkjaer et al., 1987, Intengan et al., 1999).

The role of Ang II in the vascular inflammatory process involves three steps (Cheng et al., 2005):

1. Increased vascular permeability, which may be due to endothelium layer damage as a result of blood pressure changes by Ang II.
2. Increased leukocyte infiltration of the vessel wall, due to Ang II actions which include upregulation of proinflammatory mediators and activation of circulating inflammatory cells.
3. Vascular remodelling through Ang II's cell growth and fibrotic stimulatory actions, which are mediated through increased ROS generation (Touyz, 2005, Savoia et al., 2011).

1.5.1.2.5 Vascular remodelling and the sympathetic nervous system

The SNS plays an important contributory role in the vascular structural alterations (Mancia et al., 1999, Grassi, 2009) observed in both experimental animal and human studies (Grassi, 2009, Folkow, 1982, Bevan, 1984). Mechanisms through which the SNS may affect structural alterations are vast, and may include but are not limited to: interactions with vasoactive peptides such as Ang II or ET-1, which increases ROS and other growth factors, thus leading to inflammation (Intengan and Schiffrin, 2001); increases in growth factors or vasoconstriction as a result of an increase in blood pressure (Intengan and Schiffrin, 2001); increases in the density of sympathetic nerve terminals, which result in a thickening of the arterial wall.

1.5.1.2.6 Vascular remodelling and vascular calcification

Vascular calcification is characterised by the thickening and loss of elasticity of muscular artery walls due to calcification (Oliveira et al., 2013), and is commonly associated with renal failure (Kramer et al., 2005), as well as with increased risk of cardiovascular mortality (Shanahan et al., 2011, El-Abbadi et al., 2009). Vascular calcification can independently contribute to the initiation or progression of CVD, and has significant influence on cardiovascular function and health in ESRD patients (Yilmaz et al., 2009). It is a common complication in CKD (Mizobuchi et al., 2009b, Mizobuchi et al., 2009a), reportedly present in approximately 40% of CKD patients (Russo et al., 2004), and has been found to occur within the aorta in human and animal studies (Moe et al., 2008, Wallin et al., 2001, Ng et al., 2011b), and to progress in human patients on dialysis (Bellasi et al., 2009). In addition, calcification in the smaller arteries of humans, though less extensively investigated, have been reported (Srija et al., 2012, O'Neill and Adams, 2013). In the large arteries, there are two major types of vascular calcification which are characterised by their location: atherosclerotic intimal calcification and medial artery calcification (Zhu et al., 2012). These categories of calcification are described briefly as follows:

Atherosclerotic intimal calcification: Atherosclerotic calcification is the most common form of calcific vasculopathy (Stary et al., 1995), which is the result of plaque development within the intimal layer of large vessels (Zhu et al., 2012). Hypertension is an important risk factor for atherosclerotic calcification (Allison et al., 2004), involving cellular necrosis, inflammation, and lipid deposition (Shroff and Shanahan, 2007), as well as osteogenesis, osteoblast induction and lamellar bone formation, as the vascular insult worsens (Mizobuchi et al., 2009b).

Mönckeberg sclerosis: Medial calcification, which is also known as Mönckeberg's sclerosis, occurs in the tunica media of blood vessels (Zhu et al., 2012), and is believed to be a manifestation of accelerated atherosclerosis (McCullough et al., 2008a). Medial calcification typically occurrs in patients with metabolic syndrome, diabetes, and/or CKD (Oliveira et al., 2013), and indeed is the most prevalent form of calcification in patients with CKD (Zhu et al., 2012, Mizobuchi et al., 2009b, El-Abbadi et al., 2009), resulting in the degradation of elastin fibres due to diffuse mineral deposition along elastic fibres. As a consequence of deposition,

this may increase medial VSMC migration and proliferation, ultimately leading to an encroachment of the vascular lumen (Wallin et al., 2001).

The mechanisms leading to the development of vascular calcification in CKD are complex and multifactorial, and differ between arteries (O'Neill and Adams, 2013). Though the underlying reasons for this difference in susceptibility to calcification are yet to be determined, some mechanisms have been proposed: 1) the different ontogenic origins of specific arteries may determine their susceptibility to calcification, due to altered levels of ECM degradation in response to cytokine exposure; 2) vessel characteristics, such as vessel size and vessel composition (elastic versus muscular) (Cheung et al., 2012); and 3) vessel environment, such as laminar versus turbulent flow, shear stress, presence of calcification and blood pressure levels (Schlieper, 2014).

General mechanisms of vascular calcification include cell-mediated processes such as apoptosis, osteochondrogenic differentiation, and elastin degradation (Oliveira et al., 2013). In addition to the above processes, much vascular calcification research has focused on the dysregulated mineral metabolism, namely the abnormal parathyroid hormone and vitamin D levels (McCullough et al., 2008a), and imbalanced serum phosphate and calcium levels, which can act independently or jointly to promote VSMC dysfunction and calcification in CKD (Figure 1.6) (Shanahan et al., 2011, Fang et al., 2014). A few of the main factors contributing to vascular calcification are described below.

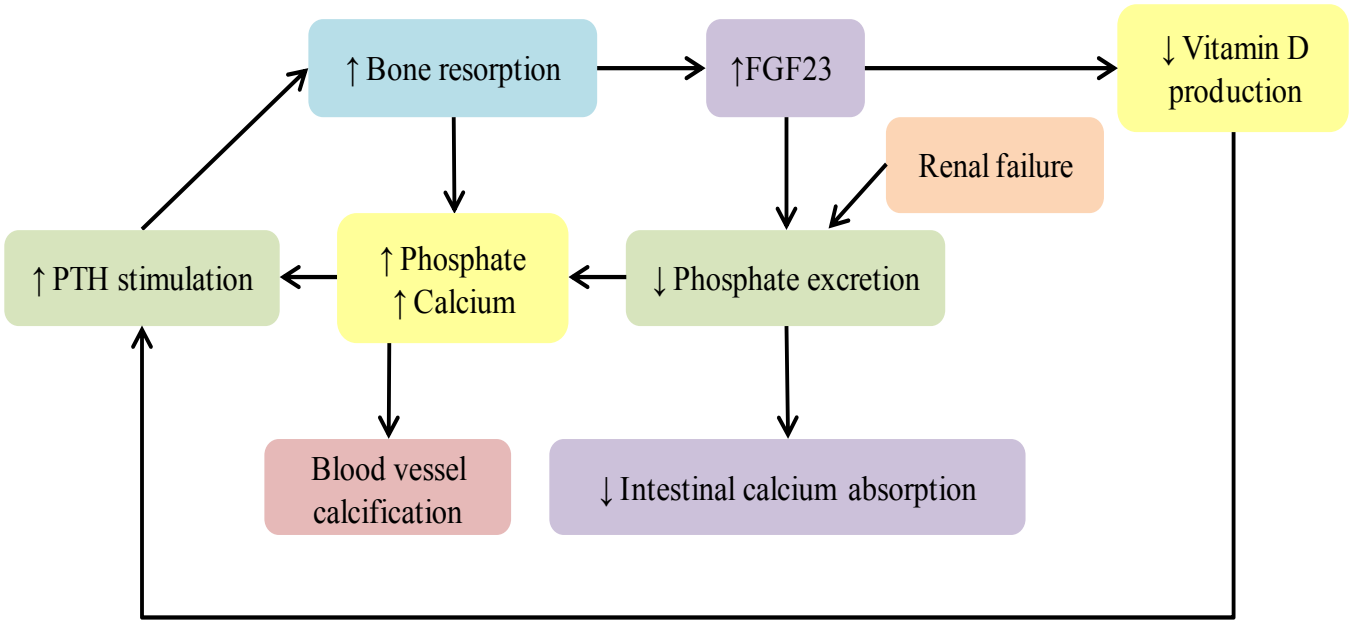

Figure 1.6. Diagram of the role of phosphate and calcium levels in chronic kidney disease. As renal failure progresses, renal P excretion decreases, which leads to a decrease in intestinal Ca absorption, as well as an increase in both P and Ca levels. Increased P and Ca levels result in blood vessel calcification and increased parathyroid hormone (PTH) stimulation, which consequently increases bone resorption and leads to a further increase in P and Ca levels. Increased bone resorption can also elevate fibroblast growth factor (FGF) -23 secretion, leading to a decrease in P levels as well as a decrease in Vitamin D production. Adapted and modified from (Seiler et al., 2009, Neven and D'Haese, 2011, Zhu et al., 2012, Shanahan et al., 2011).

Elastin degradation

In CKD patients, elastin degradation has been shown to be an important determinant of arterial stiffness as well as all-cause mortality (Smith et al., 2012). A strong correlation has been shown between matrix mellatoproteinase-mediated elastin degradation and vascular calcification (Basalyga et al., 2004). Hydroxyapatite deposits on the elastic lamellae can lead to elastocalcinosis (Oliveira et al., 2013) and elastin fibre degradation (Janzen and Vuong, 2001), which is thought to play an important role in the progression of vascular calcification and osteoblastic differentiation (Mizobuchi et al., 2009b, Oliveira et al., 2013). High calcium

levels can also lead to elastin degradation and thus vascular calcification, through its effects on nucleation complexes and enhancement of metalloproteinase-2 activity (Shroff et al., 2013, Kapustin et al., 2011). Degraded elastin facilitates the growth of hydroxyapatite along the elastic lamellae through its high affinity for calcium (Oliveira et al., 2013), and is an early and important contributor to the pathogenesis of arterial medial calcification associated with CKD (Pai et al., 2011), accelerating phosphate-induced mineralisation of VSMCs (Hosaka et al., 2009).

Mineral disorder

A dysregulation of mineral levels involving altered phosphate and calcium metabolism can promote vascular calcification (Figure 1.6) (Shroff et al., 2010). Phosphate and calcium are closely associated, where increased calcium can eventually lead to calcium-phosphate precipitation in the vasculature (Covic et al., 2010). Excess phosphate and calcium levels have been shown to promote apoptic processes (Reynolds et al., 2004, Mizobuchi et al., 2009b), and hypercalcaemia and hyperparathyroidism are associated with an increased risk of development of aortic calcification, as well as an increased risk of all-cause mortality and cardiovascular mortality (Noordzij et al., 2011).

The homeostatic balance of phosphorus and calcium involves the intestines, parathyroid gland, kidneys, and bone (Figure 1.6). Even small elevations in serum phosphate have been shown to correlate with an increased risk of mortality in CKD patients (Kestenbaum et al., 2005). Phosphate loading and uraemia have been shown to be significant contributors to arterial medial calcification, with fibroblast growth factor (FGF) -23 proposed to be one of the possible markers and/or inducers of this process (El-Abbadi et al., 2009). In the CKD state, the kidneys lose their ability to excrete phosphorus, which thus leads to an imbalance of phosphorus and ultimately vascular calcification (Eddington et al., 2010, Palmer et al., 2011, Eknoyan et al., 2003), despite the role of parathyroid hormone (PTH) and FGF-23 (Eddington et al., 2010, Palmer et al., 2011).

Vitamin D

Vitamin D is related to myriad factors which contribute to calcification and interact with each other to result in worse calcification; for example, vitamin D deficiency interacts with vascular stiffness, vascular endothelial dysfunction, and gastrointestinal absorption of calcium and phosphate, which subsequently leads to a mineral disorder (Chitalia et al., 2014, Marco et al., 2003). A variety of factors may contribute to a deficiency in vitamin D, some of which include but are not limited to, reduced sun exposure, decreased dietary intake, and impaired production of the 25-hydroxy vitamin D precursor (Holick et al., 2011, Eknoyan et al., 2003).

Patients and animal models with CKD and vascular calcification often present with either a severe deficiency or excess of vitamin D (Moe and Chen, 2004, Shroff et al., 2008, Fortier et al., 2014, Zittermann et al., 2007). Thus, interestingly, vitamin D seems to have either a potentially detrimental or protective role on the vasculature. Excess vitamin D may be related to bone resorption (Hruska et al., 2009), and has been shown to induce vascular calcification; however, supplementation with vitamin D has been shown to suppress serum tissue necrosis factor –alpha (TNF-alpha) levels, which plays an important role in intimal atherosclerosis and calcification, and leads to an increase in interleukin 10 (IL-10) levels, which have important antiatherogenic properties (Mallat et al., 1999, Schleithoff et al., 2006).

1.5.2 Functional alterations

As mentioned above, in addition to structural alterations of resistance arteries, functional alterations are of equal importance, with endothelium-dependent dysfunction for being a well-established phenomenon in CKD and ESRD patients (Wang et al., 2000, Amabile et al., 2005, Thambyrajah et al., 2000, Passauer et al., 2000).

1.5.2.1 Endothelial dysfunction

Endothelial dysfunction is characterised by and often defined as, a reduced endothelium-dependent vasodilation response to an endothelium-dependent vasodilator, such as acetylcholine (ACh) or bradykinin (BK), or to flow-mediated vasodilation (Ding and Triggle, 2010). Dysfunction of the endothelium is often associated with elevation of blood pressure,

and may be a secondary phenomenon that does not initiate blood pressure elevation; rather, it may be associated with cardiovascular risk factors that cluster with hypertension, such as smoking, obesity, dyslipidemia, metabolic syndrome, and diabetes mellitus (Schiffrin, 2012). Endothelial dysfunction occurs in both large and small arteries, and is present in early renal disease stages (Endemann and Schiffrin, 2004, Morris et al., 2001). However, specifically, it is endothelial dysfunction in resistance vessels in hypertension (Deng et al., 1995, Panza et al., 1990), that can contribute to manifestations of hypertensive target organ damage (Schiffrin et al., 2000), as well as contribute to the already existing cardiovascular mortality in mild renal insufficiency (Stam et al., 2006).

In hypertensive patients, vascular endothelial cell dysfunction could lead to reduction in endothelium-derived relaxing factors such as NO, prostacyclin, prostaglandins, and endothelium-derived hyperpolarisation (EDH), or increased production of constricting factors such as ET-1 and Ang II (described previously in section 1.4.1 Renin angiotensin system) (Foëx and Sear, 2004, Matrougui et al., 2000, Tronc et al., 1996, Huang et al., 2000, Martínez-Maldonado, 1998, Beevers et al., 2001). In addition, it is likely that inflammation and the increased oxidative stress, which probably act as important triggers, and may to a large extent be Ang II–dependent, may elicit growth factor–mediated ECM remodelling, and therefore play a role in the process leading to further structural remodelling of small vessels in hypertension (Intengan and Schiffrin, 2001). Thus, there is a complex relationship between endothelium-derived relaxing and constricting factors.

1.5.2.1.1 Nitric oxide signalling

Acetylcholine-induced vasodilation is partly mediated through NO, a potent vasorelaxant and antiproliferative molecule released from both arterial and venous endothelium (Liu et al., 2002, Waldron et al., 1999) and diffuses through the vessel wall into the VSMC causing vasodilation (Beevers et al., 2001). Nitric oxide was formerly known as endothelium derived relaxant factor (Morris et al., 2001) and is estimated to contribute to 28% of relaxation induced by ACh in rat mesenteric artery (Paulis et al., 2010). Healthy vascular endothelium acts to prevent the development of atherosclerosis principally through the production and release of NO (Morris et al., 2001).

Endothelium NO signalling has been shown to be impaired in hypertensive patients (Foëx and Sear, 2004) and in CKD-associated endothelial dysfunction (Passauer et al., 2005, Hasdan et al., 2002) – as demonstrated for example in rat mesenteric arteries by a shift of the dose-response curve to higher ACh doses, and by a decrease in the NO-dependent component of ACh-induced relaxations (Paulis et al., 2008). In addition, the presence of endothelial dysfunction in the resistance vasculature of ESRD subjects has been demonstrated by a reduced dilatation in response to ACh, yet preserved response to an NO donor (Luksha et al., 2011), and ACh-induced vasodilation has also indicated impaired endothelium *in vitro* in hypertensive rat aorta (Lockette et al., 1986, Lüscher and Vanhoutte, 1986) and small mesenteric arteries (Paulis et al., 2010). In disease states where NO production is impaired, endothelium NO may serve as a compensatory vascular NO supply, which leads to vasorelaxation via activation of potassium channels (Chauhan et al., 2003).

There are at least three isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelium NOS (eNOS) (Colasanti and Suzuki, 2000, Jaffrey and Snyder, 1995, Michel and Feron, 1997, Bruno and Taddei, 2011). Neuronal NOS is predominantly localised in central tissue and peripheral nervous system neurons (Jaffrey and Snyder, 1995, Alderton et al., 2001), where it acts as a neurotransmitter and indeed plays a key role in autonomic regulation of cardiovascular function (Patel et al., 2001, Hirooka et al., 2011); iNOS is inducible in a wide range of cells and tissues and is constitutively expressed only in select tissues such as lung epithelium (Dweik et al., 1998). It is more typically synthesised in response to inflammatory or proinflammatory mediators (Alderton et al., 2001). Endothelium NOS is found not only in vascular endothelial cells, but also in other cell types, including neuronal cells (Bruno and Taddei, 2011, Alderton et al., 2001). Neuronal NOS and eNOS are stimulated in response to increases in intracellular calcium (Jaffrey and Snyder, 1995) – that is, they are calcium dependent (Alderton et al., 2001). In contrast, iNOS is calcium-independent (Alderton et al., 2001). Endothelium NOS is a critical regulator of cardiovascular homeostasis (Sessa, 2004), synthesises small amounts of NO under physiological conditions, and is an endogenous vasodilatory mediator that continually regulates the diameter of blood vessels and maintains an anti-proliferative and anti-apoptotic environment in the vessel wall (Sessa, 2004).

As shown in Figure 1.7, NO is produced continuously under resting conditions from L-arginine and by NOS, and contributes to basal vessel tone (Morris et al., 2001, Berry et al., 2001, Rees et al., 1989, Vane et al., 1990, Palmer et al., 1988, Shimokawa and Takeshita, 1995). Once NO is produced from L-arginine, NO then causes vasodilation by stimulating the activity of soluble guanylate cyclase (sGC) within the vascular smooth muscle, thereby elevating levels of cyclic guanosine monophosphate (cGMP), via the conversion of guanosine triphosphate (GTP) to cGMP (Ignarro et al., 1987, Martinez-Revelles et al., 2008, Lincoln and Cornwell, 1991, Moncada et al., 1991).

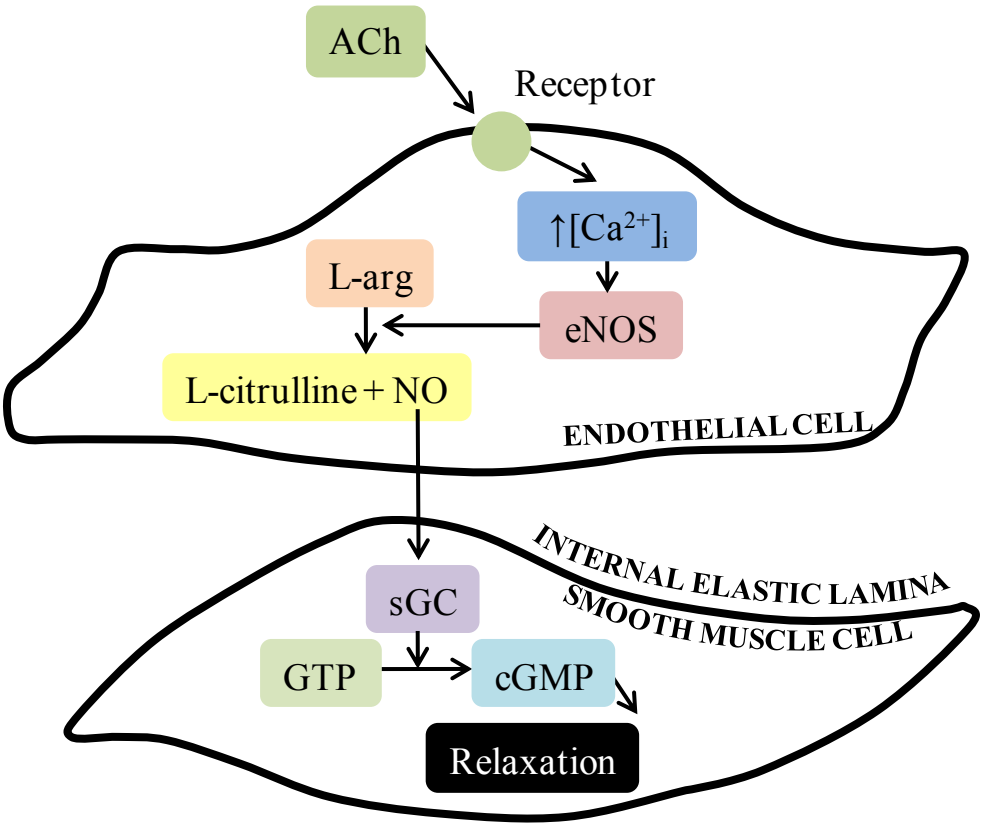

Figure 1.7. Diagram of the nitric oxide (NO) -mediated vasorelaxation pathway. Binding of a molecule such as acetylcholine (ACh) onto muscarinic receptors on the endothelial cell triggers an increase in intracellular calcium concentrations ($[Ca^{2+}]_I$), which then activates the calcium-dependent enzyme endothelium nitric oxide synthase (eNOS). Endothelium NOS then assists in the conversion of L-arginine (L-arg) to L-citrulline and NO, which results in

diffusion of the latter component across to the smooth muscle cell. Nitric oxide then activates soluble guanylate cyclase (sGC), which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which results in relaxation.

To investigate the effects of NO on vasodilation, nitrosylated L-arginine derivatives are commonly used to inhibit NOS (Rees et al., 1989, Rees et al., 1990). Previous studies have demonstrated a constrictor effect of arginine-based NOS inhibitors on isolated, pressurised arterioles with myogenic tone (Wang et al., 1999, Huang et al., 1997), implying a role for endothelium-derived NO in regulating pressure-induced constriction (Wang et al., 1999, Murphy et al., 2007). It is now well accepted that the NOS inhibitor Nω-Nitro-L-arginine methyl ester (L-NAME) has been shown to inhibit an endothelium-dependent relaxation in isolated, pressurised blood vessels in response to ACh (Murphy et al., 2007, Moore et al., 1990, Wang et al., 1999). In addition, L-NAME administration has been shown to lead to a disruption of NO signalling in small as well as large arteries (Paulis et al., 2008). Effects of the NOS inhibitors alone in a test system (i.e. in the absence of a coexisting stimulus for NO production such as L-arginine) are usually interpreted as indicating endogenous NOS activity and basal production of NO (Murphy et al., 2007).

1.5.2.1.2 Prostacyclin

Prostacyclin (also known as prostaglandin I_2 [PGI_2]) is a major member of the family of prostaglandins produced by endothelial cells (Vane et al., 1990), as well as a major product of vascular cyclooxygenase (COX), and a potent inhibitor of platelet aggregation. Prostacyclin is a powerful smooth muscle vasodilator (Vane et al., 1990) however it only contributes minimally to endothelium-dependent relaxation in resistance arteries (Moncada and Vane, 1978, Shimokawa et al., 1996).

As shown in Figure 1.8, prostacyclin production is initiated by the enzyme phospholipase A_2, which liberates arachidonic acid (AA) from membrane phospholipids (Vane et al., 1990). Arachidonic acid release is initiated via various agonists, such as NA, Ang II, and BK (Mukherjee et al., 1992, Muthalif et al., 1996). Following AA liberation, the enzyme COX then converts AA into prostaglandin cyclo-endoperoxides: prostaglandin G_2 (PGG_2) (Vane et

al., 1990) and PGG_2 becomes prostaglandin H2 (PGH_2) via peroxidise (Simmons et al., 2004). Subsequently, a variety of tissue-specific enzymes compete for the same substrate PGH_2, thus dictating the relative amounts of prostaglandins, PGI_2, or thromboxane A_2 (TxA) synthesised (Vane et al., 1990, McGiff, 1981, Simmons et al., 2004). Prostacyclin is the primary product of synthase action on PGH_2, and is a potent vasodilator (Fetalvero et al., 2007, Li et al., 2004). Prostacyclin then binds to the prostacyclin receptor (IP receptor) on the VSMC, therefore activating a Gs protein, and thus adenylate cyclase (AC) (Bos et al., 2004). Adenylate cyclase in turn converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), which stimulates protein kinase A (PKA), resulting in the inhibition of myosin light chain kinase (MLCK) and hence relaxation (Bos et al., 2004).

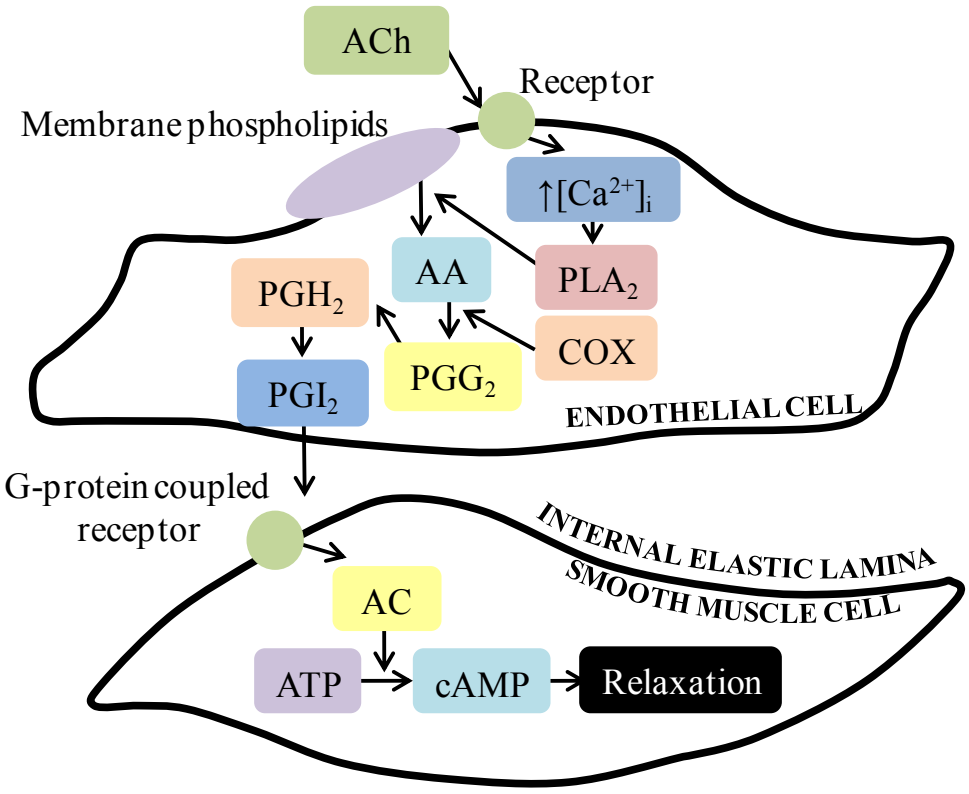

Figure 1.8. Diagram of the prostacyclin-mediated vasorelaxation pathway. Binding of a molecule such as acetylcholine (ACh) onto muscarinic receptors on the endothelial cell triggers an increase in intracellular calcium concentrations ($[Ca^{2+}]_I$), which then activates phospholipase A_2 (PLA_2). Phospholipase A_2 liberates arachidonic acid (AA) from membrane

phospholipids. Arachidonic acid is converted to prostaglandin G_2 (PGG_2) via the cyclooxygenase (COX) enzyme, and then becomes prostaglandin H_2 (PGH_2) and subsequently prostaglandin I_2 (PGI_2), also known as prostacyclin. Prostacyclin binds to the prostacyclin receptor (IP) on the smooth muscle cell which activates a G_s protein, and then adenylate cyclase (AC). Adenylate cyclase helps in the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), thus resulting in relaxation.

In the kidney, prostacyclin plays an important role in maintaining kidney function, and can induce renal fibrosis as well as vascular injury. Prostacyclin is believed to be an important opposing mechanism for vasoconstrictor pathways for renal homeostasis, which can influence a variety of mechanisms implicated in renal diseases, such as renal haemodynamic changes, GFR changes, oxidative stress and inflammatory processes (Nasrallah and Hébert, 2005).

1.5.2.1.3 Endothelium-derived hyperpolarisation

Similar to NO and prostacyclin, EDH is another prominent vasodilator factor released from the endothelium in response to endothelium-dependent vasodilators such as ACh (Figure 1.9) (Scotland et al., 2001, Chen et al., 1988, Doughty et al., 2000, Corriu et al., 1996, Zygmunt et al., 1998). Endothelium-derived hyperpolarisation causes vasodilation by hyperpolarising the adjacent smooth muscle (Scotland et al., 2001, Shimokawa et al., 1996), and is a significant component of endothelium-dependent relaxation in the resistance vasculature (Edwards et al., 1998, Lacy et al., 2000, Zygmunt et al., 1998, Shimokawa et al., 1996). Studies suggest that the role of EDH in regulation of arterial vascular tone increases as the diameter of the blood vessel decreases, such that EDH has little role in mediating endothelium-dependent vasodilation of conduit arteries but a major role in resistance arteries such as the mesenteric artery (Hwa et al., 1994, Shimokawa et al., 1996, Adeagbo and Malik, 1990, Tomioka et al., 1999, Fujii et al., 1992). It has been proposed that EDH diffuses more readily to the underlying vascular smooth muscle in small resistance arteries, and thus resistance arteries may be more responsive to EDH (Shimokawa et al., 1996), thus replacing the role of NO (Nagao et al., 1992). Furthermore, EDH activity may become up-regulated on inhibition of

NO production, functioning to compensate for a loss of NO (McCullough et al., 2008b, Waldron et al., 1999).

Endothelium-derived hyperpolarisation has been purported to be responsible for the dysfunctional endothelium-dependent hyperpolarisation typically found in hypertension. For example, Fujii (1992) found that endothelium-dependent hyperpolarisation to ACh was reduced in the mesenteric artery of adult spontaneously hypertensive rats (SHRs) (Fujii et al., 1992): however, it remains to be clarified whether the impaired hyperpolarisation is either a cause or result of hypertension (Fujii et al., 1992).

Endothelium-derived hyperpolarisation is generated by an increase in potassium conductance of the membrane, and this has some contribution to the endothelium-dependent relaxation (Chen and Suzuki, 1989, Waldron et al., 1999, Corriu et al., 1996, Adeagbo and Triggle, 1993, McCullough et al., 2008b) via inhibition of calcium influx through voltage-gated calcium channels (Waldron et al., 1999). Although it is well-established that EDH release and activity involve alteration in K+ flux of both the endothelium and VSMC, as evidenced by a mimicking of EDH effects in rat mesenteric arteries following elevation of extracellular potassium (Edwards et al., 1998, Lagaud et al., 1999, Doughty et al., 2000), the exact mechanisms involved are uncertain and highly controversial (Scotland et al., 2001, Doughty et al., 2000, Adeagbo and Triggle, 1993, Luksha et al., 2009).

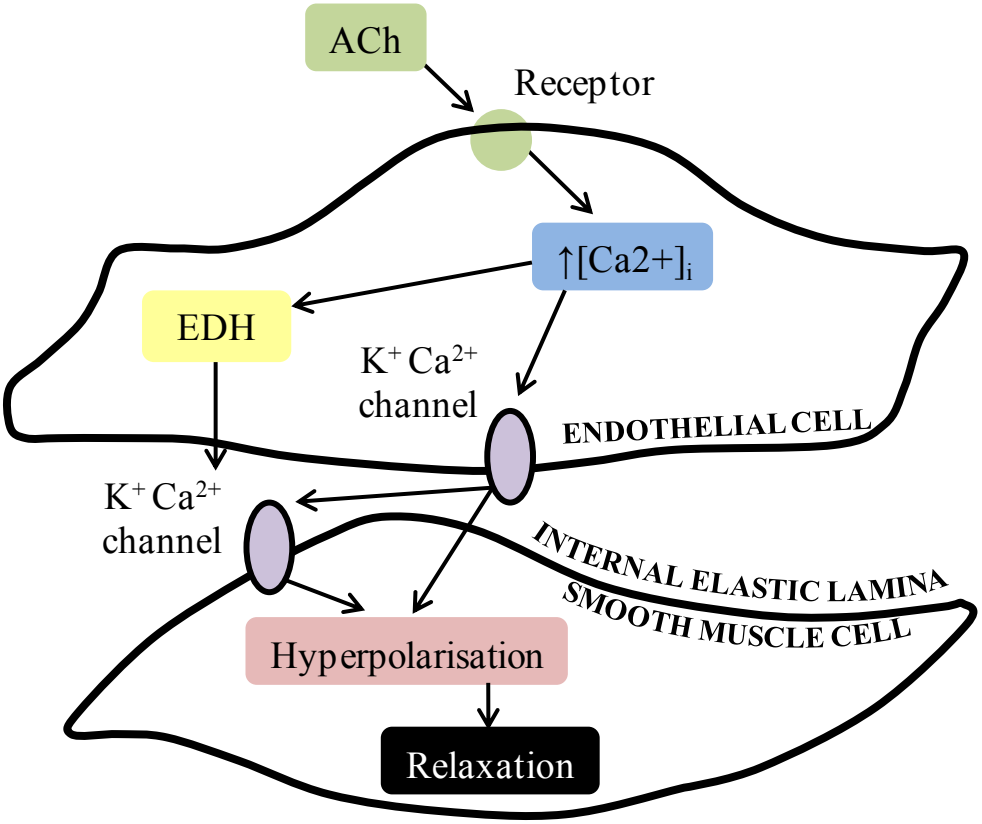

Figure 1.9. Diagram of the proposed endothelium-derived hyperpolarisation (EDH) - mediated vasorelaxation pathway. Binding of a molecule such as acetylcholine (ACh) onto muscarinic receptors on the endothelial cell triggers an increase in intracellular calcium concentrations ($[Ca^{2+}]_I$), which then activates EDH and calcium-activated potassium (K^+ Ca^2) channels in the endothelial cell. This activation then results in the activation of K^+ Ca^{2+} channels in the smooth muscle cell, and hyperpolarisation of the smooth muscle cell, thus resulting in relaxation.

The basic mechanism of the EDH-mediated response can be separated into two stages based on the place where the events occur: the endothelium stage and the VSMC stage (Luksha et al., 2009).

- **Endothelium stage**: As shown in Figure 1.9, endothelium-dependent agonists activate endothelial cell receptors, leading to the entry of extracellular and the release of intracellular calcium and synthesis of EDH (Luksha et al., 2009). The endothelium stage involves an increase in intracellular calcium ($[Ca_{2+}]_i$), activation of Ca^{2+}-

dependent K^+-channels (K^+ Ca^{2+}) and K^+ efflux followed by hyperpolarisation, as well as synthesis of EDH or generation of signals capable of diffusing through membranes or myoendothelium gap junctions to the VSMC (Luksha et al., 2009). Hyperpolarisation of endothelial cells (ECs) occurs, since calcium activates K^+ Ca^{2+}-channels and induces K^+ efflux (Luksha et al., 2009). Myoendothelium gap junctions provide the means by which hyperpolarisation of endothelial cells can subsequently be transferred to VSMCs, by either facilitating the EDH diffusion from the endothelial cells to VSMCs, or acting as a channel for electrical signal transduction (Luksha et al., 2009).

- **Vascular smooth muscle stage**: As shown in Figure 1.9, the VSMC stage involves activation of K^+ channels by transmission of EDH to the VSMCs (Luksha et al., 2009). This results in the activation of K^+ Ca^{2+}-channels and causes endothelium-dependent hyperpolarisation accompanied by closure of voltage-sensitive Ca^{2+}-channels (vCa^{2+}) that results in relaxation (Luksha et al., 2009, McGuire et al., 2001, Busse et al., 2002).

These two stages of EDH response are mediated by two different pathways: diffusible factors and contact-mediated mechanisms.

- **Diffusable factors**: Diffusible factors are endothelium-derived substances that are able to pass through the internal elastic lamina (IEL) and reach the underlying VSMC at a concentration that is sufficient to activate ion channels and initiate smooth muscle hyperpolarisation and relaxation (Luksha et al., 2009). Proposed diffusible factors are: epoxyeicosatrienoic acids, hydrogen peroxide, potassium ions and C-type natriuretic peptide (Luksha et al., 2009).
- **Contact-mediated factors**: Contact-mediated mechanisms bestow endothelium hyperpolarisation that passively spreads to the smooth muscle through intercellular coupling, and therefore endothelium-derived hyperpolarisation is considered as a solely electrical event (Luksha et al., 2009).

In order to investigate the action of EDH, NOS and COX inhibitors may be used to. Typically, inhibition of NO and prostacyclin is achieved using a combination of NOS inhibitor L-NAME, and the COX inhibitor indomethacin (McCullough et al., 2008b).

Residual relaxation in the presence of these two agents are attributed to an EDH response (Chauhan et al., 2003).

1.5.2.1.4 Endothelin-1

Endothelin-1, the predominant isoform of endothelin produced by endothelial cells, is a powerful vasoconstrictor synthesised by the endothelium (Foëx and Sear, 2004), and results in an increase in TPR mainly by vasoconstricting the mesenteric vascular bed (MacLean et al., 1989). Endothelin-1 may produce a salt-sensitive rise in blood pressure, as well as activate local RASs (Beevers et al., 2001), and plays a pivotal role in vascular remodelling due to its important mediating effects of chronic inflammation in the vasculature (Ammarguellat et al., 2002, Schiffrin, 2005, Pu et al., 2003, Barton and Luscher, 1999, Schiffrin et al., 1995b).

Endothelin mediates vasoconstriction via binding to endothelin A and B receptors in VSMCs, and vasodilation via interacting with endothelin B receptors in endothelial cells, which activates prostacyclin and NO (de Nucci et al., 1988). Though the generation of, and sensitivity to, ET-1 has been shown to be comparable between hypertensive and normotensive subjects (Foëx and Sear, 2004), the deleterious vascular effects of endogenous ET-1 may be accentuated by reduced generation of NO caused by hypertensive endothelial dysfunction (Foëx and Sear, 2004). Endothelin-1 is believed to contribute to endothelial dysfunction and atherosclerosis (Dhaun et al., 2006), as well as the pathogenesis and maintenance of hypertension, arterial stiffness and enhancement of cardiovascular risk factors such as oxidative stress and inflammation (Dhaun et al., 2006). In human CKD patients, endothelin levels are increased and the use of selective endothelin A receptor antagonist has been shown to produce a reduction in blood pressure associated with renal vasodilation (Goddard et al., 2004).

1.5.2.2 Vasoconstriction

Smooth muscle cell vasoconstriction may be initiated directly through calcium channel activation (for example, via potassium chloride [KCl] which bypasses the G protein-coupled receptor mechanism), or via binding of an agonist such as a hormone (for example, Ang II, ET-1, phenylephrine [PE]) (Figure 1.10) (Ratz et al., 2005, Webb, 2003). Upon binding of an

agonist, a G protein is activated, which increases PLC activity (Ratz et al., 2005, Webb, 2003). Phospholipase C activity results in production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3): while DAG results in activation of PKC, IP_3 triggers release of calcium from the sarcoplasmic reticulum (SR) via ryanodine receptors (Ratz et al., 2005, Webb, 2003). Following from this, an increase in intracellular calcium ($[Ca^{2+}]_i$) leads to binding of calmodulin with calcium, and subsequent activation of MLCK (Ratz et al., 2005, Webb, 2003). The active MLCK then converts inactive myosin light chain (MLC) to phosphorylated myosin, and the resultant phosphorylated myosin, along with actin, will produce cross-bridge cycling, and therefore constrict the VSMC (Ratz et al., 2005, Webb, 2003).

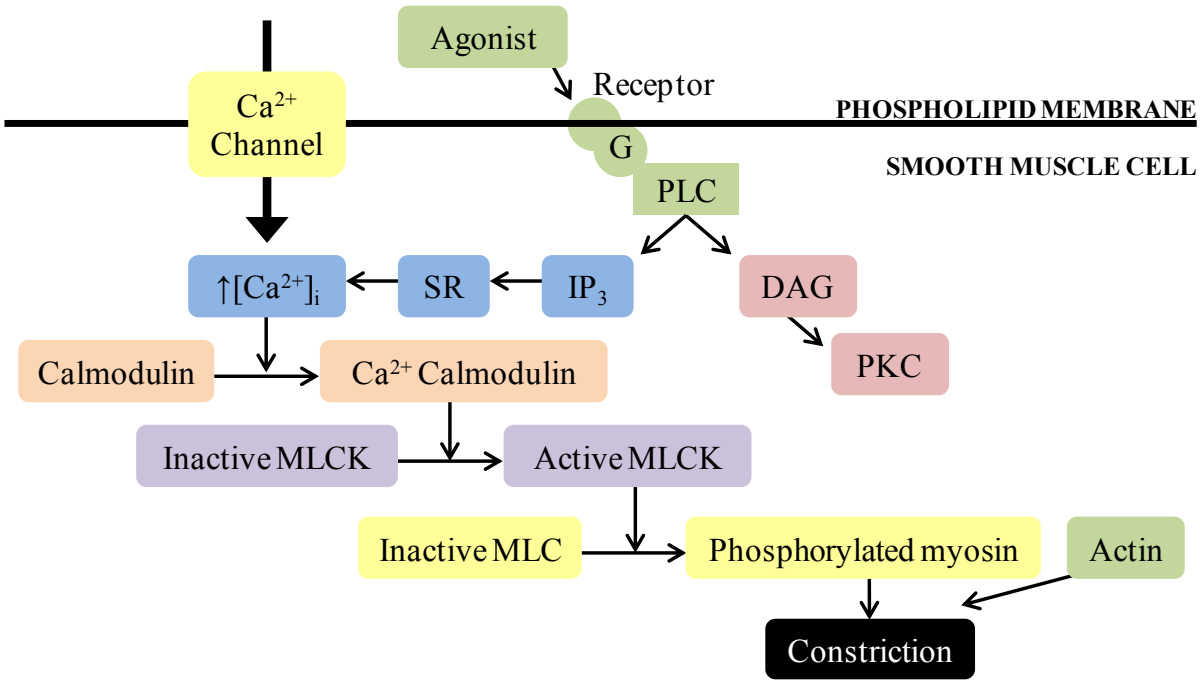

Figure 1.10: Diagram of the vasoconstriction pathway. Direct calcium channel activation, or binding of an agonist such as phenylephrine onto receptors on the phospholipid membrane ultimately triggers an increase in intracellular calcium concentrations ($[Ca^{2+}]_I$), which then activates myosin light chain kinase (MLCK). Activated MLCK leads to phosphorylation of myosin, which, together with actin, produces vasoconstriction. Adapted and modified from (Ratz et al., 2005, Webb, 2003).

1.5.2.3 Vascular amplifier effect

In 2008, Burke et al. (Burke et al., 2008) showed that depressor responses to ganglion blockade in renal denervated normotensive rabbits progressively increased over 4 weeks of Ang II infusion (Burke et al., 2008). Ganglionic blockade is typically used to demonstrate increased sympathetic vasomotor tone, with a greater fall in blood pressure assumed to be directly proportional to the level of sympathetic activity (see (Phillips et al., 2007, Abdala et al., 2012, Santajuliana et al., 1996). Burke and colleagues noted however that the depressor response to ganglion blockade should be interpreted with caution as greater depressor responses need not necessarily be attributable to increased SNA, but could alternatively be due to increased responsiveness of the vasculature to sympathetic neurotransmitters, alterations in the balance between the relative contributions of CO and TPR to mean arterial pressure (MAP), or resistance vascular remodelling resulting in a phenomena termed the "vascular amplifier effect".

According to the vascular amplifier hypothesis, there is an enhanced vascular resistance per unit constrictor stimulus in the hypertensive compared to the normal circulation (Wright and Angus, 1999). The vascular amplifier effect proposes that the vascular effector cells are more sensitive and "reactive" to excitatory influences (Folkow, 1975). A consequence of this type of change would produce intensified muscle shortenings to given stimuli, and hence exaggerated resistance and pressure increases to vasoactive influences, or to, for example, NA infusions, as seen in hypertensive subjects (Folkow, 1975). The underlying factor driving this effect is hypertrophy of the resistance vessels (Folkow, 1975, Wright and Angus, 1999). For example, Mulvany (1999) found that the relaxed peripheral resistance in primary hypertensive patients and pressor response to maximal concentrations of agonists was increased, but that the threshold concentration of agonists which caused vascular contraction was not altered. This led Mulvany (1999) to suggest that these findings could be accounted for by a slight change in the structure of the resistance vessels, such that there is a decrease in the lumen diameter and an increase in wall-lumen ratio. In addition, Folkow et al. (1974) found that rat models of primary hypertension have structurally changed precapillary resistance vessels and display the characteristic hyper-reactivity to constrictor agents. In contrast, the postcapillary vessels were structurally unchanged, and did not exhibit hyper-reactivity. Folkow et al. (1974)

suggested that the hypertensive state is not primarily a matter of generalised increase in smooth muscle sensitivity or reactivity; rather, it seems specific to the resistance vessels.

Further studies supporting a vascular amplifier effect have been conducted by Moretti and colleagues (2009). In a rabbit model of AngII-induced hypertension, Moretti and colleagues (2009) found enhanced depressor responses to ganglion blockade. Ganglionic blockade is typically used to demonstrate increased sympathetic vasomotor tone, with a greater fall in blood pressure assumed to be directly proportional to the level of sympathetic activity (Abdala et al., 2012, Santajuliana et al., 1996). However, by showing that depressor responses to the direct acting agents sodium nitroprusside (SNP) and adenosine were also enhanced, Moretti and colleagues (2009) proposed that the enhanced responses to ganglion blockade they saw were likely not reflective of an increase in SNA, but rather a 'nonspecific enhancement' consistent with the vascular amplifier effect. A similar enhanced depressor response to ganglionic blockade has been demonstrated in the LPK rodent model of CKD (Phillips et al., 2007). This was interpreted to mean increased SNA contributing to TPR and hypertension (Phillips et al., 2007), however by assessing responses to other vasodilators, a vascular amplifier effect was recently proposed to be active in this model (Ameer et al., 2014a). Notably however, the authors did not examine the resistance vasculature for any structural changes in the aforementioned studies. Hence, one of the goals of this thesis is to investigate the responsiveness of the resistance vasculature to vasoconstrictive stimuli in conjunction with assessment of structural remodelling.

1.5.3 Biomechanical property alterations

In addition to increased vascular sensitivity due to remodelling, the passive mechanical properties of the arterial wall are also altered in hypertensive animals, with the most common observation being that the wall is less compliant (Brayden et al., 1983). Vascular compliance is the ability of a vessel to buffer changes in pressure - that is, the ability of a vessel to stretch or distend (Intengan and Schiffrin, 2000, Laurant et al., 1997), and impairment of this ability is considered a characteristic of hypertension (Park and Schiffrin, 2001). Distensibility depends on the geometry of the vessel stiffness and the wall components (Intengan and Schiffrin, 2000, Laurant et al., 1997).

- *Geometry-dependent stiffness*: Geometry-dependent stiffness describes structural vascular adaptation which affects the vessels distension capacity (Behbahani, 2010).
- *Wall component stiffness*: Wall component stiffness is geometry-independent, and relates to stiffness caused by the composition of the vessel wall, namely collagen and elastin content (Intengan et al., 1999). Vascular stiffening often occurs concurrently with changes in wall composition, involving an increased proportion of less distensible (smooth muscle and elastin) to more distensible (collagen and fibronectin) elements (Baumbach and Heistad, 1989, Wolinsky, 1971, Intengan and Schiffrin, 2000): collagen and elastin are the main passive elastic components of the media (Nilsson and Aalkjær, 2003), and hence, together, are responsible for the majority of arterial stiffness. Vascular stiffness may also be modulated by adhesion molecules (Intengan et al., 1999) and integrins, which are physical connectors between the ECM and cytoskeleton, and can also mediate signal transduction (Intengan et al., 1999). Changes in ECM components may have a relevant role in the process of vascular remodelling (Intengan and Schiffrin, 2000, Intengan and Schiffrin, 2001), and may also be triggered by different haemodynamic or humoral factors: in particular, Ang II.

Processes leading to increased vessel wall component stiffness in hypertension and renal disease are likely to be multifactorial: increased oxidative stress (Oberg et al., 2004); endothelial dysfunction (Al-Nimri et al., 2003); RAS activation (Jafar et al., 2003); increased inflammation (Dogra et al., 2006); elastin degradation (Smith et al., 2012); and altered physical properties of collagen and elastin (Chirinos, 2012). Additionally, uraemia itself may have direct effects on arterial stiffness (Zoungas et al., 2004).

Of particular note are the interactions that occur between wall component stiffness and the RAS, where Ang II may directly stimulate cell production of collagen (Kato et al., 1991). In a study by Neves et al. (2003), collagen deposition in response to Ang II, mediated in part by aldosterone and which is usually associated with deposition of fibronectin, resulted in most but not all instances, increased stiffness of the vessel wall. In addition, from their confocal microscopy results, Neves et al. (2003) demonstrated an increase in collagen in the media of small resistance arteries from Ang II-infused rats, and also found that the incremental elastic modulus, which evaluates the stiffness of wall components independently of vessel geometry, was increased by Ang II. This association between Ang II and collagen is further supported

by Rizzoni et al.'s (2005) data, which suggests that the collagen content of the vascular wall may be modified by Ang II type-1 receptor blockade treatment more than by ACE inhibitor (ACEi) treatment: perhaps, because of a more extensive inhibition of the RAS, particularly of Ang II-mediated effects.

1.6 Evaluating effects of hypertension in chronic kidney disease on the resistance vasculature

A variety of arteries have been used to investigate the effects of hypertension in CKD or other disease states on the resistance vasculature, including cremaster arteries (Falcone et al., 1991, Murphy et al., 2007), subcutaneous fat biopsies (Luksha et al., 2012, Paisley et al., 2009), mid-cerebral arteries (Coats and Hillier, 1999), and mesenteric arteries (Behbahani et al., 2010).

Of the many resistance arteries available, small mesenteric arteries are often used in animal experimental studies (Neves et al., 2003), because they remodel in a similar way to hypertensive human patients (Short, 1966), and are believed to be a representative portion of the peripheral vascular bed in hypertension (Short, 1966). Mesenteric resistance arteries are lengthy and plentiful, and easily accessible for study with minimal branching. This latter feature is of particular importance, because successful pressure myograph experimentation requires the maintenance of constant luminal pressure, which is confounded when there is branching within the mounted artery. Preference is also given to these arteries because the mesenteric circulation has receptors for Ang II (McQueen et al., 1984), and because a large percentage of CO flows through the mesenteric circulation (and therefore it contributes significantly to TPR) (Behbahani et al., 2010).

1.6.1 Measuring resistance artery structural and functional properties

Assessment of the structural and functional properties of small resistance arteries is difficult to test directly *in vivo*, due to their small size; however, by mounting small resistance vessels on a pressure myograph, the *in vitro* response to various agonists can be investigated, along with structural measurements (Mulvany et al., 1978, Paisley et al., 2009).

Pressure myography involves mounting a vessel segment on size-matched cannulae, followed by pressurisation of the vessel (see Figure 1.11) (Coats and Hillier, 1999): hence, it preserves the geometry of the vessel under physiological loading, and allows the recording of diameter changes under isobaric conditions (Lu and Kassab, 2011). Diameter changes are quite large in small muscular arteries (arterioles), but relatively small in elastic arteries such as the aorta (Lu and Kassab, 2011). Hence, the pressure myograph is primarily used for small vessels that have substantial vasoreactivity (Lu and Kassab, 2011). Pressure myography is considered an advantageous technique relative to wire-mounted vessels, because findings are more easily reproduced *in vitro* in pressurised vessels (Buus et al., 1994), since the isobaric method is considered to be much closer to the *in vivo* situation than the isometric method (Buus et al., 1994, Coats and Hillier, 1999). Finally, another advantage of pressure myography is that pressurisation of an artery, within physiological limits, results in a passive generation of a physiological radial distension (Coats and Hillier, 1999).

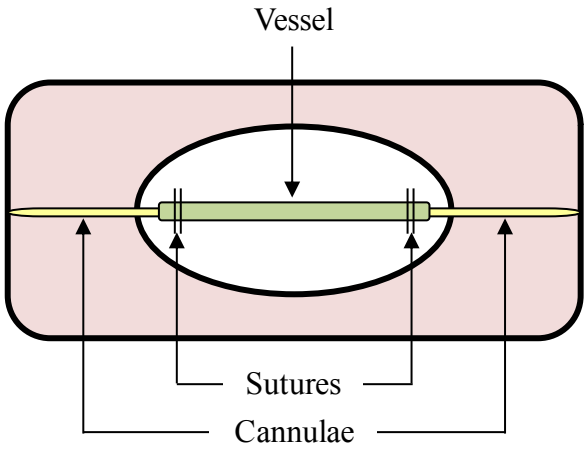

Figure 1.11. Simplified schematic illustration of a vessel mounted on two cannulae and tied by sutures in a pressure myograph.

1.6.2 Measuring resistance artery biomechanical properties

In addition to structural and functional investigations, pressure myography also allows investigations into the biomechanical properties of resistance arteries, thus making it possible to distinguish alterations in geometry due to changes in wall elasticity from those due to other

causes (i.e. remodelling) (Feihl et al., 2008). Passive pressure-diameter curves are constructed by measuring arterial internal and external lumen diameter lengths at various pressure values: a reduced internal lumen and external diameter at increased intraluminal pressures for example indicates compromised passive dilation, while a flattened pressure-diameter curve indicates incompressibility of material composition in the vessel walls.

In addition to the pressure-diameter curve, other measurements of stiffness are available, which deal with relations between forces applied to an elastic body (in this case, an artery), resulting in mechanical stress and strain in that body (Chirinos, 2012). The following are equations used to calculate *in vitro* measures of stress and strain (Schiffrin and Hayoz, 1997):

Circumferential stress (σ) is the wall tension or distending force on the vessel wall, and is calculated as pressure (P) multiplied by internal lumen diameter (D), divided by 2 multiplied by the arterial wall thickness (WT):

$$\boldsymbol{\sigma = \frac{P \times D}{2 \times WT}}$$

Strain (ε) is the relative change in internal lumen diameter in response to increased intraluminal pressure ($D-D_0$), normalised to the baseline internal lumen diameter measured at the lowest intravascular pressure (3mmHg; D_0):

$$\boldsymbol{\varepsilon = \frac{D - D_0}{D_0}}$$

From stress and strain calculations, the two variables can be plotted against each other, with strain on the x-axis and stress on the y-axis, to form a stress-strain curve, where a left-ward shift of the stress-strain curve indicates decreased compliance. From the fitting of stress-strain data to an exponential curve ($y=ae^{bx}$) using least squares analysis, the elastic modulus can be calculated. Elastic modulus describes the arterial elastic properties of the wall component stiffness from various wall components (for example, collagen, elastin and fibronectin), as well as the influence of vessel geometry on stiffness (Neves et al., 2003). In the equation below, σ_0 is the stress at D_0, and β is the constant related to the slope of the stress-strain curve.

$$\sigma = \sigma_0 \times e^{\beta\varepsilon}$$

From the above, the tangential elastic modulus is then calculated at several values of stress from the derivate of the exponential curve:

$$E_T = \frac{d_\sigma}{d_\varepsilon} = \beta\sigma_0 e^{\beta\varepsilon}$$

The above incremental elastic modulus, when plotted against stress, provides a geometry-independent measure of wall component stiffness, which includes collagen, elastin, connective tissue, endothelial cells, and smooth muscle cells (Intengan and Schiffrin, 2000). The smaller the slope of the incremental elastic modulus vs. stress plot, the lower the intrinsic stiffness of wall components.

1.7 Antihypertensive treatment in chronic kidney disease

The importance of hypertension in CKD rests on two management principles. The first principle is that hypertension confers a substantial risk for heart disease, stroke, peripheral arterial disease, and further kidney failure (Chobanian et al., 2003). This risk is amplified when proteinuria is present (Mulrow and Townsend, 2003). The second is that although hypertension ranks technically as the second most common cause of ESRD behind diabetes, it is clear that the majority of patients with diabetes and CKD also have hypertension. Therefore, the decision to lower blood pressure in CKD is undertaken to preserve target organ function on several fronts. In addition, it is more likely that a CKD patient will die from heart disease than reach ESRD (Foley, 2010). Therefore, it is critical to adequately manage hypertension in CKD, as this plays a central role in reducing the likelihood of developing CVD (Iino et al., 2003). Presently, in addition to effective control of modifiable and uraemia-specific risk factors at an early renal disease stage, clinical judgement recommends prioritisation of the maintenance of optimal or near optimal blood pressure control (Schiffrin et al., 2007, Harris and Rangan, 2005).

As mentioned previously (see section 1.5.1 Structural alterations), there exists an interactive relationship between blood pressure and the resistance artery vasculature, where the control of

blood pressure involves the activation of fast and slow processes: fast processes involve modulation of vessel tone through alterations in neurohumoral drive, while slow processes involve structural vascular remodelling of resistance vessels (Mulvany, 2002). Because of the interactions between fast and slow processes in the control of blood pressure, it is necessary to target both neurohumoral drive and resistance artery structure when trying to treat hypertension.

The majority of approaches to the treatment of hypertension in CKD involve aggressive control of blood pressure, often with inhibitors of the RAS (either ACE inhibitors or Ang II receptor blocking agents (ARBs) (Ferrari, 2007)), and/or Ca^{2+} channel antagonists which may be used in conjunction with diuretics (Cohen and Townsend, 2009, Kidney Health Australia, 2014b) or beta-blockers. There is also interest in determining whether some antihypertensive drugs may improve the structure and function of small arteries in vascular beds such as the renal or the coronary circulations, which may be involved in major long-term complications of hypertension (Brush Jr et al., 1988). For example, molecules of the Ang II signaling cascade are upregulated within the arterial wall during hypertension, and may play a causal role in vascular remodelling at the cellular level, with Ang II promoting vascular endothelial cell senescence, potentially disturbing the integrity of the vascular wall and promoting vascular injury (Shan et al., 2008, Wang et al., 2000). It seems likely that resistance vessel structural and functional correction may be necessary in hypertension to restore vascular reserve (with vascular reserve referring to the ability to increase blood flow with maximal vasodilation) (Schiffrin et al., 1995a), because reduction of blood pressure without correction of resistance vessel structure will reduce the vascular reserve (Mulvany, 2002). That is, to improve the outcome in hypertensive patients, in addition to lowering blood pressure, it may be necessary to correct resistance artery vascular remodelling and endothelial dysfunction (Schiffrin et al., 2000, Mulvany, 2008).

The use of RAS inhibitors and calcium channel blockers (CCBs), are described below in more detail.

1.7.1 Renin angiotensin system inhibitors

Renin-angiotensin system inhibitors are the preferred drugs for the treatment of hypertension in CKD: in particular, ACE inhibitors and ARBs (Kidney Health Australia, 2014a).

1.7.1.1 Angiotensin converting enzyme inhibitors

The use of ACE inhibition in the treatment of hypertension of CKD is well accepted (Cohen and Townsend, 2009), with ACE inhibition shown to improve endothelium function in resistance arteries from hypertensive patients (Rizzoni et al., 1997), as well as decrease or improve the degree of vascular remodelling seen in human patients and animal models with hypertension (Adams et al., 1990, Christensen et al., 1989, Shaw et al., 1995, Li and Schiffrin, 1996, Harrap et al., 1990, Lee et al., 1991, Thybo et al., 1994, Rizzoni et al., 2005).

In CKD patients, ramipril has been shown to reduce proteinuria, slow GFR decline, reduce serum creatinine levels, as well as reduce the progression to ESRD (Agodoa et al., 2001, The GISEN Group, 1997). Supporting these findings, the African American Study of Kidney disease (AASK) trial revealed that ACE inhibition resulted in a greater decrease in GFR and progression to ESRD or death, relative to a CCB and beta blocker (Wright Jr et al., 2002). In addition, a meta-analysis of nondiabetic CKD patients has shown that ACE inhibition is associated with a decrease in progression to ESRD, and improvement of serum creatinine levels (Jafar et al., 2001).

Further supporting evidence that ACE inhibition is effective at reducing the degree of vascular remodelling is a study by Ng and colleagues (2011a), who investigated the effect of ACE inhibition on aortic wall structure and function in the LPK rat model of CKD. Ng et al. (2011a) found that, following treatment, the relative amount of elastin increased, with no change in the number of elastin lamellae; treatment also modified the increase in collagen density. Based on their findings, Ng et al. (2011a) suggested that as compared to inhibiting a direct effect of Ang II, the influence of perindopril on vascular remodelling may be blood pressure-dependent, or it may be mediated by non-Ang II mechanisms, as ACE is responsible for both production of Ang II and degradation of the vasoactive bradykinin (Bonde et al., 2011).

1.7.1.2 Angiotensin II receptor blockers

Though ACE inhibitors and ARBs have been shown to have the same effectiveness with regards to correcting small resistance artery remodelling (ie, wall-lumen ratio), ARBs are more effective when it comes to alterations of the collagen content of the vascular wall (Rizzoni et al., 2005): perhaps because of a more extensive inhibition of the RAS, particularly of Ang II-mediated effects.

Similarly to ACE inhibition, the antihypertensive effectiveness of ARBs has been well-established (Foëx and Sear, 2004, Weber, 2001, Burnier and Brunner, 2000). In fact, evidence suggests that ACE inhibitors and ARBs have similar blood pressure controlling effects (Matchar et al., 2008). However, in contrast, ARBs are preferred over ACE inhibitors, as they appear to exhibit fewer side-effects, and have more specificity (Weber, 2001, Burnier and Brunner, 2000). In addition, the renoprotective effects of ARBs are also well-established, where two large renal outcome trials, the Reduction of Endpoints in Non-insulin dependent diabetes mellitus with the Ang II Antagonist Losartan (RENAAL) trial (de Zeeuw et al., 2004), and the Irbesartan in Diabetic Nephropathy Trial (IDNT) (Atkins et al., 2005), have shown that blockade of the Ang II receptor results in an antiproteinuric effect, with the RENAAL trial further adding that this effect seemed independent of blood pressure.

Angiotensin II binds to AT_1 and AT_2 receptors (Miura et al., 2011): Angiotensin type 1 receptor blockers have a higher affinity (10,000–30,000 times greater affinity) for the AT_1 receptor than for the AT_2 receptor, thus resulting in the blocking of Ang II effects which include vasoconstriction, sympathetic nerve activation, sodium and water retention, increase in renal perfusion pressure, aldosterone release, cell proliferation, inflammation in the vasculature and vascular remodelling (Iino et al., 2003, Chang et al., 1995, de Gasparo et al., 2000). Angiotensin II receptor blockers have also been shown to prevent stroke in hypertensive patients more successfully than other classes of antihypertensive drugs (Dahlöf et al., 2002, Schrader et al., 2005, Mochizuki et al., 2007), thus suggesting that the preventive effects of ARBs are also likely to be partially mediated by blood pressure-independent effects.

In addition to cardiovascular protection, both ACE inhibitors and ARBs have been shown to have renoprotective qualities (Ferrari, 2007, Sarafidis et al., 2007, Barnett et al., 2004), which seem to act beyond blood pressure control effects (Ferrari, 2007), with the most pronounced

effects being observed in patients with proteinuria and advanced kidney disease (Kolesnyk et al., 2010).

1.7.2 Calcium channel blockers

Calcium channel blockers are used in antihypertensive treatment and for cardiovascular complications (Gad, 2014). Because contraction of VSMCs is related to a rise in intracellular calcium concentration, drugs that block L-type calcium channels result in inhibition of calcium influx through ion-specific channels in the cell wall (Elliott and Ram, 2011) and VSMC relaxation, and therefore result in a vasodilatory and blood pressure-lowering effect (Beevers et al., 2001, Iino et al., 2003, Gad, 2014).

There are two broad categories of CCBs: dihydropyridines, which preferentially bind to L-type calcium channels in the vasculature and therefore result in vasodilation; and non-dihydropyridine CCBs, which decrease SNS activity and preferentially bind L-type calcium channels in the cardiac muscle, and therefore have negative chronotropic effects (Mugendi et al., 2014). Although all types of CCBs have peripheral vasorelaxation effects (Elliott and Ram, 2011), dihydropyridines are the more potent vasodilators (Sica, 2005).

Calcium channel blockade has been shown to be effective in hypertensive patients, irrespective of the presence or absence of CKD (Segura et al., 2005), resulting in increased GFR in human patients with ESRD (Rahman et al., 2005). For example, amlodipine, a dihydropyridine CCB, has a protective effect on renal function (Segura et al., 2005) via retarding progression of the disease (Burnier, 2013), and thus is effective in the long-term treatment of hypertension in patients with renal failure (Jeffers et al., 2015). The resultant renal protective outcomes have been purported to be due to CCB's beneficial effects on glomerular capillary pressure, SNA, the pathogenesis of renal fibrosis, and aldosterone synthesis (Sugano et al., 2013).

1.8 Polycystic kidney disease: a cause of chronic kidney disease

There are various types of renal diseases that can lead to CKD. One example, which is the focus of this thesis, is PKD. Polycystic kidney disease is a genetically driven cause of CKD,

accounting for 7–10% of all patients on dialysis, and presenting as the fourth most common form of ESRD after diabetic nephropathy, glomerulonephritis and hypertension (Harris and Torres, 2009, Wilson, 2004, ANZDATA registry, 2014).

Polycystic kidney disease is a common inherited systemic disorder, characterised by the development of multiple parenchymal cysts in the kidneys and progressive renal failure (Harris and Torres, 2009, Wilson, 2004, Fall and Prisant, 2005). Several mechanisms have been proposed regarding the pathogenesis of hypertension in PKD, including extracellular volume overload, local activation of the RAS within the renal parenchyma associated with renal cyst formation, induction of local tissue ischaemia, and increased SNS activity (Augustyniak et al., 2002, Fall and Prisant, 2005, Locatelli et al., 2003). Evidence has been presented to support an important link between sympathetic hyperactivity and cardiovascular morbidity in PKD patients (Klein et al., 2001, Neumann et al., 2002, Ecder and Schrier, 2009).

In humans, there are a number of hereditary forms of PKD including adult onset autosomal dominant (ADPKD), and autosomal recessive (ARPKD) which is an important cause of early childhood nephropathy (Harrison et al., 2010). Although cyst origination and development differs between ADPKD and ARPKD, cell-related processes associated with these two diseases seem to be similar (Torres and Harris, 2006), and rates of both cardiovascular morbidity and mortality are high as a result of CVD. These diseases belong to a family of conditions known as ciliopathies, where the primary mutation is in a gene encoding for a protein associated with the cilia (Hildebrandt et al., 2011).

Another form of autosomal recessive cystic kidney disease is nephronophthisis (NPHP). Nephronophthisis is the most frequent genetic cause of ESRD in the first three decades of life (Hildebrandt and Zhou, 2007), and acts symmetrically to result in a progressive destruction of kidney glomeruli and tubules, causing tubulointerstitial nephropathy (Torres and Harris, 2006, Wolf and Hildebrandt, 2011). Histological features of the diseased kidneys involve tubular basement membrane disintegration, tubular cyst formation, and tubulointerstitial inflammation and fibrosis (Wolf and Hildebrandt, 2011, Zollinger et al., 1980). These histological characteristics differ between the infantile and juvenile forms of NPHP, as the infantile form combines features of NPHP with features of PKD, which include enlarged

kidneys and widespread cyst development (Wolf and Hildebrandt, 2011, Gagnadoux et al., 1989).

1.8.1 Autosomal dominant polycystic kidney disease

Autosomal dominant PKD is the most common renal monogenic disease with an incidence of 1/400–1000 (Sweeney and Avner, 2006). Autosomal dominant PKD has two disease loci: PKD 1 and PKD2 (Hayashi et al., 1997, Bycroft et al., 1999), with approximately 80 to 85% of ADPKD patients having mutations in the PKD1 gene, located on chromosome 16p13 which encodes polycystin-1 (PC-1) (Sweeney and Avner, 2006). The remaining 10 to 15% of ADPKD cases have mutations in the PKD2 gene, which is located on chromosome 4q21 and encodes polycystin-2 (PC-2) (Sweeney and Avner, 2006). Autosomal dominant PKD is usually asymptomatic until the middle decades (Sweeney and Avner, 2006), with cysts originating as expansions of the renal tubule, and a rapid closure from the nephron of origin (Wilson, 2004). However, 2% to 5% of ADPKD patients present with a severe neonatal course and significant morbidity and mortality (Sweeney and Avner, 2006). In patients with ADPKD, approximately 60% develop hypertension prior to renal function impairment (Gabow et al., 1990).

1.8.2 Autosomal recessive polycystic kidney disease

In contrast to ADPKD, ARPKD only comprises 5 to 8% of all individuals requiring renal replacement therapy (dialysis and/or kidney transplantation), and only has one disease locus, the polycystic kidney and hepatic disease 1 (PKHD1) gene (Onuchic et al., 2002). Autosomal recessive PKD is, however, a significant cause of renal and liver -related morbidity and mortality in childhood (Sweeney and Avner, 2006). Estimates of the disease prevalence vary widely, but an overall frequency of 1 in 20,000 live births and a carrier level of up to 1:70 have been proposed (Zerres et al., 2004, Sweeney and Avner, 2006). The ARPKD disease gene, PKHD1, is a large gene located on chromosome 6p21.1–p12, spanning 470 kb of genomic DNA and producing a cDNA of 16 kb (Sweeney and Avner, 2006). The majority of ARPKD patients present clinically as newborn or young children (Sweeney and Avner, 2006).

However, despite dramatic improvements in neonatal and intensive care over the past decade, neonatal mortality is 25 to 35% (Sweeney and Avner, 2006), and the clinical spectrum of surviving patients is highly variable (Sweeney and Avner, 2006). Principal manifestations of the disease involve the fusiform dilation of renal collecting tubules (CT) or ducts that remain connected to the nephron of origin (Wilson, 2004), and dysgenesis of the hepatic portal triad attributable to ductal plate abnormalities (Sweeney and Avner, 2006). In ARPKD, hypertension occurs in up to 80% of affected children, with nearly all who survive the neonatal period requiring anti-hypertensive treatment (Capisonda et al., 2003, Sweeney and Avner, 2006).

1.8.3 Nephronophthisis

Nephronophthisis is one of the most frequent genetic causes of ESRD in children and adolescents (Wolf and Hildebrandt, 2011, Hildebrandt et al., 2009). It is an inherited cystic disease, displaying many clinical symptoms of ciliopathies (Zalli et al., 2012), characterised by progressive renal failure, polyuria, polydipsia, isosthenuria, anaemia, and impaired urinary concentrating ability (Hildebrandt et al., 2009, Wolf and Hildebrandt, 2011, Torres and Harris, 2006, Ala-Mello et al., 1996, O'Toole et al., 2010), along with nephron disintegration which consequently contributes to a critical tubulointerstitial nephropathy (Hildebrandt and Otto, 2005). Positional cloning has revealed more than 20 different genes (for example, NPHP1–11, NPHP1L, and TTC21B) which may underlie NPHP-related ciliopathies (Wolf and Hildebrandt, 2011, Soliman, 2012, Chaki et al., 2012, Hildebrandt et al., 2011, Sang et al., 2011). Despite this, however, the exact causative gene in approximately 70% of all NPHP patients is unknown (Hildebrandt et al., 2009). Functional characterisation of the encoded proteins (nephrocystins) of these genes has shown that all proteins mutated in humans or animal models of NPHP are expressed in the primary cilia or centrosomes of renal epithelial cells (Hildebrandt et al., 2009, Otto et al., 2003, Otto et al., 2008), therefore also identifying NPHP as a ciliopathy affecting ciliary functions, as well as epithelial cell polarity and cell-cycle control (Hildebrandt et al., 2009).

1.9 Lewis polycystic kidney rat model

The LPK rat is an autosomal recessive model of PKD first described in 2007 (Phillips et al., 2007), and is now well established as a rodent model of CKD and secondary hypertension (Hildreth et al., 2013). It is a model of nephronophthisis 9 (NPHP9), arising from a single nucleotide polymorphism in the NIMA (never in mitosis gene a)-related kinase 8 (*Nek8*) gene on chromosome 10 (McCooke et al., 2012). The *Nek8* protein localises to the proximal region of the primary cilium, where it seems to modulate ciliary targeting of polycystin-1 and polycystin-2 (Sohara et al., 2008, Hildebrandt and Otto, 2005) and this localisation is disturbed in the LPK rat model (McCooke et al., 2012).

The LPK rat has a phenotypic presentation that resembles human juvenile ARPKD (Phillips et al., 2007), similar to the juvenile cystic kidney (jck) mouse *Nek8* model (Trapp et al., 2008) (Smith et al., 2006). Important advantages to note of the LPK rat as a research model is that the CKD occurs progressively with age, and thus does not require the surgical or chemical interventions which are typically employed to induce renal failure in rat models. Such rat model examples include but are not limited to: radiation nephropathy model, which involves total-body irradiation (TBI) and is characterised by a dose-dependent decline in GFR and effective renal plasma flow (Robbins and Bonsib, 1995); unilateral ureteral obstruction (UUO) which involves complete ureteral obstruction, resulting in reduced renal blood flow and GFR (Chevalier, 2006); the 5/6 nephrectomy rat model, which requires surgical intervention (Fogo, 2003); the gentamycin-induced renal failure rat model (Amini et al., 2012); and the Thy-1 nephritis model, which may be induced by an injection of either rabbit anti-thymocyte serum, or mouse anti-Thy 1 monoclonal antibody, resulting in mesangioproliferative glomerulonephritis (MsPGN). Additionally, the naturally progressive nature of CKD in LPK rats resembles aspects of the human disease state, and hence can be considered a robust model of CKD.

1.9.1 Lewis polycystic kidney rat model characteristics

The CKD progression observed in the LPK rat, along with the associated arising CVD complications is summarised below (Phillips et al., 2007, Ding et al., 2012, Hildreth et al., 2013):

1.9.1.1 Phase 1: Precursor cystic phase

From the first week of birth, LPK rat kidneys already present with precursor cystic lesions, with focal dilations of proximal and distal tubules.

1.9.1.2 Phase 2: Cystic phase and early cardiovascular disease manifestations

From 3 to 6 weeks of age, LPK rats exhibit gross derangement of the kidney cortex and medulla, with proliferation and dedifferentiation of the tubular epithelial cell. In addition, distal tubular dilatation and interstitial inflammation are evident, and accompanied by compensatory renal function preservation. By 6 weeks of age, LPK rats have established hypertension, as well as increased cardiac mass, and increased kidney to body weight ratio (Phillips et al., 2007).

1.9.1.3 Phase 3: Cyst enlargement and severe cardiovascular disease

Lewis polycystic kidney rats demonstrate initial signs of renal dysfunction at 12 weeks of age, and progress to marked renal disease by 18–24 weeks of age, along with increased cardiac mass and left ventricular hypertrophy, and increased kidney to body weight ratio (Figure 1.12). At 12–13 weeks of age, when hypertension is well established yet renal function is not overtly impaired, the LPK rats have elevated sympathetic activity (Harrison et al., 2010, Salman et al., 2014) which presumably contributes to the hypertensive state and therefore development of CVD. At 12 weeks of age the LPK rat also exhibits features of arteriosclerosis, including increased functional aortic stiffness, remodelling, and calcification (Ng et al., 2011a), with renal deterioration accompanied by a normocytic normochromic anaemia (Phillips et al., 2007, Phillips et al., 2015). Cyst enlargement continues from 12-24 weeks of age; however, this is at a slower rate, and usually precedes development of other typical histological features of progressive renal disease. By 18-24 weeks of age, LPK rats have marked elevations in serum urea, and urinary protein creatinine (UPC) ratios, as well as decreased serum protein.

Thus, the LPK rat provides a model system where interventions to modify arteriosclerosis can be trialled under conditions that mimic the human condition in a state of accepted disease progression (Ng et al., 2011a).

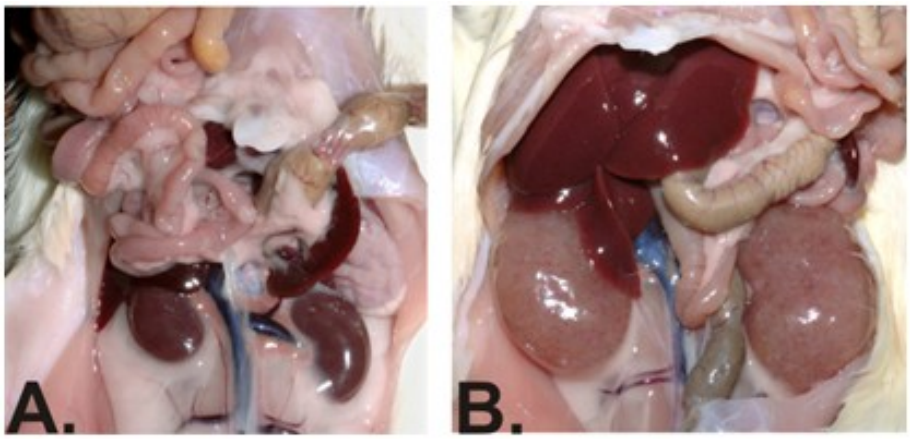

Figure 1.12. Gross kidney features in a 12 week old Lewis control (A) and Lewis polycystic kidney (LPK) (B) rat. Adapted and modified from (Phillips et al., 2007).

1.9.2 Physiological features in the Lewis polycystic kidney rat model

In addition to the characteristics mentioned above, other manifestations of CVD are evident in the LPK rats. These include but are not limited to: alterations in the RAS, SNS overactivity, and vasculature alterations.

1.9.2.1 Alterations in the renin angiotensin system

Lewis polycystic kidney rats present with low plasma renin activity and Ang II levels at 10-12 weeks of age (Phillips et al., 2007). Nevertheless, however, despite suppressed renin activity, renin is likely to play a key role in the pathogenesis of hypertension in LPK rats, as ACE inhibition treatment with perindopril results in a significant reduction in blood pressure (Ng et al., 2011a)

1.9.2.2 Sympathetic nervous system overactivity

Sympathetic nervous system overactivity is a well-established finding in the LPK rat, supported by various studies demonstrating increased baseline level of renal, lumbar and splanchnic SNA (Salman et al., 2014, Yao et al., 2015), enhanced blood pressure depressor responses to ganglionic blockade (Phillips et al., 2007, Ameer et al., 2014a), and increased low frequency (LF) power of SBP variability, which is indicative of increased sympathetic vasomotor tone (Hildreth et al., 2013, Harrison et al., 2010). Furthermore, recently, direct recordings of renal SNA in LPK animals using a telemetry based system have confirmed increased baseline levels of SNA in conscious animals (Salman et al., 2014).

1.9.2.3 Vasculature alterations

Previous work from our laboratory, demonstrated in studies by Ng et al. (Ng et al., 2011b) and Ameer et al. (Ameer et al., 2014b), found that the functional and structural properties of large arteries are markedly altered in the LPK rat model of CKD. Vascular remodelling, evidenced by increased aortic medial calcification, increased wall-lumen ratio, collagen deposition, and reduction in elastin content of the aorta was demonstrated (Ng et al., 2011b, Ameer et al., 2014b). Aortic endothelial dysfunction was also evident (Ameer et al., 2015). The altered functional and structural properties were suggested to be due to (but not limited to) three mechanisms: 1) vascular wall hypertrophy as evidenced by an increase in MCSA and wall-lumen ratio, and a decrease in elastin content and the elastin-to-collagen ratio; 2) increase in the passive stiffness of the vessel walls as PWV was markedly increased independently of blood pressure; and 3) altered vascular composition due to an increase in arterial calcium deposition (Ng et al., 2011b). These three factors likely create a self-amplification positive feedback loop, whereby active elastin degradation products and various uraemia-specific proinflammatory factors are upregulated, which in turn promote expansion of the lesions, further accelerating calcification and therefore increasing arterial stiffness (Doherty et al., 2003, Ng et al., 2011b). Although the structure, function and biomechanical properties of large conduit arteries have been studied in the LPK rat, resistance arteries in the LPK rat remain to be investigated. This is a key deficit in our knowledge, given the key role played by the resistance vasculature in the hypertensive state.

1.10 Thesis objectives

Chronic kidney disease patients are more likely to die from cardiovascular complications, as opposed to the renal disease itself. Although it is well-established that alterations in resistance arteries are likely to contribute to increased cardiovascular risk and act as a substrate for end-organ damage (Schiffrin, 2012), resistance artery alterations are still poorly understood in the CKD-mediated hypertensive state, and hence are the focus of this thesis. The hypotheses and specific aims of the present thesis are as follows:

- Chapter 3: Alterations in structural, functional and biomechanical properties have been explored in various rat models with hypertension and/or renal disease (Luksha et al., 2012, Intengan et al., 1999, Fujii et al., 1992, Behbahani et al., 2010, Laurant et al., 1997, Korsgaard and Mulvany, 1988). However, no studies have investigated the temporal relationship between all of these properties in a CKD-mediated model of hypertension. Therefore, we examined structural, functional and biomechanical changes in the LPK rat resistance arteries at three time-points: 6 weeks of age, where LPK rats are hypertensive and have gross derangement of the kidney cortex and medulla; 12 weeks of age, where LPK rats demonstrate signs of renal dysfunction in addition to hypertension and increased SNA; and 18 weeks, where LPK rats showed a marked decline in renal function concurrent with ongoing hypertension and elevated SNA (Phillips et al., 2007). Given that at 6 weeks of age the LPK rats are already hypertensive, we hypothesised structural (vascular remodelling, altered collagen/elastin ratios and calcium deposition) and biomechanical property (increased stiffness) changes would be evident at 6 weeks, but worsen with age in association with the progression of renal disease and hypertension. Consistent with such structural changes, we also expected changes in function including endothelial dysfunction, and altered sensitivity to noradrenergic stimuli.
- Chapter 4: The RAS plays a crucial role in the progression of CKD (Remuzzi et al., 2005), with Ang II playing a detrimental role at the level of the vasculature, including causing alterations of the vascular NO pathway (Lee et al., 2013), structural vascular remodelling (Bruder-Nascimento et al., 2014), VSMC hypertrophy, and inflammation (Schiffrin, 2004, Zhang et al., 2005). In addition, Ang II also contributes to the progressive loss of renal function and target organ

damage (Cohen and Townsend, 2009). Due to the detrimental effects of Ang II, we sought to investigate the effect of chronic Ang II blockade with the AT_1 receptor antagonist, valsartan, on LPK rats resistance artery structural, functional and biomechanical properties. Chronic treatment was undertaken from 4 to 18 weeks of age. We hypothesised that valsartan would lower blood pressure in the LPK rats, and ameliorate detrimental vascular changes.

- Chapter 5: If treatment with the AT_1 receptor antagonist valsartan resulted in a reduction of blood pressure, it would be unclear if any effects of valsartan on the vasculature in the LPK rats were attributable directly to the inhibition of Ang II, or due to any blood pressure-lowering effects. Hence, in order to investigate this, we performed chronic treatment using the CCB, amlodipine, from 4-18 weeks of age as an alternative antihypertensive treatment. Given the strong link between hypertension and vascular changes in the resistance arteries (Mulvany, 2011, Schiffrin, 2012), and the effectiveness of antihypertensive treatment with amlodipine observed in SHR vasculature (Sharifi et al., 1998, He et al., 2011), we hypothesised that if hypertension, as opposed to renal failure, is the main contributor to vascular changes observed in the LPK rats, that amlodipine would have comparable effects on the vasculature to valsartan.
- Chapter 6: Vascular dysfunction has been shown to be attributable to an imbalance of constricting and relaxing factors. In addition, endothelial dysfunction has been shown to be receptor-specific, for example with impairment of the EDH responses following BK but not ACh stimulation (Luksha et al., 2012). This imbalance between constricting and relaxing factors affects myogenic tone, which, in resistance arteries, plays an important role in the maintenance of internal lumen diameter. We therefore investigated the balance of constricting and relaxing factors, the receptor specificity of endothelial dysfunction, along with myogenic tone, in 18 week LPK rats. We predicted 18 week LPK rats to exhibit increased sensitivity to vasoconstrictors, and thus greater myogenic tone relative to control Lewis.

2

Chapter 2

Methodology

Quek, K.J.

2.1 Ethical approval

All studies performed during the course of this thesis were approved by the Animal Ethics Committee of Macquarie University, and conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013).

Consistent with the thesis by publication format, chapters 3, 4, 5 and 6 were written as complete papers to be submitted for publication. As such, this chapter has been included in order to provide additional detail on mesenteric artery histological staining methodologies which were otherwise not fully described.

2.2 Mesenteric artery tissue staining procedures

2.2.1 Mesenteric artery tissue harvesting

Following euthanasia, the mesentery was removed from the abdomen and second order branches of the mesenteric artery were dissected free from fat, connective tissue and the gastrointestinal tract. The tissue was superfused in Ca^{2+}-free Krebs' solution with 10 mM EGTA for 20 minutes, and was subsequently fixed in 4% formalin for 24 hours at room temperature and then stored in 70% ethanol at room temperature (Ethanol analytical grade, Fronine Lab Supplies, Riverstone, NSW, Australia), until further processing.

2.2.2 Mesenteric artery tissue processing

The mesenteric vasculature was dehydrated using a Leica tissue processor (Leica ASP200S, Nussloch, Germany). Second-order mesenteric arteries were then dissected away, and paraffin-embedded in a transverse-orientation. Mesenteric arteries mounted in paraffin blocks were sectioned cross-sectionally into 5µm thick slices using a microtome (Microm HM355, Walldorf, Germany) and mounted onto glass slides. Mounted sections were incubated at 37°C in the oven overnight to dry, and then underwent specific staining protocols (Section 2.4) in order to identify the various compositional components of the vessel wall. Lamellae and interlamellae elastin were stained with Shikata's orcein (SO), collagen deposition and smooth muscle cell nuclei were identified using Martius scarlet blue (MSB) stain, and calcium deposits were stained with Von Kossa (VK) and Alizarin red (AR). The stained slides were

then viewed using light microscopy and images acquired using a mounted video camera (Carl Zeiss Microimaging, Gottingen, Germany). All images were processed with Zeiss Axiovision software (AxioVs40 v4.8.2.0, Carl Zeiss Microimaging).

2.3 Staining procedures

2.3.1 Deparaffinisation and rehydration procedure

Prior to staining, all sections were deparaffinised by immersing in several changes of histochoice® clearing agent (AMRESCO, Inc., USA) (3x10 mins). Sections were then dipped in descending grades of alcohol (2x5 mins absolute alcohol; 2x3 mins 85% alcohol; 1x3 mins 70% alcohol), and subsequently introduced to water (3 mins).

2.3.2 Specific staining protocols

2.3.2.1 Shikata's orcein staining procedure

Sections were treated with 0.15% potassium permanganate (w/v) (Sigma-Aldrich, NSW, Australia) in 3% aqueous sulphuric acid (Univar, Ajax Finechem Pty Ltd, Australia) solution for 3 mins, then rinsed with running water. Sections were then decolourised with 1% (w/v) aqueous oxalic acid (Sigma-Aldrich) (5 dips) then washed in water. Subsequently, they were stained with 5% Shikata orcein (w/v) (Sigma-Aldrich) in 1% (v/v) alcoholic (70%) hydrochloric acid (Univar, Ajax Finechem Pty Ltd, Australia) solution for 15 mins. Sections then underwent dehydration and mounting as described below (Section 2.4.3). Shikata's orcein stained sections show elastin fibres in red or brown.

2.3.2.2 Martius scarlet blue staining procedure

Sections were immersed in preheated (60 °C) Bouin's fixative, consisting of 0.9% aqueous solution of picric acid (w/v) (Sigma-Aldrich), 5% glacial acetic acid (w/v) (Univar, APS Asia Pacific Speciality Chemicals Ltd, NSW, Australia) and 0.36% formaldehyde (w/v) (Univar, Ajax Finechem Pty Ltd, Australia) were added. Sections were immersed in Bouin's fixative in

a 60 °C oven for 1 hour. The fixative was removed from slides by washing under running tap water. Sections were then stained for 8 mins with 1% aqueous solution of Celestine blue (w/v) (Sigma-Aldrich), containing 5% ferric ammonium sulphate (w/v) (Sigma-Aldrich) and 14% glycerine (v/v) (Sigma-Aldrich). This was followed by immersion in Harris's haematoxylin solution (Clinipure Stains & Reagents for Pathology, HD Scientific Supplies, STATE Australia) for an additional 5 mins. Sections were rinsed in water then 95% alcohol, and subsequently treated with 0.5% Martius yellow (w/v) (Sigma-Aldrich) in 2% (w/v) alcoholic (95%) phosphotungstic acid (Sigma-Aldrich) solution for 3 mins. Water-rinsed sections were then stained for smooth muscle and mature fibrin by treating with 1% brilliant crystal scarlet (w/v) in 2.5% aqueous acetic acid solution for 15 mins. After washing in water to remove excess brilliant crystal scarlet stain (Sigma-Aldrich), sections were immersed in 1% aqueous phosphotungstic acid (w/v) (Sigma-Aldrich) for 10 mins. Lastly, rinsed sections were treated with 0.5% soluble blue (Sigma-Aldrich) in 1% aqueous acetic acid solution for 15 mins to stain collagen. Sections were then dehydrated and mounted. Fibrin is stained red by the brilliant crystal scarlet stain, collagen is stained blue, red blood cells are stained yellow by Martius yellow, and nuclei are stained in black or dark magenta.

2.3.2.3 Von Kossa staining procedure

To stain calcium salts, sections were first incubated with 1% aqueous silver nitrate (w/v) (Sigma-Aldrich) solution under ultraviolet light for 60 mins. Sections were then rinsed in several changes of distilled water, and the excess unreacted silver nitrate removed by submerging sections in a 5% aqueous sodium thiosulfate (w/v) (Sigma-Aldrich) for 5 mins. The sections were again washed in distilled water, then counterstained with a 0.1% nuclear fast red (w/v) (Sigma-Aldrich) in 5% aqueous aluminium sulphate (w/v) (Univar) for 5 mins. Sections were then dehydrated and mounted. Stained sections showed calcium salts in black or brown, cytoplasm in pink, and nuclei in red.

2.3.2.4 Alizarin red staining procedure

As an alternative procedure to quantify calcium, sections were immersed for 30 s in 2% Alizarin red solution (w/v) (Sigma-Aldrich), which had an adjusted pH of 4.1-4.3 obtained

using 0.5% aqueous ammonium hydroxide. Sections were then dehydrated and mounted. Stained sections showed calcium salts in orange.

2.3.3 Dehydration and mounting procedure

After staining, all sections were immersed in ascending grades of alcohol (several dips in 70% alcohol; 2x3 mins 85% alcohol; 3x10 dips in absolute alcohol), followed by histochoice (3x5 mins). Slides were then left to dry. For long-term storage and maintenance of a high refractive index for microscope examination, dehydrated sections were then mounted with a coverslip using DPX mounting media (Sigma-Aldrich).

3

Chapter 3

[1]Progressive vascular remodelling, endothelium dysfunction and stiffness in mesenteric resistance arteries in a rodent model of chronic kidney disease

Quek, K.J., Boyd, R., Ameer, O.Z., Zangerl, B., Butlin, M., Murphy, T.V., Avolio, A.P., & Phillips, J.K.

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3.1 Abstract

Chronic kidney disease (CKD) and hypertension are co-morbid conditions both associated with altered resistance artery structure, biomechanics and function. We examined these characteristics in mesenteric artery together with renal function and systolic blood pressure (SBP) changes in the Lewis polycystic kidney (LPK) rat model of CKD. Animals were studied at early (6-weeks), intermediate (12-weeks), and late (18-weeks) time-points (n=21), relative to age-matched Lewis controls (n=29). At 12 and 18-weeks, LPK rats arteries exhibited eutrophic and hypertrophic inward remodelling characterised by thickened medial smooth muscle, decreased lumen diameter, and unchanged or increased media cross-sectional area, respectively. At these later time points, endothelium-dependent vasorelaxation was also compromised, associated with impaired endothelium-dependent hyperpolarisation and reduced nitric oxide synthase activity. Stiffness, elastic-modulus/stress slopes and collagen/elastin ratios were increased in 6 and 18-week-old-LPK rats, in contrast to greater arterial compliance at 12 weeks. Multiple linear regression analysis highlighted SBP as the main predictor of wall-lumen ratio (r = 0.536, P<0.001 n = 46 pairs). Concentration-response curves revealed increased sensitivity to phenylephrine but not potassium chloride in 18-week-LPK rats. Our results indicate that impairment in LPK rats resistance vasculature is evident at 6 weeks, and worsens with hypertension and progression of renal disease.

Keywords

Mesenteric resistance artery, hypertension, polycystic kidney disease, endothelium, vascular remodelling

3.2 Introduction

Impaired function of the resistance vasculature is associated with hypertension in chronic kidney disease (CKD) (Wang et al., 2000, Martinez-Lemus et al., 2009) and as a group, CKD patients have an increased risk of mortality due to cardiovascular disease (Foley, 2010). Resistance arteries provide more than 80% of the resistance to blood flow in the body and, as a consequence, modified structural properties play a significant role in persistent increases in total peripheral resistance (Intengan and Schiffrin, 2000). The structural, biomechanical and functional attributes of resistance vessels are hypothesised as being one of the first sites of target organ damage due to increased intraluminal pressures from hypertension (Schiffrin et al., 2000, Intengan and Schiffrin, 2001).

In hypertension, resistance arteries typically undergo inward vascular remodelling, characterised by increased medial wall thickness and decreased lumen diameter that may be "eutrophic" or "hypertrophic", corresponding to unchanged or increased media cross sectional area (MCSA), respectively (Mulvany, 1999). Eutrophic remodelling is typically seen in essential hypertension (Martinez-Lemus et al., 2009) and uremic conditions (New et al., 2004), and typically occurs in the absence of stiffening (Intengan and Schiffrin, 2001). In contrast, hypertrophic remodelling has been reported in renovascular hypertension (Rizzoni et al., 1996). Vascular remodelling may initially be an adaptive compensatory mechanism to buffer pressure changes in the arteries; however, it eventually becomes maladaptive and compromises organ function, contributing to the cardiovascular complications of hypertension (Martinez-Lemus et al., 2009, Mulvany, 2011).

Structural alterations result in biomechanical property changes, which in turn worsen the degree of structural alterations, including a loss of distensibility, increase in wall stiffness, and modifications in the vessel composition (collagen-elastin ratios), consequently impairing maximal passive dilatation ability and leading to further structural remodelling (Laurant et al., 1997, Intengan et al., 1999). Arterial stiffness is increasingly being recognised as an important prognostic index and potential therapeutic target in patients with hypertension (Payne et al., 2010).

Resistance artery vascular remodelling may also exacerbate hypertension through its effects on vascular function, such as magnifying normal vasoactive inputs (vascular amplifier effect) (Wright and Angus, 1999) and vasorelaxation dysfunction, which is a well-established phenomenon in hypertension, being described in the subcutaneous resistance arteries of patients with end-stage renal disease (Luksha et al., 2012). Vasorelaxation in resistance arteries involves three components: nitric oxide (NO), prostanoids, and endothelium-dependent hyperpolarisation (EDH) (Luksha et al., 2012), with the contribution of each dependent upon the vascular bed under study, and the varying degrees of relative impairment depending on the disease state (Shimokawa et al., 1996, Kietadisorn et al., 2012, Versari et al., 2009).

We have previously established in the Lewis polycystic kidney (LPK) rodent model of CKD that animals develop large vessel stiffness, calcification, and vascular remodelling by 12 weeks of age (Ameer et al., 2014b, Ng et al., 2011) The LPK rat model is a result of an autosomal recessive mutation in NimA (Never In Mitosis Gene A)- Related Kinase 8 (*Nek8)* (McCooke et al., 2012), which in humans is responsible for nephronophthisis (NPHP) 9 (Otto et al., 2008, Frank et al., 2013). Within the nephrocystin family of proteins, multiple NPHP gene mutations have been identified and are the leading genetic cause of end-stage renal disease in children and young adults (Frank et al., 2013). In the juvenile cystic kidney (jck) mouse (Liu et al., 2002) and LPK rat (Phillips et al., 2007), the *Nek8* mutation leads to a phenotypic presentation of cystic kidney disease resembling human autosomal recessive polycystic kidney disease. The LPK animals present with established hypertension by 6 weeks of age, renal dysfunction by 12 weeks of age, and progression to marked renal disease by 18–24 weeks of age (Phillips et al., 2007). We recently demonstrated that in addition to sympathetic overactivity, a vascular amplifier effect was likely to contribute to the increased blood pressure observed in the LPK rats (Ameer et al., 2014a), consistent with the prediction that resistance artery vascular remodelling is occurring in this model. Therefore, we hypothesised that significant structural, biomechanical and functional changes would be evident in the mesenteric artery as a representative resistance vessel in the LPK rat, and further, show progressive change in parallel with the decline in renal function. Thus, the present study investigated temporal changes of resistance artery structural, functional and

biomechanical properties in association with blood pressure and renal function in a model of CKD.

3.3 Methods

3.3.1 Animals

Mixed sex LPK at 6, 12 and 18 weeks (*n*=7 for each age group), and age-matched control strain Lewis rats (6 weeks *n*=8; 12 weeks *n*=14; 18 weeks *n*=7) were obtained from the Animal Resource Centre in Western Australia, Australia, with a minimum of 3 males or females per group. A total of 43 rats were used for this study. Animals were housed in the animal house facility of Macquarie University under a 12-h light/dark cycle at 20.5°C and 57% humidity, and offered rat chow and water *ad libitum*. All experimental protocols and procedures were approved by the Animal Ethics Committee of Macquarie University and adhered to the National Health and Medical Research Council of Australia's Australian code of practise for the care and use of animals for scientific purposes (8th Edition 2013).

3.3.2 Tail cuff plethysmography and urine collection

Systolic blood pressure (SBP) was measured using tail-cuff plesmography (ADInstruments, Sydney, NSW, Australia) as described previously (Phillips et al., 2007). At least 72 hours prior to euthanasia, urine samples were collected from animals over a period of 24 hours while held individually in metabolic cages, during which animals were offered chow and water *ad libitum*.

Animals were deeply anaesthetised with 5% isofluorane (VCA I.S.O., Sydney, NSW, Australia) in 100% O_2 and decapitated. Trunk blood samples were collected in pre-cooled EDTA containing tubes (BD Microtainer®, Becton, Dickinson and Company, Macquarie Park, NSW, Australia) and centrifuged (3000 RPM for 5 min at 4C°). Plasma and urine were analysed for biochemical parameters using an IDEXX VetLab analyser (IDEXX Laboratories Pty Ltd., Rydalmere, NSW, Australia). The mesenteric vasculature, kidneys and heart were removed. The kidneys, heart, and left ventricle were weighed and respective indices (HI and KI; %) were calculated as heart or kidney weight (g)/ body weight (BW) (g) × 100.

3.3.3 Mesenteric artery isolation

Immediately following dissection, the mesenteric vasculature was immediately placed in ice-cold Krebs' physiological solution (in mM: NaCl 118.2, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $NaHCO_3$ 25, and EDTA, 0.026), continuously bubbled with carbogen (95% O_2; 5% CO_2; BOC Ltd., North Ryde, NSW, Australia) to achieve a pH value of 7.4-7.45 (Intengan et al., 1999).

After cleaning of adherent connective tissue under a dissecting microscope, a second order mesenteric artery segment (200-400 μm external diameter; ~4 mm in length) was mounted in a pressure myograph (Living Systems Instrumentation, Burlington, VT, USA) in 5 mL of carbogen-bubbled ice-cold Krebs' solution. Artery ends were cannulated with glass micropipettes (1 x 0.25 mm glass capillaries, A-M Systems, Inc., Sequim, WA, USA), and secured with 10-0 sutures (Ethilon® Nylon Suture LLC., California, USA). The remaining mesenteric vasculature was placed in 4% formalin for 24 hours, followed by 70% ethanol until further processing for histology.

3.3.4 Pressure myography

Average intraluminal pressure in the artery was increased to 120 mmHg, and the vessel length stretched until there was no lateral bowing of the vessel (Potocnik et al., 2000). Pressure was then decreased to 60 mmHg and the vessel left to equilibrate for 60 min, with Krebs' buffer superfused at a flow rate of 3 mL/min. During this period, the vessel warmed to 37 °C and constantly bubbled in carbogen via a miniature gas dispersion tube (Living Systems Instrumentation). Lumen and wall thickness dimensions were measured at a constant intraluminal pressure of 60 mmHg using the video dimension analyser within the pressure myography system for all experimental conditions. Following equilibration, vessel integrity was determined, with vessels considered viable if the α_1-adrenergic receptor agonist phenylephrine (PE; 10^{-6} M) elicited >50% constriction relative to resting lumen diameter (Behbahani et al., 2010, Luksha et al., 2011). Vascular functional and structural investigations were then performed sequentially. All data were recorded using Spike 2 v7.07a software (Cambridge Electronic Design, Cambridge, UK).

3.3.4.1 Vascular function investigations

Cumulative concentration-response curves to (i) the α_1-adrenergic receptor agonist phenylephrine (PE) and (ii) the direct muscle depolariser potassium chloride (KCl) were obtained in order to study vasoconstrictor mechanisms.

After preconstriction with PE, cumulative concentration-response curves to (i) the endothelium-dependent vasodilator acetylcholine (ACh) and (ii) –independent vasodilator sodium nitroprusside (SNP) were obtained in order to study endothelium integrity.

After preconstriction to PE, cumulative concentration-response curves to ACh were obtained after arteries had been incubated in (i) either N-$^{\omega}$nitro-L-arginine methyl ester (L-NAME) (10^{-4} M) alone or (ii) indomethacin (Indo) (10^{-5} M) and L-NAME to further delineate vasorelaxation mechanisms.

The 50% effective concentration (EC_{50}) and maximum response (R_{max}) were calculated from the concentration-response curves. The differences between Rmax responses to ACh and that in the presence of L-NAME alone was considered the NO-dependent (Paulis et al., 2008) component of the ACh-induced response; the ACh-Rmax remaining in the presence of Indo+L-NAME combined was considered the EDH (Luksha et al., 2012) component of the response. The difference between responses in the presence of Indo+L-NAME and L-NAME were considered the prostanoid component of the ACh-induced response.

Additional specific protocol details and more detailed descriptors of the data analysis for the vascular functional studies are provided in the Supplementary material (Section 3.8).

3.3.4.2 Vascular structure investigations

At the end of the experimental protocol, vessels were deactivated by superfusion with Ca^{2+}-free Krebs' solution containing 10 mM EGTA for 20 minutes. To obtain pressure–diameter relationships, intraluminal pressure was increased incrementally via the servo-controlled pump, from 3 to 180 mm Hg (3, 20, 40, 60, 80, 100, 140 and 180 mmHg). Lumen and wall thickness dimensions were measured along the length of the vessel after 3 min at each pressure level using the video dimension analyser. Media cross sectional area (MCSA), stress,

strain and elastic modulus were calculated for each animal individually using the measured diameters and wall thickness, and then values were averaged for each group (strain/age). The specific formulae are provided in Supplementary material (Section 3.8).

3.3.5 Histology

At least two of each animal's second-order mesenteric arteries were formalin-fixed second order mesenteric arteries were paraffin embedded using an automated tissue processor (Leica ASP2005, GmbH, Germany). Subsequently, at least x4 5 μm thick sections were mounted on glass microscope slides and samples stained for collagen deposition and smooth muscle cell nuclei with Martius scarlet blue (MSB) stain, for elastin with Shikata's orcein stain and for calcification with Alizarin red and Von Kossa (VK) as per published protocols (Carson and Hladik, 2009, Salman et al., 2014). Images were acquired at 20x magnification under bright field microscopy (Zeiss 1 Axiovision v4.8.2.0, Carl Zeiss Microimageing, Gottingen, Germany). Analysis was performed using the customised automated image-processing software Image J (Schneider et al., 2012). All histological parameters were calculated from an average of at least 6 fields equally distributed around the mesenteric artery segment. Specific analysis for each stain is provided in Supplementary material (Section 3.8).

3.3.6 Drugs and solutions

Phenylephrine hydrochloride (PE), acetylcholine hydrochloride (ACh), sodium nitroprusside (SNP), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin (Indo), histolene, Bouin's fixative, Celestine blue, Harris's haematoxylin, Martius yellow, brilliant crystal scarlet, aniline blue, Shikata orcein and Alizarin red were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All drugs and solutions (Krebs' and Ca^{2+}-free Krebs') were prepared on the day of the experiment. All drugs for pressure myography were dissolved in freshly made Krebs' buffer prior to experimentation.

3.3.7 Data analysis

Data were analysed using IBM Statistical Package for the Social Sciences (v22, SPSS; Chicago, Illinois USA) and GraphPad Prism (GraphPad Prism software v6 Inc., La Jolla, CA, USA). Specific details regarding statistical analysis are provided in the Supplementary

methods (Section 3.8). All results are expressed as mean ± standard error of mean (SEM). Preliminary analysis of data was performed to identify potential gender effects and unless significant, are not otherwise noted. Unless stated, males and females were grouped together within ages and strains for analyses. Data was tested for normality and if assumptions were met, two-way analysis of variance (ANOVA) was performed followed by Bonferroni *post-hoc* analysis to investigate significant differences between groups (as defined by strain and age). Data that did not meet assumptions of normality were analysed *post-hoc* with a Dunnett's T3 test.

Urine protein values less than 0.05 g/L were excluded from the data set, and corresponding UPC values were also removed. Urine protein and UPC data analyses were evaluated by one-way ANOVA, followed by Bonferroni's *post-hoc* analysis.

GraphPad Prism was used for curve-fitting and to calculate Rmax and EC_{50} values (Armitage et al., 2005). Curves were evaluated by ANOVA followed by Bonferroni's *post-hoc* analysis. Two-way ANOVA with Bonferroni's *post-hoc* analysis was used to investigate strain, age and pharmacological effects. Significance was defined as $P \leq 0.05$.

Multiple linear regression modelling, with wall/lumen ratio as the dependent variable and SBP, plasma urea and plasma creatinine as independent variables was used to derive Pearson correlation coefficients, using a one tailed test for significance ($P \leq 0.05$).

3.4 Results

3.4.1 Baseline parameters

Lewis polycystic kidney rats significantly differed from Lewis rats in body weight, SBP, HI, KI, plasma urea, plasma creatinine and urine creatinine at all ages, demonstrating lower body weight, impaired renal function, hypertension and cardiac hypertrophy (Table 3.1). In addition, both strains showed within strain age effects for body weight and SBP, which were higher at 12 and 18 weeks compared to the 6 week baseline. For LPK rats, additional within-strain increases were noted at 18 weeks compared to 6 and 12 weeks for HI, plasma urea and creatinine. The latter two plasma parameters were also increased at 12 weeks versus 6 weeks of age. A gender effect was noted for plasma urea values in the LPK, with 12 week old male LPK having larger plasma urea values than females. Very few Lewis demonstrated proteinuria, however the LPK demonstrated an age-related increase in urinary protein values between the 6 and 18 week time points. Lewis polycystic kidney rats had lower urine creatinine values than Lewis at all ages. Female 6 and 18 week Lewis had significantly lower urine creatinine values than male Lewis of the same ages. The urinary protein creatinine ratio trended towards increasing with age in the LPK but this did not reach significance.

3.4.2 Vascular function investigations

3.4.2.1 Constriction

At 18 weeks, LPK PE concentration-response curves were significantly shifted to the left relative to age-matched Lewis controls and 6 and 12 week LPK rats (Figure 3.1A and B), indicating increased sensitivity to PE. Apart from a significantly smaller PE R_{max} found in 6 week Lewis relative to 12 week Lewis, PE curve shifts were not accompanied by any other significant curve parameter changes (Supplementary table; Section 3.8). Six week Lewis had significantly smaller KCl R_{max} values relative to older Lewis (Supplementary table; Section 3.8) but no other age or strain effects were evident for the KCl concentration-response curves (Figure 3.1B, Supplementary table; Section 3.8).

3.4.2.2 Endothelium-dependent and independent vasorelaxation

Acetylcholine concentration-response curves in the LPK rats provided evidence for impaired endothelium-dependent vasorelaxation at 12 and 18 weeks, but not 6 weeks, as evidenced by a significant shift to the right relative to age-matched Lewis controls and 6 and 12 week old LPK in the 18 week old animals (Figure 3.2 A and B). In accordance with these results, 18 week LPK ACh R_{max} values were significantly smaller than age-matched Lewis controls and 6 and 12 week LPK rats (Table 3.3B). In addition, 12 week LPK ACh R_{max} values were significantly smaller than age-matched Lewis controls and 6 week LPK rats (Supplementary table; Section 3.8). Six week Lewis ACh concentration-response curves were significantly shifted to the left, relative to 18 week Lewis curves, however this was not accompanied by any changes in curve parameters (Figure 3.2 A, Supplementary table; Section 3.8).

Endothelium-independent relaxations in response to SNP showed strain and age differences at 6 weeks, such that LPK SNP concentration-response curves were significantly shifted to the left relative to Lewis and relative to 12 and 18 week LPK rats (Figure 3.2 C and D). Eighteen week Lewis curves were significantly shifted to the right relative to 6 and 12 week Lewis (Figure 3.2 C), and SNP R_{max} values were larger in 6 week Lewis relative to 12 week Lewis (Supplementary table; Section 3.8).

3.4.2.3 Vasorelaxation mechanism investigations

Relative to control ACh responses, preincubation with NOS inhibitor L-NAME reduced the ACh R_{max} in 6, 12 and 18 week Lewis, but only in the 6 week old LPK rats (Supplementary table; Section 3.8). Preincubation with a combination of the cyclooxygenase inhibitor indomethacin and L-NAME (Indo+L-NAME) resulted in a reduction in ACh R_{max} values for all ages and strains. The ACh R_{max} following preincubation with Indo+L-NAME was significantly smaller in 12 LPK rats than the ACh R_{max} after preincubation with L-NAME alone (Supplementary table; Section 3.8). The ACh R_{max} following preincubation with Indo+L-NAME in 12 week Lewis was significantly larger than 6 week Lewis, and conversely, was significantly less in 12 week LPK than in 6 week LPK rats.

3.4.2.4 Vasorelaxation component investigations

The NO-dependent and EDH component of the ACh response was calculated from the difference in the ACh R_{max} response before and after incubation L-NAME or indomethacin and L-NAME, respectively. The relative contributions in each strain and age group are illustrated in Figure 3.3. No significant age or strain effects were found for the percentage contribution to ACh R_{max} by NO or EDH between all groups however the prostanoid percentage contribution to ACh R_{max} was increased in 12 week LPK, relative to age-matched Lewis controls and 6 week LPK rats.

3.4.3 Altered resistance artery stiffness in Lewis polycystic kidney rats

Regardless of age, LPK rats pressure diameter curves were shifted downwards relative to Lewis controls. In both strains, pressure diameter curves were highest at 12 weeks. In the Lewis they shifted downwards at 18 weeks, though not to the level of the 6 week animals. Lewis polycystic kidney rats curves also shifted downwards at 18 weeks but in this case below that of the 6 week old LPK animals (Figure 3.4). Eighteen week Lewis pressure diameter curves were shifted downwards relative to 12 week Lewis, and shifted upwards relative to 6 week Lewis. In addition, 18 week LPK curves were shifted downward relative to 12 week LPK rats (Figure 3.4). Note, however, that 6 and 12 week Lewis have similar pressure-diameter curve gradients, and that the downward shift of the 6 week Lewis seems to be due to smaller mesenteric resistance vessel diameters, rather than altered stiffness.

Determination of stress-strain curves indicated that in 6 and 18 week LPK, there was a significant shifted to the left relative to age-matched Lewis control rats (Figure 3.5A, B). In 12 week LPK rats however stress-strain curves were significantly shifted to the right relative to age-matched Lewis. Overall, the Lewis demonstrated a shift to the left in the curves with increasing age. This pattern was also evident for the 6 vs. 18 week old LPK however interestingly the 12 week old LPK curves were significantly shifted to the right relative to both 6 and 18 week LPK rats. The slope of the elastic modulus vs. stress values was significantly greater in 6 week LPK vs. age matched controls, while the slope in the 18 week old LPK was greater than age matched controls and both 6 and 12 week old LPK rats (Figure 3.5 C and D).

3.4.4 Altered resistance vessel morphology in Lewis polycystic kidney rats

Both internal and external mesenteric artery diameters were significantly smaller in LPK rats compared to Lewis at 12 weeks, as was the internal diameter at 18 weeks (Table 3.2). Twelve week Lewis males had larger external diameter values than female counterparts. Regardless of age, LPK had significantly larger wall thickness and wall-lumen ratio values relative to age-matched Lewis controls, and the LPK rats demonstrated an age related increase in wall thickness between 12 and 18 weeks of age. Media cross sectional area values were increased in LPK rats relative to age-matched Lewis controls at 6 and 18 weeks of age, with an age related increase also evident in the LPK rats between 6 and 18 weeks. In summary, this indicated hypertrophic inward remodelling at 6 and 18 weeks in the LPK rats, and eutrophic inward remodelling at 12 weeks. It was also noted that the 12 week old Lewis demonstrated an increase in MCSA relative to the 6 week old Lewis animals.

3.4.5 Altered mesenteric artery composition in Lewis polycystic kidney rats

Mesenteric artery composition was altered at all ages for the LPK rats, with an upregulation of arterial stiffness components, relative to elastic components. This was evidenced by collagen density being greater in LPK than Lewis rats at all ages, and increased at 12 and 18 weeks compared to 6 weeks within LPK rats (Figure 3.6, Table 3.3). No significant alterations in nuclear density and number of nuclei per μm^2 were found other than a lower nuclear density in the 12 week Lewis relative to 6 week Lewis. An age-related decline in elastin density was evident in the Lewis and LPK animals, with elastin density significantly reduced in 12 week LPK animals relative to age-matched Lewis controls. The changes in collagen and elastin were reflected in the collagen/elastin ratios, being higher in 12 and 18 week LPK relative to age-matched Lewis, and increased in the LPK rats from age 6 to 18 weeks. No calcium deposition was evident in the mesenteric arteries of either strain.

3.4.6 Correlations between systolic blood pressure, renal function, and vessel structure

Multiple linear regression modelling revealed significant positive correlations between wall-lumen ratio and all three independent variables (plasma urea r=0.520, $P<0.001$, $n = 44$ pairs; plasma creatinine r=0.3574, $P=0.009$, $n = 44$ pairs and SBP r = 0.536, $P<0.001$ $n = 46$ pairs).

In the final model, SBP was the only significant predictor variable of structural changes (adjusted R^2=0.269, Beta=0.536, P<0.001).

3.5 Discussion

The remodelling of resistance vessels is associated with hypertension in a number of disease states, and is likely to play a key role in the development of target organ damage. In this study we report for the first time changes in resistance artery structure, function and biomechanical properties in a chronic and progressive model of kidney disease. Resistance artery structural changes involved eutrophic remodelling in the 12 week LPK rats, which then progressed with further ageing to hypertrophic remodelling associated with a relative increase in vascular smooth muscle cell size. Vascular remodelling in adult (12 and 18 weeks) LPK rats was accompanied by increased collagen/elastin ratios. Interestingly, the LPK rats exhibited increased stiffness at 6 weeks, which was followed by a compensatory rise in compliance at 12 weeks; however as age further increased, stiffness also increased. Functional changes revealed endothelium dysfunction in the adult LPK rats, and increased sensitivity to an alpha-adrenergic stimulator at18 weeks.

A significant result of this study was the significant relationship between plasma urea, creatinine and systolic blood pressure with wall-lumen ratio, whereby SBP was the main predictor of wall-lumen ratio. This suggests that both hypertension and renal dysfunction are major contributors to structural remodelling, and supports the motive for treatments in CKD patients to focus on the associated cardiovascular disease (Herzog et al., 2011). Further studies incorporating antihypertensive treatment would be required to investigate the SBP correlation in CKD to elucidate if remodelling in resistance vessels is a result of, or partial causation of increased arterial blood pressure.

Vascular remodelling has been proposed to act as a compensatory mechanism to protect the smaller capillary vessels from elevated blood pressure (Intengan and Schiffrin, 2001). In the present study, the LPK animals initially developed eutrophic inward remodelling, as indicated by an increase in arterial wall thickness, decrease in lumen diameter, unchanged MCSA and nuclei number. As the disease progressed, the resistance arteries underwent an increase in MCSA, wall thickness and wall-lumen ratio, around an artery with a smaller internal lumen diameter, relative to age-matched Lewis controls, consistent with hypertrophic inward remodelling. This finding is a well-established phenomenon in essential and secondary hypertension, and mirrors previous research by New and colleagues, who found hypertrophic

inward remodelling in mesenteric arteries from a subtotal nephrectomy model of uremic hypertension, using Wistar-Kyoto rats (New et al., 2004). The progression of remodelling in LPK rats from eutrophic to hypertrophic indicates deterioration in the resistance artery structure as hypertension ensues and renal disease progresses.

Notably, the Lewis controls also demonstrated structural change with ageing, which involved an increase in internal and external diameter, and MCSA; however, this was not accompanied by other significant parameter changes, such as wall-lumen ratio, cell number, or cell density. Given that these structural changes did not involve associated alterations in wall-lumen ratio, it is likely these Lewis changes are due to an increase in rat weight and size, rather than arterial remodelling due to mechanical stresses.

Comparison between LPK and Lewis rats demonstrated that differences in resistance arteries were already present by 6 weeks of age, including increased stiffness, as shown by an increase in collagen density, a significant leftward shift of the stress-strain curve, a downward shift of the pressure-diameter curve gradient and an increased slope of elastic modulus vs. stress slope values, an indicator of intrinsic stiffness that is independent of vessel geometry (Behbahani et al., 2010). At 12 weeks, stiffness measures in the LPK rats mesenteric artery improved, indicated by a rightward shifted stress-strain curve, relative to both other LPK rats age groups and their age-matched controls. This apparent improvement in compliance in 12 week LPK rats was despite a marked increase in the wall stiffness component, collagen. Although counterintuitive, given that collagen is an extracellular fibrillar component associated with progressive stiffening of the vascular wall (Intengan and Schiffrin, 2001), this improved compliance despite increased collagen could be due to the fact that recruitment of collagen fibres is suggested to occur in the latter portion of the pressure-diameter curve. When smooth muscle cells in series and elastin in parallel are unstretched, collagen fibres tend to be coiled and are not under tension (Intengan et al., 1999). In the remodelled artery, however, alterations in the adhesion properties of cellular and fibrillar structures occur, and potentially the presence of different types of collagen, can result in increased alignment compactness of cellular and fibrillar components (Intengan et al., 1999). Hence, collagen fibres may only be recruited when exposed to higher pressures. This realignment of collagen fibres may also explain the unchanged slope of elastic modulus vs. stress values despite greater collagen density, since intrinsic stiffness is believed to be more related to the

organisation of vessel components, as opposed to directly related to the composition of the vessel alone (Gonzalez et al., 2005). Thus, although there was increased collagen/elastin ratio which would suggest increased wall stiffness, adaptive changes through arterial smooth muscle rearrangement could be a compensatory mechanism against higher wall stress, and therefore a reason for an overall increased distensibility (Intengan et al., 1999), comparable to the adaptive vascular changes we have observed previously in the LPK rats thoracic aorta (Ameer et al., 2014b). Between 12 to 18 weeks of age, stiffness increased in the LPK animals. While the vascular remodelling at 12 weeks appeared to help counteract the detrimental effects of high blood pressure on the arterial wall, it was unable to offset the high degree of wall stress experienced at the vessel wall as the hypertension persisted and renal disease progressed, resulting in increased stiffness by 18 weeks of age.

We also assessed the composition of the arterial walls for elastin and calcification. The absence of calcification in both strains and age groups was in contrast to what we have previously described in large vessels in association with age and CKD (Ameer et al., 2014b, Ng et al., 2011). Calcification has been described in breast arterioles in women with CKD and end-stage renal disease (O'Neill and Adams, 2013). However, to the best of our knowledge, resistance artery calcification has not previously been examined in rodent models of CKD. Elastin degradation is one of the mechanisms that have been associated with vascular calcification (Pai et al., 2011), and although we document an age related decrease in elastin density in both Lewis and LPK rats, the absence of calcification may be due to the composition of the artery, with mesenteric arteries having a very small percentage of elastin (an average of ~15% in Lewis rats), and therefore a reduced likelihood of calcifying, relative to, for example, Lewis rat aortas, which are comprised of approximately 70% elastin (Salman et al., 2014).

In addition to structure and biomechanics, vascular remodelling has functional implications. In the 18-week LPK rats mesenteric artery, we saw a leftward shift in the concentration-response curves in response to the α-adrenergic receptor agonist PE, relative to both younger LPKs and age-matched Lewis rat controls. Similar results were not seen in response to KCl, which acts as a direct depolariser. This increased sensitivity to sympathetic agonist is consistent with a vascular amplifier effect. The vascular amplifier effect may be due to hypertrophy and/or remodelling of the resistance vasculature, functioning to amplify the

effect of constrictor and dilator agents on peripheral resistance, and therefore vascular conductance (Folkow, 1975, Wright and Angus, 1999, Moretti et al., 2009). In the whole animal, this translates to enhanced blood pressure responses to vasoactive agents (Wright and Angus, 1999) including ganglion blockade, such that increased depressor responses are due to enhanced vascular responsiveness to sympathetic activity, rather than increased sympathetic activity to the vasculature per se. In previous studies, our investigations revealed an augmented response to hexamethonium in the LPK rats (Phillips et al., 2007) which was determined to be primarily a reflection of increased sympathetic drive however this did not account for the full response (Ameer et al., 2014a). The current evidence of resistance vessel remodelling therefore supports the hypothesis that a vascular amplifier effect also contributes to the augmented depressor responses to ganglionic blockade in the LPK rat and to the maintenance of hypertension in this model of CKD.

Dysfunction of the endothelium is often associated with hypertension, and may be a consequence of blood pressure elevation (Schiffrin, 2012), and conversely it is a factor that increases susceptibility to the development of hypertension (Rossi et al., 2004). In the Lewis animals examined in this study, we identified an age-related shift in the endothelium-dependent and –independent vasodilatory responses. In the LPK rats we observed comparable endothelium-independent relaxation responses to SNP, but the mesenteric arteries demonstrated reduced ACh-induced endothelium-dependent relaxations at both 12 and 18 weeks relative to their Lewis counterparts. This endothelium dysfunction is in accordance with previous studies investigating resistance arteries in renovascular hypertensive and autosomal dominant polycystic kidney disease (ADPKD) patients (Porteri et al., 2003, Wang et al., 2003, Chamorro et al., 2004). It is possibly due to reduced NO availability, increased reactive oxygen species (ROS), or impaired NOS functioning, as the NOS-inhibitor L-NAME did not influence the R_{max} values in either the 12- and 18-week-old animals. Our data also indicated impairment in EDH function, with, R_{max} values in response to acetylcholine following incubation with indomethacin and L-NAME also reduced in the 18 week old LPK rats. Impairment of EDH is consistent with Borges et al. and Fuji et al. (Borges et al., 1999, Fujii et al., 1992), who found that EDH function was lost in the mesenteric vascular bed of spontaneously hypertensive rats relative to normotensive rats. It is well-established that EDH release and activity involve alterations in K^+ flux in both the endothelium and smooth muscle

cell (Luksha et al., 2009, Mombouli and Vanhoutte, 1997), resulting in hyperpolarisation of the cell and a decrease in intracellular calcium (Chen and Suzuki, 1990, Peach et al., 1987); thus, the relative reduction in contribution of EDH to the relations response found in LPK rats could be related to a defective Ca^{2+} machinery

The relative proportion of the vasorelaxation mechanism associated with EDH, NO and prostanoids was consistent in both the LPK and Lewis rats with previous findings, with EDH being the major contributor to relaxation (Luksha et al., 2012), then NO (Paulis et al., 2010), and with prostanoids playing only a minimal role (Shimokawa et al., 1996). Interestingly, despite the endothelium-dependent response declining with age in the LPK rats, when the relative contribution of each vasorelaxation component was compared, the overall percentage contribution did not vary to any substantial degree. The only notable change was an increase in the relative contribution of prostanoids to the ACh-mediated vasodilation, which increased at 12 weeks in the LPK animals. There was an indication this was maintained at 18 weeks however it was not statistically significant. This increase may be a possible compensatory response to the impaired NOS and EDH functioning. A similar mechanism was proposed by Sun et al. who describe an upregulation of prostaglandins in the skeletal muscle arterioles from endothelium NOS knockout mice, and suggested that it could be a vascular adaptation mechanism to maintain normal peripheral resistance (Sun et al., 1999).

Important to note is a possible limitation of this study, regarding the blood pressure recording method of choice, tail cuffing. Although rats were trained for tail cuffing frequently in order to ensure consistent results could be measured from them, it is important to still consider the possibility that measuring blood pressure via the tail cuff method could induce autonomic activation in the animals, due to the small confined space that rats are placed in for measurements. In addition, the use of PE as a preconstrictor for subsequent vasodilatory investigations may be a possible limitation for this study, given that increased sensitivity to PE concentration-response curves was found in LPK rats. The present study used PE to remain consistent with previous literature which used α-adrenergic stimulation to preconstrict vessels (for example, see Shahid and Buys, 2013). This is because use of an alternative preconstrictor such as KCl may affect our investigations of endothelium-dependent relaxation, considering that EDH vasorelaxation mechanisms play a large role in resistance artery vasodilation and involve activation of calcium-activated potassium (K^+ Ca^2) channels.

To further investigate the effects of preconstriction with PE compared with KCl, and the resultant effects on vasorelaxation in LPK resistance arteries, future studies may employ different preconstrictors.

In conclusion, our data show altered and progressive changes in the structural, functional and biomechanical properties of mesenteric resistance arteries from the LPK rodent model of CKD. Structural alterations were characterised by eutrophic and hypertrophic inward remodelling at intermediate and later renal disease time points, respectively, and these structural alterations significantly correlated with plasma urea, plasma creatinine and hypertension. These findings strongly support current treatment strategies focusing on both hypertension and renal function in the control of CKD (Harris and Rangan, 2005). Increased vessel stiffness in the LPK rats was accompanied by greater collagen/elastin ratios as well as amplified elastic modulus vs. stress slopes. Functional alterations were characterised by impaired endothelium-dependent relaxation in older animals, contributed to by an impairment in both the EDH and NO vasodilatory components. Other functional observations revealed enhanced sensitivity to sympathetic receptor activation, that together with the vascular remodelling findings, supports a vascular amplifier hypothesis. Hypertension in CKD has multiple effects on resistance arteries, resulting in the activation of compensatory structural mechanisms which eventually become maladaptive, thus exacerbating the disease, while functional mechanisms involve endothelium dysfunction and impairment of vasorelaxation. Notably, impairments in the LPK rats vasculature arise as early as 6 weeks when hypertension is first established, and generally worsen with persistence of the hypertension and decline in renal function. This study provides insight to the changes that resistance arteries undergo in a state of CKD-mediated hypertension, and therefore will assist in guiding the development of treatments to prevent disease progression.

3.6 Acknowledgements

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3.7 Conflict of interest

None declared.

Tables

Table 3.1: Baseline morphometric and biochemical parameters of 6, 12 and 18 week old Lewis and Lewis polycystic kidney rats.

	6 week		12 week		18 week	
Parameter	Lewis	LPK	Lewis	LPK	Lewis	LPK
n	8	7	11	7	6	7
BW(g)	131±6	81±4 [a*]	300±17 [a]	165 ±18 [b* d]	312±45 [c]	190±31 [c* f]
SBP (mmHg)	107±2	127±4 [a*]	126±2 [a]	195±2 [b* d]	126±3 [c]	203±8 [c* f]
HI (%)	0.39±0.02	0.59±0.03 [a*]	0.33±0.01	0.55±0.04 [b*]	0.34±0.01	0.79±0.02 [c* e f]
KI (%)	1.13±0.02	7.95±0.24 [a*]	0.75±0.01	8.10±0.48 [b*]	0.80±0.01	7.29±0.45 [c*]
Plasma urea (mmol/L)	7.86±0.40	16.86±0.71 [a*]	7.17±0.31	33.1±3.61 [b* d]	6.45±0.23	44.20±2.20 [c* e f]
Plasma creatinine (μmol/L)	26.43±3.72	29.00±3.06 [a*]	27.71±6.98	85.00±13.96 [b* d]	21.70±2.52	218.00±43.74 [c* e f]
Urine Protein (g/L)	nd	0.40±0.09	nd	1.72±0.61	0.23 [(n = 1)]	2.74±0.63 [f]
Urine Creatinine (g/L)	1.67±0.56	0.23±0.03 [a*]	1.64±0.12	0.21±0.03 [b*]	1.41±0.47	0.31±0.07 [c*]
UPC	-	3.03±1.43	-	7.03±1.84	0.07 [(n = 1)]	12.27±3.93

BW, body weight; SBP, systolic blood pressure; HI, heart index; KI, kidney index; UPC, urine protein creatinine ratio; LPK, Lewis polycystic kidney rat. nd: not detected. Apart from urine protein and UPCs, which were evaluated by one-way ANOVA, data were evaluated by two-way ANOVA. Data that met parametric assumptions were followed by Bonferroni's *post-hoc* analysis, or Dunnett's T3 test if assumptions were violated. Results are expressed as mean ± SEM. Significant difference between groups where indicated $P<0.05$. Within strain age effect: (a) 6 and 12 weeks Lewis; (b) 12 and 18 weeks Lewis; (c) 6 and 18 weeks Lewis; (d) 6 and 12 weeks LPK; (e) 12 and 18 weeks LPK; (f) 6 and 18 weeks LPK. Within ages strain effect: (a*) 6 week Lewis and LPK; (b*) 12 week Lewis and LPK; (c*) 18 week Lewis and LPK.

Table 3.2: Mesenteric artery structural parameters of 6, 12 and 18 week old Lewis and Lewis polycystic kidney rats.

	6 week		12 week		18 week	
Parameter	Lewis	LPK	Lewis	LPK	Lewis	LPK
n	8	7	14	7	7	7
Internal diameter (μm)	200.6±15.2	151.4±16.5	292.4±18 [a]	174.9±21.6 [b*]	240.3±12.3	147.1±14.5 [c*]
External diameter (μm)	258.1±14.1	243.9±13.9	350.8±17.6 [a]	265.1±12.6 [b*]	297.0±9.0 [b]	261.6±11.1
Wall thickness (μm)	28.8±1.6	46.2±3.6 [a*]	29.2±1.3	45.14±6.3 [b*]	28.36±1.9	57.2±3.0 [c* e]
Wall-lumen ratio	0.15±0.02	0.34±0.06	0.11±0.01	0.30±0.07 [b*]	0.12±0.02	0.43±0.07 [c*]
MCSA ($\times 10^3$) (μm^2)	20.5±1.4	28.3±2.2 [a*]	27.8±1.5 [a]	29.7±2.9	23.6±0.9	36.3±2.2 [c* f]

MCSA, media cross sectional area; LPK, Lewis polycystic kidney rat. Results are expressed as mean ± SEM. For two-way ANOVA analyses, data that met parametric assumptions were followed by Bonferroni's *post-hoc* analysis, or Dunnett's T3 test if assumptions were violated. Significant difference between groups where indicated $P<0.05$. Within strain age effect: (a) 6 and 12 weeks Lewis; (b) 12 and 18 weeks Lewis; (c) 6 and 18 weeks Lewis; (d) 6 and 12 weeks LPK; (e) 12 and 18 weeks LPK; (f) 6 and 18 weeks LPK. Within ages strain effect: (a*) 6 week Lewis and LPK; (b*) 12 week Lewis and LPK; (c*) 18 week Lewis and LPK.

Table 3.3: Histological parameters of 6, 12 and 18 week old Lewis and Lewis polycystic kidney rats.

	6 week		12 week		18 week	
Parameter	Lewis	LPK	Lewis	LPK	Lewis	LPK
n	8	7	14	7	7	8
Collagen density (%)	9.4±0.4	18.4±0.5 [a*]	17.8±1.1 [a]	33.3±2.6 [b* d]	19.8±2.4 [c]	32.2±5.0 [c* f]
Nuclear density (%)	4.4±0.4	3.3±0.3	2.2±0.3 [a]	2.6±0.6	2.9±0.3	4.2±0.6
Number of nuclei per μm^2	0.09±0.01	0.12±0.01	0.08±0.02	0.12±0.04	0.13±0.03	0.13±0.03
Elastin density (%)	19.2±0.6	18.5±0.8	14.7±1.1 [a]	8.3±1.4 [b* d]	9.6±0.9 [b c]	6.4±2.3 [f]
Collagen/elastin ratio	0.5±0.03	1.0±0.04	1.3±0.15	4.8±0.78 [b* d]	2.1±0.35	8.9±2.21 [c* e f]

LPK, Lewis polycystic kidney rat. Collagen density, nuclear density and number of nuclei per μm^2 were quantified using Martius scarlet blue (MSB) staining, while elastin density was quantified using Shikata's orcein. For two-way ANOVA analyses, data that met parametric assumptions were followed by Bonferroni's *post-hoc* analysis, or Dunnett's T3 test if assumptions were violated. Results are expressed as mean ± SEM. Significant difference between groups where indicated $P<0.05$. Within strain age effect: (a) 6 and 12 weeks Lewis; (b) 12 and 18 weeks Lewis; (c) 6 and 18 weeks Lewis; (d) 6 and 12 weeks LPK; (e) 12 and 18 weeks LPK; (f) 6 and 18 weeks LPK. Within ages strain effect: (a*) 6 week Lewis and LPK; (b*) 12 week Lewis and LPK; (c*) 18 week Lewis and LPK

Figures

Figure 3.1: Vasoconstriction curves to phenylephrine and potassium chloride in Lewis and Lewis polycystic kidney rat mesenteric artery.

Six, 12 and 18 week old Lewis (A, C) and Lewis polycystic kidney rat (LPK, B, D) mesenteric artery contraction responses (%) to cumulative additions of the α_1-adrenergic receptor agonist phenylephrine (PE; A, B) and the direct depolariser vasoconstrictor potassium chloride (KCl; C, D). Results are expressed as mean ± SEM. Curves were evaluated by two–way ANOVA followed by Bonferroni's *post-hoc* analysis. Significant differences between concentration-response curves where indicated when $P<0.05$. Within strain age effect: (a) 6 and 12 weeks Lewis; (b) 12 and 18 weeks Lewis; (c) 6 and 18 weeks Lewis; (d) 6 and 12 weeks LPK; (e) 12 and 18 weeks LPK; (f) 6 and 18 weeks LPK. Within ages strain effect: (a*) 6 week Lewis and LPK; (b*) 12 week Lewis and LPK; (c*) 18 week Lewis and LPK. n = 7 minimum number of animals for each group (strain/age).

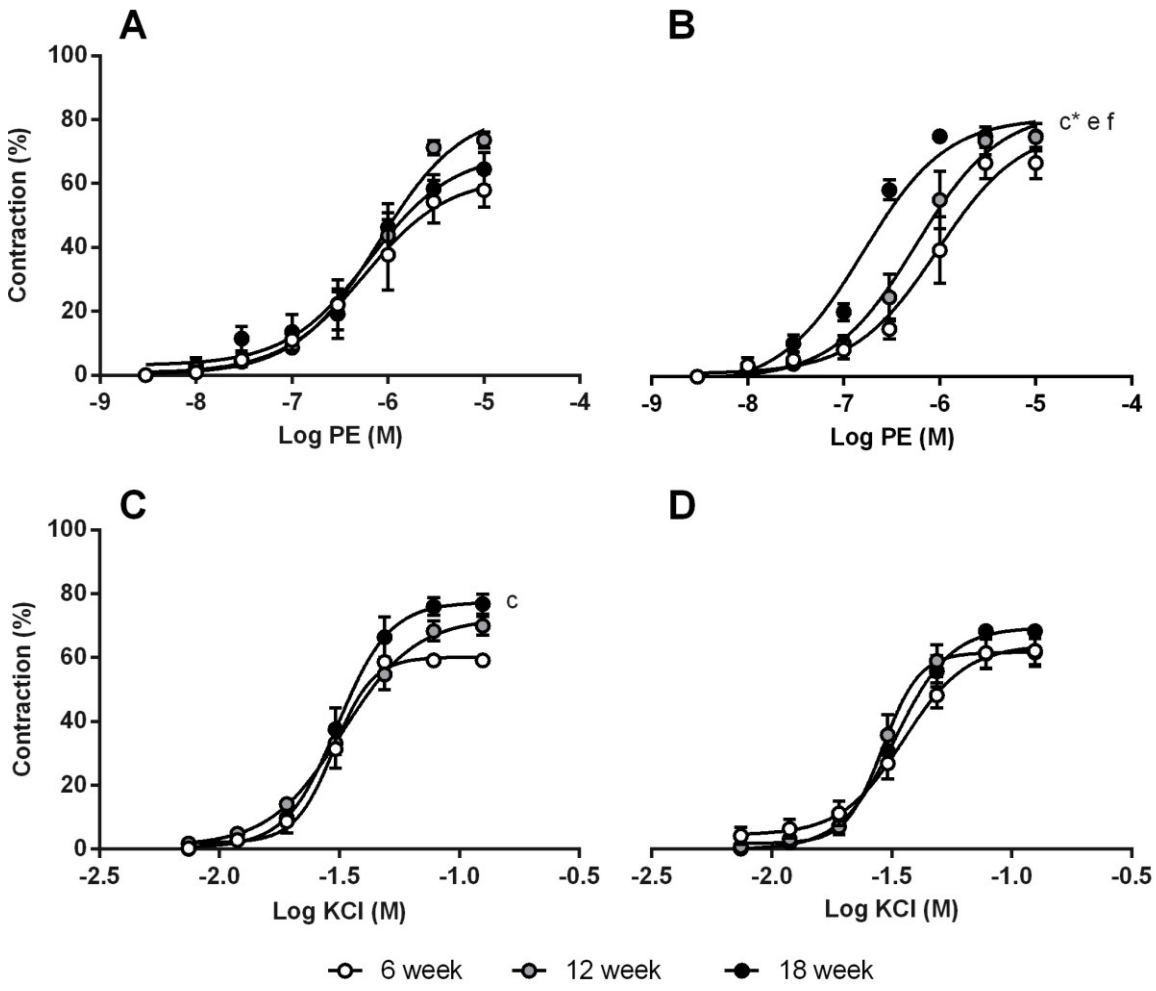


Figure 3.2: Vasodilation curves to acetylcholine and sodium nitroprusside in Lewis and Lewis polycystic kidney rat mesenteric artery.

Six, 12 and 18 week old Lewis (A, C) and Lewis polycystic kidney rat (LPK, B, D) second order mesenteric artery relaxation responses (%) to cumulative additions of endothelium-dependent and –independent vasodilators, (A, B) acetylcholine (ACh) and (C, D) sodium nitroprusside (SNP), respectively, following preconstriction with 1μM phenylephrine (PE). Results are expressed as mean ± SEM. Curves were evaluated by two–way ANOVA followed by Bonferroni's *post-hoc* analysis Significant differences between concentration-response curves where indicated when $P<0.05$. Within strain age effect: (a) 6 and 12 weeks Lewis; (b) 12 and 18 weeks Lewis; (c) 6 and 18 weeks Lewis; (d) 6 and 12 weeks LPK; (e) 12 and 18 weeks LPK; (f) 6 and 18 weeks LPK. Within ages strain effect: (a*) 6 week Lewis and LPK; (b*) 12 week Lewis and LPK; (c*) 18 week Lewis and LPK. n = 7 minimum number of animals for each group (strain/age).

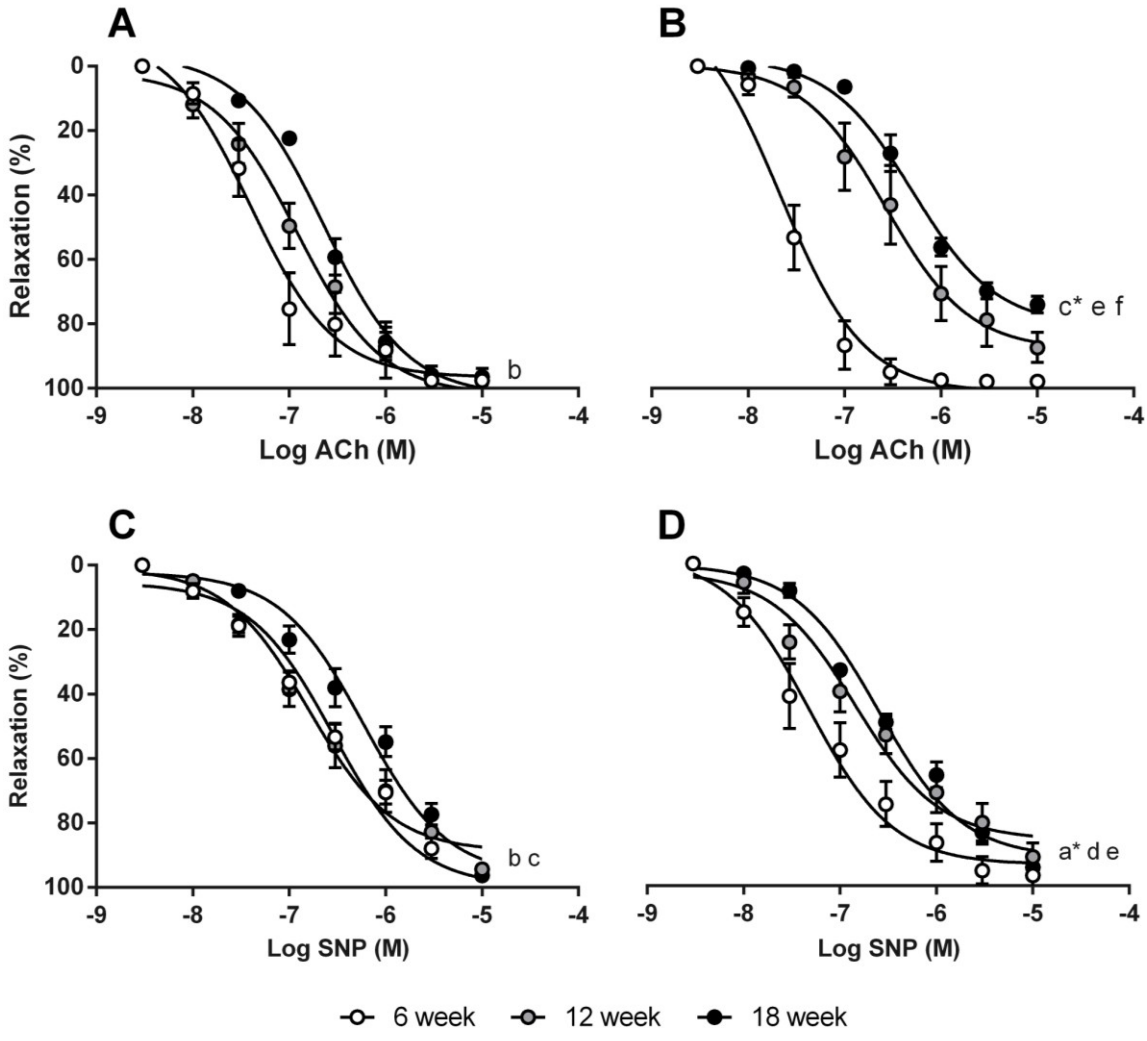


Figure 3.3: The relative contribution of vasodilatory mediators to acetylcholine responses in Lewis and Lewis polycystic kidney rat mesenteric artery.

Contribution of each vasodilatory component [nitric oxide (NO); prostanoid and endothelium-dependent hyperpolarisation (EDH)] to acetylcholine (ACh) -induced relaxation in 6, 12 and 18 week old Lewis and Lewis polycystic kidney rat (LPK) mesenteric artery. Results are expressed as mean ± SEM. Data were evaluated by two-way ANOVA followed by Bonferroni's *post-hoc* analysis. Significant difference between groups where indicated $P<0.05$. Within strain age effect: (a) 6 and 12 weeks Lewis; (b) 12 and 18 weeks Lewis; (c) 6 and 18 weeks Lewis; (d) 6 and 12 weeks LPK; (e) 12 and 18 weeks LPK; (f) 6 and 18 weeks LPK. Within ages strain effect: (a*) 6 week Lewis and LPK; (b*) 12 week Lewis and LPK; (c*) 18 week Lewis and LPK. n = 7 minimum number of animals for each group (strain/age).

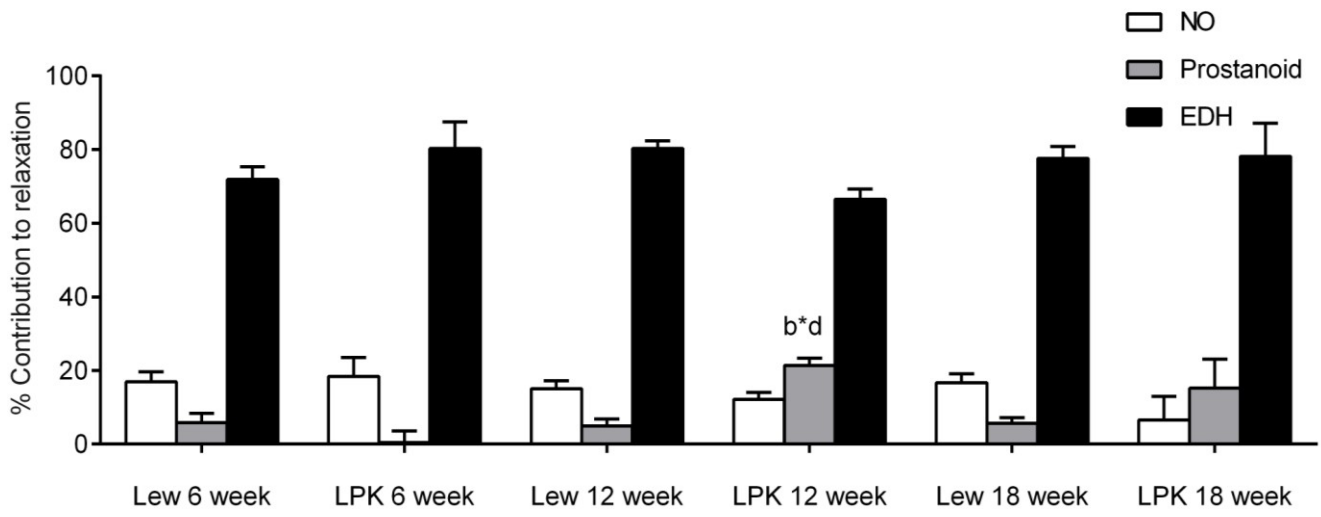


Figure 3.4: Pressure-diameter curves in Lewis and Lewis polycystic kidney rat mesenteric artery.

Passive pressure curves in 6, 12 and 18 week Lewis (A) and Lewis polycystic kidney rat (LPK; B) second order mesenteric arteries. Results are expressed as mean ± SEM. Overall curve comparisons were evaluated by two-way ANOVA, followed by Bonferroni's *post-hoc* analysis. Significant difference between groups where indicated $P<0.05$. Within strain age effect: (a) 6 and 12 weeks Lewis; (b) 12 and 18 weeks Lewis; (c) 6 and 18 weeks Lewis; (d) 6 and 12 weeks LPK; (e) 12 and 18 weeks LPK; (f) 6 and 18 weeks LPK. Within ages strain effect: (a*) 6 week Lewis and LPK; (b*) 12 week Lewis and LPK; (c*) 18 week Lewis and LPK. $n = 7$ minimum number of animals for each group (strain/age).

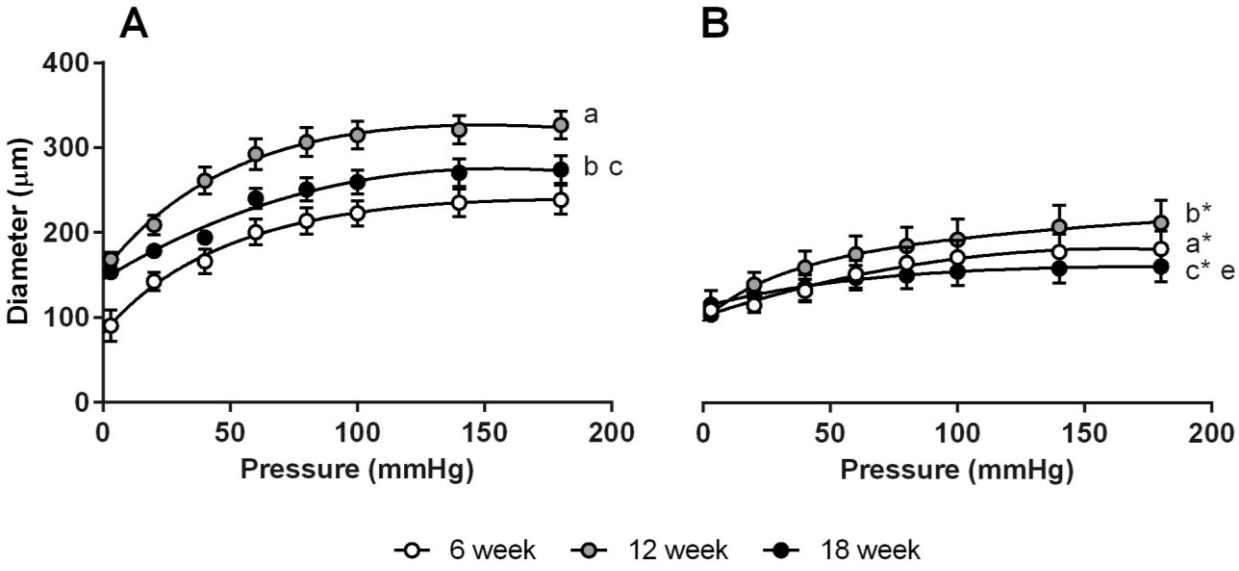


Figure 3.5: Stress-strain curves in Lewis and Lewis polycystic kidney rat mesenteric artery.

Stress-strain data fitted to an exponential curve for 6, 12 and 18 week old Lewis (A) and Lewis polycystic kidney rat (LPK; B) second order mesenteric artery. Panels C and D provide slope of elastic modulus vs. stress, calculated from stress-strain curve data for each strain. Results are expressed as mean ± SEM. Data were evaluated by two-way ANOVA, followed by Bonferroni's *post-hoc* analysis. Significant difference between groups where indicated $P<0.05$. Within strain age effect: (a) 6 and 12 weeks Lewis; (b) 12 and 18 weeks Lewis; (c) 6 and 18 weeks Lewis; (d) 6 and 12 weeks LPK; (e) 12 and 18 weeks LPK; (f) 6 and 18 weeks LPK. Within ages strain effect: (a*) 6 week Lewis and LPK; (b*) 12 week Lewis and LPK; (c*) 18 week Lewis and LPK. n = 7 minimum number of animals for each group (strain/age).

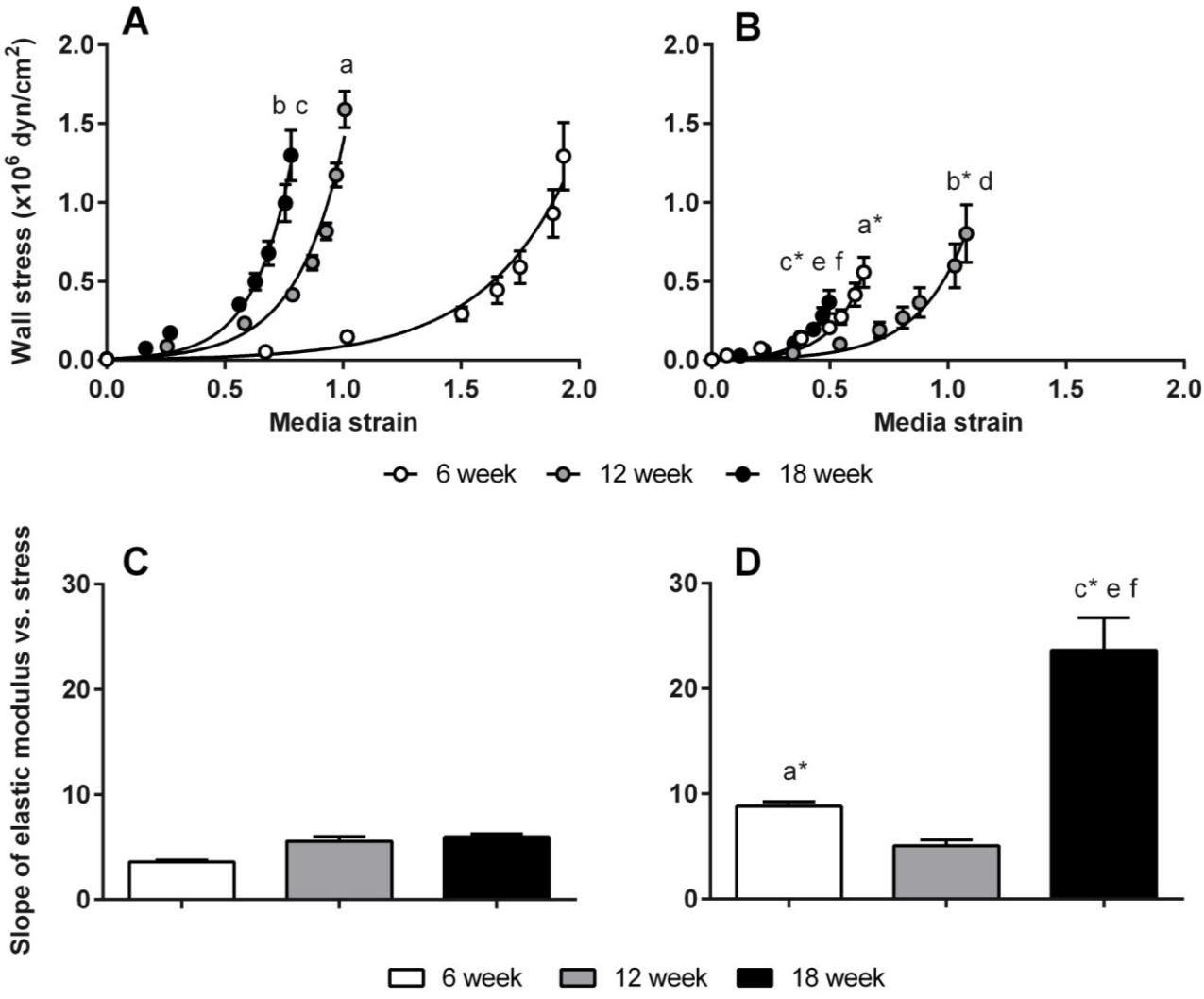
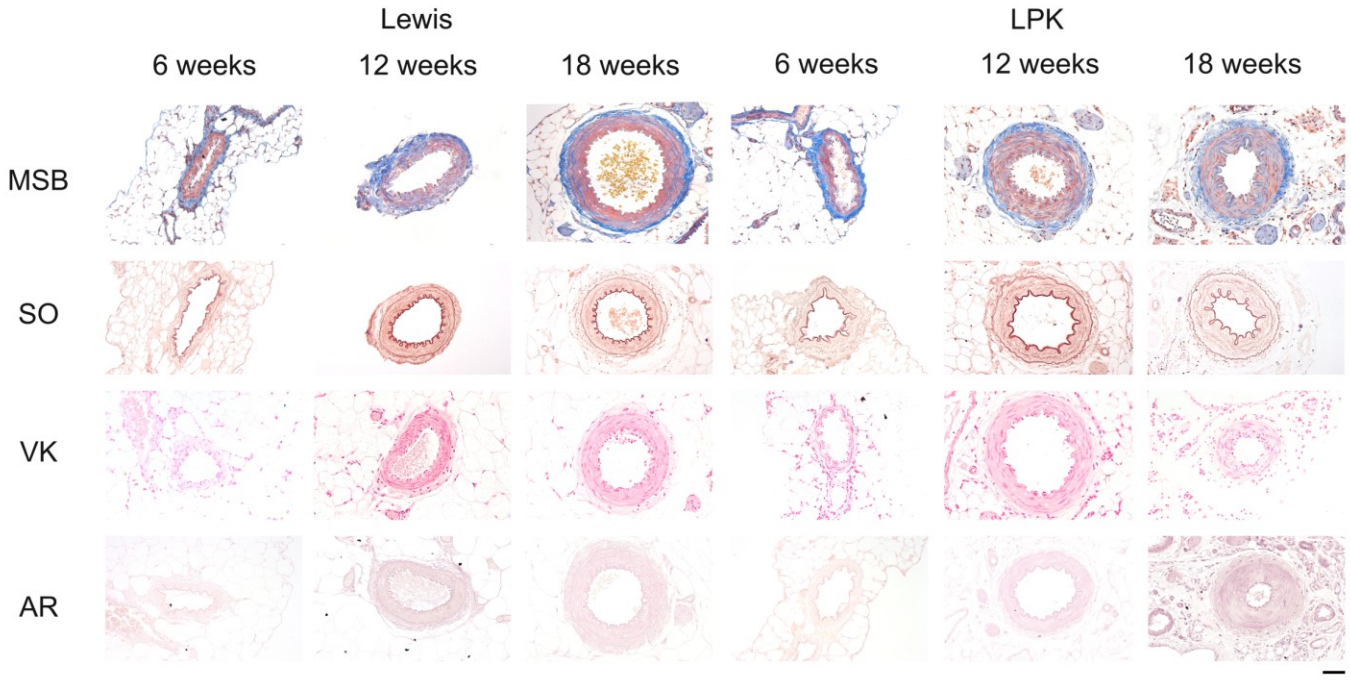


Figure 3.6: Vascular composition in Lewis and Lewis polycystic kidney rat mesenteric artery.

Representative images of 6, 12 and 18 week old Lewis and Lewis polycystic kidney rat (LPK) second order mesenteric artery wall, showing the collagen component in blue in sections stained with Martius scarlet blue (MSB), the elastin in red/brown in section stained with Shikata's orcein (SO), and lack of calcification as indicated by no dark brown/black deposits with Von Kossa (VK) or staining orange staining with Alizarin red (AR). Scale bar (bottom right panel) is 50 μm for all panels.



3.8 Supplementary material

Supplementary methods

3.8.1 Vascular function investigations: Protocols and data analysis

Unless otherwise specified, each concentration of pharmacological agent was extraluminally perfused for 3 min, after which vessel dimensions were measured (Luksha et al., 2011). Twenty min washouts were performed between each protocol:

3.8.1.1 Vasoconstriction investigations protocols

Concentration dependent responses to the cumulative addition of (i) PE (10^{-8}–10^{-5} M) and (ii) the direct muscle depolariser potassium chloride (KCl) (7.45×10^{-3}–1.25×10^{-1} M) were recorded.

3.8.1.2 Vasorelaxation investigations protocols

Endothelium integrity: Arteries were preconstricted with PE (10^{-6} M), and concentration dependent responses to the cumulative addition of (i) the endothelium-dependent vasodilator acetylcholine (ACh; 10^{-8}–10^{-5} M) and (ii) –independent vasodilator sodium nitroprusside (SNP; 10^{-8}–10^{-5} M), respectively, were recorded.

Vasorelaxation mechanisms: Arteries were incubated in (i) either N^{ω} nitro-L-arginine methyl ester (L-NAME) alone or (ii) indomethacin (Indo) and L-NAME for 30 min. Following incubation, arteries were then preconstricted with PE (10^{-6} M), and concentration dependent responses to the cumulative addition of ACh (10^{-8}–10^{-5} M) constructed.

3.8.1.3 Data analysis

All drug concentrations were log transformed for graphical representation and curve analysis. To evaluate sensitivity to pharmacological agents, the 50% effective concentration (EC_{50}) was calculated from each concentration-response curve, with concentration-responses fitted to a sigmoidal curve ($Y=\text{Lower plateau}+(R_{max}\text{-Lower plateau})/(1+10^{(\text{LogEC50-X})})$), where Y is the

percentage relaxation or constriction of the vessel and X is the concentration (Armitage et al., 2005).

The largest response induced by agonists (PE, KCl, ACh and SNP) was considered the maximum response (R_{max}).

Percentage vasoconstriction was calculated as a percentage change in internal diameter relative to the passive resting diameter. Percentage vasorelaxation was calculated as a percentage change in internal diameter, divided by the change in internal diameter before and after PE (10^{-6} M) constriction (Luksha et al., 2011).

3.8.2 Vascular structure investigations: Formulae

3.8.2.1 Media cross sectional area

Subtraction of the internal cross sectional area (CSA) from the external CSA. $MCSA=\pi(D_e^2-D_i^2)/4$, where D_e is the external diameter, and D_i is the internal diameter (Laurant et al., 1997, Souza-Smith et al., 2011, Schiffrin and Hayoz, 1997).

3.8.2.2 Media stress

$\sigma=P\times D/(2\times WT)$, where P is the intraluminal pressure, and D and WT are the internal diameter and media thickness, respectively. Pressure is converted as 1 mm Hg = 1.334×10^3 dyn/cm^2 (Laurant et al., 1997, Souza-Smith et al., 2011).

3.8.2.3 Media strain

$\varepsilon=(D-D_o)/D_o$, where D is the observed lumen diameter for a given intraluminal pressure and D_o is the baseline diameter measured at 3 mm Hg (Laurant et al., 1997, Souza-Smith et al., 2011, Schiffrin and Hayoz, 1997).

3.8.2.4 Elastic modulus

Stress-strain data from each vessel was fitted to an exponential curve: (y = aebx): $\sigma = \sigma_o e^{\beta\varepsilon}$, where σ_o is the stress at the baseline diameter and β is a constant related to the rate of increase

of the stress-strain curve. As a measure of arterial elasticity and stiffness, elastic modulus (ET) was calculated at several values of stress from the derivative of the exponential curve: $ET = d\sigma/d\varepsilon = \beta\sigma_o e^{\beta\varepsilon}$ (Laurant et al., 1997, Souza-Smith et al., 2011, Schiffrin and Hayoz, 1997).

For each of the above parameters (MCSA, media stress, strain or elastic modulus) the result for each individual animal was calculated and then averaged for each group to provide mean ± standard error of mean (SEM) data.

3.8.3 Histology: Stain analysis

3.8.3.1 Shikata's orcein

Analogue images were digitised and separated into 3 coloured images (red, blue and green). Subsequent image processing was performed on the red image only, indicating the elastin component of the mesenteric artery. The images were then binarised to extract relative measures of elastin (%) in the examined field.

3.8.3.2 Martius scarlet blue

MSB stained images were analysed by setting a background threshold, which allowed for visualisation and quantification of the blue staining, indicating the total collagen density (% area). All images were then binarised to extract relative measures of collagen and the smooth muscle cell nuclei in the studied field. Collagen/elastin ratios were calculated from the ratio of collagen density (%) divided by elastin density (%). Nuclei were detected by setting a minimum threshold on the binarised image. Within each field examined, the total number of nuclei and nuclear density (percentage area of field occupied by nuclei) were calculated.

3.8.3.3 Alizarin red

Images were analysed by setting a threshold, which allowed for visualisation and quantification of the orange staining, indicating the total calcification density (% area). All images were then binarised to extract relative measures calcification in the studied field.

3.8.3.4 Von Kossa

Von Kossa stained images were analysed by setting a threshold, which allowed for visualisation and quantification of the dark brown / black staining, indicating the total calcification density (% area). All images were then binarised to extract relative measures of collagen and the smooth muscle cell nuclei in the studied field. For both Alizarin red and VK staining, LPK aortic tissue, which we have previously shown demonstrates calcification (Ameer et al., 2014) was used as a positive control.

3.8.4 Statistical analysis

Data were analysed using IBM Statistical Package for the Social Sciences (v22, SPSS; Chicago, Illinois USA) and GraphPad Prism (GraphPad Prism software v6 Inc., La Jolla, CA, USA). All results are expressed as mean ± SEM. Preliminary analysis of data was performed to identify potential gender effects, using a univariate general linear model against the fixed factors of strain, age and gender. Unless significant, gender effects are not otherwise noted. Data were tested for homogeneity of variance with Levene's test, normality with the Kolmogorov-Smirnov test, and for skewness and kurtosis. If assumptions of normality were met, two-way analysis of variance (ANOVA) was performed followed by Bonferroni *post-hoc* analysis to investigate significant differences between groups (as defined by strain and age). Data that did not meet assumptions of normality were analysed *post-hoc* with a Dunnett's T3 test.

Time-control studies revealed no significant differences for vasoconstriction responses to PE (10^{-6}M) at the beginning and end of the study, for all strains and age groups (P>0.05).

Concentration response curves were evaluated by two–way ANOVA followed by Bonferroni's *post-hoc* analysis. Two-way ANOVA with Bonferroni's *post-hoc* analysis was used to investigate strain, age and pharmacological effects (ACh alone vs. ACh+L-NAME preincubation and ACh+Indo+L-NAME preincubation, and ACh+L-NAME alone vs. ACh+Indo+L-NAME) for the concentration-response curve parameters R_{max} and EC_{50}.

Two-way ANOVA with Bonferroni's *post-hoc* analysis was also used to investigate strain and age effects for percentage component contribution to vasorelaxation (NO; prostanoid; EDH). Significance was defined as $P \leq 0.05$.

Multiple linear regression modelling, with wall/lumen ratio as the dependent variable and SBP, plasma urea and plasma creatinine as the independent variables, was undertaken using a stepwise selection method of entry and pairwise exclusion of missing values. Pearson correlation coefficients were derived from the model, using a one tailed test for significance ($P \leq 0.05$) for grouped data (ages and strains combined).

Supplementary table

Table 3.4: Concentration-response curve 50% effective concentration and maximum response parameters in Lewis and Lewis polycystic kidney second order mesenteric arteries.

Age	6 week		12 week		18 week	
Strain (*n*)	Lewis (8)	LPK (7)	Lewis (13)	LPK (6)	Lewis (7)	LPK (7)
EC_{50}						
PE ($\times10^{-6}$ M)	1.54±0.62	1.23±0.25	1.07±0.23	0.79±0.28	1.10±0.43	0.16±0.01
KCl	0.03±0.00	0.05±0.01	0.05±0.01	0.03±0.00	0.04±0.01	0.04±0.01
ACh ($\times10^{-7}$ M)	2.41±2.06	0.30±0.12	2.93±0.94	10.3±7.85	2.38±0.22	5.62±0.93
SNP ($\times10^{-7}$ M)	3.58±0.76	1.27±0.69	8.12±4.71	2.15±0.98	7.32±1.92	2.59±0.16
ACh+ L-NAME ($\times10^{-7}$ M)	11.7±4.29	27.7±27.6	2.31±0.53	7.49±4.01	3.47±0.67	5.30±1.92
ACh+ Indo+L-NAME ($\times10^{-7}$ M)	10.0±2.73	8.86±5.40	5.33±0.89	7.36±3.64	6.10±1.45 [h]	6.63±1.29
R_{max}						
PE	57.96±5.23	66.96±4.85	73.59±2.51 [a]	75.05±4.33	64.55±5.22	75.32±1.35
KCl	59.10±1.71	62.06±4.37	69.95±3.01 [a]	61.50±4.42	76.75±3.08 [c]	68.19±2.01
ACh	97.58±1.64	97.84±1.67	102.68±1.78	87.36±4.66 [b* d]	96.43±2.53	74.05±2.57 [c* e f]
SNP	105.05±0.85	96.82±3.54	94.40±1.88 [a]	91.05±4.23	96.28±0.68	94.25±3.13
ACh+ L-NAME	80.82±1.84 [g]	79.61±4.56 [g]	83.81±4.27 [g]	77.16±5.58	80.23±2.64 [g]	68.69±3.93
ACh+ Indo+L-NAME	70.04±3.09 [h]	78.78±7.04 [h]	82.62±2.66 [a h]	58.36±4.75 [d h i]	74.88±3.71	53.34±3.63 [f h i]

LPK, Lewis polycystic kidney rat; PE, phenylephrine; KCl, potassium chloride; ACh, acetylcholine; SNP, sodium nitroprusside; L-NAME, N^{ω}-nitro-L-arginine methyl ester; Indo, indomethacin; EC_{50}, effective concentration at 50%; R_{max}, maximum response. All data were evaluated by two-way ANOVA, followed by Bonferroni's *post-hoc* analysis. Results are expressed as mean ± SEM. Significant difference between groups where indicated $P<0.05$.

Within strain age effect: (a) 6 and 12 weeks Lewis; (b) 12 and 18 weeks Lewis; (c) 6 and 18 weeks Lewis; (d) 6 and 12 weeks LPK; (e) 12 and 18 weeks LPK; (f) 6 and 18 weeks LPK. Within ages strain effect: (a*) 6 week Lewis and LPK; (b*) 12 week Lewis and LPK; (c*) 18 week Lewis and LPK. Within strain pharmacological agent effect: (g) between ACh and ACh+L-NAME; (h) between ACh and ACh+Indo+L-NAME; (i) between ACh+L-NAME and ACh+Indo+L-NAME.

4

Chapter 4

Angiotensin II Type 1 receptor antagonism improves structural, functional and biomechanical properties in resistance arteries from a rodent model of chronic kidney disease

Quek, K.J., Ameer, O.Z., Avolio, A.P., Murphy, T.V., & Phillips, J.K.

4.1 Abstract

The renin angiotensin system, in particular Angiotensin II (Ang II), plays a significant role in the development of hypertension in chronic kidney disease (CKD). Effects of chronic Ang II blockade via AT_1 receptor antagonism were investigated in a genetic hypertensive rat model of CKD, the Lewis polycystic kidney (LPK) rat. Mixed-sex LPK and Lewis control rats (total n=31) were split between treated (valsartan 60mg/kg/day p.o. from 4-18 weeks) and vehicle groups. Animals were phenotyped for systolic blood pressure and urine biochemistry. After euthanasia, blood was collected for urea and creatinine analysis, and mesenteric vasculature was collected for pressure myography and histology. Valsartan treatment improved LPK rats vascular structure by increasing internal (untreated vs. treated: 118.25±15.76 vs. 211.43±16.65; P<0.0001) and external diameter values (untreated vs. treated: 224.63±13.93 vs. 279.29±18.62; P=0.0003), and normalising wall thickness (untreated vs. treated: 53.19±3.29 vs. 33.93±2.17; P<0.0001) and wall-lumen ratios (untreated vs. treated: 0.52±0.09 vs. 0.16±0.01; P<0.0001). Endothelium dysfunction as measured by acetylcholine responses was evident in untreated LPK rats, and abolished with treatment. Blocking AT_1-mediated actions in LPK rats improved the nitric oxide -dependent and endothelium-dependent hyperpolarisation vasorelaxation components, and down-regulated the prostanoid vasorelaxation component. Biomechanical properties were also improved with treatment, as indicated by an increase in compliance, decrease in intrinsic stiffness, and alterations in the artery wall composition, which included decreases in collagen density and collagen/elastin ratio. Our results indicate that treatment with valsartan is highly effective in ameliorating impairments seen in the resistance vasculature in a model of CKD, and improving detrimental vascular outcomes.

Keywords

Mesenteric resistance artery, hypertension, chronic kidney disease, endothelium function, vascular stiffness, AT_1 receptor blockade.

4.2 Introduction

Chronic kidney disease (CKD) is strongly linked to an increased risk of mortality due to cardiovascular disease (CVD) (Foley, 2010). In fact, patients with CKD are more likely to die from CVD before progressing to end-stage renal disease (ESRD), with high blood pressure being a key contributing factor to this risk (Keith et al., 2004, McCullough et al., 2008, Gullion et al., 2006, Sarnak et al., 2003). Resistance arteries provide more than 80% of the resistance to blood flow in the body and, as a consequence, play a key contributing role to the development and maintenance of CVD in CKD (Martinez-Lemus et al., 2009). Impaired resistance artery properties (such as altered structure and/or function) are known to play a significant role in persistent increases in total peripheral resistance (Intengan and Schiffrin, 2000), and their impaired function is associated with hypertension in CKD (Wang et al., 2000).

The renin-angiotensin system (RAS) plays an important regulatory role in the development of hypertension and progression of CKD (Cohen and Townsend, 2009, Remuzzi et al., 2005), as evidenced by the effectiveness of RAS inhibitors in controlling blood pressure in renal disease (Martínez-Maldonado, 1998). Angiotensin II (Ang II) in particular plays a detrimental role at the vasculature level, resulting in alterations of the vascular nitric oxide (NO) pathway (Lee et al., 2013), structural vascular remodelling (Bruder-Nascimento et al., 2014), vascular smooth muscle cell (VSMC) hypertrophy, and inflammation (Schiffrin, 2004, Zhang et al., 2005). Angiotensin II is the major effector peptide of the RAS, binding to two receptor subtypes, the AT_1 and AT_2 receptors (Miura et al., 2011). Gender differences of AT_1 and AT_2 receptor gene expression has been shown in rats with essential hypertension, where male rats showed higher ratios of AT_1 to AT_2 receptors (Silva-Antonialli et al., 2004). Irrespective of these differences between genders, in the vasculature the majority of Ang II pressor actions occur via the AT_1 receptors (Carey and Siragy, 2003). In addition to the effects that Ang II mediated hypertension can have on renal function, Ang II also has direct effects on the kidney (Tamura et al., 2015) which can contribute to proteinuria (Ferrari, 2007), and the progressive loss of renal function in CKD (Cohen and Townsend, 2009). Angiotensin II is greatly implicated in the development and progression of structural alterations (vascular remodelling) in small resistance arteries (Touyz, 2005): it has been demonstrated that long-term infusions of non-pressor doses of Ang II are able to induce resistance vessel vascular remodelling,

involving significantly increased media width (vessel wall thickness), media cross sectional area (MCSA), and wall-lumen ratio (Griffin et al., 1991). Furthermore, Ang II via AT_1 receptors may induce remodelling of the arterial wall through smooth muscle growth (Touyz et al., 1999, Gibbons et al., 1992) and collagen deposition (Benetos et al., 1997), as found in resistance arteries in essential hypertensive humans and rats (Aalkjaer et al., 1987, Intengan et al., 1999).

Using the Lewis polycystic kidney (LPK) rodent model of CKD, we have previously established both large and resistance vessel structural and functional abnormalities (Ameer et al., 2014a; Chapter 3; Ng et al., 2011a). The LPK rat model arose from a spontaneous mutation in the *Nek8* gene (McCooke et al., 2012), resulting in a form of nephronophthisis 9 (NPHP9), and has a phenotypic representation resembling that of human autosomal recessive polycystic kidney disease (Phillips et al., 2007). Lewis polycystic kidney rats have established hypertension by 6 weeks of age, demonstrate initial signs of renal dysfunction at 12 weeks of age, and progress to late-stage renal disease by 18–24 weeks of age, concurrent with increased cardiac mass and left ventricular hypertrophy (LVH) (Phillips et al., 2007). Thus, the LPK rat is likely to provide a model system for the natural progression of CKD (Ng et al., 2011a). In parallel with the hypertension and renal dysfunction, we have demonstrated altered resistance artery function at 18 weeks, as indicated by increased sensitivity to phenylephrine (PE) and impaired endothelium-dependent vasorelaxation, and altered structural and biomechanical properties, as indicated by hypertrophic inward remodelling, increased collagen/elastin ratios and increased stiffness (see Chapter 3). Furthermore, previous investigations in LPK aorta have demonstrated that LPK rats also develop large vessel stiffness, calcification, vascular remodelling, and alterations in biomechanical properties (Ng et al., 2011b, Ameer et al., 2014).

The effects of RAS disruption on LPK rat aorta have also been studied, showing that treatment with the angiotensin converting enzyme (ACE) inhibitor, perindopril, results in a reduction in blood pressure and corrects the evidence of abnormal aortic stiffness in the LPK rats (Ng et al., 2011a). Angiotensin converting enzyme inhibitors result in blockade of Ang II production, as opposed to specific blockade of Ang II actions on the AT_1 and AT_2 receptors, and the mechanism of this beneficial effect has not been characterised in detail. In contrast to ACE inhibitors, AT_1 receptor antagonists have opposing effects on bradykinin (BK) receptors

(Wilson et al., 2013), and do not affect Ang II concentrations, nor oppose the binding of Ang II to AT_2 receptors (Taal and Brenner, 2000, Campbell et al., 1994) and Ang-(1-7), which provide beneficial cardiovascular effects (Ruilope et al., 2005, Castro et al., 2005).

Angiotensin II results in effects which include vasoconstriction, blood pressure increase, aldosterone release, cell proliferation, inflammation in the vasculature, and vascular remodelling (Iino et al., 2003, Chang et al., 1995, de Gasparo et al., 2000). Angiotensin II receptor blockers have been shown to prevent stroke in hypertensive patients more successfully than other classes of antihypertensive drugs (Dahlöf et al., 2002, Schrader et al., 2005, Mochizuki et al., 2007), thus suggesting that the preventive outcomes of Ang II receptor blockers are also likely to be partially mediated by blood pressure-independent effects. Inhibiting the RAS via targeting Ang II may be a particularly effective treatment method in the mesenteric resistance arteries, because these arteries have receptors for Ang II (McQueen et al., 1984), and molecules of the Ang II signalling cascade have been shown to be upregulated within the arterial wall during hypertension, and may thus play a causal role in vascular remodelling (Wang et al., 2010).

Given the critical role of resistance vasculature in the determination of high blood pressure in CKD, and the detrimental effects of Ang II, we sought therefore to investigate the role of AT_1 receptors on LPK male and female rats mesenteric artery structure, function and biomechanical properties. Our previous studies showed that LPK rats at 18 weeks reveal the most pronounced mesenteric artery vascular changes (Chapter 3). Hence, chronic treatment with the AT_1 receptor antagonist valsartan was performed from 4 weeks till 18 weeks of age. We hypothesised that valsartan would ameliorate the detrimental structural, functional and biomechanical vascular changes seen in aged LPK rats.

4.3 Methods

4.3.1 Animals

Mixed sex LPK and age-matched control strain Lewis rats were obtained from the Animal Resource Centre in Western Australia, Australia, with a minimum of 3 males or females per group. A total of 31 rats were used for this study, but not all sets of data were collected from each animal. Animals were acclimatised and housed in the animal house facility of Macquarie University under a 12-h light/dark cycle at 20.5°C and 57% humidity, and offered chow and water *ad libitum*. All experimental protocols and procedures were approved by the Animal Ethics Committee of Macquarie University and adhered to the National Health and Medical Research Council of Australia's Australian code of practise for the care and use of animals for scientific purposes (8th Edition 2013).

Animals were trained to voluntarily take condensed milk from a 1 ml syringe, as described previously (Soeters et al., 2008), being the vehicle for treatment administration. Littermate Lewis or LPK rats were then equally allocated between control (vehicle only) and treatment groups. Animals were treated from 4-18 weeks of age with valsartan 60 mg/kg/day (Diovan®, Novartis, Switzerland) once daily, in up to 1 mL condensed milk. Accordingly, vehicle animals were fed once daily with up to 1 mL condensed milk.

4.3.2 Tail cuff plethysmography and urine collection

Systolic blood pressure (SBP) was measured using tail-cuff plesmography (ADInstruments, Sydney, NSW, Australia) as described previously (Phillips et al., 2007). Systolic blood pressure and urine (protein and creatinine) were measured at the 18 week time point, at least 72 hours prior to euthanasia. Urine samples were collected from animals over a period of 24 hours while held individually in metabolic cages, during which animals were offered chow and water *ad libitum*, and their water consumption was measured.

4.3.3 Tissue harvesting and biochemical analyses

Animals were deeply anaesthetised till loss of consciousness with 5% isofluorane (VCA I.S.O., Sydney, NSW, Australia) in 100% O_2 and decapitated. Trunk blood samples were

collected in pre-cooled EDTA containing tubes (BD Microcontainer®, Becton, Dickinson and Company, Sydney, NSW, Australia), and subsequently centrifuged (3000 RPM for 5 min at 4C°). Plasma and urine were analysed for biochemical parameters using an IDEXX VetLab analyser (IDEXX Laboratories Pty Ltd., Rydalmere NSW, Australia). The thorax was dissected open and the mesenteric vasculature removed, followed by the kidneys and heart. The kidneys, heart, and left ventricle were weighed and respective indices (kidney index [KI], heart index [HI] and left ventricle index [LVI]; %) were calculated as heart or kidney weight (g)/ body weight (BW) (g) × 100. The LVI was calculated as left ventricle (g)/ heart weight (g) × 100.

4.3.4 Mesenteric artery isolation

The mesenteric vasculature was isolated and immediately placed in ice-cold Krebs' physiological solution (in mM: NaCl 118.2, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $NaHCO_3$ 25, and EDTA, 0.026), continuously bubbled with carbogen (95% O_2; 5% CO_2; BOC Ltd., North Ryde, NSW, Australia) to achieve a pH value of 7.4-7.45 (Intengan et al., 1999).

After cleaning of adherent connective tissue under a dissecting microscope, a second order mesenteric artery segment (200-400 μm external diameter; ~4 mm in length) was mounted in a pressure myograph (Living Systems Instrumentation, Burlington, VT, USA) in 5 mL of carbogen-bubbled cold Krebs solution. Artery ends were cannulated with glass micropipettes (1 x 0.25 mm glass capillaries, A-M Systems, Inc., Sequim, WA, USA), and secured with 10-0 sutures (Ethilon® Nylon Suture LLC., California, USA). The remaining mesenteric vasculature was placed in 4% formalin for 24 hours, followed by 70% ethanol until further processing for histological investigations.

4.3.5 Pressure myography and histology

Pressure myography protocols for vascular functional and structural investigations were undertaken as described previously in Chapter 3 (sections 3.3.4 and 3.8). Histology protocols are as described previously in Chapter 2 (sections 2.3 and 2.4) and Chapter 3 (section 3.8.3).

4.3.6 Drugs and solutions

Phenylephrine hydrochloride (PE), acetylcholine hydrochloride (ACh), sodium nitroprusside (SNP), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin (indo), histolene, Bouin's fixative, celestine blue, Harris's haematoxylin, Martius yellow, brilliant crystal scarlet, aniline blue, Shikata orcein and Alizarin red were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All drugs and solutions (Krebs' and Ca^{2+}-free Krebs') were prepared on the day of the experiment. All drugs for pressure myography were dissolved in freshly made Krebs' buffer.

4.3.7 Data analysis

Data were analysed using IBM Statistical Package for the Social Sciences (v22, SPSS; Chicago, Illinois USA) and GraphPad Prism (GraphPad Prism software v6 Inc., La Jolla, CA, USA). All results are expressed as mean ± standard error of mean (SEM).

Preliminary analysis of all data was performed to identify potential gender effects, using a univariate general linear model against the fixed factors of strain, age and gender. Unless significant, gender effects are not otherwise noted. Data were tested for homogeneity of variance with Levene's test, normality with the Kolmogorov-Smirnov test, and skewness and kurtosis. If assumptions of normality were met, two-way analysis of variance (ANOVA) was then performed using Bonferroni's *post-hoc* analysis to investigate significant differences between groups (as defined by strain and age). Data that did not meet assumptions of normality were analysed with Dunnett's T3 test.

Urine protein values less than 0.05 g/L were excluded from the data set, and corresponding UPC values were also removed. As no protein was detected in the Lewis urine samples, LPK rats samples (untreated and treated) were compared using a two-tailed unpaired *t*-test ($P<0.05$)

The 50% effective concentration (EC_{50}) and maximum response (R_{max}) were calculated from the concentration-response curves. Concentration-response curves were fitted to a sigmoidal curve ($Y = \text{Lower plateau} + (R_{max}\text{-Lower plateau}) / (1+10^{(\text{LogEC50-X})})$) to calculate EC_{50} values, where Y is the percentage relaxation or constriction of the vessel and X is the concentration

(Armitage et al., 2005). After testing for the normal distribution of data and gender effects as detailed above, two-way ANOVA with Bonferroni *post-hoc* analysis was used to investigate strain, treatment and pharmacological effects (ACh alone vs. ACh+L-NAME preincubation and ACh+Indo+L-NAME preincubation, and ACh+L-NAME alone vs. ACh+Indo+L-NAME) for concentration-response curve parameters (R_{max}, EC_{50}), in Lewis and LPK rats. Two-way ANOVA with Bonferroni's *post-hoc* analysis was also used to investigate strain and treatment effects for percentage component contribution to vasorelaxation (NO; prostanoid; endothelium-derived hyperpolarisation [EDH]). Significance was defined as $P \leq 0.05$.

4.4 Results

4.4.1 Baseline parameters

Lewis polycystic kidney rats exhibited smaller body weights than Lewis (Table 4.1), with male animals weighing more than females in Lewis strains (untreated Lewis males vs. females: 413.25±8.02 g vs. 241.25±8.27 g, $P<0.001$; treated Lewis males vs. females: 432.00±9.35 g vs. 245.25±6.93 g, $P<0.001$), irrespective of treatment group. At 18 weeks of age, SBP was elevated in the LPK rats relative to Lewis and there was a significant treatment effect, with valsartan reducing blood pressure in the LPK rats. However, SBP measurements were not normalised to Lewis values. Regardless of treatment group, HI, LVI and KI were elevated in the LPK rats relative to Lewis. In addition, there was a treatment effect, where treated LPK had lower KI values than untreated LPK rats. Plasma analyses revealed significantly higher urea and creatinine values in untreated and treated LPK rats. Urine analysis revealed lower urine creatinine values in LPK relative to Lewis, regardless of treatment group. Negligible protein was detected in the urine of Lewis animals. Urine protein was elevated in both LPK groups, and while there was a trend towards a reduction after treatment ($P=0.0647$), this was not statistically significant, as reflected in the UPC analysis, which was elevated and overall influenced by treatment in the LPK animals. Water intake was increased in untreated LPK compared to untreated Lewis, and valsartan treatment reduced water intake in LPK.

4.4.2 Altered resistance vessel morphology in Lewis polycystic kidney rats

Mesenteric arteries from untreated LPK demonstrated evidence of geometrical remodelling, with significantly smaller internal and external diameter values, increased wall thickness and wall-lumen ratio compared to untreated Lewis (Table 4.2). Treatment did not alter structural parameters in the Lewis animals, however in the LPK, valsartan increased internal diameter and reduced both wall thickness and wall-lumen ratio, such that these two latter parameters were not significantly different to treated Lewis animals. There was no strain or treatment effect noted for MCSA. Gender effects revealed that untreated LPK males had significantly smaller wall thickness values than corresponding females (males vs. females: 44.63±2.39 μm vs. 61.75±0.50 μm, $P=0.043$).

4.4.3 Vascular function investigations

4.4.3.1 Constriction

The untreated LPK PE concentration-response curves were significantly shifted to the left relative to untreated Lewis and treated LPK (Figure 4.1A). This increase in potency of PE was reflected as a lower EC_{50} value in untreated LPK relative to untreated Lewis (Table 4.3). The untreated LPK KCl concentration-response curve was also significantly left-shifted relative to treated LPK (Figure 4.1B); however, this was not accompanied by any alterations in concentration-response curve parameters.

4.4.3.2 Endothelium-dependent and independent vasorelaxation

The untreated LPK had impaired relaxation: the endothelium-dependent ACh concentration-response curve was significantly shifted to the right relative to untreated Lewis and treated LPK (Figure 4.2A). In accordance with these results, untreated LPK had significantly smaller ACh R_{max} values than untreated Lewis and treated LPK (Table 4.3). Overall, valsartan treatment normalised ACh-dependent vasorelaxation in LPKs.

The endothelium-independent SNP concentration-response curve in untreated LPK was significantly shifted to the right relative to untreated Lewis (Figure 4.2B). Following valsartan treatment, however, there were no significant differences between Lewis and LPK. Any shifts in SNP concentration-response curves however were not accompanied by alterations in concentration-response curve parameters (Table 4.3).

4.4.3.3 Vasorelaxation mechanism investigations

Valsartan treatment in LPK resulted in vasorelaxation responses comparable to Lewis. Relative to control ACh responses, preincubation with the nitric oxide synthase (NOS) inhibitor L-NAME reduced the ACh R_{max} in untreated and treated Lewis, and treated LPK (Table 4.3). Preincubation with a combination of the cyclooxygenase inhibitor indomethacin and L-NAME (Indo+L-NAME) resulted in a reduction in ACh R_{max} values relative to the ACh response for all treatment groups and strains. The ACh R_{max} following preincubation

with Indo+L-NAME was significantly smaller in untreated LPK than the ACh R_{max} after preincubation with L-NAME alone (Table 4.3). No significant changes in EC_{50} were found.

Untreated LPK had significantly smaller ACh R_{max} values after preincubation with L-NAME, relative to untreated Lewis and treated LPK, respectively. In addition, untreated LPK had significantly smaller ACh R_{max} values after preincubation with indo+L-NAME, relative to untreated Lewis. No significant changes in EC_{50} were found.

4.4.3.4 Vasorelaxation component investigations

When the relative contribution of each vasorelaxation pathway was assessed, the percentage contribution to ACh R_{max} by NO was increased in treated LPK, relative to untreated LPK, while the percentage contribution to ACh R_{max} by prostanoids was reduced, such that it was no longer significantly different to the Lewis animals (treated or untreated). The percentage contribution to ACh R_{max} by EDH was less in untreated LPK relative to untreated Lewis controls, but this difference was not evident in the treated animals.

4.4.4 Altered resistance artery stiffness in Lewis polycystic kidney rats

Regardless of treatment group, LPK pressure diameter curves were lower relative to Lewis controls. After treatment, the LPK pressure diameter curve was significantly higher relative to untreated LPK (Figure 4.4).

The stress-strain curves and the corresponding slope of elastic modulus vs. stress calculations for untreated LPK were significantly shifted to the left and had larger slope of elastic modulus vs. media stress values, respectively, relative to untreated Lewis controls (Figure 4.5 A and B). Treatment shifted Lewis and LPK stress-strain curves to the right, relative to their corresponding untreated matches. This was not accompanied by significant changes in slope of elastic modulus vs. media stress values, however the slope in treated LPK was no longer significantly different to treated Lewis controls.

4.4.5 Altered resistance vessel composition in Lewis polycystic kidney rats

Representative images of Lewis and LPK images are provided in Figure 4.6. Histological analysis indicated that treatment significantly reduced collagen density in the LPK animals such that it was no longer greater than Lewis control animals (Table 4.4). In addition, collagen/elastin ratios were normalised by treatment in the LPK. A gender effect was noted, with untreated male LPK having significantly higher collagen/elastin ratio values than untreated female LPK (males vs. females: 18.57±1.21 vs. 3.12±1.03, P=0.028). No significant difference in nuclear density, number of nuclei per μm^2, elastin density and calcium density were found between strains or treatment groups.

4.5 Discussion

The alterations of resistance vessels due to Ang II is associated with hypertension in a number of disease states, and is likely to play a key role in the development of target organ damage. In this study we report for the first time the effects of AT_1 receptor antagonism on resistance artery structure, function and biomechanical properties in a model of CKD. Chronic treatment with an AT_1 receptor antagonist improved detrimental vascular remodelling, vascular amplification, endothelium dysfunction and stiffness in LPK.

Chronic treatment with valsartan resulted in significant antihypertensive effects in LPK, supporting previous research on the effectiveness of RAS inhibitors in blood pressure control and use in the treatment of hypertension, and importantly, the key role of Ang II as a primary contributor to pathologies seen in CVD and CKD (Chapman et al., 1990, Baltatzi et al., 2011). An increase in Ang II levels results in an increased risk of CVD in CKD through a variety of mechanisms. One such mechanism is LVH, which is a well-established finding in hypertensive CKD patients (Schiffrin et al., 2007). Left ventricular hypertrophy is a significant predictor of sudden cardiac death, and is important clinically because its regression with treatment has been shown to improve survival outcomes (Saravanan and Davidson, 2010). In the present study, AT_1 receptor antagonism did not improve cardiac hypertrophy, despite Ang II receptor blockade being shown to reduce heart workload, fibrosis, or progression of LVH in patients with nonobstructive hypertrophic cardiomyopathy (Shimada et al., 2013). However, in accordance with previous research (Jiang et al., 2015), valsartan treatment decreased water intake in LPK. This effect may be due to valsartan's ability to intracerebroventricularly affect Ang II levels in rats, and therefore mediate polydipsia centrally (Braszko et al., 2005).

In addition to antihypertensive outcomes, another mechanism through which valsartan can exert its effectiveness is through its renal anti-proteinuric actions (Lee et al., 2011). In the present study, although systemic AT_1 receptor antagonism improved hypertension, no obvious renoprotective effects were found, although there was a trend towards decreased urine protein levels in treated LPK. Valsartan's ability to correct blood pressure and improve water intake as well as polydipsia is of clinical importance, because proteinuria is a major predictor of developing ESRD (Plum et al., 1998), and also its progression (Gu et al., 2012).

A key finding of this study was that treatment with an AT_1 receptor antagonist significantly improved the degree of structural vascular remodelling seen in LPK. In the present study, untreated LPKs exhibited eutrophic inward remodelling[1], as indicated by an increase in arterial wall thickness, decrease in lumen diameter, and unchanged MCSA (Intengan et al., 1999). Vascular remodelling was not associated with a change in the number of nuclei or nuclear density, indicating no hyperplasia. Treatment improved vascular structure, as indicated by larger internal and external diameter values relative to untreated LPK, however these values were still reduced, relative to untreated and treated Lewis resistance vessels. Nonetheless, overall, treatment was effective in improving vascular remodelling outcomes, resulting in a decrease in wall thickness and wall-lumen ratio values in LPK, to levels that were comparable with Lewis. This outcome of improved wall-lumen ratio values in LPK is of particular importance, as increased wall-lumen ratio is believed to contribute to the maintenance of hypertension (Mulvany, 2002). Alongside its' actions as a potent vasoconstrictor, Ang II has the ability to directly induce cell growth, in part via formation of reactive oxygen species (ROS). Angiotensin II can also activate inflammatory mechanisms contributing to vascular remodelling (Luft, 2001), and is implicated in the development and progression of structural alterations (vascular remodelling) in small resistance arteries (Touyz, 2005). Given its blood pressure-lowering effectiveness in LPK, valsartan's ability to improve resistance vessel structure and correct remodelling in LPK is likely to be due to a combination of the blood pressure-lowering effect of valsartan, as well as the blockade of AT_1 receptors, which therefore prevents Ang II's cell proliferating and inflammatory effects.

Blockade of the AT_1 receptor improved vascular compliance and decreased stiffness and matrix deposition in the artery wall. Untreated LPK exhibited increased stiffness relative to age-matched Lewis, as shown by the significant leftward shift of the stress-strain curve and less steep pressure-diameter curve gradient. In accordance with these findings, slope of elastic

[1] These findings contrast with the hypertrophic inward remodelling findings in 18 week LPKs in Chapters 3 and 6. This discrepancy is discussed in the final discussion (Chapter 7).

modulus vs. media stress values, an indicator of intrinsic stiffness independent of vessel geometry (Behbahani et al., 2010), was increased in untreated LPK, and collagen density and collagen/elastin ratio was also increased. Treatment with valsartan improved, although did not normalise, compliance in the LPK rats. Specifically, AT_1 receptor antagonism seemed to improve intrinsic stiffness, as indicated by LPK elastic modulus vs. stress slope values and collagen and collagen/elastin ratio values becoming comparable with Lewis controls. The effectiveness of valsartan in this regard may be due to the inhibition of Ang II, which has been shown to act via AT_1 receptors to induce remodelling of the arterial wall through smooth muscle growth (Touyz et al., 1999, Gibbons et al., 1992) and collagen deposition (Benetos et al., 1997). Also important to note is that in addition to hypertension, renal disease itself may also exert detrimental effects on collagen and elastin functionality, which can thus lead to the loss of fibre flexibility (Luksha et al., 2011), and increase stiffness even further.

Other investigations of the composition of the arterial wall investigated elastin and calcification. Consistent with our previous findings (Chapter 3), no alterations in elastin and calcification were found in LPK. The absence of calcification in both strains and age groups is in contrast to what we and others have previously described in large vessels in association with age and CKD (Ng et al., 2011b), as well as in breast arterioles of women with CKD and ESRD (O'Neill and Adams, 2013). However, no investigations of resistance artery calcification have been reported in the LPK. Given that elastin degradation is strongly associated with vascular calcification and is one of the main mechanisms of calcification (Pai et al., 2011), and that mesenteric arteries have a very small percentage of elastin (an average of ~10% in Lewis rats), mesenteric arteries therefore have a reduced likelihood of calcifying, relative to, for example, the thoracic and abdominal aorta, which is comprised of approximately 70% and 65% of elastin, respectively (Ameer et al., 2014b).

In addition to structure and biomechanics, AT_1 receptor antagonism improved functional alterations noted in the LPK. Consistent with Chapter 3, evidence for a vascular amplifier effect was apparent in the untreated LPK resistance vessels, which suggests increased sensitivity to PE, relative to Lewis controls. This was found to be specific to the α_1-adrenergic receptor agonist, as similar results were not found for concentration-response curves to a receptor-independent agent. Although no significant differences were found between untreated Lewis and LPK for responses to a receptor-independent agent (potassium chloride),

following valsartan administration, LPK were less sensitive to potassium chloride. The underlying mechanism contributing to this finding is unknown, and could possibly related to changes in potassium channel expressions in the mesenteric resistance artery following treatment. Further studies are certainly warranted to understand this finding. In addition to treatment affecting potassium chloride sensitivity, valsartan also ameliorated the enhanced constrictor effect to PE, returning to a response comparable to Lewis.

Dysfunction of the endothelium is often associated with hypertension, and may be a consequence of blood pressure elevation (Schiffrin, 2012), as well as a factor that increases susceptibility to hypertension (Rossi et al., 2004). The present study found impaired ACh-induced endothelium-dependent relaxation in untreated LPK resistance vessels, which is in accordance with previous studies investigating resistance arteries in renovascular hypertensive and autosomal dominant polycystic kidney disease (ADPKD) patients (Porteri et al., 2003, Wang et al., 2003). In addition, consistent with Chapter 3 and previous research, the present study found that EDH contributed to ~70% of relaxation (Luksha et al., 2012), NO to 30% of relaxation (Paulis et al., 2010), and prostanoids a minimal role (Shimokawa et al., 1996) in Lewis controls, irrespective of treatment. When deficiency of a vasorelaxation mechanism occurs, such as in the case of hypertension, the endothelium may upregulate other mechanisms as compensatory physiological pathways (Versari et al., 2009). In the present study, incubation with a NOS inhibitor significantly attenuated ACh-induced relaxation in the Lewis (regardless of treatment group) and treated LPK, but not in the untreated LPK, as indicated by smaller ACh R_{max} values following preincubation with L-NAME. The NOS inhibitor's ability to attenuate relaxation in the Lewis and treated LPK indicates functional NOS activity, while its inability to affect ACh-mediated relaxation in the untreated LPK suggests defective NOS activity (Wang et al., 2003), or perhaps reduced bioavailability of NO through scavenging by ROS, given the impaired endothelium-independent vasorelaxation in LPK. Further investigations of the vasorelaxation mechanisms revealed significant strain effects for prostanoid and EDH: untreated LPK prostanoid components were up regulated relative to Lewis controls, and untreated LPK EDH components were down regulated compared to Lewis cohorts. Treatment with an AT_1 receptor blocker resulted in an increased relative contribution of NO to ACh R_{max}, as well as improved EDH response and corrected prostanoid components. Valsartan's ability to improve these vasorelaxation components may

be related to Ang II's role in contributing to impaired EDH-mediated vasorelaxation (Goto et al., 2004), or its upregulating effects on prostaglandin production in various tissues (Gohlke et al., 1996). Nonetheless, treatment with an AT_1 receptor blocker was effective in improving endothelium function in the LPK. Angiotensin II has vascular inflammatory effects, involving increased vascular permeability, which may be due to endothelium layer damage as a result of blood pressure changes (Cheng et al., 2005). Therefore, AT_1 receptor blocker treatment's effectiveness may be exerted through the inhibition of detrimental Ang II effects on endothelium function. Chronic valsartan treatment could increase availability of inactive versus active endothelium NOS (eNOS), since the activity levels of eNOS have been shown to be impaired in a cystic kidney model (Peterson et al., 2013); or, due to valsartan's blood pressure-lowering effect, which reduces stress against the arterial wall, its therapeutic outcomes may be due to its ability to improve endothelium integrity. Alternatively, the direct antagonism of AT_1 receptors can lead to increased availability of NO as a result of either stimulation of NO through AT_1 receptor antagonism mechanisms (Gohlke et al., 1996), or the decreased availability of superoxide anions (López et al., 2003).

In conclusion, our data show that in a rat model of CKD, AT_1 receptor antagonism has antihypertensive effects, reduces water intake, and improves altered mesenteric artery structural, functional and biomechanical properties. The exact mechanisms by which valsartan treatment acts to exert its effectiveness remains to be determined. Valsartan may directly improve responses by blocking those actions of Ang II which promote vascular injury (Shan et al., 2008), or alternatively, it may be the blood pressure lowering effects of valsartan which result in favourable outcomes, due to decreased wall stress, and therefore improved endothelium function (Mizuno et al., 2008). Hence, further investigations (Chapter 5) into the effects of blood pressure lowering on the vasculature via a non-Ang II mechanism were performed. The present investigation into the effects of blocking Ang II actions by AT_1 receptor antagonism highlight the importance of Ang II in the contribution to resistance artery structural, functional and biomechanical properties, and provide an explanation as to how angiotensin receptor blockers exert their therapeutic effectiveness in CKD.

4.6 Acknowledgements

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4.7 Conflict of interest

None declared.

Tables

Table 4.1: Baseline morphometric and biochemical parameters.

	Untreated		Treated	
Parameter	Lewis	LPK	Lewis	LPK
n	8	5	8	7
BW (g)	327±33	183±7[a]	339±36	190±8[b]
SBP (mmHg)	118±2	200±8[a]	108±1	154±3[b d]
HI (%)	0.34±0.01	0.63±0.13[a]	0.30±0.01	0.68±0.04[b]
LVI (%)	67.42±0.57	77.53±2.07[a]	63.25±1.48	75.39±2.47[b]
KI (%)	0.80±0.01	7.00±0.70[a]	0.77±0.01	5.49±0.42[b d]
Plasma urea (mmol/L)	6.13±0.19	44.72±1.68[a]	6.01±0.27	45.76±0.64[b]
Plasma creatinine (µmol/L)	24.25±4.12	288.40±95.91[a]	19.13±2.41	327.29±38.05[b]
Urine protein (g/L)	nd	1.89±0.66	nd	0.73±0.28
Urine creatinine (g/L)	2.57±0.59	0.24±0.05[a]	3.50±0.00	0.28±0.08[b]
UPC	nd	7.84±2.90	nd	4.61±1.87
Water intake (mL)	10.64±2.69	31.60±3.60[a]	17.10±4.04	17.03±3.27[d]

BW, body weight; SBP, systolic blood pressure; HI, heart index; LVI, left ventricle index; KI, kidney index; nd, not determined; UPC, urine protein creatinine ratio; LPK, Lewis polycystic kidney rat. Apart from urine protein and UPCs, data that met parametric assumptions were followed by Bonferroni's *post-hoc* analysis, or Dunnett's T3 test if assumptions were violated. Urine protein and UPC were evaluated by unpaired t-test. Results are expressed as mean ± SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

Table 4.2: Mesenteric artery structural parameters of untreated and valsartan treated 18 week old Lewis and Lewis polycystic kidney rats.

	Untreated		Treated	
Parameter	Lewis	LPK	Lewis	LPK
n	8	8	6	7
Internal diameter (μm)	270.50±9.78	118.25±15.76[a]	294.33±29.38	211.43±16.65[b d]
External diameter (μm)	324.63±8.66	224.63±13.93[a]	352.17±26.94	279.29±18.62[b]
Wall thickness (μm)	27.06±1.38	53.19±3.29[a]	28.92±2.97	33.93±2.17[d]
Wall-lumen ratio	0.10±0.01	0.52±0.09[a]	0.11±0.02	0.16±0.01[d]
MCSA ($x10^3$) (μm^2)	25.19±1.22	28.35±2.51	28.83±3.05	26.48±2.79

MCSA, media cross sectional area; LPK, Lewis polycystic kidney rat. Second order mesenteric artery structural parameter data was obtained at 60 mmHg following Ca^{2+}-free superfusion. Data that met parametric assumptions were followed by Bonferroni's *post-hoc* analysis, or Dunnett's T3 test if assumptions were violated. Results are expressed as mean ± SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

Table 4.3: Concentration-response curve parameters for untreated and valsartan treated 18 week old Lewis and Lewis polycystic kidney rats.

	Untreated		Treated	
(n)	Lewis (8)	LPK (8)	Lewis (8)	LPK (7)
EC_{50} (M)				
PE ($x10^{-7}$ M)	13.80±4.80	1.39±0.18[a]	6.66±1.74	10.33±3.76
KCl ($x10^{-3}$ M)	37.86±4.12	24.70±2.71	32.69±3.73	37.48±4.94
ACh ($x10^{-7}$ M)	4.16±1.97	6.95±1.19	4.59±2.22	3.99±0.81
SNP ($x10^{-8}$ M)	12.08±4.66	4.90±0.89	6.05±1.60	10.47±2.05
L-NAME ($x10^{-7}$ M)	3.86±0.40	4.32±1.39	5.29±1.09	5.28±0.83
Indo+L-NAME ($x10^{-7}$ M)	6.36±2.22	8.88±4.27	11.66±9.19	5.46±1.06
R_{max}				
PE	64.45±6.34	66.92±4.89	55.35±6.73	68.71±4.07
KCl	72.28±2.45	69.06±2.49	62.09±5.69	65.15±5.24
ACh	98.49±1.89	69.56±4.34[a]	97.26±4.54	103.05±4.13[d]
SNP	92.02±3.16	93.67±4.43	96.99±2.78	95.37±1.66
L-NAME	82.84±2.00[e]	68.89±4.67[a]	86.89±2.40[e]	85.75±3.10[d e]
Indo+L-NAME	79.52±3.80[f]	48.22±3.12[a f g]	88.70±3.10[f]	80.56±3.56[d f]

LPK, Lewis polycystic kidney rat; PE, phenylephrine; KCl, potassium chloride; ACh, acetylcholine; SNP, sodium nitroprusside; L-NAME, N^{ω}-nitro-L-arginine methyl ester; Indo, indomethacin; EC_{50}, effective concentration at 50%; R_{max}, maximum response. Data that met parametric assumptions were followed by Bonferroni's *post-hoc* analysis, or Dunnett's T3 test if assumptions were violated. Results are expressed as mean ± SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK. Within strain

pharmacological agent effect: (e) between ACh and ACh+L-NAME; (f) between ACh and ACh+Indo+L-NAME; (g) between ACh+L-NAME and ACh+Indo+L-NAME.

Table 4.4: Histological parameters of untreated and valsartan treated 18 week old Lewis and Lewis polycystic kidney rats.

	Untreated		Treated	
Parameter	Lewis	LPK	Lewis	LPK
n	6	8	7	6
Collagen density (%)	18.0±1.3	33.6±4.7[a]	13.4±1.1	21.9±3.5[d]
Nuclear density (%)	2.8±0.4	3.4±0.2	3.4±0.4	4.2±0.3
Number of nuclei per μm^2	0.19±0.01	0.16±0.03	0.20±0.01	0.19±0.02
Elastin density (%)	8.6±2.3	6.8±2.5	7.4±2.4	6.8±1.8
Collagen/elastin ratio	2.6±0.4	9.7±3.2[a]	2.7±0.5	4.0±0.9
Calcium density (%)	0.01±0.01	0.06±0.03	0.01±0.01	0.00±0.00

LPK, Lewis polycystic kidney rat. Collagen density, nuclear density and number of nuclei per um^2 were quantified using Martius scarlet blue (MSB) staining, while elastin density was quantified using Shikata's orcein (SO). Data were evaluated by two-way ANOVA, followed by Bonferonni's *post-hoc* analysis. Results are expressed as mean ± SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

Figures

Figure 4.1: The effect of valsartan on vasoconstriction curves to phenylephrine and potassium chloride in Lewis and Lewis polycystic kidney rat mesenteric artery.

Untreated and valsartan treated 18 week old Lewis and Lewis polycystic kidney rat (LPK) mesenteric artery contraction responses (%) to cumulative additions of the α_1-adrenergic receptor agonist PE (A) and direct depolariser KCl (B). Results are expressed as mean ± SEM. Data were evaluated by two–way ANOVA followed by Bonferroni's *post-hoc* analysis. Significant differences between groups overall concentration-response curves where indicated $P<0.05$. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

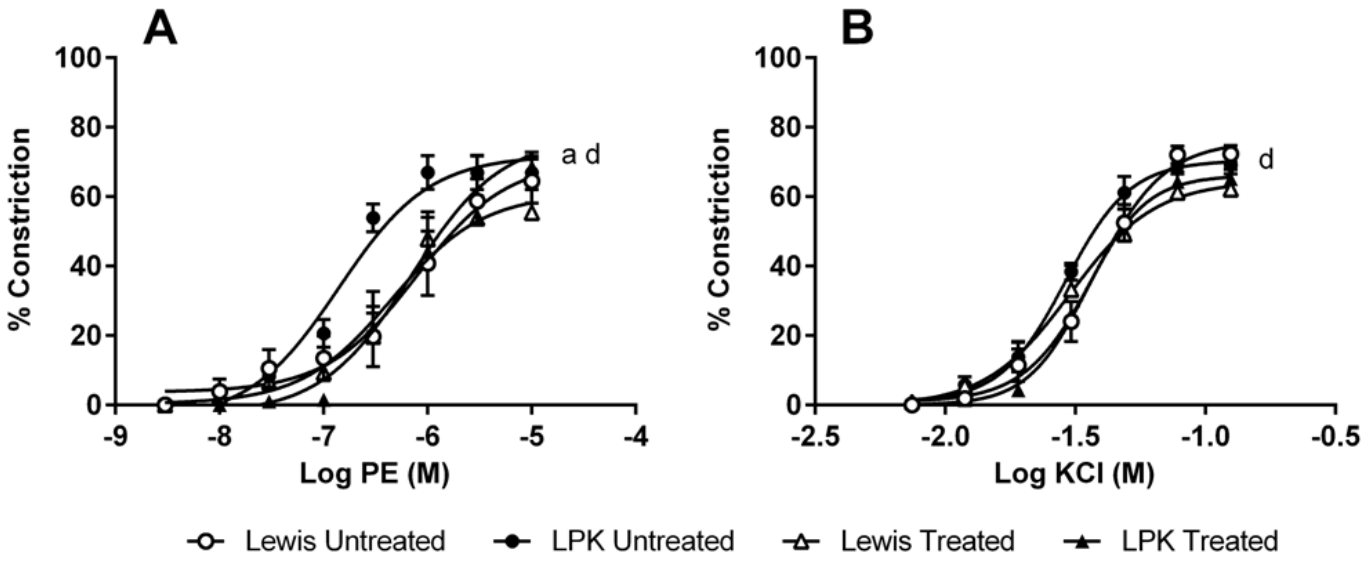


Figure 4.2: The effect of valsartan on vasorelaxation curves to acetylcholine and sodium nitroprusside in Lewis and Lewis polycystic kidney rat mesenteric artery.

Untreated and valsartan treated 18 week old Lewis and Lewis polycystic kidney rat (LPK) second order mesenteric artery relaxation responses (%) to cumulative additions of endothelium-dependent and –independent vasodilators, (A) acetylcholine (ACh) and (B) sodium nitroprusside (SNP), respectively, following preconstriction with 1μM phenylephrine (PE). Results are expressed as mean ± SEM. Significant difference between groups where indicated $P<0.05$. Data were evaluated by two–way ANOVA followed by Bonferroni's *post-hoc* analysis. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

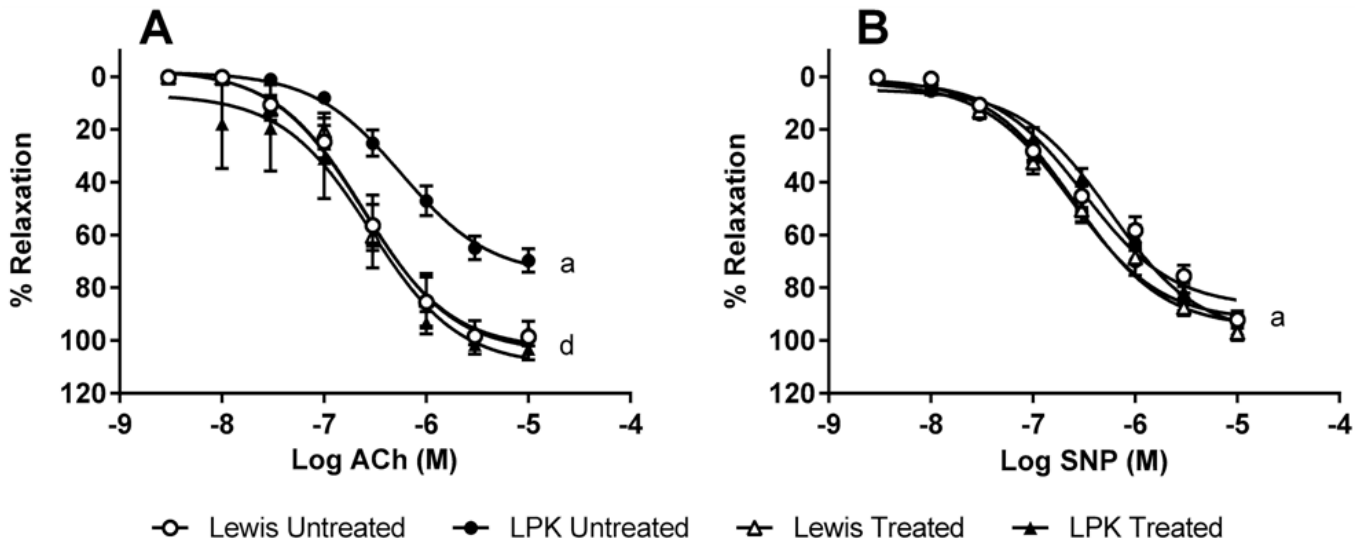


Figure 4.3: The effect of valsartan on the relative contribution of vasodilatory mediators to acetylcholine responses in Lewis and Lewis polycystic kidney rat mesenteric artery.

Contribution of each vasodilatory component (nitric oxide, NO; prostanoid and endothelium-derived hyperpolarisation, EDH) to acetylcholine (ACh) -induced relaxation in untreated and treated Lewis and Lewis polycystic kidney rat (LPK) mesenteric artery. Data were evaluated by two-way ANOVA. Results are expressed as mean ± SEM. Significant difference between groups where indicated $P<0.05$. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

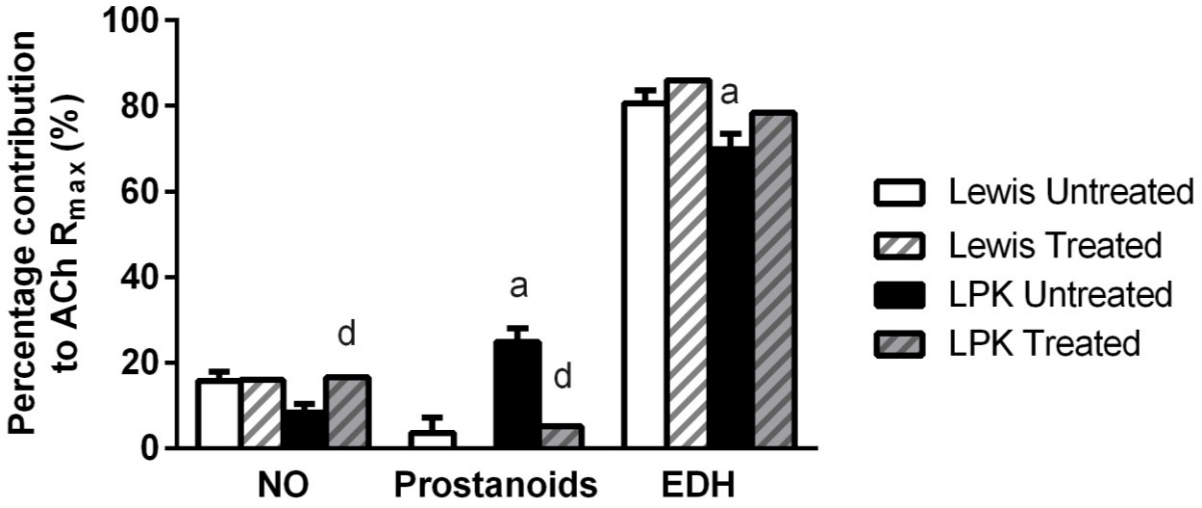


Figure 4.4: The effect of valsartan on pressure-diameter curves in Lewis and Lewis polycystic kidney rat mesenteric artery.

Passive pressure curves in untreated and treated 18 week Lewis and Lewis polycystic kidney rat (LPK) second order mesenteric arteries. Curves represent diameter passive pressure (diameter, μm) in response to changing pressure (mmHg) in Ca^{2+}-free Krebs' solution. Overall curve comparisons were evaluated by two-way ANOVA, followed by Bonferroni's *post-hoc* analysis. Results are expressed as mean ± SEM. Significant difference between groups where indicated $P<0.05$. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

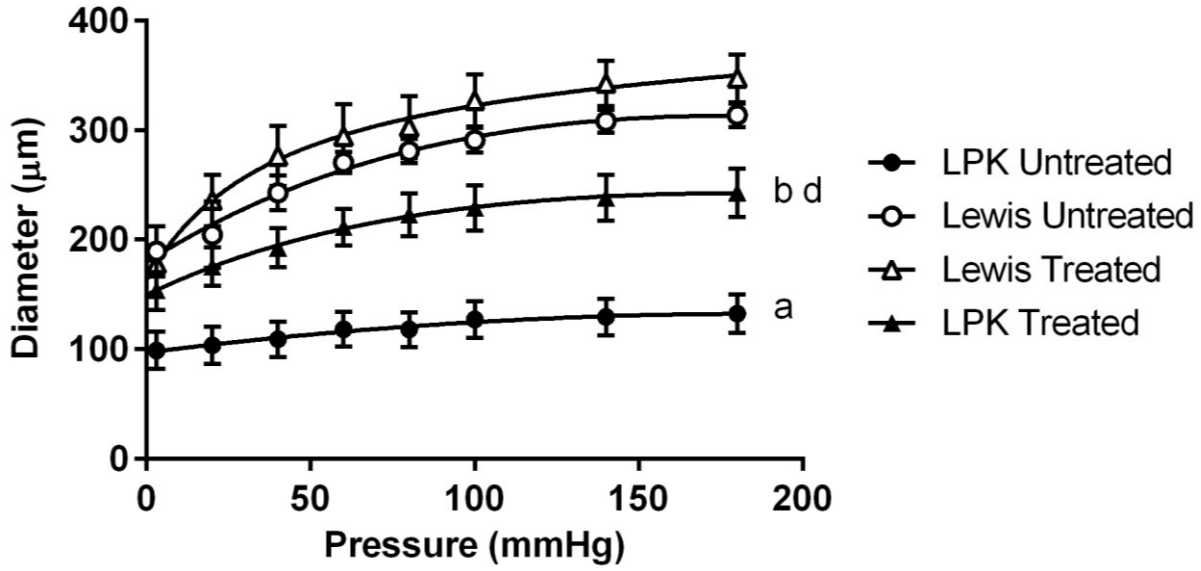


Figure 4.5: The effect of valsartan on stress-strain curves in Lewis and Lewis polycystic kidney rat mesenteric artery.

Stress-strain data fitted to an exponential curve for untreated and treated 18 week old Lewis and Lewis polycystic kidney rat (LPK) second order mesenteric artery (A). Slope of elastic modulus vs. stress, calculated from stress-strain curve data (B). Data were evaluated by two-way ANOVA, followed by Bonferroni's *post-hoc* analysis. Results are expressed as mean ± SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

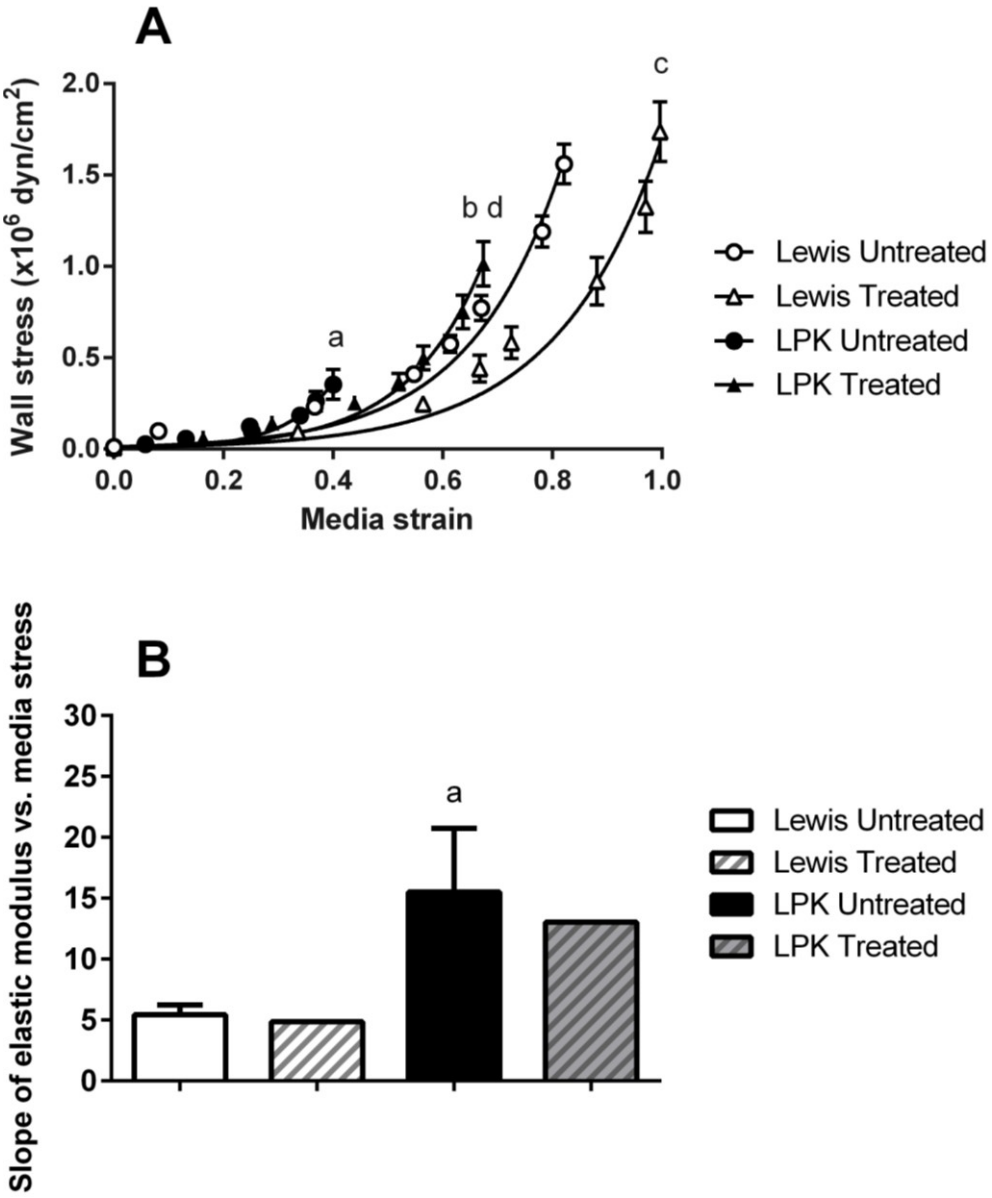
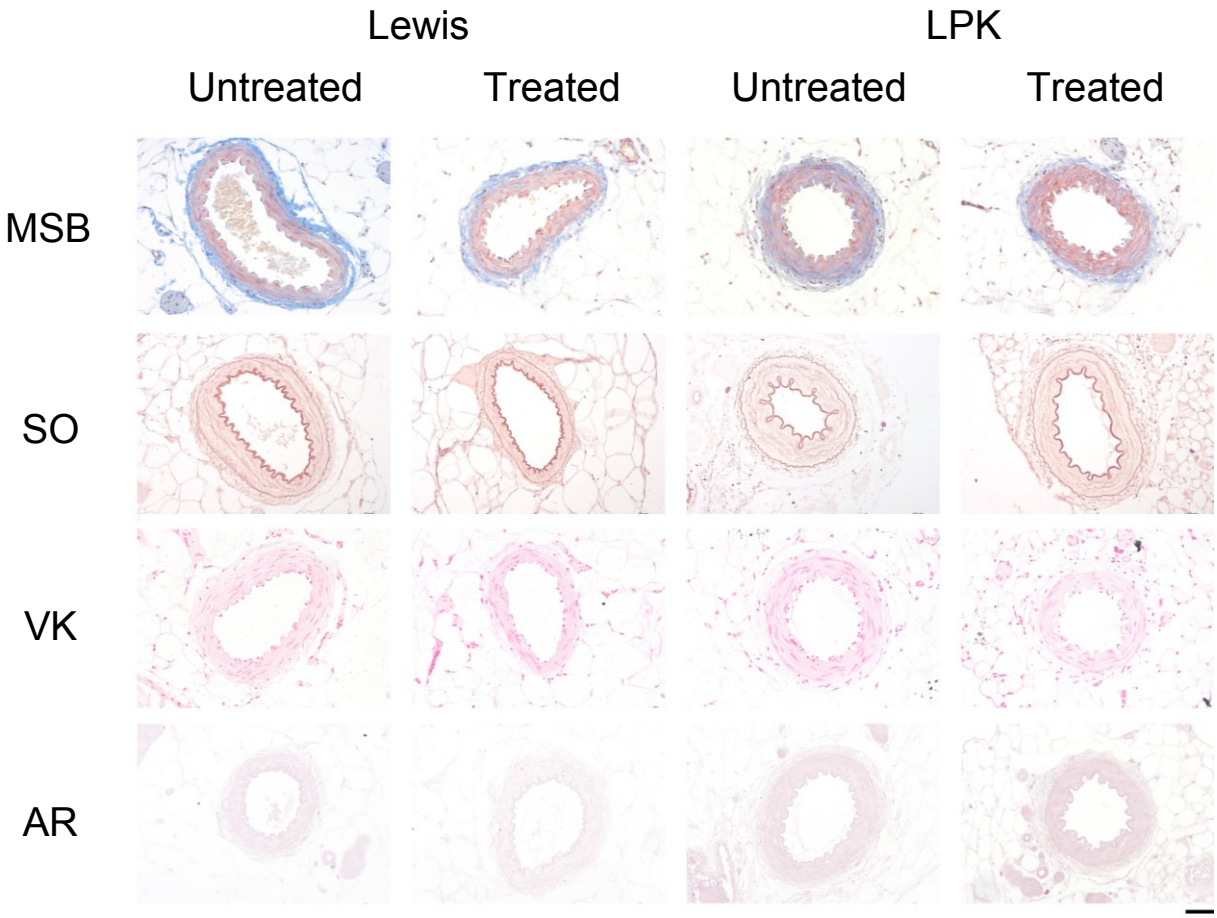


Figure 4.6: The effect of valsartan on vascular composition in Lewis and Lewis polycystic kidney rat mesenteric artery.

Representative untreated and treated 18 week old Lewis (left panels) and Lewis polycystic kidney rat (LPK) (right panels) second order mesenteric artery wall, stained with Martius scarlet blue (MSB; top row), Shikata's orcein (SO; second row), Von Kossa (VK; third row), and Alizarin red (AR; bottom row) showing the collagen component in blue (MSB), the elastin in red/brown (SO), and absence of calcification as indicated by no dark brown/black deposits (VK) and no orange staining (AR). Scale bar is 50 μm.



5

Chapter 5

Calcium channel blockade alters resistance artery structure and function in a rodent model of chronic kidney disease

Quek, K.J., Avolio, A.P., Murphy, T.V., & Phillips, J.K.

5.1 Abstract

Hypertension is a common comorbidity associated with chronic kidney disease (CKD), and anti-hypertensive therapy in patients often involves the inclusion of L-type Ca^{2+} channel (LTCC) blockers. The effect of chronic treatment with such a compound on resistance vascular structure and function was investigated in a genetic hypertensive rat model of CKD, the Lewis polycystic kidney (LPK) rat. Mixed sex LPK and Lewis control rats (total n=38) were split between treated (amlodipine 20mg/kg/day p.o. from 4-18 weeks) and vehicle groups. Animals were phenotyped for systolic blood pressure and renal function. After euthanasia, the mesenteric vasculature was collected for pressure myography and histology. Treatment with amlodipine reduced LPK rat blood pressure (untreated vs. treated: 185±5 vs. 165±9 mmHg; P=0.019) and improved renal function (plasma creatinine: untreated vs. treated: 197±17 vs. 140±16 μmol/L; P=0.002). The mesenteric artery demonstrated increased internal (Lewis vs. LPK: 270±21 vs. 118±19 μm; P<0.0001) and external diameters (Lewis vs. LPK: 327±18 vs. 200±19 μm; P=0.0001) and wall-lumen ratios (Lewis vs. LPK: 0.12±0.02 vs. 0.51±0.15; P=0.0001) in the untreated LPK. Endothelium dysfunction, as determined by vasorelaxation to acetylcholine, was evident in untreated LPK, and corrected with treatment. Blocking LTCCs in LPK improved the nitric oxide-dependent and endothelium-derived hyperpolarisation-dependent vasorelaxation components, and down regulated the prostanoid vasorelaxation component. Biomechanical properties of compliance and intrinsic stiffness were unaltered after treatment in LPK, and no change was seen in artery wall composition. Our results indicate that blockade of LTCCs with amlodipine is effective in improving detrimental vascular features of resistance arteries in CKD.

Keywords

Resistance artery, hypertension, chronic kidney disease, structure, function, stiffness, calcium channel blockade.

5.2 Introduction

Cardiovascular disease (CVD) and chronic kidney disease (CKD) are common comorbidities, and are linked to an increased risk of mortality (Foley, 2010). Cardiovascular disease is of particular importance in CKD, as it is the main cause of mortality in these patients (Keith et al., 2004, McCullough et al., 2008, Gullion et al., 2006, Sarnak et al., 2003).

Resistance arteries provide more than 80% of the resistance to blood flow in the body, and structural, biomechanical and functional properties of resistance vessels are hypothesised as being one of the first sites of target organ damage due to increased intraluminal pressures from hypertension (Schiffrin et al., 2000, Intengan and Schiffrin, 2001). Impaired resistance artery properties are known to play a significant role in persistent increases in total peripheral resistance (TPR) (Intengan and Schiffrin, 2000), and their impaired function is associated with hypertension in CKD (Wang et al., 2000).

A hallmark characteristic of hypertension is altered expression of calcium channels in the vasculature (Cox et al., 2002). In normal vascular smooth muscle cells (VSMCs), voltage-dependent L-type Ca^{2+} channels (LTCCs) are the dominant Ca^{2+} channels, and thus the primary pathway responsible for the influx of intracellular Ca^{2+} (Kudryavtseva et al., 2014). Therefore, LTCCs play an important regulatory role in the maintenance of vascular resistance and therefore blood pressure (Sonkusare et al., 2006). In disease states such as hypertension, LTCC function has been shown to be enhanced, and notably, their expression upregulated in the mesenteric vasculature (Lawton et al., 2012). The importance of LTCCs in the maintenance of vascular structure is demonstrated by the ability of LTCC blockers to normalise wall-lumen ratios in resistance arteries, in both hypertensive humans (Sihm et al., 1998) and rats (Korsgaard et al., 1990). For example, calcium channel blockers (CCBs) are often used to block LTCCs in antihypertensive treatment (Gad, 2014). By inhibiting the LTCCs found on cardiac myocytes and peripheral VSMCs, CCBs have been shown to dilate the microvasculature, and thereby improve regional circulation (Iino et al., 2003, Gad, 2014).

In previous investigations, using a rodent model of CKD, the Lewis polycystic kidney (LPK) rat, we have shown that in parallel with hypertension and renal dysfunction, vascular changes are also evident (Chapter 3; Phillips et al., 2007). At 18 weeks of age, LPK resistance arteries

demonstrate altered function, as indicated by increased vasoconstriction to phenylephrine (PE), impaired endothelium-dependent vasorelaxation, and altered structural and biomechanical properties, as indicated by vascular remodelling, increased collagen/elastin ratios and increased stiffness. Treatment with an AT_1 receptor antagonist, valsartan, improved vascular parameters, even normalising some to levels comparable to Lewis controls (Chapter 4). Valsartan treatment, however, also resulted in a reduction of blood pressure. Therefore it is unclear whether valsartan's effectiveness in improving vasculature parameters in the LPK was attributable to the direct inhibition of angiotensin II's (Ang II) deleterious effects, or due to valsartan's blood pressure-lowering effects. Hence, to further investigate this treatment effect, and because of the significant role that calcium plays in the maintenance of vascular tone alongside the proven effectiveness of CCBs in clinical settings, we sought to investigate the effect of amlodipine on LPK resistance artery structure, function and biomechanical properties.

We hypothesised that amlodipine would lower blood pressure in the LPK rats, ameliorate vascular remodelling, and therefore correct the increased stiffness, increased sensitivity to PE, and endothelium dysfunction found in 18 week old LPK rats (Chapter 3). Relative to valsartan, however, we predicted that the effectiveness of amlodipine to be less, because Ang II is a major contributor to myriad of effects on the vasculature (Iino et al., 2003, Chang et al., 1995, de Gasparo et al., 2000).

5.3 Methods

5.3.1 Animals

Eighteen week old mixed sex LPK and age-matched Lewis control strain rats were obtained from the Animal Resource Centre in Western Australia, Australia, with a minimum of 3 males or females per group. Thirty eight rats were used for this study, however not all sets of data were collected from each animal. Animals were housed in the animal house facility of Macquarie University under a 12-h light/dark cycle at 20.5°C and 57% humidity, and offered chow and water *ad libitum*. All experimental protocols and procedures were approved by the Animal Ethics Committee of Macquarie University and adhered to the National Health and Medical Research Council of Australia's Australian code of practise for the care and use of animals for scientific purposes (8th Edition 2013).

Animals were trained to voluntarily take condensed milk from a 1 ml syringe, (Soeters et al., 2008). Condensed milk served as the vehicle for treatment administration. Littermate Lewis or LPK rats were randomly and equally allocated between control (vehicle only) and treatment groups. Treatment with amlodipine 20 mg/kg/day (Ozlodip®, Ranbaxy, Australia) was performed from 4-18 weeks of age, once daily, made up in up to 1 mL condensed milk. Vehicle animals were fed once daily with 1 mL condensed milk.

5.3.2 Tail cuff plethysmography and urine collection.

Tail-cuff plesmography was used to measure systolic blood pressure (SBP) (ADInstruments, Sydney, NSW, Australia) as described previously (Phillips et al., 2007). At the 18 week timepoint, SBP and urine (protein and creatinine) were measured, at least 72 hours prior to euthanasia. Over a period of 24 hours, urine samples were collected from animals while held individually in metabolic cages; during which animal's water consumption was measured, and animals were offered chow and water *ad libitum*.

5.3.3 Tissue harvesting and biochemical analyses

All animals were deeply anaesthetised with 5% isofluorane (VCA I.S.O., Sydney, NSW, Australia) in 100% O_2 until loss of consciousness, and subsequently decapitated. Trunk blood

samples were collected in pre-cooled EDTA containing tubes (BD Microcontainer®, Becton, Dickinson and Company, Sydney, NSW, Australia), and subsequently centrifuged (3000 RPM for 5 min at 4C°). Plasma and urine were analysed for biochemical parameters using an IDEXX VetLab analyser (IDEXX Laboratories Pty Ltd., Rydalmere NSW, Australia). To remove the mesenteric vasculature, the thorax was dissected open. Following mesenteric vasculature removal, the kidneys and heart were also collected. The kidneys, heart, and left ventricle were weighed and respective indices (kidney index [KI], heart index [HI] and left ventricle index [LVI]; %) were calculated as heart or kidney weight (g)/ body weight (BW) (g) × 100. The LVI was calculated as left ventricle (g)/ heart weight (g) × 100.

5.3.4 Mesenteric artery isolation

Following mesenteric vasculature isolation, it was immediately placed in ice-cold Krebs' physiological solution of the following composition in mM (NaCl 118.2, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $NaHCO_3$ 25, and EDTA, 0.026), continuously bubbled with carbogen (95% O_2; 5% CO_2; BOC Ltd., North Ryde, NSW, Australia) to achieve a pH value of approximately 7.4-7.45 (Intengan et al., 1999).

After cleaning of adherent connective tissue under a dissecting microscope, a second order mesenteric artery segment (200-400 μm external diameter; ~4 mm in length) was mounted in a pressure myograph (Living Systems Instrumentation, Burlington, VT, USA) in 5 mL of carbogen-bubbled cold Krebs' solution. Artery ends were cannulated with glass micropipettes (1 x 0.25 mm glass capillaries, A-M Systems, Inc., Sequim, WA, USA), and secured with 10-0 sutures (Ethilon® Nylon Suture LLC., California, USA). The remaining mesenteric vasculature was placed in 4% formalin for 24 hours, followed by 70% ethanol until further processing for histological studies.

5.3.5 Pressure myography and histology

Pressure myography and histology protocols were undertaken as described previously (Chapter 3; sections 3.3.4, 3.3.5, and 3.8).

5.3.6 Drugs and solutions

Phenylephrine hydrochloride (PE), acetylcholine hydrochloride (ACh), sodium nitroprusside (SNP), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin (indo), histolene, Bouin's fixative, Celestine blue, Harris's haematoxylin, Martius yellow, brilliant Crystal Scarlet, aniline blue, Shikata orcein and Alizarin red were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All drugs and solutions (Krebs' and Ca^{2+}-free Krebs') were prepared on the day of the experiment. All drugs for pressure myography were dissolved in freshly made Krebs' buffer.

5.3.7 Data analysis

Data were analysed using IBM Statistical Package for the Social Sciences (v22, SPSS; Chicago, Illinois USA) and GraphPad Prism (GraphPad Prism software v6 Inc., La Jolla, CA, USA). All results are expressed as mean ± standard error of mean (SEM).

Preliminary analysis of all data was performed to identify potential gender effects, using a univariate general linear model against the fixed factors of strain, age and gender. Unless significant, gender effects are not otherwise noted. Data were tested for homogeneity of variance with Levene's test, normality with the Kolmogorov-Smirnov test, and skewness and kurtosis. If assumptions of normality were met, two-way analysis of variance (ANOVA) was then performed using Bonferroni's *post-hoc* analysis to investigate significant differences between groups (as defined by strain and age). Data that did not meet assumptions of normality were analysed with Dunnett's T3 test.

Urine protein values less than 0.05 g/L were excluded from the data set, and corresponding UPC values were also removed. As no protein was detected in the Lewis urine samples, LPK samples (untreated and treated) were compared using a two-tailed unpaired *t*-test ($P<0.05$)

The 50% effective concentration (EC_{50}) and maximum response (R_{max}) were calculated from the concentration-response curves. Concentration-response curves were fitted to a sigmoidal curve ($Y = \text{Lower plateau} + (R_{max}\text{-Lower plateau}) / (1+10^{(\text{LogEC50-X})})$) to calculate EC_{50} values, where Y is the percentage relaxation or constriction of the vessel and X is the concentration (Armitage et al., 2005). After testing for the normal distribution of data and gender effects as

detailed above, two-way ANOVA with Bonferroni *post-hoc* analysis was used to investigate strain, treatment and pharmacological effects (ACh alone vs. ACh+L-NAME preincubation and ACh+Indo+L-NAME preincubation, and ACh+L-NAME alone vs. ACh+Indo+L-NAME) for concentration-response curve parameters (R_{max}, EC_{50}), in Lewis and LPK. Two-way ANOVA with Bonferroni's *post-hoc* analysis was also used to investigate strain and treatment effects for percentage component contribution to vasorelaxation (NO; prostanoid; endothelium-derived hyperpolarisation [EDH]). Significance was defined as $P \leq 0.05$.

5.4 Results

5.4.1 Baseline parameters

Untreated LPKs exhibited smaller body weights than Lewis, with male animals weighing more than females in Lewis regardless of treatment group (Table 5.1) (untreated Lewis males vs. females: 405.71±6.38 g vs. 236.75±3.94 g, $P<0.001$; treated Lewis males vs. females: 388.75±3.71 g vs. 236.00±2.48 g, $P<0.001$). In addition, irrespective of treatment group, SBP was elevated in the LPK relative to Lewis. However, treatment with amlodipine reduced blood pressure in the LPK.

The HI values were elevated in LPK relative to Lewis in both groups. After treatment HI values were elevated in the Lewis compared to untreated controls. Regardless of treatment group, LVI and KI were elevated in the LPK relative to Lewis, and treatment caused a further increase in KI in the LPK animals. Plasma analyses revealed significantly higher urea and creatinine values in LPK regardless of treatment group, as well as a treatment effect, with amlodipine treatment reducing plasma creatinine values in the LPK. Treated male LPK showed significantly higher plasma creatinine values than treated female LPK. Urine analysis revealed lower urine creatinine values in LPK relative to Lewis, for both untreated and treated cohorts. Treatment with amlodipine significantly reduced urinary protein and UPC in the LPK animals. Water intake was significantly higher in untreated LPK than untreated Lewis, and trended towards higher values in treated LPK relative to treated Lewis ($P=0.059$).

5.4.2 Altered resistance vessel morphology in Lewis polycystic kidney rats

Untreated LPK had significantly smaller internal and external diameter values than corresponding untreated Lewis, as well as elevated wall-lumen ratio values (Table 5.2). Regardless of treatment group, LPK had significantly larger wall thickness values relative to Lewis. Treatment normalised LPK structural parameters, resulting in no significant differences between treated LPK and treated Lewis internal diameter, external diameter, and wall-lumen ratios. In addition, no significant changes in media cross sectional area (MCSA) values were found.

5.4.3 Vascular function investigations

5.4.3.1 Constriction

The untreated LPK PE concentration-response curves were significantly shifted to the left relative to untreated Lewis and treated LPK (Figure 5.1 A). This was accompanied by a shift in the PE EC_{50} concentration-response curve parameter, with untreated LPK exhibiting smaller values than untreated Lewis (Table 5.3). No significant differences were found for LPK potassium chloride (KCl) concentration-response curve comparisons; however, treated Lewis KCl EC_{50} values were significantly larger than corresponding untreated Lewis (Figure 5.1 B; Table 5.3).

5.4.3.2 Endothelium-dependent and independent vasorelaxation

The untreated LPK endothelium-dependent ACh concentration-response curve was significantly shifted to the right relative to untreated Lewis and treated LPK (Figure 5.2 A). In accordance with these results, untreated LPK had significantly smaller ACh R_{max} values than untreated Lewis and treated LPK, and larger ACh EC_{50} values than untreated Lewis (Table 5.3). Overall, amlodipine treatment in LPK resulted in ACh-dependent vasorelaxation responses comparable to Lewis.

No significant strain or treatment differences were found in response to endothelium-independent SNP concentration-response curve (Figure 5.2 B). In addition, there were no alterations in concentration-response curve parameters (Table 5.3).

5.4.3.3 Vasorelaxation mechanism investigations

Relative to control ACh responses, preincubation with the nitric oxide synthase (NOS) inhibitor L-NAME reduced the ACh R_{max} in both groups of Lewis and amlodipine treated LPK but not the in the untreated LPK (Table 5.3). Preincubation with a combination of the cyclooxygenase inhibitor indomethacin and L-NAME (Indo+L-NAME) resulted in a reduction in ACh R_{max} values for all treatment groups and strains (Table 5.3). Preincubation with Indo+L-NAME also resulted in significantly larger EC_{50} values in treated Lewis relative to untreated Lewis.

Both treated groups had a lower ACh R_{max} after Indo+L-NAME preincubation relative to their untreated controls. Although untreated LPK had significantly smaller ACh R_{max} values after preincubation with indo+L-NAME, relative to untreated Lewis, this difference was no longer evident in the treated animals (Table 5.3).

5.4.3.4 Vasorelaxation component investigations

The percentage contribution to ACh R_{max} by NO showed no strain or treatment effects (Figure 5.3). The prostanoid component was increased in untreated LPK, relative to untreated Lewis controls, and this was significantly reduced after treatment. The percentage contribution to ACh R_{max} attributed to EDH was impaired in untreated LPK relative to untreated Lewis controls (Figure 5.3), and this was normalised after amlodipine treatment.

5.4.4 Effect of amlodipine on altered resistance artery stiffness in Lewis polycystic kidney rats

Regardless of treatment group, LPK pressure diameter curves were shifted downwards relative to Lewis controls. In addition, after treatment, the LPK pressure diameter curve was shifted upward, while the treated Lewis pressure diameter curve was shifted downward, relative to untreated Lewis (Figure 5.4).

For both untreated and treated cohorts, LPK stress-strain curves and the corresponding slope of elastic modulus vs. stress calculations were significantly shifted to the left, and had larger slope of elastic modulus vs. stress values, respectively, relative to Lewis controls (Figure 5.5 A and B). Treatment shifted Lewis stress-strain curves to the right relative to untreated Lewis, but this was not accompanied by significant changes in slope of elastic modulus vs. stress values. Treatment did not impact the LPK stress-strain curves or slope of elastic modulus vs. stress calculations (Figure 5.5 A and B).

5.4.5 Effect of amlodipine on altered resistance vessel wall composition in Lewis polycystic kidney rats

Irrespective of treatment group, LPK had significantly greater collagen density than corresponding Lewis controls (Figure 5.6; Table 5.4). In addition, elastin density was

significantly less in untreated LPK relative to untreated Lewis. This difference however was corrected in the treated LPK animals. Collagen/elastin ratios were significantly higher in LPK relative to Lewis, regardless of treatment group. There were no significant differences in nuclear density, number of nuclei per μm^2 or calcium density between any of the study groups (Figure 5.6; Table 5.4).

5.5 Discussion

In this study we report for the first time the effects of calcium channel blockade on resistance artery structure, function and biomechanical properties in a model of CKD. Chronic treatment with amlodipine resulted in significant antihypertensive effects, improved renal function, and ameliorated detrimental vascular remodelling, vascular amplification, and endothelium dysfunction in LPK. These results contribute to previous research on the effectiveness of CCBs in blood pressure control, supporting the use of CCBs in the treatment of hypertension, and highlighting the importance of LTCCs as primary contributors to pathologies seen in CVD and CKD.

In this study, we demonstrated the effectiveness of amlodipine in producing antihypertensive outcomes in the LPK animals. The important role of LTCCs in VSMC contraction due to its regulation of the Ca^{2+} influx pathway is well-established (Kudryavtseva et al., 2014), and there is previous evidence for their playing a role in driving hypertension, including patch-clamping studies which have shown that mesenteric VSMCs from SHRs exhibit enhanced calcium current movement (Ohya et al., 1998, Cox and Lozinskaya, 1995, Cox et al., 2002), and increased expression of LTCCs in resistance arteries (Sonkusare et al., 2006). As a consequence, more binding sites are available for CCBs, therefore amplifying their vasodilator action proportional to the degree of hypertension (Sonkusare et al., 2006).

Although calcium channel blockade did not improve water intake in LPK, and had no effect on LVH in LPKs, it did have cardiac remodelling effects on Lewis controls, where amlodipine treatment resulted in an increased heart index. Another key finding of this study was the improvement in renal function in the LPK animals after treatment with amlodipine, as evidenced by decreased plasma creatinine and urinary protein excretion. Interestingly, this was concurrent with a relative increase in the size of the kidneys in the LPK animals, which could perhaps be due to an increase in cyst size, as previous research has found accelerated cyst growth in the Han:SPRD Cy/+ rat, following calcium channel inhibition via verapamil treatment, due to upregulated cell-signalling pathways (Nagao et al., 2008).

Treatment with a CCB also improved the degree of structural vascular remodelling seen in LPK, in accordance with previous research in SHRs which found regression of vascular

remodelling in small arteries after treatment with amlodipine (Sharifi et al., 1998). The LPK animals demonstrate eutrophic inward remodelling with increased arterial wall thickness, decreased lumen diameter and unchanged MCSA. Importantly, this vascular remodelling was not associated with a change in the number of nuclei or nuclear density, suggesting the arteries underwent a re-arrangement of smooth muscle cells around a smaller diameter, as opposed to hypertrophy or hyperplasia. Amlodipine improved vascular remodelling, with the normalisation of wall-lumen ratio values being of particular importance, as wall-lumen ratio is believed to significantly impact the continuation of the hypertensive state (Mulvany, 2002).

Interestingly, use of the CCB in Lewis animals increased compliance, as indicated by the increased gradient of the pressure-diameter curve and rightward shift of the stress-strain curve. However, there was no effect on intrinsic stiffness, which is an indicator of stiffness independent of vessel geometry (Behbahani et al., 2010). The effectiveness of amlodipine in increasing compliance in Lewis controls may be due to the vasodilatory effect of calcium channel blockade, through its stimulation of the release of NO from blood vessels via an increase in kinin activity (Xu et al., 2002, Zhang et al., 2002). In contrast to Lewis, however, the CCB did not significantly affect LPK stress-strain curves, nor intrinsic stiffness. In accordance with these findings, collagen density and collagen/elastin ratio was increased in both LPK groups. In the SHR, amlodipine treatment has been shown to normalise the collagen-elastin ratio in small arteries, relative to Wistar Kyoto (WKY) controls (Sharifi et al., 1998). A lack of effect in the LPK could perhaps be due to treatment being insufficient to overcome the deleterious effects of a combination of hypertension and renal disease in LPK.

Our other investigations into the composition of the arterial wall were consistent with our previous findings (Chapters 3 and 4), with LPK showing reduced elastin levels and no evidence of calcification. The absence of calcification in both strains and age groups was in relative difference to what we and others have previously described in large vessels in association with age and CKD (Ameer et al., 2014b), as well as in breast arterioles of women with CKD and end-stage renal disease (ESRD) (O'Neill and Adams, 2013). Previous research has shown elastin degradation to be strongly associated with vascular calcification, and one of the main mechanisms of calcification (Pai et al., 2011). Hence, given that mesenteric arteries already have a very small percentage of elastin (an average of ~10% in Lewis rats), our report finding of calcification absence is not unexpected.

L-type Ca^{2+} channels are important for not only the regulation of vascular structure, but also VSMC contractility (Kudryavtseva et al., 2014). Hence, in addition to structure, calcium channel blockade also improved functional alterations noted in the LPK. In the present study, a leftward shift in the concentration-response curves to the α_1-adrenergic receptor agonist PE, as well as decreased PE EC_{50} values relative to Lewis controls was evident. This enhanced sensitivity to PE is consistent with a vascular amplifier effect, as similar results were not evident for the direct depolariser KCl. Amlodipine ameliorated the response to levels comparable to Lewis, and is likely to be due to a combination of reduced availability of intracellular calcium, as well as a reduction in the degree of structural alteration (Kudryavtseva et al., 2014).

Impaired endothelium functioning is not only a contributing factor to the development of high blood pressure (Rossi et al., 2004), but also an outcome of hypertension (Schiffrin, 2012). In accordance with Chapters 3 and 4, and previous investigations of resistance arteries in both renovascular hypertensive and autosomal dominant polycystic kidney disease (ADPKD) patients (Porteri et al., 2003, Wang et al., 2003), the present study found impaired ACh endothelium-dependent relaxation in untreated LPK mesenteric resistance vessels. Incubation with the NOS inhibitor, L-NAME, had no effect on ACh R_{max} values in untreated LPK, which therefore suggests defective NOS activity (Wang et al., 2003). In contrast, L-NAME impaired ACh-induced relaxation in the Lewis (regardless of treatment group) and treated LPK. Thus collectively, these findings show that amlodipine treatment in the LPK restores impaired NOS functioning, resulting in vasorelaxation responses to ACh resembling that of Lewis controls.

Further investigations into other vasorelaxation components revealed that consistent with previous findings, in untreated and treated Lewis controls, EDH contributed to ~70% of relaxation (Luksha et al., 2012), NO to 30% of relaxation (Paulis et al., 2010), and prostanoids a minimal role (Shimokawa et al., 1996). When deficiency of a vasorelaxation component occurs, such as in the case of hypertension, the endothelium may upregulate other mechanisms to serve as compensatory physiological pathways (Versari et al., 2009). This phenomenon was evident in untreated LPK, which exhibited up regulated prostanoid components relative to Lewis controls, and down regulated EDH components relative to Lewis. Treatment with the CCB increased the contribution of NO to ACh R_{max}, and normalised the EDH and prostanoid components. Possible pathways by which amlodipine

may have acted indirectly through an antioxidant activity-mediated mechanisms, or via its oxygen free radical-scavenging properties (Sobal et al., 2001, On et al., 2002): experimental data has demonstrated that calcium antagonism has an antioxidant effect, preserving NO availability by protecting endothelium cells against free radical injury and oxidative stress (Mak et al., 1992). Alternatively, through its direct vasodilatory effects, amlodipine may exert its effectiveness via an increase in kinin activity, and stimulation of NO independent of its calcium channel blockade effects (Xu et al., 2002, Zhang et al., 2002), as demonstrated by amlodipine's ability to increase nitrite production, which therefore reflects an increase in NO biosynthesis (Zhang et al., 1999). Nonetheless, irrespective of the pathway through which amlodipine is effective, when considering the present study and Chapter 4's findings together, it is clear that blood pressure dependence is a significant factor in determining both structural and functional vascular outcomes, and it seems that valsartan and amlodipine treatment may affect upregulation of the prostanoid vasodilatory component by similar or overlapping mechanistic actions.

In conclusion, our data demonstrate the effectiveness of calcium channel blockade in altering resistance vessel structural and functional properties in a rat model of CKD. Amlodipine treatment normalised vascular remodelling parameters, and improved endothelium dysfunction. However, unlike valsartan treatment, calcium channel blockade did not improve resistance artery stiffness nor water intake, and had antihypertensive effects which were of a lesser extent. Overall, the present study highlights the importance of LTCCs and calcium in the contribution to vascular tone and structure, and therefore resistance artery properties. These findings contribute to our understanding of the role of LTCCs in the CKD state, and may help direct future investigations of treatment options.

5.6 Acknowledgements

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The authors would like to thank Professor Ian Wright (University of Wollongong) for reviewing this chapter.

5.7 Conflict of interest

None declared.

Tables

Table 5.1: Baseline morphometric and biochemical parameters.

	Untreated		Treated	
Parameter	Lewis	LPK	Lewis	LPK
n	8	7	7	6
BW(g)	351±24	221±21[a]	312±29	228±17
SBP (mmHg)	109±1	185±5[a]	123±1	165±9[b d]
HI (%)	0.20±0.04	0.48±0.01[a]	0.33±0.01[c]	0.47±0.01[b]
LVI (%)	75.19±1.40	89.95±0.73[a]	66.61±0.79	81.70±5.12[b]
KI (%)	0.57±0.11	6.71±0.39[a]	0.81±0.02	8.93±0.45[b d]
Plasma urea (mmol/L)	7.79±0.25	42.78±1.85[a]	7.74±0.42	38.31±2.84[b]
Plasma creatinine (μmol/L)	32.08±1.42	196.50±16.61[a]	27.88±1.78	140.25±15.95[b d]
Urine Protein (g/L)	nd	1.25±0.34	2.00±0.38[(n=3)]	0.40±0.09[d]
Urine Creatinine (g/L)	1.83±0.32	0.13±0.03[a]	2.11±0.36	0.26±0.05[b]
UPC	nd	13.73±4.20	1.65±0.65	4.23±2.17[a]
Water intake (mL)	22.00±1.39	38.57±5.54[a]	24.13±3.73	40.17±5.87

BW, body weight; SBP, systolic blood pressure; HI, heart index; LVI, left ventricle index; KI, kidney index; nd, not determined; UPC, urine protein creatinine ratio; LPK, Lewis polycystic kidney rat. Note: n=3 for urine protein values, and that all other rats within this treatment group had urine protein values ≤0.05. Apart from urine protein and UPCs, data that met parametric assumptions were followed by Bonferroni's *post-hoc* analysis, or Dunnett's T3 test if assumptions were violated. Urine protein and UPC in untreated vs. treated LPK were evaluated by unpaired *t*-test. Results are expressed as mean ± SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

Table 5.2: Mesenteric artery structural parameters of untreated and amlodipine treated 18 week old Lewis and Lewis polycystic kidney rats.

	Untreated		Treated	
Parameter	Lewis	LPK	Lewis	LPK
n	14	8	8	8
Internal diameter (μm)	270.00±21.12	117.75±19.00 [a]	222.13±25.91	164.75±17.40
External diameter (μm)	326.58±18.24	199.50±19.28 [a]	284.25±24.22	246.25±19.39
Wall thickness (μm)	28.29±2.53	40.88±3.40[a]	31.06±2.34	40.75±2.43[b]
Wall-lumen ratio	0.12±0.02	0.51±0.15[a]	0.15±0.02	0.26±0.02
MCSA ($x10^3$) (μm^2)	25.53±1.97	20.43±3.35	24.24±2.38	26.71±3.40

MCSA, media cross sectional area; LPK, Lewis polycystic kidney rat. Second order mesenteric artery structural parameter data was obtained at 60 mmHg following Ca^{2+}-free superfusion. Data that met parametric assumptions were followed by Bonferroni's *post-hoc* analysis, or Dunnett's T3 test if assumptions were violated. Results are expressed as mean±SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

Table 5.3: Concentration-response curve parameters for untreated and amlodipine treated 18 week old Lewis and Lewis polycystic kidney rats.

	Untreated		Treated	
(n)	Lewis (11)	LPK (8)	Lewis (8)	LPK (8)
EC_{50} (M)				
PE ($x10^{-7}$ M)	18.43±5.37	2.00±0.42[a]	10.56±1.58	11.27±2.73
KCl ($x10^{-3}$ M)	28.14±3.87	35.73±5.26	45.78±2.48[c]	33.89±2.94
ACh ($x10^{-8}$ M)	29.51±6.14	55.32±7.55[a]	35.50±7.04	35.41±11.05
SNP ($x10^{-8}$ M)	10.80±2.86	7.02±1.46	5.75±0.72	8.25±2.02
L-NAME ($x10^{-7}$ M)	3.89±0.81	16.53±9.38	15.62±3.24	9.46±0.25
Indo+L-NAME ($x10^{-7}$ M)	5.40±1.11	4.47±1.12	15.27±4.96[c]	7.30±2.22
R_{max}				
PE	62.11±4.32	65.61±1.90	69.54±1.98	57.69±3.60
KCl	62.53±3.90	65.21±2.69	71.19±2.08	65.87±1.96
ACh	99.16±1.77	57.52±4.57[a]	90.69±3.33	97.20±3.96[d]
SNP	95.38±1.79	102.75±1.72	102.62±3.50	95.75±3.00
L-NAME	68.64±8.41[e]	52.85±5.02[a]	74.04±6.40[e]	65.21±4.29[e]
Indo+L-NAME	80.20±2.15[f]	38.08±3.63[a f]	60.41±4.20[c f]	64.33±6.70[d f]

Concentration-response curve parameters (EC_{50} and R_{max}) to various pharmacological agents in untreated and treated 18 week old Lewis and LPK second order mesenteric arteries. LPK, Lewis polycystic kidney rat; PE, phenylephrine; KCl, potassium chloride; ACh, acetylcholine; SNP, sodium nitroprusside; L-NAME, N^{ω}-nitro-L-arginine methyl ester; Indo, indomethacin; EC_{50}, effective concentration at 50%; R_{max}, maximum response. All data were evaluated by two-way ANOVA, followed by Bonferroni's *post-hoc* analysis. Results are expressed as mean±SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d)

untreated and treated LPK. Within strain pharmacological agent effect: (e) between ACh and ACh+L-NAME; (f) between ACh and ACh+Indo+L-NAME; (g) between ACh+L-NAME and ACh+Indo+L-NAME.

Table 5.4: Histological parameters of untreated and amlodipine treated 18 week old Lewis and Lewis polycystic kidney rats.

	Untreated		Treated	
Parameter	Lewis	LPK	Lewis	LPK
n	8	8	8	6
Collagen density (%)	12.36±1.57	27.30±2.91 [a]	10.61±0.65	29.03±0.88 [b]
Nuclear density (%)	3.64±0.24	3.54±0.68	3.90±0.49	2.31±0.31
Number of nuclei per μm^2	0.76±0.44	0.12±0.01	0.11±0.01	0.20±0.02
Elastin density (%)	9.89±0.51	7.80±0.38 [a]	9.56±0.55	8.06±0.24
Collagen/elastin ratio	1.01±0.16	4.01±0.45 [a]	1.14±0.09	3.61±0.14 [b]
Calcium density (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

LPK, Lewis polycystic kidney rat. Collagen density, nuclear density and number of nuclei per um^2 were quantified using Martius scarlet blue (MSB) staining, while elastin density was quantified using Shikata's orcein (SO). Data that met parametric assumptions were followed by Bonferroni's *post-hoc* analysis, or Dunnett's T3 test if assumptions were violated. Results are expressed as mean±SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

Figures

Figure 5.1: The effect of amlodipine on vasoconstriction curves to phenylephrine and potassium chloride in Lewis and Lewis polycystic kidney rat mesenteric artery.

Untreated and treated 18 week old Lewis and Lewis polycystic kidney rat (LPK) mesenteric artery contraction responses (%) to cumulative additions of α_1-adrenergic receptor stimulator PE (A) and the direct depolarising vasoconstrictor KCl (B). Results are expressed as mean±SEM. Significant differences between groups overall concentration-response curves where indicated P<0.05. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

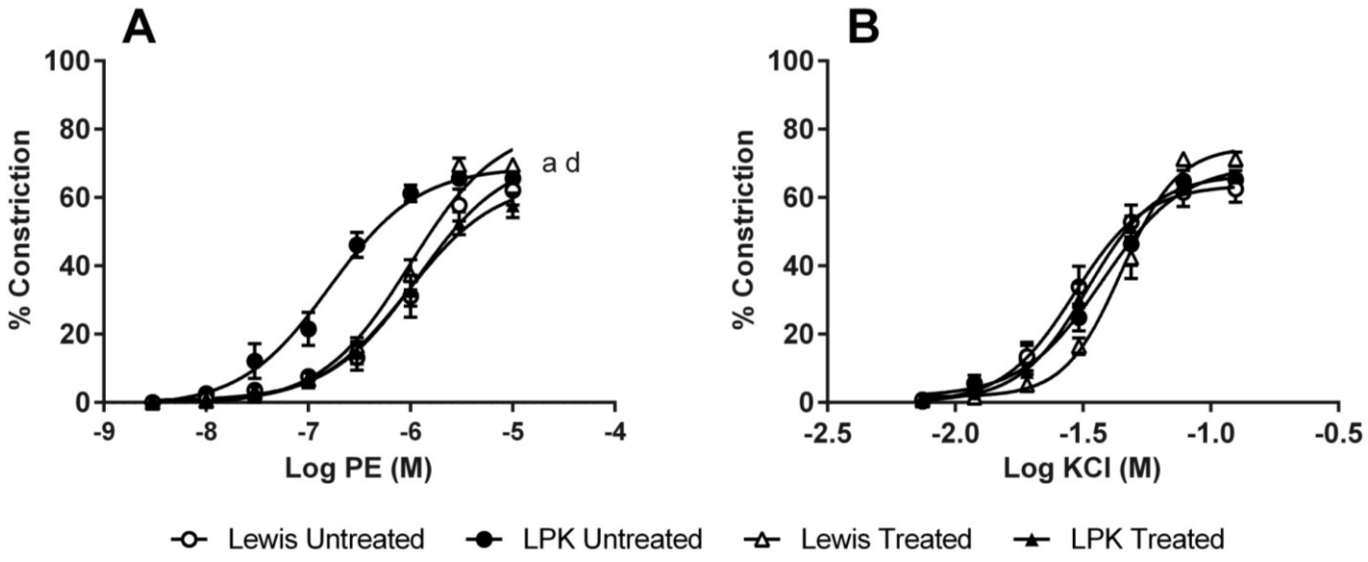


Figure 5.2: The effect of amlodipine on vasorelaxation curves to acetylcholine and sodium nitroprusside in Lewis and Lewis polycystic kidney rat mesenteric artery.

Untreated and treated 18 week old Lewis and Lewis polycystic kidney rat (LPK) second order mesenteric artery relaxation responses (%) to cumulative additions of endothelium-dependent and –independent vasodilators, (A) acetylcholine (ACh) and (B) sodium nitroprusside (SNP), respectively, following preconstriction with 1μM phenylephrine (PE). Results are expressed as mean±SEM. Significant difference between groups where indicated $P<0.05$. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

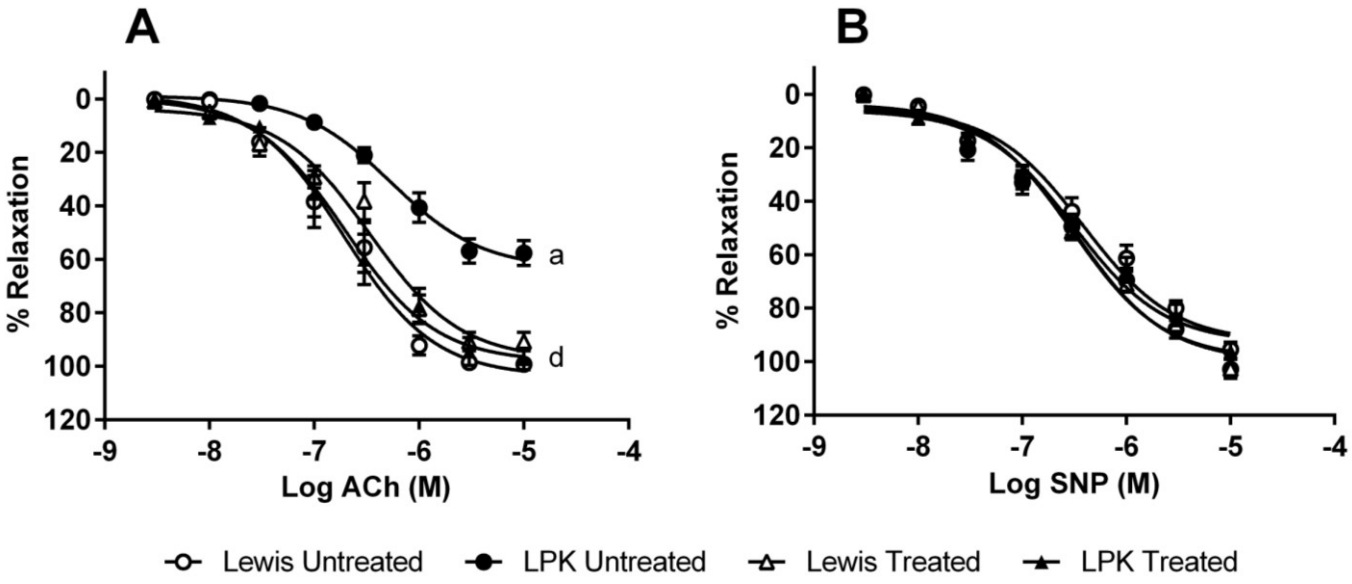


Figure 5.3: The effect of amlodipine on the relative contribution of vasodilatory mediators to acetylcholine responses in Lewis and Lewis polycystic kidney rat mesenteric artery.

Contribution of each vasodilatory component (nitric oxide, NO; prostanoid and endothelium-derived hyperpolarisation, EDH) to acetylcholine (ACh) -induced relaxation in untreated and treated Lewis and Lewis polycystic kidney rat (LPK) mesenteric artery. Results are expressed as mean±SEM. Significant difference between groups where indicated $P<0.05$. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

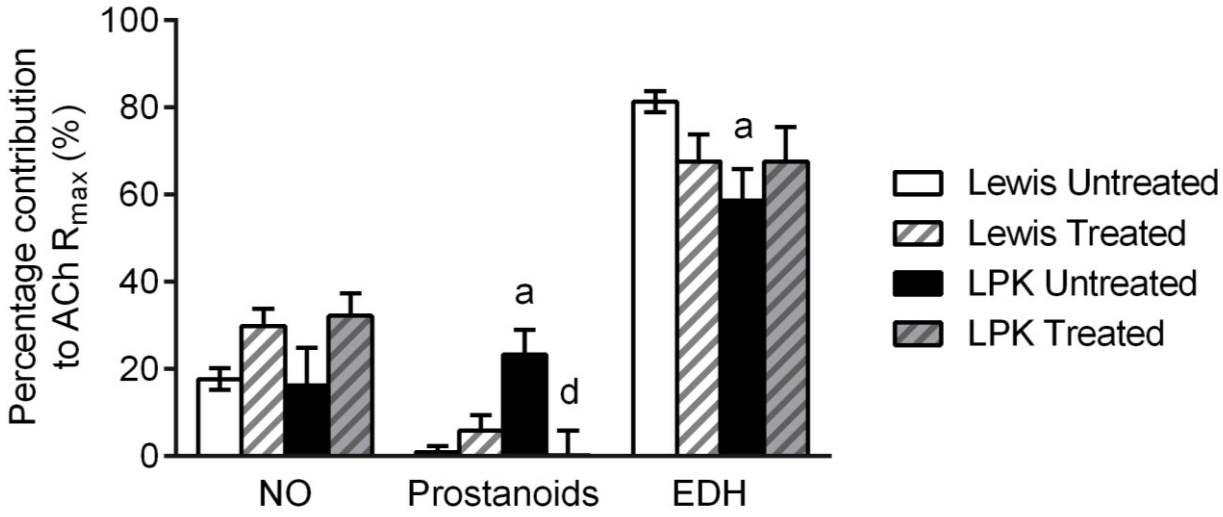


Figure 5.4: The effect of amlodipine on pressure-diameter curves in Lewis and Lewis polycystic kidney rat mesenteric artery.

Passive pressure curves in untreated and treated 18 week Lewis and Lewis polycystic kidney rat (LPK) second order mesenteric arteries. Curves represent diameter passive pressure (diameter, μm) in response to changing pressure (mmHg) in Ca^{2+}-free Krebs' solution. Overall curve comparisons were evaluated by two-way ANOVA, followed by Bonferroni's *post-hoc* analysis. Results are expressed as mean±SEM. Significant difference between groups where indicated $P<0.05$. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

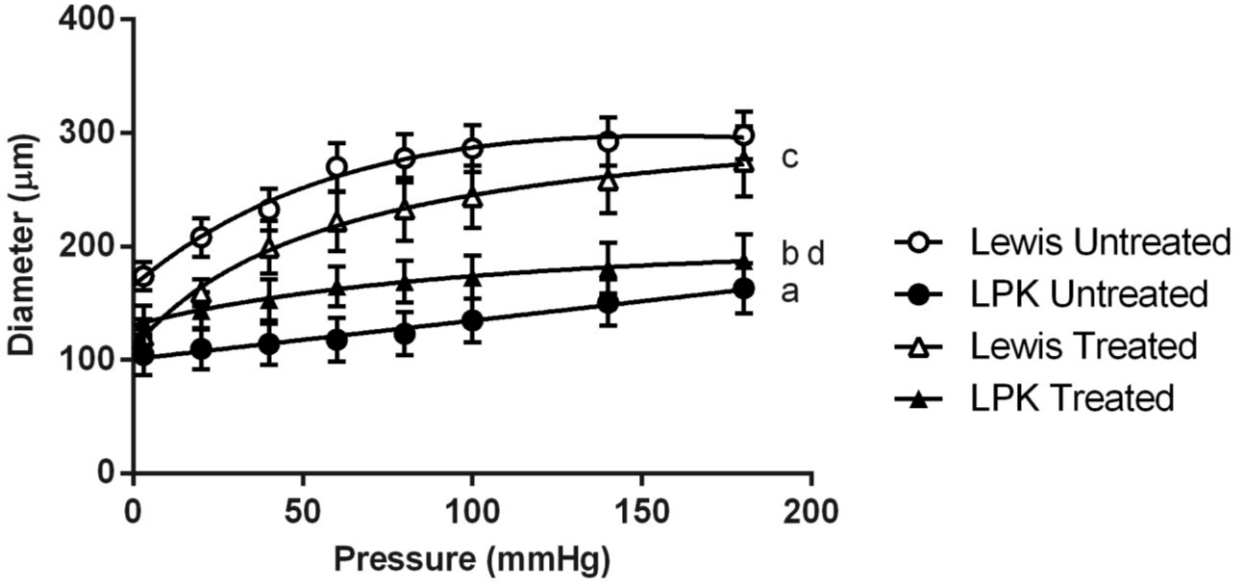


Figure 5.5: The effect of amlodipine on stress-strain curves in Lewis and Lewis polycystic kidney rat mesenteric artery.

Stress-strain data fitted to an exponential curve for untreated and treated 18 week old Lewis and Lewis polycystic kidney rat (LPK) second order mesenteric artery (A). Slope of elastic modulus vs. stress, calculated from stress-strain curve data (B). Data were evaluated by two-way ANOVA, followed by Bonferroni's *post-hoc* analysis. Results are expressed as mean±SEM. Within treatment strain effect: (a) untreated Lewis and LPK; (b) treated Lewis and LPK. Within strain treatment effect: (c) untreated and treated Lewis; (d) untreated and treated LPK.

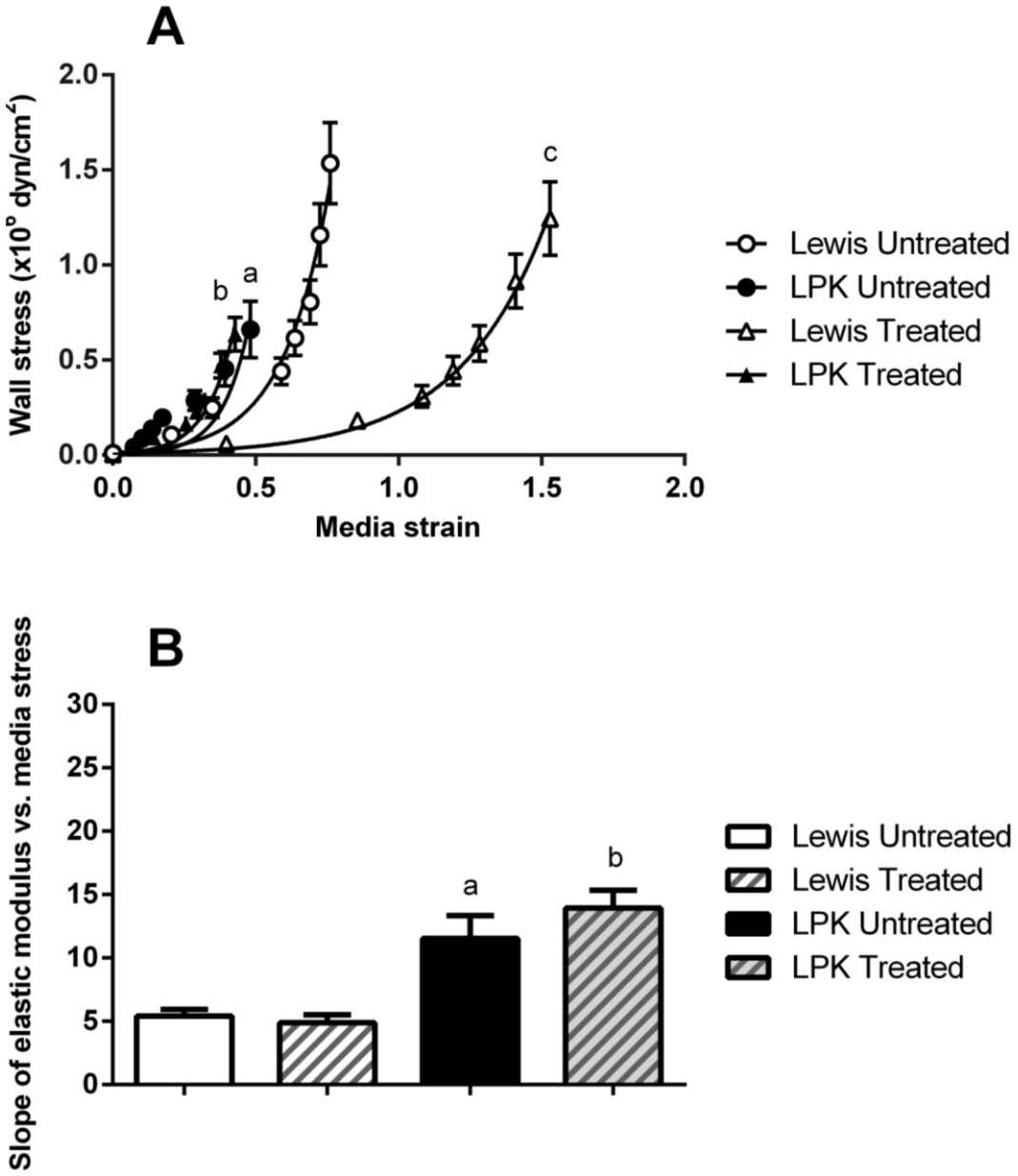
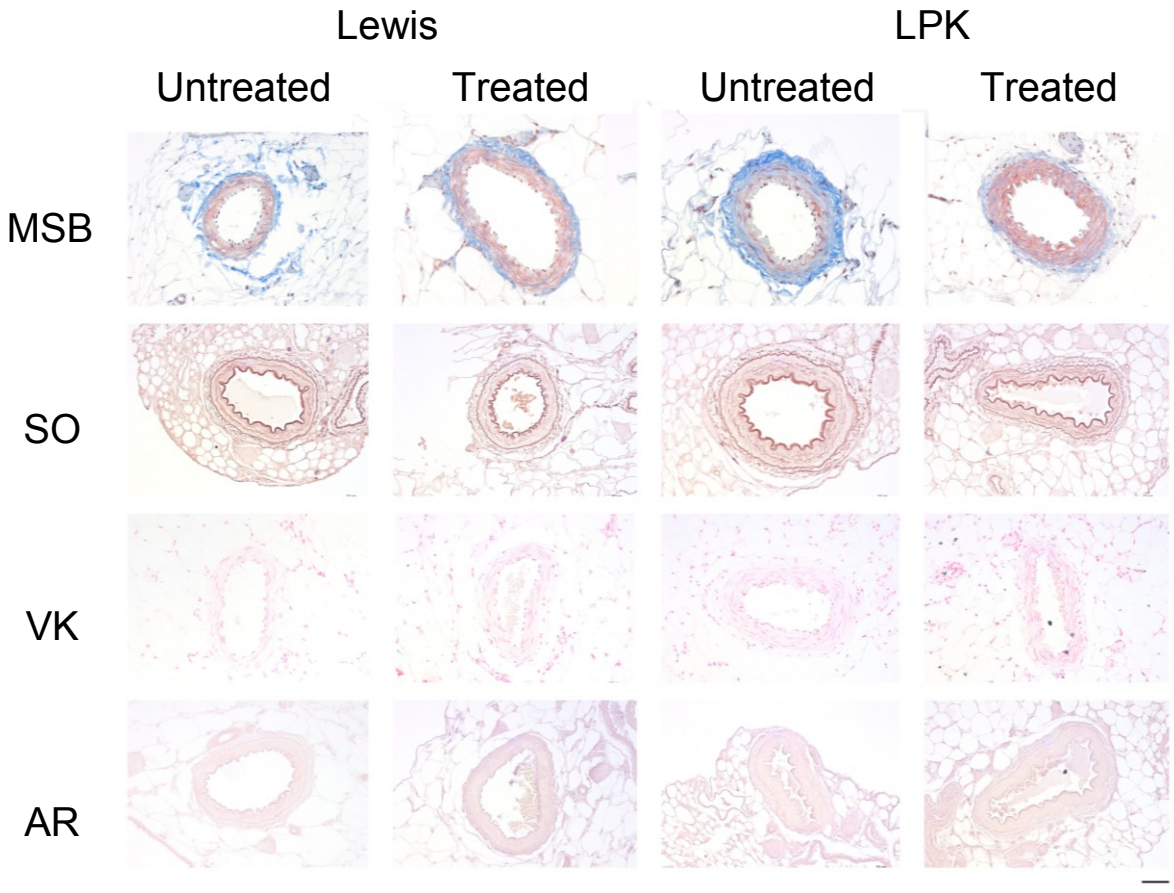


Figure 5.6: The effect of amlodipine on vascular composition in Lewis and Lewis polycystic kidney rat mesenteric artery.

Representative untreated and treated 18 week old Lewis (left panels) and Lewis polycystic kidney rat (LPK) (right panels) second order mesenteric artery wall, stained with Martius scarlet blue (MSB; top row), Shikata's orcein (SO; second row), Von Kossa (VK; third row), and Alizarin red (AR; bottom row) showing the collagen component in blue (MSB), the elastin in red/brown (SO), and absence of calcification as indicated by no dark brown/black deposits (VK) and no orange staining (AR). Scale bar is 50 μm.



6

Chapter 6

Mechanisms underlying impaired vascular function in resistance arteries in association with hypertension in a rodent model of chronic kidney disease

Quek, K.J., Avolio, A.P., Murphy, T.V., & Phillips, J.K.

6.1 Abstract

Hypertension is a significant complication in chronic kidney disease (CKD), often resulting in increased morbidity in these patients. Vascular dysfunction is a likely contributor to the disease pathology. We examined changes in constricting and relaxing factors in the mesenteric artery from the genetic hypertensive rat model of CKD, the Lewis polycystic kidney (LPK) rat, at a late stage CKD time-point (18 weeks; n=8), relative to age-matched Lewis controls (18 weeks; n=8). Animals were phenotyped for systolic blood pressure and urine biochemistry. After euthanasia, blood was collected for urea and creatinine analysis, and mesenteric vasculature collected for pressure myography. Lewis polycystic kidney rat demonstrated increased myogenic tone relative to Lewis and an increased percentage vasoconstriction response to Angiotensin II but not phenylephrine or endothelin-1. Endothelium dysfunction was evident in response to the vasoactive agonists acetylcholine (ACh) and bradykinin (BK) (%: ACh, Lewis vs. LPK: 88.75±5.21 vs. 66.19±4.49, *P*=0.020; BK, Lewis vs. LPK: 95.09±4.61 vs. 65.02±4.84, *P*=0.005). Correlations between renal function (plasma creatinine and urea) and vasorelaxation values revealed a significant negative relationship (plasma creatinine and ACh, r=-0.689, *P*=0.003; urea and ACh, r=-0.689, *P*=0.003; plasma creatinine and BK, r=-0.725, *P*=0.002; urea and BK, r=-0.749, *P*=0.001). Histological analysis of the LPK mesenteric arteries revealed hypertrophic inward remodelling characterised by increased medial smooth muscle thickness, decreased lumen diameter, and unchanged and increased media cross-sectional area, respectively. Our results highlight the importance of constricting and relaxing factors to overall vascular function, suggest an absence of agonist-specificity for endothelium-dependent vasorelaxation investigations in rats with hypertension and advanced renal disease.

Keywords

Resistance artery, hypertension, chronic kidney disease, myogenic tone, vascular function.

6.2 Introduction

Myogenic tone is a smooth muscle cell's ability to contract in response to an increase of transmural pressure, independent of any neural, metabolic or hormonal mediation (Davis and Hill, 1999, Davis et al., 2001, Feihl et al., 2006, Sonoyama et al., 2007). The contribution of myogenic tone in the regulation of blood vessels increases as vessel size decreases (Davis, 1993), and hence plays a particularly important role in resistance arteries (Davis, 1993, Uchida and Bohr, 1969).

The endothelium is believed to be an important modulator of myogenic tone (Meininger and Davis, 1992), where vasoactive factors from the endothelium can increase or decrease myogenic tone levels and thus affect vascular resistance (Cipolla, 2009). This is supported by studies of mesenteric arteries denuded of endothelium from spontaneously hypertensive rats (SHRs), which exhibited enhanced myogenic constriction (Garcia et al., 1997) – perhaps due to the absence of endothelium-derived nitric oxide (NO) (Scotland et al., 2001). The endothelium plays a crucial role in the pathophysiology of hypertension, being responsible for the regulation and release of endothelium-derived relaxing factors such as NO and endothelium-derived hyperpolarisation (EDH), and endothelium-derived constricting factors such as endothelin-1 (ET-1) (Kietadisorn et al., 2012). In disease conditions such as hypertension, the endothelium loses its protective role and this is typically associated with a decrease in NO availability (Vanhoutte, 1989). When this occurs, the endothelium activates compensatory physiological pathways, which may involve upregulation or downregulation of other vasodilatory mechanisms, such as prostanoids and EDHs (Versari et al., 2009).

Dysfunctional endothelium can produce and release vasoconstrictor substances such as Angiotensin II (Ang II), ET-1 and vasoconstrictor prostanoids which may further exacerbate endothelium dysfunction (Taddei et al., 2003, Kietadisorn et al., 2012). Angiotensin II for example is able to produce a local, potent vasoconstriction, and therefore an increase in blood pressure (Vanhoutte, 1989, Griffin et al., 1991, Haber et al., 1975). Beyond contraction of smooth muscle, Ang II is implicated in the development of the progression of structural alterations (vascular remodelling) in small resistance arteries (Touyz, 2005): it has been demonstrated that long-term infusions of non-pressor doses of Ang II are able to induce resistance vessel vascular remodelling, involving significantly increased media width, media cross-sectional area (MCSA), and wall-lumen ratio (Griffin et al., 1991). In addition, Ang II

stimulates ET-1 production which leads to further increased vasoconstriction (Schiffrin, 2005) and vascular remodelling, due to ET-1's mediating effects of chronic inflammation in the vasculature (Ammarguellat et al., 2002, Schiffrin, 2005, Pu et al., 2003, Barton and Luscher, 1999, Schiffrin et al., 1995).

Endothelin-1 is the predominant isoform of endothelin and is a powerful vasoconstrictor (Foëx and Sear, 2004), and results in an increase in total peripheral resistance (TPR) mainly by vasoconstricting the mesenteric vascular bed (MacLean et al., 1989). Though the generation of, and sensitivity to, ET-1 has been shown to be comparable between hypertensive and normotensive subjects in some studies (Foëx and Sear, 2004), other research has demonstrated that despite normal circulating levels of ET-1, hypertensive patients present with augmented vasoconstriction to ET-1 in peripheral circulation, in association with defective NO availability (Schiffrin, 1999). For example, as demonstrated in a rat model of polycystic kidney disease (PKD), endothelin activity was upregulated alongside a deficiency in NO, which together was likely to have contributed to enhanced vasoconstriction and renal dysfunction in this model (Al-Nimri et al., 2003), Further, the use of selective endothelin A receptor antagonist in CKD patients produces a reduction of blood pressure associated with renal vasodilation (Goddard et al., 2004). Hence, in hypertension, it seems that complex interactions between NO and ET-1 can contribute to and/or exacerbate endothelium dysfunction (Foëx and Sear, 2004, Versari et al., 2009).

Dysfunction of relaxing factors is another well-established aspect of endothelium dysfunction: for example, endothelium-dependent relaxation in subcutaneous resistance arteries from autosomal dominant polycystic kidney disease (ADPKD) patients have an impaired ability to generate bioactive NO (Wang et al., 2003), and the mesenteric vascular bed of SHRs exhibit impaired EDH functioning (Borges et al., 1999, Fujii et al., 1992). In addition, a study investigating endothelium dysfunction in resistance arteries from end-stage renal disease (ESRD) patients suggested that impairments of EDH responses are receptor-specific, and revealed that different outcomes can be seen in response to factors such as bradykinin (BK) or acetylcholine, ACh) (Luksha et al., 2012).

The Lewis polycystic kidney (LPK) is a rodent model of CKD, which has a phenotypic representation resembling that of human autosomal recessive polycystic kidney disease (Phillips et al., 2007). In parallel with the hypertension and renal dysfunction, vascular

changes are also evident: as shown in the preceding chapters, LPK resistance arteries at 18 weeks undergo vascular remodelling and exhibited impaired endothelium-dependent vasorelaxation. Given the importance of myogenic tone in the regulation of vascular resistance and therefore blood pressure (Davis and Hill, 1999), we investigated myogenic tone in Lewis and LPK rats, and examined specific mechanisms that may underlie our previous observed changes in vascular function . Specifically, we examined any differences in agonist-specificity of endothelium-dependent vasodilation, hypothesising that the endothelium dysfunction observed in LPK rats (Chapters 3, 4, and 5) is mediated by an enhancement of endothelium-derived constricting factors, and downregulation of endothelium-derived relaxing factors. In addition, and in accordance with previous findings (Luksha et al., 2012), we also hypothesised that EDH responses will be agonist-specific, being exaggerated in response to BK but not ACh.

6.3 Methods

6.3.1 Animals

Mixed sex 18 week old LPK and their age-matched control strain Lewis rats were obtained from the Animal Resource Centre in Western Australia, Australia, with equal ratios of males to females. A total of 16 rats were used for this study. Animals were offered chow and water *ad libitum*, housed under a 12-h light/dark cycle at 20.5°C and 57% humidity in the animal house facility of Macquarie University. All experimental protocols and procedures were approved by the Animal Ethics Committee of Macquarie University and adhered to the National Health and Medical Research Council of Australia's Australian code of practise for the care and use of animals for scientific purposes (8th Edition 2013).

6.3.2 Tail cuff plethysmography and urine collection

Systolic blood pressure (SBP) was measured using tail-cuff plesmography (ADInstruments, Sydney, NSW, Australia) as described previously (Phillips et al., 2007). At least 72 hours prior to euthanasia, urine samples were collected from animals over a period of 24 hours while held individually in metabolic cages, during which animals were offered chow and water *ad libitum*.

6.3.3 Tissue harvesting and biochemical analyses

Animals were deeply anaesthetised with 5% isofluorane (VCA I.S.O., Sydney, NSW, Australia) in 100% O_2 and decapitated. Trunk blood samples were collected in pre-cooled EDTA containing tubes (BD Microtainer®, Becton, Dickinson and Company, Macquarie Park, NSW, Australia) and centrifuged (3000 RPM for 5 min at 4C°). An IDEXX VetLab analyser (IDEXX Laboratories Pty Ltd., Rydalmere, NSW, Australia) was used to analyse plasma and urine for biochemical parameters. The mesenteric vasculature, kidneys and heart were removed. The kidneys, heart, and left ventricle were weighed and respective indices (kidney index [KI], heart index [HI] and left ventricle index [LVI]; %) were calculated as heart or kidney weight (g)/ body weight (BW) (g) × 100. The LVI was calculated as left ventricle (g)/ heart weight (g) × 100.

6.3.4 Mesenteric artery isolation

After dissection, the mesenteric vasculature was immediately placed in ice-cold Krebs' physiological saline solution (in mM: NaCl 118.2, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $NaHCO_3$ 25, and EDTA, 0.026). Cold Krebs' was continuously bubbled with carbogen (95% O_2; 5% CO_2; BOC Ltd., North Ryde, NSW, Australia) to achieve a pH value of 7.4-7.45 (Intengan et al., 1999).

Adherent connective tissue was removed under a dissecting microscope, following which, a second order mesenteric artery segment (200-400 μm external diameter; ~4 mm in length) was mounted in a pressure myograph (Living Systems Instrumentation, Burlington, VT, USA) in 5 mL of continuously carbogen-bubbled ice-cold Krebs' solution. Two artery segments were dissected from each animal. Artery ends were cannulated with glass micropipettes (1 x 0.25 mm glass capillaries, A-M Systems, Inc., Sequim, WA, USA), and secured with 10-0 sutures (Ethilon® Nylon Suture LLC., California, USA). The remaining mesenteric vasculature was placed in 4% formalin for 24 hours, followed by 70% ethanol until further processing for histological studies.

6.3.5 Pressure myography

Average intraluminal pressure in the artery was increased to 120 mmHg, and the vessel length stretched till there was no lateral bowing of the vessel (Potocnik et al., 2000). Pressure was then decreased to 60 mmHg and the vessel left to equilibrate for 60 min, with Krebs' buffer superfused at a flow rate of 3 mL/min. During this period, the vessel was gradually warmed to 37 °C and constantly bubbled via a miniature gas dispersion tube (Living Systems Instrumentation). Lumen and wall thickness dimensions were measured at a constant intraluminal pressure of 60 mmHg using the video dimension analyser within the pressure myography system for all experimental conditions. Following equilibration, vessel integrity tests, followed by vascular functional and structural investigations were performed sequentially. All data were recorded using Spike 2 v7.07a software (Cambridge Electronic Design, Cambridge, UK). Vessels were considered viable if the α_1-adrenergic receptor agonist phenylephrine (PE; 10^{-6} M) elicited > 50% constriction relative to resting lumen diameter (Behbahani et al., 2010, Luksha et al., 2011).

6.3.5.1 Protocol 1

Following the vessel integrity test, the following protocol was performed sequentially on a second order mesenteric artery segment. Unless otherwise specified, each concentration of the pharmacological agent was extraluminally perfused for 3 minutes, after which vessel dimensions were recorded (Luksha et al., 2011). The concentration used for each drug was determined from previous studies (Chapters 3-5), based on the maximal response as determined from dose-response curves. Twenty min washouts were performed between each protocol.

1. To investigate myogenic tone, intraluminal pressure was increased incrementally via the servo-controlled pump, from 3 to 180 mm Hg (3, 20, 40, 60, 80, 100, 140 and 180 mmHg). Lumen and wall thickness dimensions were measured along the length of the vessel after 3 minutes at each pressure level using the video dimension analyser.
2. Endothelium integrity: Arteries were preconstricted with PE (10^{-6} M), then dilated with the endothelium-dependent vasodilator ACh (10^{-5} M).
3. Vasorelaxation mechanisms: Arteries were incubated for 30 minutes in (i) either N-nitro-L-arginine methyl ester (L-NAME) alone (10^{-4} M) or (ii) indomethacin (indo) (10^{-5} M) and L-NAME combined. Following incubation, arteries were then preconstricted with PE (10^{-6} M), then dilated with the endothelium-dependent vasodilator ACh (10^{-5} M).
4. Vasoconstriction: Arteries were constricted with Ang II (10^{-7} M). Angiotensin II was superfused in the pressure myograph for a minimum of 10 minutes or until steady state constriction was achieved, before vessel dimensions were recorded.
5. At the end of the experimental protocol, vessels were deactivated by superfusion with Ca^{2+}-free Krebs' solution (in mM: NaCl 118.2, KCl 4.7, EGTA 10, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $NaHCO_3$ 25, and EDTA, 0.026) for 20 minutes. To obtain pressure–diameter relationships, intraluminal pressure was increased incrementally via the servo-controlled pump, from 3 to 180 mm Hg (3, 20, 40, 60, 80, 100, 140 and 180 mmHg). Lumen and wall thickness dimensions were measured along the length of the vessel after 3 minutes at each pressure level using the video dimension analyser.

6.3.5.2 Protocol 2

Following the vessel integrity test, the below protocol was performed sequentially on new second order mesenteric artery segment. Unless otherwise specified, each concentration of the pharmacological agent was extraluminally perfused for 3 minutes, after which vessel dimensions were recorded (Luksha et al., 2011). Twenty min washouts were performed between each protocol.

1. Endothelium integrity: Arteries were preconstricted with PE (10^{-6} M), then dilated with the endothelium-dependent vasodilator BK (10^{-6} M).
2. Vasorelaxation mechanisms: Arteries were incubated for 30 minutes in (i) either N-nitro-L-arginine methyl ester (L-NAME) alone or (ii) indomethacin (indo) and L-NAME combined. Following incubation, arteries were then preconstricted with PE (10^{-6} M), then dilated with the endothelium-dependent vasodilator BK (10^{-6} M).
3. Vasoconstriction: Arteries were constricted with ET-1 (10^{-8} M). Endothelin-1 was superfused in the pressure myograph for a minimum of 10 minutes or until steady state constriction was achieved, before vessel dimensions were recorded.

The response induced by the agonists (PE, Ang II, ET-1, ACh and BK) was determined. The differences between responses to ACh and that in the presence of L-NAME alone was considered the NO-dependent (Paulis et al., 2008) component of the ACh-induced response; the ACh-R remaining in the presence of Indo+L-NAME combined was considered the EDH (Luksha et al., 2012) component of the response. The difference between responses in the presence of Indo+L-NAME and L-NAME were considered the prostanoid component of the ACh-induced response. The aforementioned calculations for ACh-induced responses were also performed for BK-induced responses.

6.3.6 Formulas

Media cross sectional area (MCSA): Subtraction of the internal cross sectional area (CSA) from the external CSA. MCSA = π (D_e^2–D_i^2) / 4, where De is the external diameter, and Di is the internal diameter (Laurant et al., 1997, Souza-Smith et al., 2011, Schiffrin and Hayoz, 1997).

6.3.7 Drugs and solutions

Phenylephrine hydrochloride (PE), acetylcholine hydrochloride (ACh), sodium nitroprusside (SNP), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin (indo), bradykinin (BK), angiotensin II (Ang II), and endothelin-1 (ET-1) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All drugs and solutions (Krebs' and Ca^{2+}-free Krebs') were prepared on the day of the experiment. All drugs for pressure myography were dissolved in freshly made Krebs' buffer.

6.3.8 Data analysis

Data were analysed using GraphPad Prism (GraphPad Prism software v6 Inc., La Jolla, CA, USA). All results are expressed as mean ± standard error of mean (SEM). Preliminary analysis of data was performed to identify potential gender effects. Unless significant, gender effects are not otherwise noted.

For comparisons between Lewis and LPK baseline parameters and structural parameters, independent samples *t*-tests were performed. Pearson correlations were performed between ACh or BK response and plasma urea or plasma creatinine.

Data were tested for homogeneity of variance with Levene's test, normality with the Kolmogorov-Smirnov test, and skewness and kurtosis. If assumptions of normality were met, two-way analysis of variance (ANOVA) was then performed using Bonferroni's *post-hoc* analysis to investigate significant differences between groups (as defined by strain and age). Data that did not meet assumptions of normality were analysed with Dunnett's T3 test.

Two-way ANOVA was used to assess myogenic tone differences between Lewis and LPK. Two-way ANOVA were also used to investigate strain and pharmacological effects (ACh/BK alone vs. ACh/BK + L-NAME preincubation and ACh/BK + Indo + L-NAME preincubation, and ACh/BK + L-NAME alone vs. ACh/BK + Indo + L-NAME) for overall concentration-response curves. Significance was defined as $P \leq 0.05$.

6.4 Results

6.4.1 Baseline parameters

Relative to Lewis, LPKs exhibited smaller body weights (Table 6.1). Significant within strain effects were found, where male Lewis animals weighed more than females (males vs. females: 406.75±13.41 g vs. 254.00±6.78 g, $P<0.001$). Lewis polycystic kidney rats had higher blood pressure values relative to Lewis controls. In addition, higher HI, LVI and KI values were observed in LPK, relative to Lewis. Plasma analyses revealed significantly higher urea and creatinine values in LPK relative to Lewis, and urine analysis revealed urinary protein in the LPK samples, and elevated creatinine values when compared to the Lewis control rats.

6.4.2 Increased myogenic tone in Lewis polycystic kidney rats

Lewis polycystic kidney rats exhibited increased myogenic tone relative to Lewis, as shown by the significantly decreased diameter at the highest pressure (180 mmHg) value (Figure 6.1A). In addition, passive pressure-diameter curves revealed significant differences between LPK and Lewis, where LPK were shifted downwards relative to Lewis (Figure 6.1B).

6.4.3 Altered resistance vessel morphology and function in Lewis polycystic kidney rats

Significantly larger wall thickness, wall-lumen ratio and MCSA values were evident in LPK, relative to Lewis controls (Table 6.2). However, internal and external diameter values were not significantly different.

Vasoconstriction in response to PE and ET-1 were not significantly different between strains (Figure 6.2). However, vasoconstriction in response to Ang II was significantly elevated in the LPK rats relative to Lewis, therefore indicating increased responsiveness.

Regardless of agonist used (ACh or BK), LPK response values were significantly smaller relative to Lewis controls (Table 6.3; Figure 6.3). In addition, LPK also had smaller ACh and BK response values in the presence of indo+L-NAME relative to Lewis.

Relative to control ACh or BK responses, preincubation with NOS inhibitor L-NAME reduced the ACh response or BK response for Lewis (Table 6.3). In contrast, preincubation

with a combination of the cyclooxygenase inhibitor indomethacin and L-NAME (indo+L-NAME) resulted in a reduction in ACh response and BK response values for both strains. Preincubation with indo+L-NAME also significantly reduced ACh response and BK response values in LPK, relative to vasorelaxation induced by ACh or BK alone.

The percentage contribution to ACh response and BK response by prostanoid was increased in LPK, relative to Lewis controls, while the percentage contribution to ACh response and BK response by EDH was impaired in LPK relative to Lewis controls (Figure 6.4). In contrast, no significant strain effects were found for the percentage contribution to ACh response by NO.

6.4.4 Correlations between maximum vasorelaxation and renal function

Pearson correlations revealed significant negative correlations between vasorelaxation responses and renal function parameters (plasma urea and plasma creatinine), irrespective of the agonist used (ACh or BK; Table 6.4).

6.5 Discussion

In this study we report for the first time resistance artery myogenic tone, sensitivity of endothelium-derived constrictor (ET-1) and dilators (ACh, BK), and agonist-specificity of endothelium-dependent vasorelaxation in a rat model of CKD. Increased myogenic tone was found in LPK rats at higher pressure values, and resistance artery structural changes involved hypertrophic remodelling, and were accompanied by functional changes involving increased vasoconstriction responses to Ang II and endothelium dysfunction. The degree of endothelium dysfunction was associated with the degree of renal impairment, and was unaffected by different agonists.

The endothelium is an important modulator of myogenic tone (Meininger and Davis, 1992), which plays an important role in the regulation of vascular resistance and therefore blood pressure (Davis and Hill, 1999). Previous studies in mesenteric arteries of adult SHRs have demonstrated enhanced myogenic tone, most notably at pressures beyond physiological range (Izzard et al., 1996). In accordance with these findings, the present study found increased myogenic tone in LPK at the highest pressure value of 180 mmHg, as indicated by the decreased diameter. This means that LPK have increased ability to generate resistance to flow, which is a protective mechanism to prevent end-organ damage, and allows constant blood perfusion through arteries (Kauffenstein et al., 2012).

The LPK exhibited increased maximum percentage vasoconstriction to Ang II, whereas percentage vasoconstriction to PE and ET-1 was not different to control animals. Because Ang II is a potent vasoconstrictor and thus causes a rise in blood pressure (Griffin et al., 1991, Haber et al., 1975), these results build on previous findings (Chapter 4), supporting an important role for Ang II in the mediation of hypertension in the LPK – perhaps via an increased sensitivity or receptor-upregulation mechanism.

In both essential and secondary hypertension, mesenteric arteries from a subtotal nephrectomy model of uraemic hypertension using Wistar Kyoto rats (WKY) have been shown to undergo hypertrophic inward remodelling (New et al., 2004). The LPK exhibited comparable changes, with hypertrophic inward vascular remodelling characterised by an increased wall thickness, increase in wall-lumen ratio, and increased MCSA. Considering these results in association with the effectiveness of AT_1 antagonism treatment found in Chapter 4, it seems likely that in

the LPK model, Ang II via AT_1 receptors may induce remodelling of the arterial wall through smooth muscle growth (Touyz et al., 1999, Gibbons et al., 1992).

Although the present study did not demonstrated enhanced constriction responses to ET-1 in the LPK rats, this does not rule out the possibility that ET-1, together with Ang II, may still contribute to vascular remodelling, given that hypertrophic remodelling is purported to be a characteristic of ET-1 involvement in the hypertensive process (Schiffrin et al., 1996). Angiotensin II stimulates ET-1, which mediates chronic inflammation and has growth-promoting effects on the resistance vasculature (Ammarguellat et al., 2002, Schiffrin, 2005, Pu et al., 2003, Barton and Luscher, 1999, Schiffrin et al., 1995). Some studies have not found increased constrictor responses to ET-1 (Intengan and Schiffrin, 1999, Laviades et al., 1998), suggesting that the augmented vasoconstriction observed in hypertension may be attributable to vascular remodelling around a reduced lumen diameter, whereas other research has argued against this hypothesis (Hahn et al., 1995). Because we found no change in the maximal vasoconstriction to ET-1 and PE in LPK compared to Lewis, but increased responses to Ang II, it seems likely that other factors beyond the vascular remodelling we observed, play a role in contributing to the Ang II response. Perhaps interactions between ET-1 and Ang II may have contributed to the increased vasoconstriction to Ang II; therefore, further studies are necessary to investigate the contribution of and interactions between ET-1 and Ang II in resistance vasculature in the LPK model.

Endothelium dysfunction is a typical feature of hypertension, and may be a consequence of blood pressure elevation (Schiffrin, 2012), as well as being a factor that increases susceptibility to hypertension (Rossi et al., 2004). Consistent with previous chapters, the present study found impaired maximum ACh-induced endothelium-dependent relaxation in LPK. Correlations between responses to ACh or BK and renal function parameters (plasma urea and plasma creatinine) across all animals revealed significant negative correlations between high renal function parameter values and lower response values, regardless of agonist. These findings are in accordance with previous research showing that patients with even mild renal impairment have associated impaired ACh-mediated relaxation (Perticone et al., 2004). The more severe the uraemia, the worse the endothelium dysfunction due to oxidative stress and reduced stimulation of NO (Morris et al., 2001; Passauer et al., 2005).

Significant strain differences were also seen for the prostanoid and EDH components of the vasodilatory response, where prostanoid and EDH components were up and down regulated, respectively, in the LPK relative to Lewis controls. Although the role of prostanoids is primarily a vasodilatory effect, in disease conditions such as hypertension, they have been purported to contribute to endothelium-derived constricting factors (Rapoport and Williams, 1996, Gluais et al., 2005). In the present study, prostanoid components may have been up regulated in the LPK in an attempt to improve vasorelaxation due to deficient NOS and EDH functioning, as a possible compensatory physiological pathway (Versari et al., 2009). The impairment of EDH, on the other hand, is consistent with Borges et al. and Fuji et al., who found that EDH function was lost in the mesenteric vascular bed of SHR (Borges et al., 1999, Fujii et al., 1992).

Studies investigating endothelium dysfunction in resistance arteries from ESRD patients and SHRs have suggested that impairments of responses are receptor-specific (Wirth et al., 1996, Luksha et al., 2012). That is, depending on the agonist used (BK or ACh), different degrees of endothelium dysfunction and vasorelaxation mechanism impairments may be evident. In the present study, in addition to using ACh we also employed BK in our investigations of endothelium vasorelaxation. In contrast to previous research, however, our findings indicated that the degree of impairment was the same in the LPK, regardless of which agonist was used (ACh or BK). In addition, when vasorelaxation components were investigated individually, agonist-specific mechanisms of endothelium dysfunction were still not evident. These results could perhaps be compared to Wirth et al.'s findings in older SHR, which revealed comparable vasorelaxation responses to ACh and BK (Wirth et al., 1996). Wirth et al. also found that increased sensitivity of BK-induced responses are evident in the early stages of hypertension (Wirth et al., 1996). Therefore, given that our LPK were investigated at 18 weeks of age, when advanced renal disease and hypertension are well established, perhaps our findings are due to the significant degree of vascular injury advancement in LPK.

In conclusion, this study shows enhanced myogenic tone which is evident at high pressure values in the LPK. Our study of potential underlying mechanisms suggests this may be modulated by an imbalance of endothelium-derived constricting and relaxing factors. Lewis polycystic kidney rats exhibited increased maximum vasoconstriction responses to Ang II along with hypertrophic remodelling, yet comparable vasoconstriction responses to Lewis for

PE and ET-1. Endothelium dysfunction was evident in LPK, with the degree of renal impairment being associated with the extent of endothelium dysfunction, regardless of agonist used (BK or ACh). In addition, impaired vasorelaxation was evident for either agonist, and seemed to be largely due to an impairment of the EDH vasodilatory component, and partially due to impaired NOS activity, and was accompanied by an upregulation of the prostanoid component. In summary, the present highlights the importance of endothelium-derived constricting and relaxing factors, which may be a significant contributor to the impaired modulation of myogenic tone, and suggest an absence of agonist-specificity for endothelium-dependent vasorelaxation investigations in rats with established hypertension and severe renal disease.

6.6 Acknowledgements

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6.7 Conflict of interest

None declared.

Tables

Table 6.1: Baseline and biochemical parameters of 18 week old Lewis and Lewis polycystic kidney rats.

Parameter	Lewis	LPK
n	7	7
BW(g)	330±30	254±19*
SBP (mmHg)	113±2	190±9*
HI (%)	0.28±0.01	0.55±0.05*
LVI (%)	79.61±1.04	86.02±0.99*
KI (%)	0.74±0.03	8.43±0.32*
Plasma urea (mmol/L)	7.83±0.34	45.02±1.38*
Plasma creatinine (µmol/L)	33.94±1.69	214.00±18.70*
Urine Protein (g/L)	nd	1.13±0.25
Urine Creatinine (g/L)	1.58±0.20	0.34±0.06*
UPC	nd	4.06±1.39

BW, body weight; SBP, systolic blood pressure; HI, heart index; LVI, left ventricle index; KI, kidney index; nd, not determined; UPC, urine protein creatinine ratio; LPK, Lewis polycystic kidney rat. Data were evaluated by independent samples *t*-tests. Results are expressed as mean ± SEM. *, $P<0.05$ strain effect between Lewis and LPK.

Table 6.2: Mesenteric artery geometrical parameters from 18 week old Lewis and Lewis polycystic kidney rats.

Parameter	Lewis	LPK
n	8	8
Internal diameter (μm)	219.75±23.10	177.06±9.55
External diameter (μm)	237.88±12.92	272.92±14.21
Wall thickness (μm)	28.38±1.63	52.30±2.54*
Wall-lumen ratio	0.15±0.02	0.30±0.03*
MCSA ($x10^3$) (μm^2)	21.68±1.48	38.79±1.73*

MCSA, media cross sectional area; LPK, Lewis polycystic kidney rat. Second order mesenteric artery structural parameter data was obtained at 60 mmHg following Ca^{2+}-free superfusion. Data were evaluated by independent samples *t*-tests. Results are expressed as mean ± SEM. *, $P<0.05$ strain effect between Lewis and LPK.

Table 6.3: 18 week old Lewis and Lewis polycystic kidney second order mesenteric artery vasodilatory responses to acetylcholine or bradykinin alone, or following incubation in various pharmacological agents.

Pharmacological agent	ACh response		BK response	
	Lewis	LPK	Lewis	LPK
n	7	8	8	8
ACh or BK	88.75±5.21	66.19±4.49*	95.09±4.61	65.02±4.84*
L-NAME	66.92±5.94[a]	55.48±4.73	66.86±6.95[a]	49.17±5.86
Indo+L-NAME	63.08±5.98[b]	33.90±7.06* [b c]	71.64±7.81[b]	23.94±7.06* [b c]

LPK, Lewis polycystic kidney rat; L-NAME, N^{ω}-nitro-L-arginine methyl ester; Indo, indomethacin; ACh, acetylcholine; BK, bradykinin. All data were evaluated by two-way ANOVA, followed by Bonferroni's *post-hoc* analysis. Results are expressed as mean ± SEM. *, $P<0.05$ strain effect between Lewis and LPK; a, $P<0.05$ pharmacological agent effect within strains between ACh or BK and L-NAME; b, $P<0.05$ pharmacological agent effect within strains between ACh or BK and indo+L-NAME; c, $P<0.05$ pharmacological agent effect within strains between L-NAME and indo+L-NAME.

Table 6.4: Mesenteric artery correlations between acetylcholine response or bradykinin response and plasma creatinine or plasma urea values from 18 week old Lewis and Lewis polycystic kidney rats combined.

Kidney function parameter		ACh response	BK response
n		14	14
Plasma creatinine (μmol/L)	Pearson r	-0.689	-0.725
	P value	0.003	0.002
Urea (mmol/L)	Pearson r	-0.689	-0.749
	P value	0.003	0.001

ACh, acetylcholine; BK, bradykinin. Pearson correlation (r) values provided for maximum relaxation responses between endothelium-dependent (ACh or BK) mechanisms and kidney function parameters (plasma creatinine and plasma urea) in mesenteric arteries of Lewis and Lewis polycystic kidney (LPK) rats combined. Data was analysed using linear regression with a one tailed test for significance ($P \leq 0.05$).

Figures

Figure 6.1: Myogenic tone and pressure-diameter curves in Lewis and Lewis polycystic kidney rat mesenteric artery.

Eighteen week old Lewis and Lewis polycystic kidney rat (LPK) mesenteric resistance artery myogenic tone graphs (A) showing changes in internal diameter in response to increases in pressure (mmHg). Panel B illustrates passive pressure-diameter (diameter, μm) curves in response to changing pressure (mmHg) in Ca^{2+}-free Krebs' solution. Data were evaluated by two-way ANOVA. Results are expressed as mean ± SEM. *, $P<0.05$ strain effect between Lewis and LPK.

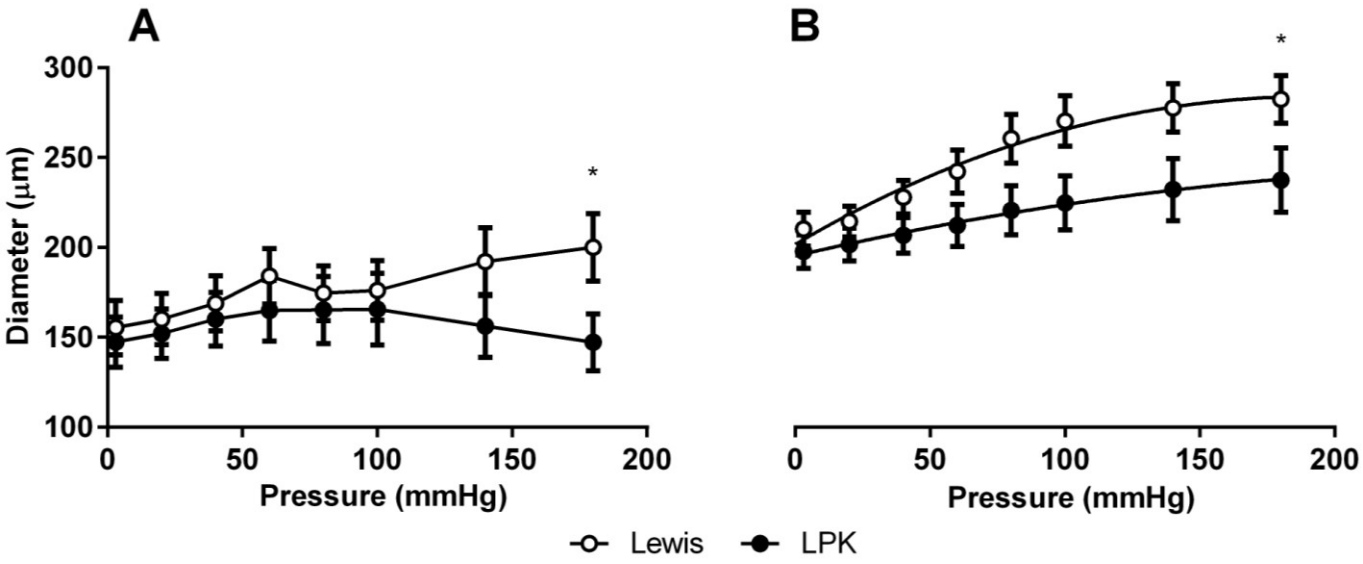


Figure 6.2: Percentage vasoconstriction in response to various constrictors in Lewis and Lewis polycystic kidney rat mesenteric artery.

Eighteen week old Lewis and Lewis polycystic kidney rat (LPK) mesenteric resistance artery percentage vasoconstriction (%) in response to single doses of phenylephrine (PE 10^{-6} M), angiotensin II (Ang II, 10^{-7} M), or endothelin-1 (ET-1, 10^{-8} M). Data were evaluated by independent samples *t*-tests. Results are expressed as mean ± SEM. *, $P<0.05$ strain effect between Lewis and LPK.

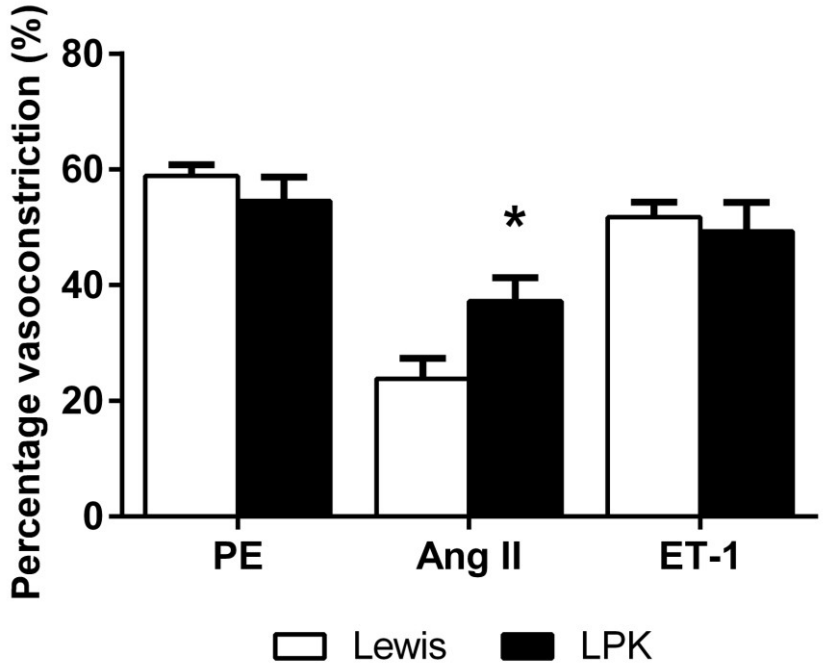


Figure 6.3: Percentage relaxation in response to vasodilators in Lewis and Lewis polycystic kidney rat mesenteric artery.

Eighteen week old Lewis and Lewis polycystic kidney rat (LPK) mesenteric resistance artery percentage relaxation (%) in response to acetylcholine (ACh, 10^{-5} M) and bradykinin (BK, 10^{-6} M). Data were evaluated by independent samples *t*-tests. Results are expressed as mean ± SEM. *, $P<0.05$ strain effect between Lewis and LPK.

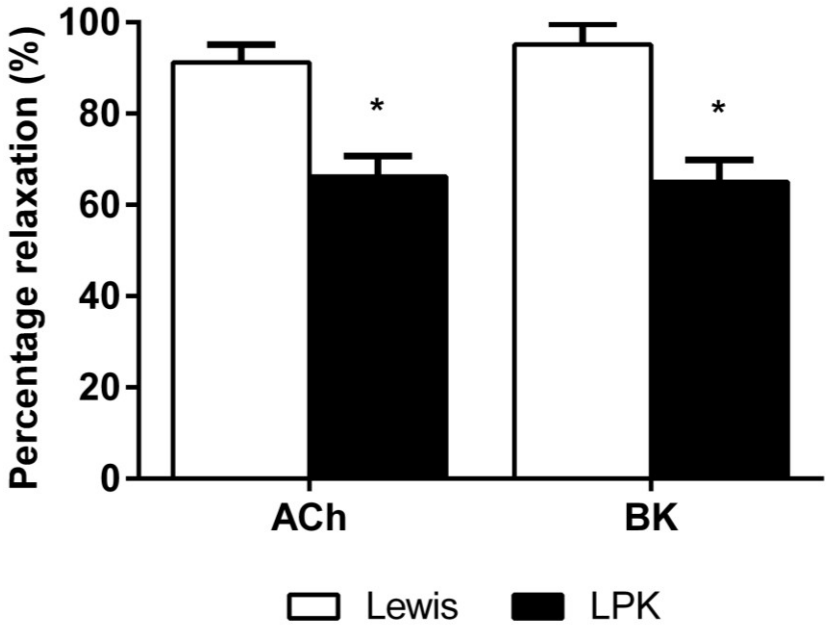
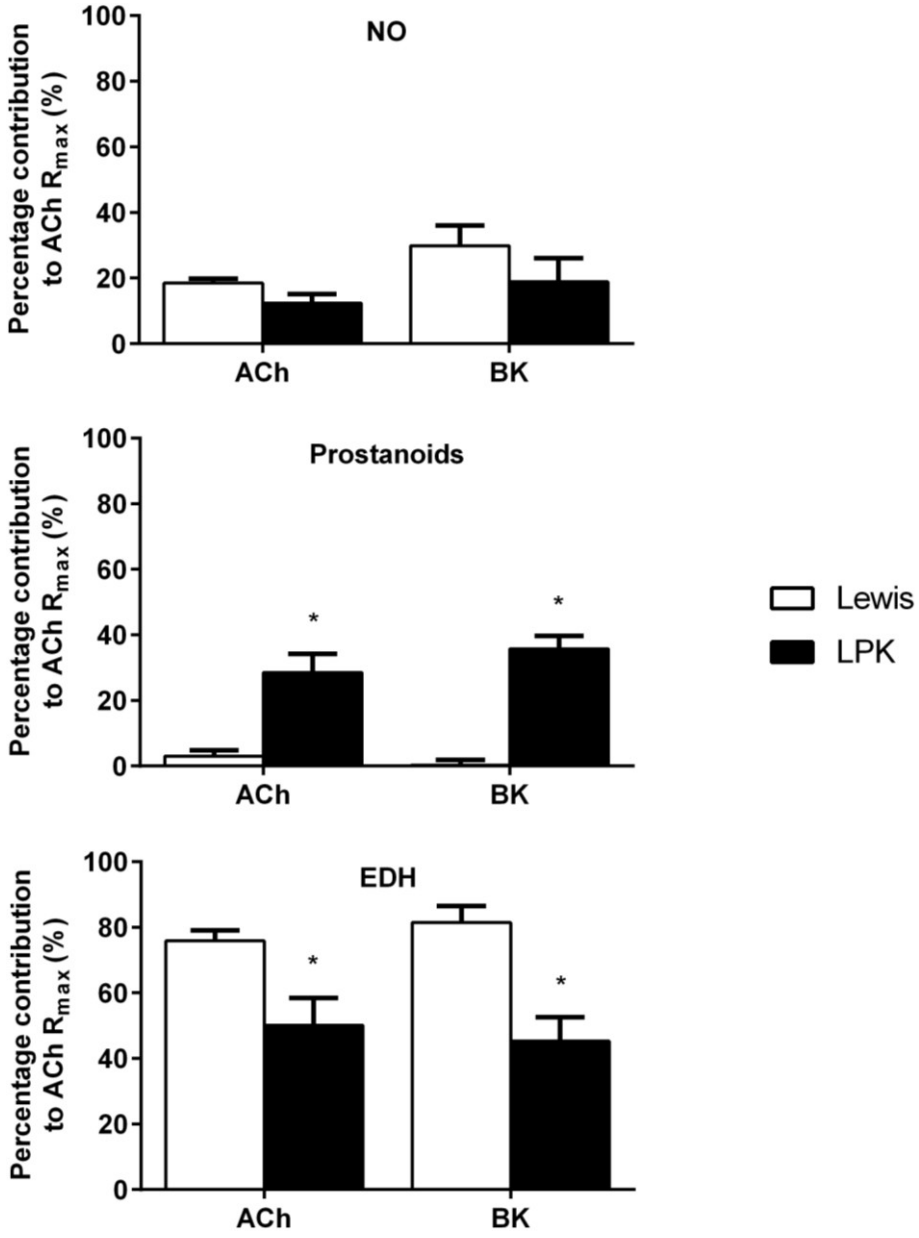


Figure 6.4: Relative contribution of vasodilatory mediators to acetylcholine responses in Lewis and Lewis polycystic kidney rat mesenteric artery.

Contribution of each vasodilatory component (nitric oxide, NO; prostanoid and endothelium-derived hyperpolarisation, EDH) to acetylcholine (ACh) or bradykinin (BK) -induced relaxation in 18 week old Lewis and Lewis polycystic kidney rat (LPK) mesenteric artery. Data were evaluated by two-way ANOVA. Results are expressed as mean ± SEM. Significant difference between groups where indicated $P<0.05$. Data were evaluated by independent samples *t*-tests. Results are expressed as mean ± SEM. *, $P<0.05$ strain effect between Lewis and LPK.



7

Chapter 7

Final discussion

Quek, K.J.

7.1 Discussion

Hypertension is associated with more deaths and disease than any other biomedical risk factor (Lopez et al., 2006, AIHW, 2011), and results in increased morbidity and mortality because of its negative effects on end-organ damage, which lead to several severe pathological outcomes such as kidney and cardiovascular disease (CVD). Hypertension is of particular importance in chronic kidney disease (CKD) patients, who are more likely to die from CVD than CKD itself: thus making kidney disease the most common "secondary" form of hypertension (Cohen and Townsend, 2009, Ross and Banerjee, 2013). Hence, further research in this area is required to understand and improve pathophysiological outcomes in CKD, and ultimately provide more effective therapeutic outcomes.

Mesenteric resistance arteries are believed to be a representative portion of the peripheral vascular bed in hypertension, and remodel in a similar way to hypertensive human patients (Short, 1966). This thesis provides valuable insight into the vascular changes that mesenteric arteries undergo in the CVD and CKD states, and also helps to reveal the unique therapeutic outcomes that different treatments have on these resistance arteries. This thesis presented 4 studies that investigated alterations in the resistance vasculature in a rat model of CKD, the Lewis polycystic kidney (LPK), with a specific focus on structure, function and biomechanical properties. This research demonstrated that following the early onset of both hypertension and renal dysfunction in the LPK rats, alterations in the resistance arteries develop that appear to be an attempt to protect end-organs and mitigate the increased wall stress against the arterial wall. These compensatory mechanisms, however, eventually become maladaptive changes and lead to an exacerbation of the disease state, and likely contribute to the maintenance and progression of hypertension. We further aimed to investigate the specific changes that resistance arteries undergo to contribute to the maintenance of hypertension in CKD, and the effectiveness of treatment in mitigating these deleterious effects.

Previous investigations have established that the LPK, a rodent model of CKD, arose due to a spontaneous mutation in the *Nek8* gene (McCooke et al., 2012), resulting in a form of nephronophthisis 9 (NPHP9) (Phillips et al., 2007). The LPK has a phenotypic representation resembling that of human autosomal recessive polycystic kidney disease (ARPKD), presenting with established hypertension by 6 weeks of age, and demonstrating initial signs of

renal dysfunction at 12 weeks of age, with renal function significantly declining by 18–24 weeks of age (Phillips et al., 2007). Hence, the LPK rat provides a very useful model system for the study of the natural progression of CKD (Ng et al., 2011a), and was used for all experimental chapters in this thesis.

Previous studies investigating LPK aorta by Ng et al. (2011b) and Ameer et al. (2014), demonstrated that in association with established hypertension and renal failure, LPK rats develop large vessel stiffness, calcification, vascular remodelling, and alterations in biomechanical properties by 12 weeks of age. Research by others which examined resistance arteries in the renal impairment milieu found eutrophic inward remodelling in uraemic resistance arteries (cremaster and mesenteric) (New et al., 2004), and vascular dysfunction in the subcutaneous resistance arteries of patients with end-stage renal disease (ESRD) (Luksha et al., 2012). Therefore, we hypothesised that similar changes would be evident in the small mesenteric resistance arteries of LPK rats, and specifically investigated the temporal changes of resistance artery 1) structural, 2) functional and 3) biomechanical properties, in association with blood pressure and renal function (Chapter 3). Our data showed that resistance arteries exhibited progressive dysfunction that was not necessarily associated with the progression of hypertension and indicators of renal functioning. The structural alterations were characterised by eutrophic and hypertrophic inward remodelling at established (12 week) and late (18 week) renal disease time points, respectively, with hypertension being a significant, main predictor of structural alterations. Functional alterations were characterised by increased vasoconstriction sensitivity to phenylephrine (PE) in 18 week LPK, and impaired endothelium-dependent relaxation in 12 and 18 week LPK. This impairment seemed to be largely due to dysfunction of the endothelium-dependent hyperpolarisation (EDH) vasodilatory component, partially due to impaired nitric oxide synthase (NOS) activity, and accompanied by an upregulation of prostanoid activity. Increased stiffness in 6 and 18 week LPK was accompanied by increased collagen/elastin ratios, as well as increased elastic modulus vs. stress slopes. In contrast, at 12 weeks, LPK vasculature showed increased compliance. Overall, Chapter 3 revealed that hypertension in CKD results in myriad of effects on resistance arteries, which changes as hypertension and renal disease progresses, and interact with one another. Structural vascular remodelling, although an initially adaptive mechanism, eventually becomes maladaptive, thus exacerbating the disease state through its impairment of endothelial functioning, and its effects on biomechanical properties and vessel

composition, leading to an increase in the stiffer collagen components of the arterial wall. Critical impairments in the LPK vasculature arise at as early as 6 weeks, and generally worsen as hypertension pursues and with progression to late stage renal disease. This indicates the importance of resistance artery remodelling in contributing to the severity of disease.

After establishing the presence of resistance artery impairments in the LPK vasculature, we sought to next investigate the effectiveness of chronic pharmacological treatments (4-18 weeks of age) in mitigating this wide variety of alterations observed (Chapters 4 and 5). Given the important contributory role of the renin angiotensin system (RAS) and L-type Ca^{2+} channels (LTCCs) in hypertension and renal disease, as well as the proven effectiveness of angiotensin type 1 (AT_1) receptor antagonism and calcium channel blockade in the treatment of hypertension in CKD, Chapters 4 and 5 sought to examine the effects of valsartan and amlodipine on the resistance vasculature.

The effects of RAS antagonism on the LPK aorta have been studied previously, with the angiotensin converting enzyme (ACE) inhibitor perindopril correcting evidence of abnormal aortic stiffness (Ng et al., 2011a). Valsartan is an angiotensin type 1 receptor antagonist, enabling specific targeting of angiotensin II (Ang II), which plays an important role at the vasculature level, in driving alterations of the vascular nitric oxide (NO) pathway (Lee et al., 2013), stimulation of reactive oxygen species (ROS) (Montezano et al., 2014), structural vascular remodelling (Bruder-Nascimento et al., 2014), vascular smooth muscle cell (VSMC) hypertrophy, and inflammation (Schiffrin, 2004, Zhang et al., 2005). Previous research has shown that blocking Ang II results in myriad of effects, including decreased vasoconstriction, blood pressure reduction, decreased sympathetic nerve activation, decreased aldosterone release, reduced collagen deposition, reduced cell proliferation and inflammation in the vasculature, and reduced vascular remodelling (Benetos et al. 1997, Iino et al., 2003, Chang et al., 1995, de Gasparo et al., 2000). Our data showed that in a rat model of CKD, AT_1 receptor antagonism improved resistance artery outcomes in LPK: 1) valsartan corrected vascular remodelling in LPK, resulting in normalisation of wall-lumen ratio and wall thickness values, and an increase in internal and external diameter values; 2) valsartan treatment ameliorated increased sensitivity to PE (Chapter 3) and normalised endothelial dysfunction; 3) compliance was increased with treatment although not normalised, and intrinsic stiffness was corrected to levels comparable to the Lewis normotensive rats. Overall, Chapter 4 highlighted the

importance of Ang II and AT_1 receptors in contributing to abnormalities in LPK resistance artery structural, functional and biomechanical properties. Valsartan also effectively reduces the hypertension, however it is unclear what mechanisms valsartan treatment acts through to exert its effectiveness. Valsartan may directly improve LPK resistance vessels functionality, through its blockade of vascular Ang II actions which promote vascular injury (Shan et al., 2008), and can therefore improve vascular NO mechanisms, or, valsartan may also affect the vasculature through its enhancement of local Ang II's effects, which can consequently stimulate unblocked AT_2 receptors, which have vasorelaxant effects (Bayraktutan, 2003). Alternatively, valsartan may indirectly improve vascular outcomes because of its blood pressure-lowering effects decreasing shear stress against the arterial wall, and consequently improving endothelial function (Mizuno et al., 2008). Therefore, to further investigate whether these beneficial outcomes of valsartan treatment were a direct result of Ang II inhibition or indirectly through beneficial effects that are beyond blood pressure-reduction, we performed treatment with amlodipine, a calcium channel blocker (CCB), to investigate response of the resistance vasculature to a drop in blood pressure independent of the RAS.

Under normal vascular conditions, LTCCs play an important regulatory role in Ca^{2+} influx levels, and in the maintenance of vascular resistance and therefore blood pressure (Sonkusare et al., 2006). However, in a disease state such as hypertension, LTCC function has been shown to be enhanced, and its expression upregulated in the mesenteric vasculature (Lawton et al., 2012). Therefore, we sought to investigate the effect of CCBs on LPK resistance artery structure, function and biomechanical properties. Our data showed that calcium channel blockade with amlodipine in the LPK resulted in positive outcomes: 1) amlodipine improved vascular remodelling, normalising internal and external diameter, and wall-lumen ratio; 2) endothelial dysfunction was improved with amlodipine treatment, and increased sensitivity to PE was abolished.

Interestingly, although amlodipine treatment improved compliance in Lewis controls, it had no effect on LPK stiffness. The blood pressure-lowering effects of amlodipine may have resulted in the favourable outcomes we documented, due to decreased wall stress against the arterial wall, and therefore improved endothelial function (Mizuno et al., 2008). Amlodipine may directly improve LPK resistance vessel functionality via blockade of calcium influx and/or its ability to stimulate NO release independent of calcium channels, its antioxidant

activity and preservation of endothelial integrity (He et al., 2011). Alternatively, amlodipine may also affect the vasculature through its consequent vasodilatory effects. Overall, Chapter 5 highlighted the importance of blood pressure-reduction via LTCC blockade in producing favourable outcomes for LPK resistance arteries. Relative to valsartan treatment (Chapter 4), the effectiveness of amlodipine was less. This may be due to the greater antihypertensive outcome of valsartan, or it could support the hypothesis that Ang II actions are a major contributor to myriad of effects on the vasculature (Iino et al., 2003, Chang et al., 1995, de Gasparo et al., 2000), that result in structural and functional changes in the vasculature in disease states, which are independent of its blood pressure-lowering effects.

Although both valsartan and amlodipine reduced sensitivity to PE, the mechanisms through which these treatments exerted their effects may differ. In the normal vasculature, Ang II potentiates the sympathetic system; therefore, valsartan's antagonism at the AT_1 receptor may have decreased Ang II's effects, and consequently resulted in less sensitivity to sympathetic stimulation. On the other hand, because α_1-mediated vasoconstriction requires calcium, treatment with a CCB results in reduced calcium availability, and therefore decreased degree of constriction to PE.

The initiation of treatment at 4 weeks was because this was the earliest age that LPKs could be weaned from their mothers and subsequently trained to voluntarily consume the pharmacological agents (valsartan or amlodipine). Given that Chapter 3 investigated LPKs at 6 weeks, whereas treatment interventions started at 4 weeks of age, it is difficult to conclude whether the interventions performed in Chapters 4 and 5 are preventative, reversing, or a combination of both. Therefore, future investigations of the effectiveness of pharmacological agent interventions may involve the initiation of treatment at later time points (such as 12 weeks of age), and compare these later interventions with the earlier drug administrations (from 4 weeks of age). In addition to this, perhaps further studies may involve combination treatment regimens, of both valsartan and amlodipine, to investigate the potential synergistic effects of these agents, and experimentation of denuded mesenteric vessels and other resistance arteries may be performed to explore any potential parallel changes in vascular beds.

The endothelium is believed to be an important modulator of myogenic tone (Meininger and Davis, 1992), where vasoactive factors from the endothelium can affect vascular resistance (Cipolla, 2009). Further, research has shown that endothelial dysfunction findings may vary in different disease states, depending on the agonist used (acetylcholine [ACh] or bradykinin [BK]). Therefore, we further investigated the signalling mechanisms underlying the vascular pathology that was identified in previous chapters. Results indicated that increased myogenic tone was evident in LPK, and while increased maximum vasoconstriction responses to Ang II were also present in the LPK, maximal vasoconstriction responses were not different between strains for PE and endothelin-1 (ET-1). Interestingly, Chapter 6's PE response findings (to a single dose of PE) revealed no significant differences between Lewis and LPK rats, whereas Chapters 3-5 showed leftward shifted concentration-response curves for LPK rats relative to Lewis controls. Collectively, these findings indicate that vasoconstriction to a single dose of PE (at a concentration of 10^{-6} M) does not reveal strain differences; however, in response to increasing concentrations of PE, LPK show increased sensitivity. This gives rise to the possibility that the present study may not have found significant vasoconstriction responses to Ang II and ET-1 due to administration of these pharmacological agents via a single dose. Therefore, future studies could construct concentration-response curves to Ang II and ET-1, to further investigate possible strain differences. Other findings in Chapter 6 showed that the degree of renal impairment correlated with the extent of endothelium dysfunction, regardless of agonist used (ACh or BK): perhaps because of increased oxidative stress and decreased NO in the severe uraemic state, resulting in impaired endothelium-mediated vasorelaxation (Morris et al., 2001; Passauer et al., 2005). Overall, findings from Chapter 6 highlighted the importance of endothelial-derived constricting and relaxing factors, and suggested an absence of agonist-specificity for endothelium-dependent vasorelaxation investigations in rats with established hypertension and advanced renal disease.

A discrepancy in findings noted between chapters is the nature of the remodelling observed in the mesenteric artery of LPK at 18 weeks: Chapters 3 and 6 found hypertrophic remodelling, whereas Chapters 4 and 5 found eutrophic remodelling. One possible explanation for these different findings is variation due to sampling variation between litters, with some LPK litters more likely to undergo one type of remodelling relative to the other. Note that, although LPK were ordered in litters (approximately 4-6 rats), and divided equally into treatment conditions,

in an attempt to reduce the likelihood of variability between batches affecting overall findings, the possibility of between-litter variability cannot be ruled out. Nonetheless, while hypertrophic remodelling is considered more severe and advanced, both eutrophic and hypertrophic inward remodelling are detrimental, and both result in the exacerbation and maintenance of hypertension due to a decreased lumen diameter and increased wall thickness.

7.2 Perspectives

The pathogenesis of CVD in CKD is complicated, with various factors playing contributing or exacerbating the maintenance of hypertension, including endothelial dysfunction (Wang et al., 1999, Wang et al., 2000), RAS over-activation (Boudoulas et al., 2012), and vascular remodelling (Mulvany, 2011, Schiffrin, 2012). These factors interact with each other to result in a worsening of CKD, and thus increase cardiovascular complications. This thesis has identified structural, functional and biomechanical property alterations that resistance arteries undergo in a hypertensive model of CKD, and reports the superior effectiveness of AT_1 receptor antagonism over LTCC blockade treatment in the amelioration of vascular/renal alterations, due to the disruption of Ang II's effects in addition to general antihypertensive results. As a result of the uncertainty of these comorbid disease states, clearly more investigations are still necessary to understand the mechanisms that lead to the development of vascular damage, and the subsequent maintenance and exacerbation of CVD and CKD. Extending our findings of the resistance vasculature in CKD, future research may involve investigations of changes in receptor expression (for example, ET-1, AT_1, Ang II muscarinic and kinin receptors) in LPK resistance arteries, and hind limb blood flow to further examine vascular resistance. Insight of receptor expression may assist in the development of future treatment targets, while hind limb blood flow investigations will provide *in vivo* insight of vasculature in the CVD and CKD states. In addition, further investigations may be performed to understand the underlying mechanisms behind reduced EDH and NO functioning, and upregulated prostanoid contributions seen in endothelium-dependent vasorelaxation in LPK: perhaps, for example, via conducting channel-specific studies to probe the role of EDH. Finally, following from the series of chapters presented in this thesis, the role of ROS and its

potential contribution to endothelium dysfunction is largely unexplored, and hence further studies of its expression levels may be interesting for future directions.

8

Chapter 8

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Appendix 1

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2011/044 – 14 **Date of Expiry:** 11 September 2014

Full Approval Duration: *12 September 2011 to 11 September 2014 (36 months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
Prof. Jacqueline Phillips
Australian School of Advanced Medicine
Macquarie University NSW 2109
0409 225 707
Jacqueline.phillips@mq.edu.au

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above,
Animal Welfare Officer - 9850 7758 / 0439 497 383,
or **Manager, CAF** - 9850 7780 / 0428 861 163

Associate Investigators:

Alberto Avolio	0408 657 616
Cara Hildreth	0412 266 420
Mark Butlin	0422 908 895
Melissa Farnham	0415 821 096
Qi-Jian Sun	0413 733 250

Other people participating:

Ms Alice Ding	0433 665 967
Ms Divya Sarma Kandukuri	0432 435 596
Mr Omar Al-Adhami	0426 292 791
Mr Ibrahim Salman	0426 286 182
Barbara Zangerl	0426 849 669
George Lindesay	(02) 9850 9812
Yimin Yao	0422 672 507
Rochelle Boyd	0409 322 382
Ko Jin Quek	0404 124 209
Manash Saha	0469 344 124

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Do stiff blood vessels or overactive nerves cause long-term high blood pressure in kidney disease?**

Purpose: 4 – Research: human or animal biology

Aim: This project aims to use an animal model to determine if drugs which block the hormone angiotensin II therapy for hypertension reduces the stiffness of blood vessels or elevated nerve activity.

Surgical Procedures category: 3 (Minor conscious intervention)

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved:

Species	Strain	Sex	*Age*	*TOTAL*	Supplier/Source
Rat	Lewis	M/F	3-18 wks	156	ARC Perth
Rat	LPK	M/F	3-18 wks	156	ARC Perth/Westmead Hospital
Rat	SHR	M/F	4-24 wks	156	ARC Perth
Rat	WKY	M/F	4-24 wks	156	ARC Perth
		TOTAL		**624**	

Location of research:

Location	*Full street address*
Australian School of Advanced Medicine	Level 1, Clinic Building, 2 Technology Place, Macquarie University NSW 2109
Central Animal Facility	Building F9A, Research Park Drive, Macquarie University NSW 2109

Conditions of Approval: N/A

Amendments approved by the AEC since initial approval:

1. Addition of new personnel to protocol: Barbara Zangerl (approved May 2012).
2. Addition of new personnel to protocol: George Lindesay (approved June 2012).
3. Addition of new personnel to protocol: Yimin Yao subject to adequate supervision (approved July 2012).
4. Addition of Melissa Farnham as Associate Investigator (approved July 2012).
5. Modification of experimental procedure (approved 16 August 2012).
6. Change of age of animals used (approved 16 August 2012).
7. Addition of new personnel to protocol: Marie-Ange Kouassi (approved September 2012).
8. Addition of new personnel to protocol: Rochelle Boyd (approved & ratified December 2012).
9. Addition of new personnel to protocol: Ko Jin Quek (approved February 2013).
10. Addition of new personnel to protocol: Manash Saha as Student (Executive approved 17 April 2013, ratified by AEC 18 April 2013).
11. Increase dose range of Valsartan to 60mg/kg/day for LPK rats (Executive approved 23 May 2013, ratified by AEC 13 June 2013)
12. Add procedures performed under anaesthesia to include manipulation of blood pressure & venous return (Approved 13 June 2013)
13. Addition of Qi-Jian Sun as Associate investigator (Executive approved 6 August 2013, ratified by AEC 15 August 2013)
14. Alternative method to provide drug treatment (Executive approved 5 August 2013, ratified by AEC 15 August 2013)
15. Increase maximum dose of Amlodipine to 20mg/kg/day and begin BP treatment for SHR at the earlier age of 8 weeks (Executive approved 30 August 2013, ratified by AEC 19 September 2013)

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal supplier's license.

Prof Mark Connor (Chair, Animal Ethics Committee) **Approval Date:** 19 September 2013

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2014/048 -3 **Date of Expiry:** 28 October 2015

Full Approval Duration: *28 October 2014 to 28 September 2017*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
Professor Jacqueline Phillips
Australian School of Advanced Medicine
Macquarie University, NSW 2109
0409 225 707
Jacqueline.Phillips@mq.edu.au

Associate Investigator:
Omar Al – Adhami 0426 292 791

Other:
Rochelle Boyd 0409 322 382
Ko Jin Quek 0404 124 209

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or Manager, CAF: 9850 7780 / 0428 861 163 and **Animal Welfare Officer**: 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Arterial structural and functional alterations in chronic kidney disease: a study in rodents**

Purpose: **5 - Research: Human or Animal Health and Welfare**

Aims: The small arteries of the body (resistance vessels) play a significant role in the maintenance of blood pressure. In CKD (Chronic Kidney Disease), these vessels can become thickened, stiff and non-responsive, however the mechanisms underlying these changes are not well understood. We want to define these mechanisms and investigate how they relate to high blood pressure and the decline in renal function, and how hormones such as angiotensin II contribute to the disease process.

Surgical Procedures category: 2 - Animal Unconscious without Recovery

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex/Weight	Total	Supplier/Source
02 – Rattus	Lewis	4 weeks/any	60	ARC Perth
02 – Rattus	Lewis Polycystic Kidney	4 weeks/any	60	ARC Perth
		TOTAL	120	

Location of research:

Location	*Full street address*
Central Animal Facility	Building F9A, Research Park Drive, Macquarie University, NSW 2109
ASAM	Level 1, F10A, 2 Technology Place, Macquarie University, NSW 2109

Amendments approved by the AEC since initial approval:
1, Amendment #1 – Addition of Omar Al –Adhami as Associate Investigator(Executive approved, ratified by AEC 11 December 2014)
2, Amendment #2 – Request to change usage of animal (Executive approved, ratified by AEC 11 December 2014)

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Dr Karolyn White (Acting Chair, Animal Ethics Committee) **Approval Date:** 11 December 2014