UNIVERSITY OF LEICESTER

Investigating The Roles That G Protein-Coupled Receptor Kinase 2 Plays in Arterial Smooth Muscle Proliferation

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ABSTRACT ASMA S ALONAZI

Investigating The Roles That G Protein Coupled Receptor Kinase 2 Plays in Arterial Smooth Muscle Cell Proliferation

Vascular smooth muscle cell (VSMC) proliferation plays a key role in the development of hypertensive vascular remodelling, a process strongly associated with increased circulating vasoconstrictor concentrations such as angiotensin-II (AngII), endothelin-1 (ET1) and uridine-5'-triphosphate (UTP), leading to continuous activation of their cognate $G_{\alpha q}$-coupled/G protein-coupled receptors (GPCR). Furthermore, hypertension is associated with elevated G protein coupled receptor kinase 2 (GRK2) expression in arterial smooth muscle cells, which negatively regulates $G_{\alpha q}$/GPCR signalling. Indeed, GRK2 has been shown to play a key role in cell growth and development of the cardiovascular system, and the regulation of cell cycle progression. Therefore this study aimed to investigate the potential roles that GRK2 plays in VSMC proliferation, and in particular on two signalling pathways that have been implicated in this process: mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/Akt.

Initial studies indicated that inhibition of MAPK/ERK and PI3K/Akt prevented VMSC growth stimulated by ET1 and AngII, while UTP did not stimulate proliferation. Similarly, GRK2 depletion or catalytic inhibition prevented ET1/AngII-stimulated VSMC proliferation. Furthermore, prolonged ET1 and AngII-stimulated ERK signalling was dependent on GRK2 expression and catalytic activity, whereas these treatments enhanced UTP-stimulated signals. Moreover, ET1-stimulated ERK signals were arrestin3-dependent, whilst AngII signals were arrestin2-dependent. Conversely, GRK2 expression but not its catalytic activity was essential to facilitate ET1 and AngII-stimulated PI3K/Akt signals, whereas UTP signals were unaffected. Interestingly, ET1/AngII-stimulated PI3K/Akt signalling was arrestin-independent. Similarly, ET1/AngII signalling to the Akt target GSK3 was ablated following GRK2 knockdown.

Collectively, these data highlight two different molecular mechanisms underlying GRK2-mediated regulation of VSMC proliferation; with ERK-stimulated growth being dependent on GRK2 kinase activity and arrestin recruitment, and the other presumably utilizing GRK2 as a PI3K scaffold to facilitate efficient PI3K/Akt/GSK3 signalling. Thus, increased GRK2 expression in hypertension could be an underlying factor promoting long-term proliferative pathways and consequently, contribute to VSMC proliferation, vascular wall thickening, and hypertensive vascular remodelling.

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This Thesis Dedicated to..

My Mother's Soul

&

My Father's Soul..

Asma

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ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium concentration
7-TM	Seven transmembrane
AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
Akt	Protein kinase B
Arrestins	β-arrestins
AngII	Angiotensin II
ANOVA	Analysis of variance
AT_1	Angiotensin II type 1 receptor
ATP	Adenosine triphosphate
BP	Blood pressure
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
cAMP	Adenosine cyclic-3´,5´-monophosphate
CDK2	Cyclin-dependent kinase 2
COMP101	Compound 101
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle`s medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EC_{50}	Concentration given 50% of the maximal response
EDTA	Ethylenediaminetetraacetic acid
ET1	Endothelin-1
ET_A	Endothelin type A receptor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FDA	Food and Drug Administration

Fluo4-AM	Fluo-4-acetoxymethyl ester
g	Gram
G_0 phase	Gap 0/quiescence or resting phase
G_1 phase	Gap 1/first growth phase
G_2 phase	Gap 2/second growth phase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
G protein	Heterotrimeric guanine nucleotide-binding protein
GRK	G protein-coupled receptor kinase
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
h	Hour
ICC	Immunocytochemistry
IGF	Insulin-like growth factor
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
kDa	Kilodalton
LPA	Lysophosphatidic acid
M	Molar
M phase	Mitotic phase
MAPK	Mitogen activated protein kinase
MFI	Mean nuclear fluorescence intensity
min	Minutes
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
ml	Milliliter
mM	Millimolar
µM	Micromolar
mmHg	The millimeter of mercury
mg	Milligram

µg	Microgram
MSMCs	Mesenteric arterial smooth muscle cells
NA	Noradrenaline
NC	Negative control
NO	Nitric oxide
nM	Nanomolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCNA	Proliferating cell associated nuclear antigen
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptors
PDK1	Phosphoinositide dependent-kinase 1
pERK	Phosphorylated extracellular signal-regulated kinase
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
Pin1	Prolyl-isomerase promotes
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLC-β	Phospholipase C-β
$P2Y_2$	Purinergic P2Y receptor type 2
***P* value**	Probability value
R1	First response
R2	Second response
R_{max}	Maximum response
RAAS	Renin-angiotensin-aldosterone system
RASM	Rat aortic smooth muscle
RFP	Red fluorescent protein
RhoA	Ras gene homolog family member A
RGS	Regulator of G protein signalling
ROS	Reactive oxygen species

RTK	Receptor tyrosine kinases
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s	Second
SEM	Standard error of mean
siRNA	Small/short interfering RNA
Ser	Serine
S phase	DNA synthesis phase
SNS	Sympathetic nervous system
SHR	Spontaneously hypertensive rat
SSRI	Selective serotonin reuptake inhibitor
SR	Sarcoplasmic reticulum
TBST	Tris buffered saline with Tween 20
TCA	Trichloroacetic acid
TEMED	N, N, N`, N`-tetramethylethylenediamine
tERK	Total extracellular signal-regulated kinase
Thr	Threonine
TGF-β	Transforming growth factor-β
UTP	Uridine-triphosphate
ULTR	Uterine smooth muscle cell lines
V	Vasopressin
v/v	Volume/volume
VSMCs	Vascular smooth muscle cells
WKY	Wistar Kyoto rat
w/v	Weight/volume

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Chapter One

1. General Introduction

1.1. Hypertension

1.1.1. Hypertension prevalence and definition

Hypertension is one of the most prevalent chronic diseases contributing to the development of cerebrovascular and cardiovascular disease-related morbidity and mortality (Mahmood et al., 2014). The world health organization considers elevated blood pressure (BP) to be one of the most common major risk factors for ischemic heart disease, kidney disease, stroke, morbidity and mortality. Indeed, hypertension is responsible for ~45% of heart disease mortality and ~51% of stroke-related death (WHO, 2013). Worldwide, the prevalence of hypertension is high and continues to increase. In 2015, the estimated global incidence of hypertension was 1.13 billion, with an estimated breakdown of 24% of male and 20% of female within the total population. Moreover, it is expected by 2025 that the number of hypertensive patients will rise up to 1.56 billion (Kearney et al., 2005; Collaboration, 2017; Williams et al., 2018). The prevalence of hypertension is an alarming statistic which indicates a major growing global health problem, demanding need for research and new therapies to overcome its complications.

The term 'hypertension' or systemic arterial hypertension generally refers to a biological condition characterised by a chronic increase of the systemic arterial blood pressure (BP) above a certain threshold value (Giles et al., 2009). BP is expressed as the ratio of the systolic BP to diastolic BP, where systolic BP is the pressure that the blood applies to the arterial walls during heart contraction and diastolic BP is the pressure of the blood on the arterial walls while the heart is relaxed (Oparil et al., 2018). International guidelines of arterial hypertension management, define BP threshold levels as the systolic BP value of ≥140 mmHg and/or diastolic BP value ≥90 mmHg (Bhagani et al., 2018; Williams et al., 2018). Hypertension can be categorised according to BP values into high-normal BP, grade 1 hypertension, grade 2 hypertension, grade 3 hypertension, isolated systolic hypertension, white-coat hypertension and masked hypertension; more details on BP classification and definition of hypertension according to BP values are found in **Table 1.1** (Bhagani et al., 2018). Giles et al. 2009, described elevated BP as one manifestation of hypertensive disease while the early markers of hypertension such as

cardiovascular disease risk factors, pre-clinical and clinical cardiovascular manifestations exist before BP becomes sustainably elevated. The meaning of hypertension has been extended to include a progressive cardiovascular syndrome developing from complex interconnected causes. Hypertension progression is strongly linked with structural and functional abnormalities in vascular cells and the myocardium that lead to end-organ damage and increased morbidity (Giles et al., 2005; Giles et al., 2009).

Table 1.1 Definitions and classification of BP (Bhagani et al., 2018).

Category	BP (mmHg)	
	Systolic BP	**Diastolic BP**
Optimal BP	<120	<80
Normal BP	<130	<85
High-normal BP	130-139	85-89
Grade 1 hypertension	140-159	90-99
Grade 2 hypertension	160-179	100-109
Grade 3 hypertension	>180	>110
Isolated systolic hypertension	>140	<90
White-coat hypertension	>140	>90
Masked hypertension	<140	<90

1.1.2. Hypertension aetiology and risk factors

Hypertension can be categorised according to its aetiology into essential hypertension and secondary hypertension (Rogier van der Velde et al., 2015). Essential hypertension (also called primary or idiopathic hypertension) represents ~90-95% of hypertensive cases and can be defined as elevated BP with unknown specific cause (Carretero and Oparil, 2000). Essential hypertension is a multifactor disease developing from genetic, environmental and lifestyle factors (Bolivar, 2013). Although the specific cause of essential hypertension is unclear, there are different contributing factors to elevated BP, such as genetic inheritance, obesity, dyslipidaemia, insulin resistance, ageing, stress, high alcohol and salt intake, low potassium intake and inactive lifestyle (Carretero and Oparil, 2000; Bhagani et al., 2018). The remaining ~5-10% of hypertensive patients have the secondary form of hypertension with an underlying

reversible, identifiable pathophysiological cause. (Charles et al., 2017). Common underlying origins of secondary hypertension are categorized into renal, vascular, endocrine and neural causes such as chronic kidney disease, renovascular diseases, aldosteronism, Cushing's syndrome, hypothyroidism, hyperthyroidism, atherosclerotic renal artery stenosis and phaeochromocytoma (Charles et al., 2017; Bhagani et al., 2018). Secondary hypertension can also manifest through the use of some drugs that elevate BP. Common examples of drugs considered as a cause of hypertension are sympathomimetic agents, steroids, serotonin/noradrenaline reuptake inhibitors, tricyclic antidepressants, oral contraceptives, some chemotherapeutic and immunosuppressive agents (Charles et al., 2017). Recognition of the exact cause of secondary hypertension is critical to enable reversal of the problem. If undiagnosed, secondary hypertension may result in resistant hypertension and development of complications (Puar et al., 2016).

1.1.3. BP measurement and evaluation

Normally hypertension is an asymptomatic silent disease, even though some patients suffer from symptoms like headaches, dizziness and lethargy as a result of their elevated BP (Bhagani et al., 2018). Because BP is unstable, variable and subject to immediate internal 'e.g. hormonal' and external 'e.g. stress' influences, diagnosis of hypertension should be based on several frequent BP measurements taken on separate days (Messerli et al., 2007). The ideal technique for measuring BP is using auscultatory or oscillometric sphygmomanometers, either automatic or semiautomatic according to standardized conditions and protocols (Williams et al., 2018). Furthermore, the *European Society of Cardiology (2018)* guidelines for the management of arterial hypertension support the use of out-of-office BP measurement devices, i.e. ambulatory BP monitoring or home BP monitoring devices as additional methods for repeat BP measurements to confirm the diagnosis of hypertension (Williams et al., 2018). Moreover, these guidelines recommend cardiovascular risk assessment with the SCORE (Systematic Coronary Risk Evaluation) system for patients who were not previously at high or very high risk of cardiovascular disease (Williams et al., 2018). SCORE is a clinical evaluation system of cardiovascular mortality risk in asymptomatic individuals to determine the possible prognosis of the condition, by estimating the 10 year risk of a first serious atherosclerotic incident in relation to other risk factors such as age, sex, smoking, diabetes, total cholesterol level, and BP to estimate appropriate treatment decisions (Aktas et al., 2004; Williams et al., 2018).

1.1.4. BP regulation mechanisms and pathophysiology

BP regulation involves a complex interaction of many interrelated mechanisms. Various systems integrate into BP regulation including the sympathetic nervous system, cardiac output, peripheral vascular resistance, the renin-angiotensin-aldosterone system (RAAS) and endothelial function. In addition to genetic background and family history for hypertension, disturbance or malfunction in one or more components of factors controlling BP participate in an increase in mean BP. Consequently, over time this can cause target-organ damage (**Figure 1.1**) (Jeunemaitre et al., 1992; Oparil et al., 2018).

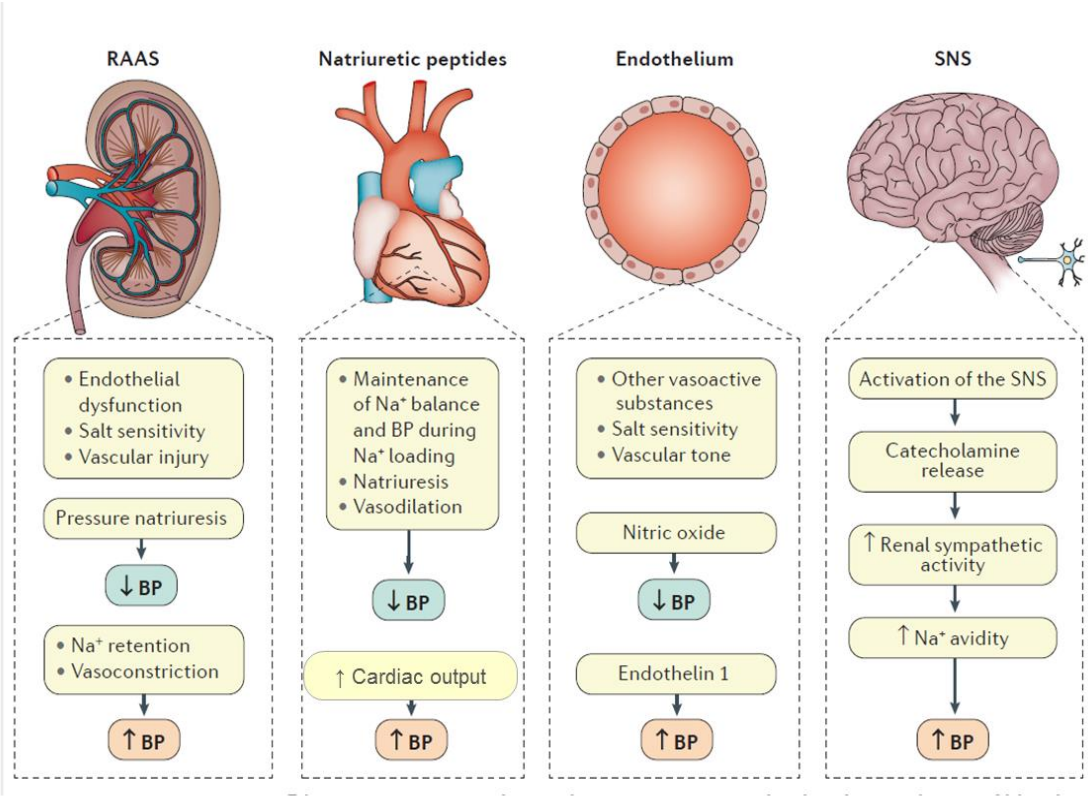


Figure 1.1 Main physiological systems involved in BP regulation.

Regulatory systems involved in the maintenance of physiological BP including RAAS: renin-angiotensin-aldosterone system; SNS: sympathetic nervous system, natriuretic peptides and endothelial cells. Modified from Oparil et al., 2018.

1.1.4.1. Cardiac output and peripheral vascular resistance

Cardiac output and peripheral vascular resistance are two essential factors that maintain normal BP. There is a proportional relation between BP and cardiac output/peripheral vascular resistance, which can be expressed by the following equation: MAP = CO × PVR (where MAP: mean arterial pressure, CO: cardiac output, PVR: total peripheral vascular resistance) (Mayet and Hughes, 2003). MAP defined as the average arterial pressure throughout one cardiac cycle which reflect the average pressure that drives blood thru the systemic circulation (Henry et al., 2002). Low MAP can cause insufficient blood supply to the organs. Conversely, raised MAP contributes to vascular remodelling, ventricular remodelling and end-organ damage (Wehrwein and Joyner, 2013). MAP is influenced by cardiac output and peripheral vascular resistance, each of which is control by several factors. This relationship indicates that maintenance of a normal BP is based on the balance between cardiac output and peripheral vascular resistance. Elevated BP can occur through increased cardiac output, increased peripheral vascular resistance, or a combination of both (Mayet and Hughes, 2003). Cardiac output is defined as the volume of blood pumped by the left ventricle per minute, which depends on stroke volume and heart rate which can be regulated by many factors such as the sympathetic nervous system. Peripheral vascular resistance is controlled predominantly by the smooth muscle cells of small arterioles, i.e. changes in arterial diameter which consequently affect blood flow and peripheral resistance (Mayet and Hughes, 2003). In the early stages of hypertension, sympathetic hyperactivity mediates increases in cardiac output and consequently increase in BP, a result of this, peripheral arteriolar resistance also increases as a compensatory protective mechanism to prevent the harmful effects of elevated BP (Beevers et al., 2001). Prolonged contraction of small arteriole smooth muscle alters the structure of arterial vessel walls leading to arterial wall thickening, resulting in an irreversible rise in peripheral vascular resistance and consequently a sustained elevation in BP (Beevers et al., 2001).

1.1.4.2. Sympathetic nervous system

The autonomic nervous system has a central role in maintaining normal BP as it regulates vascular contraction and relaxation. Likewise, it is responsible for the mediation of short-term changes in BP in response to external factors such as stress and physical activity (Beevers et al., 2001). Mancia and Grassi (2014) described that autonomic nervous system dysregulation plays a critical role in the development of essential

hypertension. The sympathetic nervous system is commonly more activated in hypertensive patients than in normotensive people (Julius and Nesbitt, 1996; Mancia and Grassi, 2014). Sympathetic hyperactivity which over-stimulates adrenoceptor activity and causes vascular smooth muscle contraction, appears in the early stages of hypertension and becomes more noticeable with disease progression and severity over time. Moreover, adrenergic over-activation can contribute to hypertension-induced end-organ damage (Smith et al., 2004; Mancia and Grassi, 2014). The autonomic nervous system can also control BP via the baroreceptor reflex system. Baroreceptors are mechanoreceptors located in various areas of the arterial network, but are mainly found in the aortic arch and carotid sinuses (Lovic et al., 2014). They act as BP sensors and modulators of autonomic tone as they are highly sensitive to any change in BP. Baroreceptors are stimulated by the increased arterial stretch that accompanies elevated BP. Activated baroreceptors send signals to the central nervous system to diminish sympathetic outflow and increase vagal tone, which leads to a reduction in peripheral vascular resistance and thus decreases BP (Lovic et al., 2014). Conversely, lower BP reduces baroreceptor activity, which reduces vagal output and increases sympathetic nervous system activity to raise BP (Lovic et al., 2014). Decreasing baroreceptor sensitivity or disruption of appropriate baroreceptor reflex system functioning is implied to be critical factor in the pathogenesis of essential hypertension (Lovic et al., 2014; Mancia and Grassi, 2014).

1.1.4.3. Renin-angiotensin-aldosterone system (RAAS)

The renin-angiotensin aldosterone system (RAAS) plays a central role in the regulation of arterial blood pressure. RAAS refers to a series of hormonal cascades that controlling organ perfusion, extracellular volume and consequently arterial pressure (Atlas, 2007). This pathway is initiated by the secretion of renin from the juxtaglomerular apparatus of the kidney as a result of sympathetic nervous system stimulation or low blood pressure/flow through the kidney. Renin catalyses the conversion of angiotensinogen (released from the liver) into angiotensin I, an inactive biological substance which is rapidly hydrolysed by angiotensin converting enzyme (ACE) to the physiologically active peptide angiotensin II (AngII) (Harrison and Luther, 2013; Karnik et al., 2015). ACE is a physiologically important enzyme localized on the plasma membranes of numerous cell types including vascular endothelial cells, renal proximal tubule epithelial cells and neuronal epithelial cells. ACE also metabolizes other

vasodilatory peptides such as bradykinin and kallidin to inactive metabolites, which results in increased vasoconstriction and decreased vasodilation (Turner and Hooper, 2002). AngII can be further converted to smaller biologically active peptides such as angiotensin III, angiotensin IV, angiotensin 1-7 (Reudelhuber, 2005).

AngII is an octapeptide and the primary active product of the RAAS. It acts as a potent vasoconstrictor and plays an essential role in BP regulation. AngII mediates several physiological and pathophysiological process through its effect on four different angiotensin receptor subtypes. The vast majority of AngII effects on the cardiovascular system are mediated via the angiotensin type 1 (AT_1) receptor (Carey and Siragy, 2003). Stimulation of AT_1 receptors results in vasoconstriction, increased BP, increased cardiac contractility and vascular wall and cardiac hypertrophy (Gavras and Gavras, 2002). Furthermore, the ability of AngII to induce cell proliferation, inflammatory responses, stimulation of aldosterone synthesis and oxidative stress is also reported to be mediated through the AT_1 receptor (Carey and Siragy, 2003). The angiotensin type 2 (AT_2) receptor shows low levels of expression in the adult, and is reported to mediate vasodilation, anti-proliferative and apoptotic effects (Stanton, 2003). The role of angiotensin type 3 (AT_3) receptors is unknown, while angiotensin type 4 (AT_4) receptors are thought to play role in plasminogen activator inhibitor 1 release (Stanton, 2003; Atlas, 2007). Unsurprisingly, dysregulation or sustained activation of the RAAS contributes to the pathophysiology of hypertension (Laragh, 2001). The pathophysiological role of AngII in hypertension is mediated through its potent vasoconstrictor effect that enhances BP. Also, the RAAS interacts with the central nervous system as AngII has a role in maintaining sympathetic outflow to the vasculature, facilitates sympathetic ganglia transmission and prevents noradrenaline reuptake at nerve terminals. Furthermore, AngII signalling can cause production of reactive oxygen species (ROS), impaired vascular insulin metabolic signalling and endothelial dysfunction (Raasch et al., 2001; Mehta and Griendling, 2007). Additionally, AngII stimulates aldosterone release from the adrenal gland, a major regulator of extracellular volume via regulation of sodium and potassium balance via its effect on mineralocorticoid receptors, thus enhancing sodium and water retention from kidney tubules as well as from other organs such as the colon, salivary and sweat glands. This process also encourages potassium and hydrogen ion excretion resulting in increased extracellular (systemic) volume and consequently increased BP (Beevers et al., 2001; Atlas, 2007).

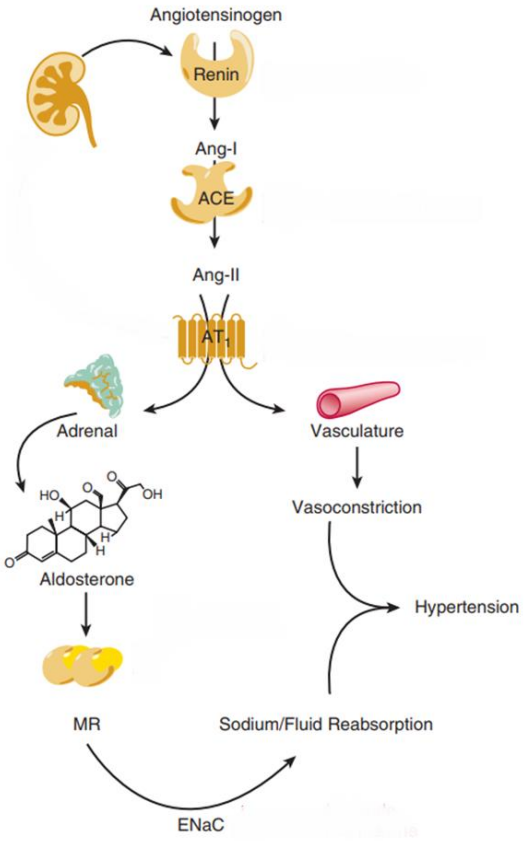


Figure 1.2 Schematic diagram showing the role of the renin-angiotensin-aldosterone-system (RAAS) in BP regulation.

Activation of the RAAS results in generation of the potent vasoconstrictor angiotensin II (AngII). AngII also stimulates aldosterone release, resulting in sodium and fluid retention and thus elevates BP. ACE, angiotensin-I converting enzyme; Ang: angiotensin; AT_1, angiotensin II type 1 receptor; ENaC, epithelial sodium channel; MR, mineralocorticoid receptor. Adapted from Harrison and Luther, 2013.

1.1.4.4. Endothelial function

In healthy humans the intact vascular endothelium layer plays an important role in regulating vascular tone and BP. Endothelial cells produce numerous vasoactive substances which regulate vascular tone. For example, nitric oxide (NO) is a crucial vasodilator released from endothelial cells in response to elevated shear stress associated with increased BP (Spieker et al., 2006). In addition to its role as a suppressor of abnormal vascular smooth muscle cell migration and proliferation, NO acts as potent vasodilator, and inhibitor of platelet adhesion and aggregation, (Oparil et al., 2003). Indeed, previous studies have reported that reduction in NO production (defects in the endothelium NO release) in hypertensive patients, results in an elevation in the BP and hypertension development (Panza et al., 1993; Shiekh et al., 2011).

Moreover, another critical endothelium-derived vasoactive substance is endothelin-1 (ET1). A short 21 amino acid peptide, ET1 is a potent vasoconstrictor which mediates its effect through binding to two endothelin receptor isoforms, ET_A and ET_B. ET1 acts on ET_A receptors expressed in vascular smooth muscle promoting vasoconstriction, cell proliferation and matrix accumulation (Kohan and Barton, 2014). Conversely, activation of ET_B receptors has vasodilatory, anti-proliferative and anti-fibrotic effects (Agapitov and Haynes, 2002; Kohan and Barton, 2014). The endothelium can also release additional vasodilators, for example, prostacyclin and endothelium-derived hyperpolarizing factors. In addition, further vasoconstrictors can be secreted from the endothelium such as locally generated AngII, prostanoids, thromboxane A2 and prostaglandin A2 (Kohan and Barton, 2014). The ability of the endothelium to control vascular tone can be determined by the balance between endothelium-derived vasodilators and vasoconstrictors especially NO and ET1 respectively (Agapitov and Haynes, 2002). Dysfunction or dysregulation of the endothelium-dependent regulatory systems may be involved in hypertension pathophysiology (Rajendran et al., 2013). Indeed studies have reported that ET1 infusion results in an increased in the BP level (Vierhapper et al., 1990) and block of ET_A-receptors using antagonists extensively decreases BP in patients with essential hypertension indicating their important role in hypertensive pathophysiology (Krum et al., 1998).

1.1.4.5. Other factors which affect BP

High salt intake results in high sodium concentrations in the serum, which in turn stimulates water retention, increases systemic blood volume and thus increases BP. This form of elevated BP called salt sensitivity, shows a marked increase in BP (≥10 mmHg) a few hours after high sodium (≥5 g) ingestion. Normally, compensatory haemodynamic alterations including a reduction in peripheral vascular resistance and increased endothelial production of NO to maintain normal BP. If the patient has a genetic predisposition for endothelial dysfunction or the effect of NO is diminished, an increase in BP occurs. Moreover, long-term high salt consumption can trigger endothelial dysfunction in non-salt-sensitive people (Feng et al., 2017). Other important BP regulators are natriuretic peptides including atrial natriuretic peptide and brain natriuretic peptide. These peptides mediate a hypotensive action via their natriuretic and vasodilator effects that assist in the maintenance of normal sodium balance, decreased plasma volume and BP regulation (Woodard and Rosado, 2008). Natriuretic peptides promote the transfer of fluid from the intravascular to the interstitial compartment, increase the glomerular filtration rate, inhibit renal sodium reabsorption with a concomitant reduction in renin, and aldosterone plasma levels (Brenner et al., 1990; Woodard and Rosado, 2008). Disruptions in the natriuretic peptide system or a reduction in natriuretic peptide production promote hypertension (John et al., 1995). For instance, corin (a key enzyme in natriuretic peptides synthesis) deficiency has been associated with volume overload and salt-sensitive hypertension (Armaly et al., 2013). Regulation of BP is also affected by ageing. The Framingham Heart Study reported that systolic BP continuously rises between 30 and 84 years of age (Franklin, 1999). Age-related increases in BP are mostly related to changes in arteriolar stiffness, alteration in RAAS, decreased baroreceptor responsiveness, increased sensitivity to the sympathetic nervous system activity, altered renal function and sodium balance (Weber et al., 1989; Pinto, 2007).

1.1.5. Management of hypertension

Since hypertension is considered a chronic disease, early treatment is beneficial to reduce end-organ damage and subsequent decreases in hypertension-associated morbidity and mortality (Sear, 2019). According to the *2013 ESH/ESC* hypertension guidelines BP target of <140/90 mmHg is recommended regardless of the cardiovascular risk factors that patients have (Mancia et al., 2013). In 2018, *ESH/ESC* recommended the same BP target for all patients. Furthermore, they recommend a systolic BP target of 120-129

mmHg (aged <65 years) and 130-139 (aged ≥65 years) for patient receiving antihypertensive treatment (Williams et al., 2018). Hypertension management includes lifestyle modification and use of BP lowering drugs according to the hypertension stage and patient condition.

1.1.5.1. Non-pharmacological management

Healthy lifestyle modification is counselled for all hypertensive patients. Good lifestyle choices can stop or delay the onset of hypertension, diminish cardiovascular risk and decrease the need for drug therapy in grade 1 hypertensive patients (Williams et al., 2018). The recommended lifestyle behaviours that have been shown to reduce BP are reducing sodium intake (<5 g per day), increasing potassium intake (3.5–5.0 g per day), moderate alcohol consumption (~3.5 units for male and ~1.75 units for female per day) and rich fruit, vegetable and low-fat diets, increased physical activity and regular exercise (≥ 30 minutes/day) (Whelton et al., 1997; Stevens et al., 2001; Whelton et al., 2002; Roerecke et al., 2017).

1.1.5.2. Pharmacotherapy management (antihypertensive drugs)

Antihypertensive drugs comprise numerous drug classes. There are multiple different drugs within each class with various pharmacological effect, therapeutic benefits and side-effects. Therapeutic targeting of hypertension management aims to control BP and prevent end-organ damage (Jackson and Bellamy, 2015). Antihypertensive drugs can be categorised into several wide-ranging groups. The first group being those which block the RAAS, including ACE inhibitors, angiotensin receptor antagonists and direct renin inhibitors. The second major antihypertensive group of drugs mediate their BP lowering effects by increasing vasodilatation and reducing peripheral vascular resistance. This group includes calcium channel blockers, α_1-adrenoceptor blockers, some types of β-adrenoceptor blockers and direct acting vasodilators. Moreover, non-vasodilatory β-adrenoceptor blockers lower BP via reducing cardiac output. Additionally, centrally acting agents such as hydralazine decrease sympathetic outflow and thereby decrease BP (Laurent, 2017). Another group of antihypertensive drugs, the diuretics, act by reducing intravascular volume via increasing water and sodium excretion (Ram, 2002; Jackson and Bellamy, 2015; Laurent, 2017). Examples of the various antihypertensive drugs, their pharmacological classes, subclasses and effects on the physiological systems, mechanisms of action and associated side effects are described in **Table 1.2**. The choice of antihypertensive medications has to be based on the patient's condition, individual

efficacy and tolerability. First-line antihypertensive medications are usually ACE inhibitors, angiotensin receptor blockers, calcium channel blockers and diuretics. In heart failure patients or post-myocardial infarction patients, β-adrenoceptor blockers are recommended. Calcium channel blockers and diuretics are recommended for Afro-Caribbean individual and α-methyldopa for pregnant women (Oparil et al., 2018; Williams et al., 2018).

Table 1.2 Pharmacological classes of antihypertensive drugs and corresponding mechanisms of action.

Antihypertensive Class		Physiologic Effect and Mechanisms of Action	Subclasses/Examples	Main Side Effect/Contraindication	References
I	**Angiotensin-Converting Enzyme Inhibitors**	Potent vasodilators; ↓ total peripheral resistance. Act by decreasing angiotensin II formation through inhibiting ACE activity. In addition, they prevent bradykinin breakdown and potentiate NO generation.	Captopril, Enalapril Lisinopril, Ramipril	Fall in BP associated with intravascular volume depletion. Cough (dry, hacking, non-productive). Angioneurotic oedema, renal impairment and hyperkalemia.	Elliott, 1996; Brown and Vaughan, 1998.
II	**Angiotensin II Receptor Antagonists**	Act as competitive antagonists at AT_1 receptors, leads to vasodilation, ↓ total peripheral resistance. Cardiac output remains unchanged.	Candesartan, Irbesartan Losartan, Valsartan	Generally well-tolerated drugs. Can cause functional renal insufficiency. Contra-indicated in pregnancy, in patients with previous hyperkalemia or bilateral renal artery stenosis.	Barreras and Gurk-Turner, 2003; Nakamura et al., 2005.

Antihypertensive Class		Physiologic Effect and Mechanisms of Action	Subclasses/Examples	Main Side Effect/Contraindication	References
III	**Renin inhibitors**	Acts as highly potent and selective inhibitor of renin enzyme.	Aliskiren	Contra-indicated in pregnancy, in patients with previous hyperkalemia, angioneurotic oedema or bilateral renal artery stenosis.	Rahuel et al., 2000.
IV	**β-Adrenoceptor Antagonists**	↓ Cardiac output, ↓ sympathetic activity, ↓ renin secretion leading to reduction in vasomotor tone. Additionally, β-blockers exhibit antianginal and anti-ischemic effects.	*Selective β_1-blockers:* Atenolol, Metoprolol. *Selective β_2-blockers:* Nebivolol (vasodilator activity via NO) *Non-selective β_1/β_2-blockers:* Propranolol *Combined α- and β-blockers (vasodilator effect):* Carvedilol, Labetalol	β-blockers can cause glucose intolerance and mask hypoglycaemic symptoms. Insomnia, hallucinations, and depression (with the highly lipid-soluble β-blockers). β-blockers should not be used in patients with asthma, or unstable heart failure.	Gottlieb et al., 1998; Frishman and Alwarshetty, 2002; Ahmet et al., 2008; Prisant, 2008.

Antihypertensive Class		Physiologic Effect and Mechanisms of Action	Subclasses/Examples	Main Side Effect/Contraindication	References
V	**α_1-Adrenoceptor Antagonists**	Selective α_1-adrenoceptor antagonists: long term use causes significant ↓ BP with no change in cardiac output.	Prazosin, Terazosin, Doxazosin	First-dose phenomenon; sudden severe orthostatic hypotension which commonly occurs during the 90 min following the first dose, or when the dose is increased.	Kirby, 2003.
VI	**Diuretics**	Inhibit sodium and chloride reabsorption at the distal convoluted tubule or thick ascending loop of Henle, ↓ extracellular fluid volume and cardiac output.	*Thiazides:* Hydrochlorothiazide *Thiazide-related compounds:* Indapamide *Loop Diuretics:* Furosemide *Potassium-sparing agents:* Spironolactone	Hypokalemia, polyuria, insulin resistance, also may cause hypomagnesemia, hypercalcemia, and hyperuricemia. Long-term high dosage (>50 mg/day) thiazide therapy may cause hyperlipidaemia.	Brater, 2000; Greenberg, 2000.

Antihypertensive Class		Physiologic Effect and Mechanisms of Action	Subclasses/Examples	Main Side Effect/Contraindication	References
VII	**Calcium Channel Blockers**	Vasodilatory effects are mainly mediated by the blockade of L-type calcium channels; binding to calcium channels affects their duration of action.	*Dihydropyridines:* Amlodipine, Nifedipine *Non-dihydropyridines* Diltiazem**,** Verapamil	Dihydropyridines can cause headaches, flushing, and ankle oedema. Nifedipine can cause reflex tachycardia, myocardial ischemia. Verapamil can cause constipation and AV block.	Psaty et al., 1995; Alderman et al., 1997.
VIII	**α_2-Adrenoreceptor Agonists**	Stimulation of central α_2-adrenoceptors in the vasomotor centre which causes ↓sympathetic outflow, ↓peripheral vascular resistance and a moderate ↓ in the heart rate and cardiac output.	Clonidine	Decreased sympathetic activity, leading to postural hypotension, sedation and drowsiness. Contra-indicated in patients with severe depression.	Sica and Grubbs, 2005.

Antihypertensive Class		Physiologic Effect and Mechanisms of Action	Subclasses/Examples	Main Side Effect/Contraindication	References
VIIII	**Direct Vasodilators**	Acts as direct vasodilator of resistance arterioles, ↓ total peripheral resistance.	Hydralazine Minoxidil	Hydralazine causes several serious adverse effects, no longer indicated except for urgent control of severe hypertension in pregnancy with β-adrenoceptor blockers. Because of the severity of minoxidil adverse effects, it is not indicated except in severe hypertension in unresponsive patients.	Campese, 1981; Cohn et al., 2011; Mancia et al., 2013.

1.1.6. Hypertension and end organ damage

It is a well-known fact that hypertension is the leading risk factor for morbidity and mortality typically as a results of end organ damage (Kannel, 2004; Mahmood et al., 2014). Hypertension-induced main organ damage presents as damage in the brain, retina, heart and kidney. Uncontrolled BP produces alterations of the microcirculation supplying these organs resulting in retinopathy, cerebral infarction, intracranial haemorrhage, dementia, coronary heart disease, atherosclerosic stenosis, myocardial infarction, left ventricular hypertrophy and heart failure (Schmieder, 2010). Moreover, this can also induce micro-albuminuria, glomerulopathy and end stage renal disease (Cohuet and Struijker-Boudier, 2006; Schmieder, 2010). The pathophysiological mechanisms of hypertension-induced end organ damage probably results from complex interactions among hypertension pathogenesis, such as hypertension-related hemodynamic over-load (Schmieder, 2010), endothelial dysfunction (Hsueh and Anderson, 1992) as well as presence of risk factors such as obesity, diabetes, hyperlipidaemia, smoking and physical inactivity (Israili et al., 2007). Furthermore, previous evidence shows that RAAS activation plays a role in end organ damage, and blocking of RAAS may provide protection from hypertension-induced end organ damage (Weir, 2007; Cohn and Goldman, 2008). Additionally, previous studies reported that hypertension correlates with structural and functional modifications in the vasculature (Laurent and Boutouyrie, 2015). Moreover, there is a correlation between BP regulatory systems and vascular structural and functional alterations in hypertension (Byrom, 1974; Byrom, 1976; Cohuet and Struijker-Boudier, 2006). These vascular changes also contribute to the long term maintenance of hypertension and development of end organ damage (Cohuet and Struijker-Boudier, 2006). Additionally, many clinical studies have reported relationships between alterations in vascular wall structure and cardiovascular disease development (O'Leary et al., 1999; Nambi et al., 2012; Magnussen, 2017). Although, antihypertensive agents that target the RAAS could provide some degree of protection from end-organ damage in addition to BP control (Dahlöf, 2003), it should be emphasized that the overall goal of most of current antihypertensive drugs is to normalize or stabilise BP (Luscher et al., 1996; Israili et al., 2007). However, the main cause for end-organ damage, for instance changes in blood vessel structure remain untreated. Understanding the association between additional factors (not only BP) implicated in the pathogenesis of end organ damage would extensively help to change the hypertension therapeutic strategy and

possibly improve hypertension therapy. Therefore, I will focus on hypertension-associated vascular structural changes in the next section.

1.2. Vascular remodelling

1.2.1. Definition of vascular remodelling

In 1987, Glagov and his colleagues reported the unpredictable finding that atherosclerosis-induced arterial lumen narrowing is not simply the outcome of atherosclerotic lesion enlargement. They described many changes in arterial wall structure including narrowed lumen, increased outside diameter and changes in the wall mass as an adaptive response to preserve blood flow (Glagov et al., 1987). Arterial wall adaptive responses such as controlling wall mass, lumen diameter and contractile capacity allow normal blood supply for organ requirements. Thus, the failure of the blood vessel to adapt, i.e. inability to remodel the vessels appropriately has been suggested as vascular failure (Schwartz et al., 2003). In disease states, such as in hypertension, these arterial adaptive changes do not return to normal and initiate the pathological vascular wall alterations seen with cardiovascular disease. This defective change is termed as vascular remodelling (Savoia et al., 2011), which is an active process of structural and functional modifications, involving changes in cell growth, cell migration, and extracellular matrix synthesis or degradation. Remodelling occurs in response to prolonged interactions between growth factors, vasoactive substances, and hemodynamic stimuli and consequently contribute to vascular pathophysiology (Gibbons and Dzau, 1994).

1.2.2. Classification of vascular remodelling

1.2.2.1. Normal arterial wall structure

Basically, the arterial vascular wall contains three distinctive layers called tunics that contain various cellular components. The first inner layer is the tunica intima, also called the tunica interna, consisting of a single layer of endothelial cells, whereas the tunica externa, also called tunica adventitia, is the strong supportive outer layer of the blood vessel wall and is formed from an extracellular matrix of mainly elastin and collagen. Between the tunica intima and tunica externa, there are layers of vascular smooth muscle cells (VSMCs), called the tunica media, which constitutes the thickest layer in the atrial wall and is responsible for altering the blood vessel diameter in response to neuronal or humoral stimuli (Rubenstein et al., 2012; Greif, 2013; Laurent and

Boutouyrie, 2015). As shown in **Figure 1.3**, different types of arteries vary slightly in their structures according to their distribution in the body. The aorta and its major branches are termed conducting or elastic arteries, and their tunica media has more elastin and collagen than VSMC content, which facilitates vascular compliance to high BP flow from the cardiac output. As the blood flows further from the heart the arterial structure changes to more muscular arteries with relatively high contractile ability as they have a higher VSMC quantity with decreased extracellular matrix content in the tunica media layer. Finally, resistance arteries branch off from the muscular arteries, were the tunica media consists of VSMCs layers and there is an absence of extracellular matrix (Tennant and McGeachie, 1990; Rubenstein et al., 2012).

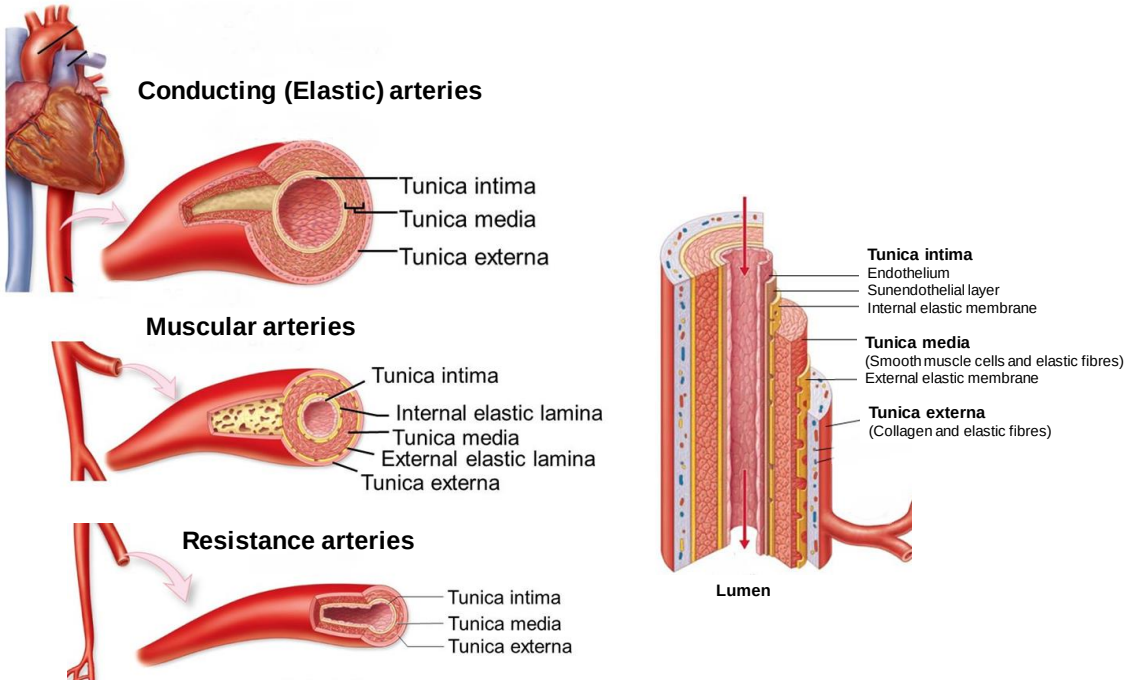


Figure 1.3 Anatomical and histological structure of conducting, muscular and resistance arteries.

Structural difference of the arterial vascular walls in different types of arteries. The internal layer, the tunica intima, is a single layer of endothelial cells that are in direct contact with blood, then tunica media, which is composed of smooth muscle cells, control vascular tone. The tunica externa is composed of elastin and collagen fibres which give structural support to the arteries, Modified from Marieb, 2013.

1.2.2.2. Hypertensive vascular remodelling

There are multiple different forms of vascular remodelling, which vary depending on the vessel type, disease state and progression. Hypertensive vascular remodelling can develop in large and small arteries walls and include changes in vascular wall cellular components such as endothelial cells, VSMCs, elastin and collagen content (Savoia et al., 2011; Duca et al., 2016). It cac be outward, inward or eutrophic remodelling; processes of increase, decrease or no change the arterial lumen diameter (cross-sectional area), respectively. Vascular remodelling can be also hypertrophic or hypotrophic as a result of an increase or decrease in vascular wall cellular content, respectively (Gibbons and Dzau, 1994; Carretero, 2005; Humphrey, 2008).

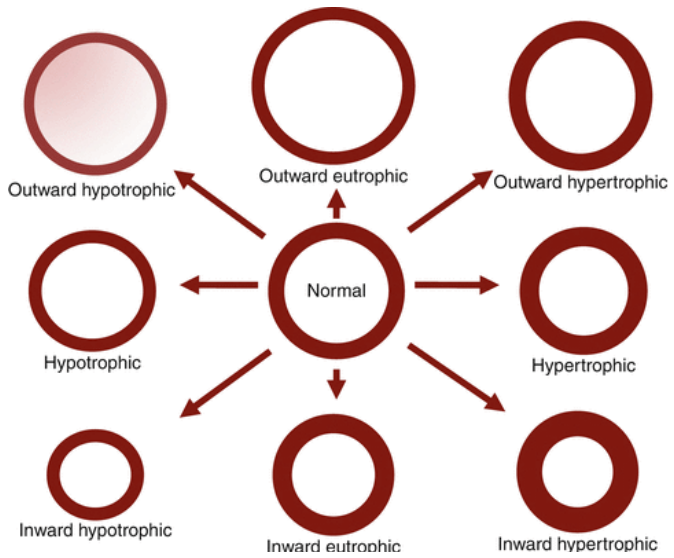


Figure 1.4 Diagram presenting cross-section of different types of arterial remodelling.

The vessel in the centre represents a normal artery cross-sectional area, eutrophic, with no change in cross-sectional area. Hypotrophic remodelling shows a reduced cross-sectional area of the arterial wall (left); or hypertrophic, shows increased cross-sectional area (right). These kinds of remodelling can be inward (lower line), showing decreased lumen diameter or outward (upper line), with increased lumen diameter. Adapted from Touyz and Montezano, 2015.

In hypertension, large arteries display hypertrophic remodelling while the smaller vessels such as muscular and resistances arteries undergo a combination of cellular hypertrophy and lumen reduction (**Figure 1.5**) (Owens et al., 1981; Owens and Schwartz, 1983; Owens and Reidy, 1985; Korsgaard and Mulvany, 1988; Prewitt et al., 2002). In response to prolonged hypertension, large conductive arteries show a total increase in the vessel wall thickness due to increased thickness of the intima media layer in the range of 15-40% (Roman et al., 1992; Laurent and Boutouyrie, 2015). Moreover, structural alterations observed in large arteries during hypertension are similar to those seen in elderly patients, suggesting that hypertension accelerates age-related large arterial wall remodelling (Mitchell et al., 2007; Harvey et al., 2015). Many previous studies in hypertensive animal models and hypertensive patients report that hypertensive vascular remodelling of resistance arteries can be either eutrophic and/or hypertrophic, depending on the disease severity (Mulvany, 1999). Resistance arteries of spontaneously hypertensive (SHR), renovascular hypertensive rats, in addition to mild essential hypertensive patients show mainly inward eutrophic remodelling (Schiffrin and Deng, 1992; Korsgaard et al., 1993; Schiffrin et al., 1993; Li et al., 1996b). Furthermore, hypertrophic remodelling is associated with severe hypertensive conditions, such as those observed in deoxycorticosterone acetate (DOCA)-salt hypertensive rats and renal hypertensive rats (Korsgaard and Mulvany, 1988; Deng and Schiffrin, 1992). Additionally, Baumbach and colleagues reported that hypertension is associated with structural changes in the resistance arteries, resulting in a reduction in lumen diameter and increase in media/lumen (M/L) ratio (Baumbach and Heistad, 1989; Baumbach and Ghoneim, 1993). Collectively, a wealth of evidence indicates that hypertension stimulates structural modifications to resistance arteries and arterioles, leading to a reduction in lumen diameter, which plays a major role in long-term or chronic systemic vascular resistance and BP elevation in hypertension (Schiffrin et al., 1993; Mulvany, 2002; Simon, 2004; Feihl et al., 2006). Furthermore, small artery remodelling is a hallmark of hypertension pathophysiology and could be the earliest manifestation of end organ damage, and also shows a positive correlation with the incidence of cerebrovascular and cardiovascular events (Park and Schiffrin, 2001; Rizzoni et al., 2003; Mulvany, 2012).

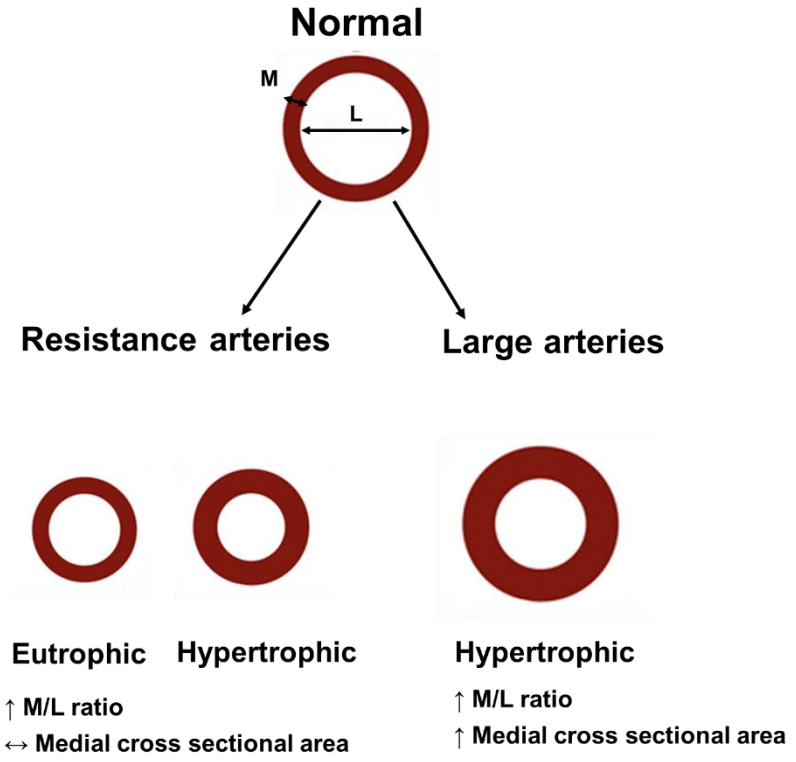


Figure 1.5 Different forms of hypertensive vascular remodelling in large and resistance arteries.

Hypertension induces changes to large and resistance arterial wall structure, with hypertrophic remodelling occurring in large arteries, while either hypertrophic or eutrophic remodelling can occur in resistance arteries. M: media, L: lumen, ↔: no change. Figure adapted from Grassi, 2003; Duprez, 2006.

1.2.3. Pathophysiology of hypertensive vascular remodelling

Vascular remodelling is generated by several inter-related processes, comprising of endothelial dysfunction, VSMC proliferation, differentiation and migration, elastin fibre degradation, and deposition of extracellular matrix materials (van Varik et al., 2012). Initially, the endothelium acts as sensor for any change in haemodynamic state since endothelial cells are directly exposed to physical forces, humoral factors and inflammatory mediators (Gibbons and Dzau, 1994). Blood flow and shear stress stimulate endothelial cells to produce NO and other mediators, which in turn adjust vascular tone. Vascular mechanical homeostasis is referred to as the ability of vessels to adapt in response to the amount of wall stresses and tensions from blood flow (van Haaften et al., 2017). In the case of pathological conditions, such as cardiovascular diseases, the ability of the endothelium to control vascular tone is reduced (Panza et al., 1990; Csiszar et al., 2009; Urschel et al., 2012), indicating that disruption of vascular mechanical homeostasis may, in fact, initiate early pathways involved in pathological vascular remodelling (Humphrey, 2008). Furthermore, the endothelium contributes directly to vessel structural changes by releasing and activating many different substances which influence VSMC growth, apoptosis, migration and alter the composition of the extracellular matrix (Gibbons and Dzau, 1994). VSMCs are the major regulators of vessels' diameter, and their functions are controlled by the autonomic nervous system innervation, endothelium released vasodilators or vasoconstrictors, reactive oxygen species, hormones and vasoactive peptides together with a cell-cell connection through gap junctions (Davis, 2012; Ma et al., 2014). Furthermore, at the molecular level, the previously highlighted factors influence VSMC inflammation, proliferation and rearrangement; processes that are implicated in changes to the vascular wall architecture (Davis, 2012; Tuna et al., 2012). Considering that the main feature of hypertensive vascular remodelling is increased arterial wall thickness and altered M/L ratio i.e. increase in the mass content of VSMCs layer, the mechanisms underlying VSMC proliferation will be discussed in the next section.

1.2.3.1. Pathological VSMC proliferation in hypertension

Abnormal VSMC proliferation is one of the main causes of arterial remodelling that contributes to several types of cardiovascular diseases such as hypertension, atherosclerosis, restenosis and pulmonary hypertension (De Mey, 1995; Rudijanto, 2007; Tajsic and Morrell, 2011). In normal conditions, contractile phenotype of VSMC regulates arterial tone and diameter (Alexander and Owens, 2012). They are characterized by specific phenotypic markers such as α-smooth muscle actin, smooth muscle 22-α, calponin and smoothelin. Expression of these contractile proteins allows VSMC to control vascular tone, and regulate systemic and local BP (Rensen et al., 2007; Coll-Bonfill et al., 2016). In disease states, VSMC undergo phenotype modulation, differentiating from contractile 'quiescent' to synthetic 'proliferative' types, a process referred to as phenotypic switching, in response to stress or vascular injury, which is considered to be one of the main drivers of arterial wall remodelling (Owens, 1995; Alexander and Owens, 2012; Coll-Bonfill et al., 2016). In addition, inflammatory mediators, growth factors and cytokines stimulate phenotypic alteration of VSMC (Willis et al., 2004). Interestingly, previous data show that >80% of VSMC at the sites of vascular remodelling or sites of arterial injury exhibit features of phenotypic differentiation (Shankman et al., 2015). Moreover, uncontrolled, differentiated smooth muscle cells accumulate in the vascular wall leading to media thickening and vascular stiffness (Nemenoff et al., 2011). Proliferation of VSMC also plays a central role in neointimal hyperplasia associated with atherosclerosis and pulmonary artery hypertension (Nemenoff et al., 2011). When VSMC switch to the synthetic phenotype, they release matrix metalloproteinases enzymes, which facilitate migration of VSMC from the media to the intima, further enhancing intimal VSMC hyperplasia and proliferation, resulting in arterial wall thickening (Willis et al., 2004; van Varik et al., 2012). Additionally, proliferative or synthetic VSMC express low levels of contractile proteins and high levels of signalling molecules which promote cell proliferation, migration, inflammation and fibrosis (Owens et al., 2004). The most obvious VSMC's proliferative stimuli are growth factors including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin and insulin-like growth factor-1. Furthermore, vasoactive substances such as AngII, ET1 and serotonin (5-HT) substantially stimulate the proliferation of VSMC through their effects on corresponding G protein-coupled receptors (GPCR). Also, calcium, reactive oxygen species and inflammatory cytokines such as interleukins and tumour necrosis factor-α considerably stimulate the proliferation of VSMC (Wang et al.,

2018a). These pro-proliferative stimuli activate signal transduction cascades that trigger VSMC growth and thus remodelling of the arterial wall. Prominent pathways involved in these processes include Ras/MAPK/ERK, Src, phosphatidylinositol 3-kinases/Akt, phospholipase C-γ and JAK/STAT molecules and their signalling pathways (Zhang and Liu, 2002; Stabile et al., 2003; Wang et al., 2018a).

VSMC are considered key regulators of arterial tone since normal BP is maintained by ensuring a balance between the circulating concentrations and actions of vasodilators and vasoconstrictors. Together with vasoconstrictors-related molecular downstream signalling pathways which are implicated in the proliferation of VSMC and hypertensive vascular remodelling progression, the control of the proliferation of VSMC could be a possible therapeutic target to prevent development and progression of hypertensive vascular remodelling. Since the majority of vasodilators and vasoconstrictors mediate their effects via GPCRs, the next section will focus on GPCR structure and function.

1.3. GPCR

1.3.1. GPCR definition, structure and activation

GPCRs are a superfamily (>800 identified human GPCR sequences) of heptahelical integral membrane proteins which respond to various stimuli and are involved in the transmission of numerous signals across cell membranes. They are responsible for controlling a plethora of signalling networks which initiate a wide range of physiological and pathological actions (Gether, 2000; Fredriksson et al., 2003). GPCRs are important therapeutic targets and approximately 40% of Food and Drug Administration (FDA) approved therapeutics drugs target GPCRs or their associated signalling pathways (Schlyer and Horuk, 2006; Zhang and Xie, 2012). GPCRs are also called seven transmembrane (7-TM) receptors as they have a common basic structure consisting of seven transmembrane helices (TM I-TM VII) linked together by three intracellular (i1-i3) and three extracellular (e1-e3) loops, with both extracellular N-terminal and intracellular C-terminal domains (Baldwin, 1993; Bockaert and Philippe Pin, 1999). The structure and function of the extracellular N-terminal and intracellular C-terminal domains dictate the variations between GPCRs structures (Bockaert and Philippe Pin, 1999). For example the extracellular N-terminal domain is responsible for ligand recognition and modifying ligand access. The 7-TM helices are responsible for ligand binding which results in conformational changes which facilitate transduction of extracellular ligand receptor signals to the intracellular region. In addition, the intracellular C-terminal domain interacts with cytosolic G proteins, GPCR regulatory proteins and downstream signalling effectors (Zhang et al., 2015). A total of 826 GPCR members can be classified according to 7-TM helical segments sequences into five different subfamilies or classes. Class A (rhodopsin family, 701 members), class B1 (secretin family, 15 members), class B (adhesion family, 24 members), class C (glutamate family, 15 members) and class F (frizzled/taste family, 24 members) (Fredriksson et al., 2003; Zhang et al., 2015). GPCRs recognize and transduce messages in response to a variety of stimuli such as neurotransmitters, hormones, chemokines, peptides, small molecule odorants, photons, purines, calcium, and peptides (Baldwin, 1993). Upon receptor activation, a relatively large scale rearrangement of the 7-TM helices occurs, leading to different receptor conformational changes and a variety of effects on downstream signalling pathways (Nygaard et al., 2009). The classical GPCR signalling transduction process is dependent on receptor-mediated activation of heterotrimeric G proteins. Heterotrimeric G proteins are guanylyl nucleotide-binding proteins that

comprise of three subunits, G_α, G_β, and G_γ. The G_α subunit (~40-46 kDa) contains the guanine nucleotide binding site and possesses intrinsic GTPase activity. G_β and G_γ subunits (~36 kDa and ~9 kDa) are associated with each other as a dimer (Hollmann et al., 2005). During the inactive state, GDP bound G_α associates with the $G_{\beta\gamma}$ dimer to form complex $G_{\alpha/\beta\gamma}$ heterotrimers. Receptor activation promotes the exchange of GDP for GTP on the G_α subunits, promoting a conformational change that causes dissociation of the G_α from $G_{\beta\gamma}$ subunits. Both G_α and $G_{\beta\gamma}$ subunits have been shown to initiate and modulate diverse downstream effector proteins and intracellular signalling response (Nygaard et al., 2009; Zhang et al., 2015). G_α subunits are classified into $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$. Each subunit targets different effectors; $G_{\alpha s}$ activate adenylyl cyclase, $G_{\alpha i}$ inhibit adenylyl cyclase and $G_{\alpha q}$ activate phosolipase C leading to generation of second messengers that mediate various cellular responses (Downes and Gautam, 1999) (**Figure 1.6**). The $G_{\alpha 12/13}$ mediated signalling pathway via interaction and/or activation of diverse proteins at various levels that regulate various cellular responses such as activation of the small monomeric GTPase RhoA (Siehler, 2007; Suzuki et al., 2009). G protein signalling can be terminated by G_α subunit's intrinsic GTPase activity, which hydrolyzes bound GTP to GDP results in re-association of G_α and $G_{\beta\gamma}$ subunits and termination of classical signals (Druey et al., 1998). G_α subunit deactivation process is further accelerated by regulator of G protein signaling (RGS) proteins which act as GTPase-activating proteins. They regulate G_α subunit by increasing the rates of hydrolysis of GTP to GDP and thus return G_α subunit to the inactive state (Druey et al., 1998; Ross and Wilkie, 2000). Additionally, GPCR activity and cellular responses can also be terminated by receptor desensitization, a process involving active receptor phosphorylation of the GPCR by G protein coupled receptor kinases and arrestin recruitment which will be discussed later (*Section 1.3.4*).

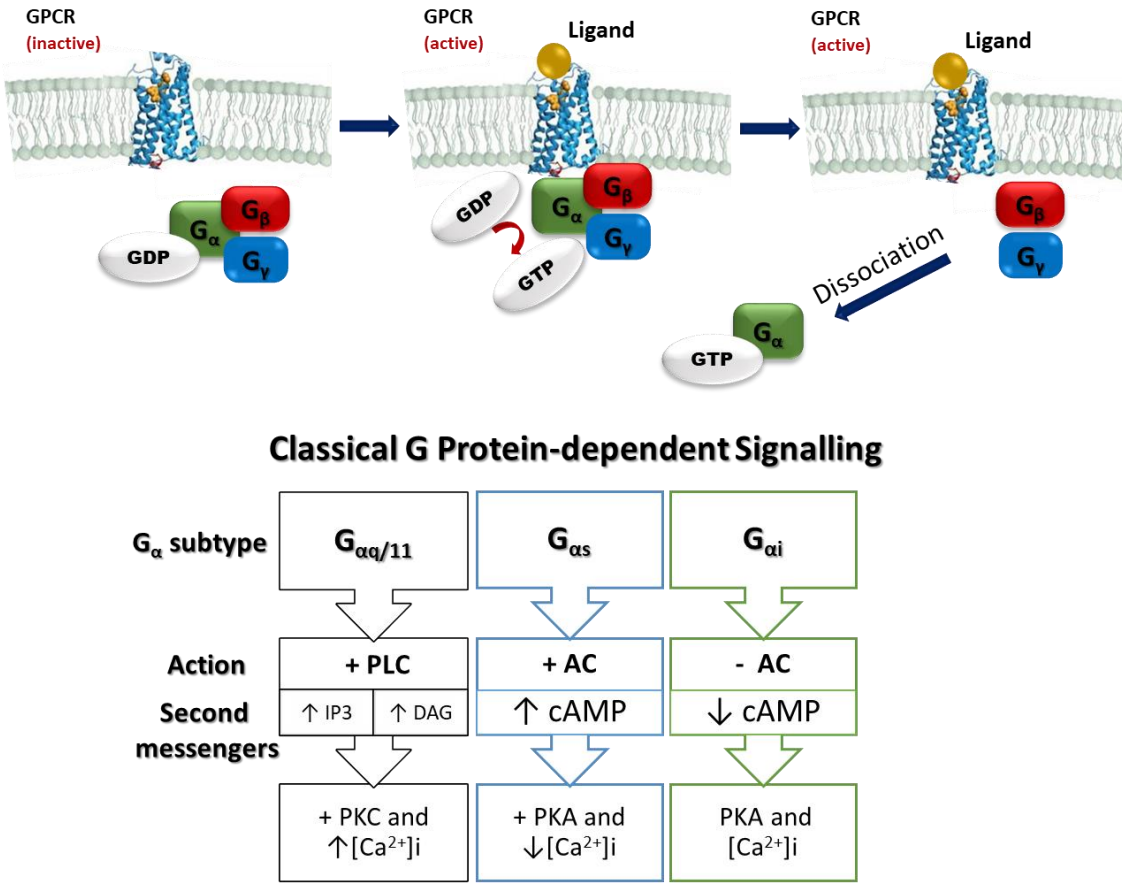


Figure 1.6 Schematic diagram showing GPCR activation and related classical signalling pathways.

Upon ligand binding, the receptor becomes activated which recruits heterotrimeric G proteins, causing the GTP/GDP exchange on the G_{α}, resulting in $G_{\alpha/\beta\gamma}$ dissociation, and activation of classical G protein dependent signalling pathways. PLC, phospholipase C; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKC, protein kinase C; PKA, protein kinase A; $[Ca^{2+}]_i$, intracellular calcium concentration.

1.3.2. Role of vascular GPCRs in hypertension

1.3.2.1. G protein-mediated calcium-dependent/independent mechanisms of VSMC contraction and relaxation

Vascular diameter and tone are determined by activation (phosphorylation) and inactivation (dephosphorylation) of contractile proteins such as actin and myosin (Touyz et al., 2018), which are expressed in VSMC. Phosphorylation of the myosin light chain (MLC) is the main process that promotes VSMC contraction (Somlyo and Somlyo, 2003), and the mechanisms that control MLC phosphorylation can be Ca^{2+}-dependent or Ca^{2+}-independent (Touyz et al., 2018). Ca^{2+}-dependent mechanism are activated by stimulation of VSMCs by vasoconstrictors such as neurohumoral stimuli (e.g. noradrenaline) and vasoactive peptides such as AngII, ET1, vasopressin and uridine-triphosphate (UTP). Such induce activation of their corresponding $G_{\alpha q}$ coupled GPCR. $G_{\alpha q}$ activation results in stimulation of phospholipase C (β-isoform), leading to the formation of the second messengers inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3, acts on inositol trisphosphate receptors (IP_3Rs) located in the sarcoplasmic reticulum, mediating efflux of Ca^{2+} from intracellular stores into the cytoplasm. At the same time, DAG activates protein kinase C (PKC). PKC potentiates vascular Ca^{2+} channels activity such as voltage-dependent L-type Ca^{2+} channels, promoting Ca^{2+} influx and increased intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. Higher $[Ca^{2+}]_i$ increases Ca^{2+} binding to calmodulin, generating Ca^{2+}-calmodulin complex which induce a conformational change in MLC kinase (MLCK), converting it to an active form. Activated MLCK phosphorylates target proteins that stimulate myosin-actin interactions, which causes force, shortening of myosin-actin filaments and consequent smooth muscle contraction leading to vasoconstriction (Allen and Walsh, 1994; Lozinskaya et al., 1997; Harris et al., 2008; Hill-Eubanks et al., 2011; Lymperopoulos and Bathgate, 2013; Ghosh et al., 2017). Furthermore, vasoconstrictors can mediate smooth muscle contraction in a Ca^{2+}-independent manner, via regulating the dephosphorylation of MLC phosphatase (MLCP) (Puetz et al., 2009). Two different signalling pathways are involved in the Ca^{2+}-independent mechanism. The first mechanism utilises the PLC-DAG-PKC pathway, whereby activated PKC phosphorylates CPI-17, a smooth muscle-specific inhibitor of MLCP, which in turn inhibits phosphatase activity and thus stimulates persistent contraction (Ringvold and Khalil, 2017). It has been reported that PKCα and PKCε phosphorylate calmodulin and calponin, thus promoting actin and myosin interaction and subsequently VSMC contraction (Khalil et al., 1992; Walsh et al., 1996; Khalil, 2013). A

second Ca^{2+}-independent mechanism uses the RhoA-Rho kinase pathway. RhoA is a small GTPase binding protein, highly expressed in VSMCs which is rapidly activated by vasoconstrictors. Activation of RhoA stimulates Rho-kinase which phosphorylates MLCP and inhibits its activity (Puetz et al., 2009; Loirand and Pacaud, 2010). Moreover, Rho-kinase stimulates phosphorylation of myosin phosphatase target subunit 1 (MYPT1) which then interferes with binding of MLCP to MLC and accordingly decreases MLCP activity resulting in sustained contraction of VSMC (Rahman et al., 2014). On the other hand, relaxation of VSMC requires a low $[Ca^{2+}]_i$ level and increased activity of MLC phosphatase, which occurs as a result of either the removal of the vasoconstrictor or by the action of vasodilators (Webb, 2003). Vasodilators such as adrenaline act on β-adrenoceptors, recruiting $G_{\alpha s}$ to stimulate adenylyl cyclase, leading to the formation of cAMP and subsequently activation of protein kinase A (PKA). PKA phosphorylates MLCK leading to activation of calcium pumps in the plasma membrane and sarcoplasmic reticulum to decrease $[Ca^{2+}]_i$ concentrations, and promotes cell hyperpolarization by opening K^+ channels. Collectively these functions of PKA cause increased smooth muscle relaxation and induce vasodilation (Consigny, 1991; Harris et al., 2008; Fukunaga et al., 2016) by decreasing the ability of VSMC to contract.

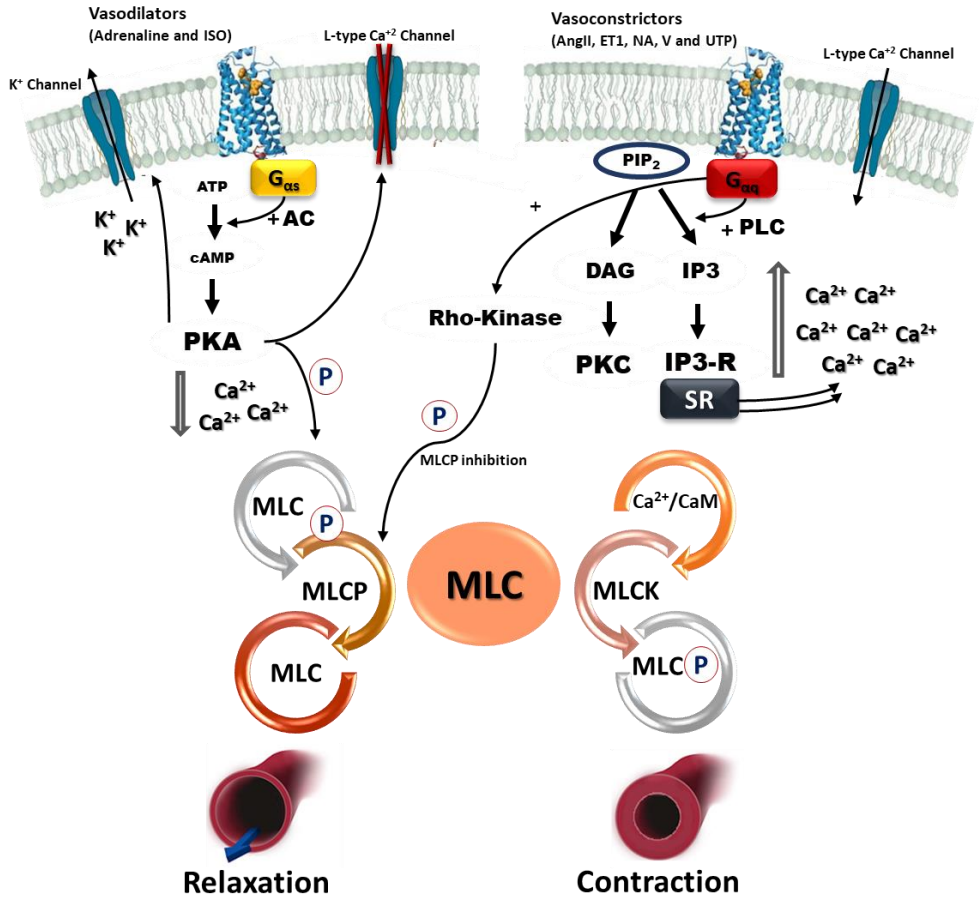


Figure 1.7 G protein-mediated regulation of contraction and relaxation of VSMC.

Vasoconstrictors bind to $G_{\alpha q}$ coupled receptors to activate PLC, hydrolysing PIP_2 creating the second messengers DAG and IP_3, which increase $[Ca^{2+}]_i$ resulting in phosphorylation of specific target proteins, the activation of MLCK and thereby the phosphorylation of MLC and smooth muscle cell contraction. However, vasodilators bind to $G_{\alpha s}$ coupled receptors, activate AC, produce the second messenger cAMP resulting in decreased $[Ca^{2+}]_i$, activation of MLCP and thereby dephosphorylation of MLC and smooth muscle cell relaxation. AngII, angiotensin II; ET1, endothelin-1; NA, noradrenaline; V, vasopressin: UTP, uridine triphosphate; ISO, isoprenaline; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptors; SR, sarcoplasmic reticulum; AC, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKC, protein kinase C; PKA, protein kinase A; Ca^{2+}/CaM, Ca^{2+}-calmodulin complex; MLC, myosin light chain; MLCK, MLC: myosin light chain kinase; MLCP, myosin light chain phosphatase.

1.3.2.2. G protein-mediated elevated vascular tone in hypertension

GPCRs expressed in the VSMC play a major role in maintaining the balance between contraction and relaxation of the blood vessels (Harris et al., 2008). Hyper-contractility of VSMC is one feature of essential hypertension (Brinks and Eckhart, 2010). Hypertension is characterized by sustained vasoconstriction combined with impaired vasorelaxation (Feldman and Gros, 1998). Alterations in the regulatory processes of cell signalling pathways that maintain normal $[Ca^{2+}]_i$ and MLC phosphorylation are likely to be a major contributor to the abnormal contraction of arterial smooth cells, which results in hypertension (Qin et al., 2004; Walsh, 2011). Indeed, previous studies reported that hypertension is associated with abnormalities in arterial myogenic responses (Immink et al., 2004; Jarajapu and Knot, 2005). In addition, adenylyl cyclase activity is reported to be altered in various hypertensive animal models, and the ability of $G_{\alpha s}$ to activate adenylyl cyclase reduced, probably due to the over-expression of $G_{\alpha i}$ (Anand-Srivastava et al., 1991; Anand-Srivastava, 1992). Moreover, hypertension is strongly associated with increased circulating levels of the vasoconstrictors which increase arterial contraction and ultimately elevate BP (Schiffrin, 1995; Bader et al., 2001). Activation of the PLC-DAG-IP_3 pathway, increased $[Ca^{2+}]_i$ and amplified contractile responses of VSMC to vasoconstrictors, have been determined in hypertensive animals models and in hypertensive patients (Bazan et al., 1992; Silver et al., 1992; Touyz et al., 1995; Kosch et al., 2001). Furthermore, increased expression and activity of PKC has been reported in animal models of hypertension (Salamanca and Khalil, 2005) such as spontaneously hypertensive heart failure (SHHF) (Johnsen et al., 2005), and the SHR (Bazan et al., 1992). Collectively, these findings highlight a positive correlation between elevated vasoconstrictor concentrations, amplified GPCR/$G_{\alpha q}$ signalling and hypertension. Over-stimulation of $G_{\alpha q}$ signalling pathways is likely to not only enhance contraction of VSMC, but also activate signalling pathways associated with vascular remodelling (Mehta and Griendling, 2007; Huang et al., 2011).

1.3.3. Vasoconstrictors GPCR relevant signalling pathways on VSMC proliferation

Hypertension is associated with increased circulating levels of vasoconstrictors which correlates with increased vasoconstrictor GPCR activity and an enhancing of their downstream signal transduction (Schiffrin, 1995; Bader et al., 2001; Kubo et al., 2002). In VSMCs, AngII mediates its effect mainly via AT_1, a $G_{\alpha q}$ coupled receptor that stimulates various downstream signalling cascades promoting proliferation of VSMC (Watanabe et al., 2001; Paul et al., 2006; Mehta and Griendling, 2007; Kawai et al., 2017). Many previously published studies report that AngII induced proliferation of aortic smooth muscle cells derived either from spontaneously hypertensive rats, Sprague Dawley rats or from white rabbits (Geisterfer et al., 1988; Paquet et al., 1990; Watanabe et al., 2001; Qin et al., 2004). Furthermore, data show that attenuation of AngII action via inhibition of ACE or blocking of AT_1 receptors suppressed proliferation of VSMC (Uehara et al., 1993; Mabrouk et al., 2001). In VSMCs, ET1 exerts its effects through ET_A, a $G_{\alpha q}$ coupled receptor which stimulates several downstream signalling pathways that induce proliferation of VSMC (Hirata et al., 1989; Daou and Srivastava, 2004; Huang et al., 2011). Previous reports show that the powerful mitogenic effect of ET1 on VSMC derived from hypertensive rats can be reversed by pharmacological blockade of ET_A receptors (Schiffrin et al., 1993; Li et al., 1996b; Li et al., 1996a; Ljuca and Drevensek, 2010). Although AngII and ET1 are known to activate a plethora of intracellular signalling pathways, both agonists are known to active the MAPK/ERK and PI3K/Akt pathways, both of which are centrally linked to cell proliferation (Takahashi et al., 1999; Zhao et al., 2005; Ljuca and Drevensek, 2010; Huang et al., 2011). Therefore, the next section will focus on the roles that MAPK/ERK and PI3K/Akt signalling pathways play in cellular proliferation.

1.3.3.1. $G_{\alpha q}$ modulates MAPK/ERK signalling pathway

Mitogen activated protein kinase (MAPK) cascades are essential signal transduction pathways that regulate a diverse number of cellular functions including cell proliferation (Zhang and Liu, 2002). MAPKs are serine-threonine kinases that are activated by a wide-range of stimuli, and have been characterised into three cascades: extracellular signal-regulated kinase (ERK; including ERK1/2, ERK3/4, ERK5 and ERK7); Jun kinase (JNK and SAPK) and; p38 kinase. MAPK cascades involve activation of a series of enzymes including a MAPK kinase kinase, a MAPK kinase and a MAPK (Widmann et al., 1999). One critical MAPK cascade implicated in cell cycle progression and mitogenesis is the p44/p42 MAPK (ERK1/2) pathway (Pagès et al., 1993). The majority of $G_{\alpha q}$ coupled receptors have been shown to activate ERK1/2 via the Ras/Raf/MEK cascade. $G_{\alpha q}$ subunits transduce signals through PLC-DAG-PKC and PLC-IP_3-Ca^{2+} mechanisms (**Figure 1.8**) (Muthalif et al., 1996; Schonwasser et al., 1998). Both mechanisms activate Ras and lead to activation of the MAPK kinase Raf, which in turn activates MEK1/2, which then phosphorylates ERK1/2 at threonine 202 and tyrosine 204, ultimately enhancing their enzymatic activity (Xu et al., 2001). Subsequently, phosphorylated/active ERK1/2 translocates to the nucleus and mediates the regulation and activation of a large number of genes, which stimulate cell growth and differentiation (Zhang and Liu, 2002). Another mechanism for the Ras/Raf/MEK cascade activation through $G_{\alpha q}$ coupled receptors-mediated receptor of tyrosine kinase transactivation. For example, $G_{\alpha q}$ coupled receptors transactivate EGFR (Prenzel et al., 1999; Ohtsu et al., 2006). This transactivation plays an essential role in propagation of down-stream signalling. For instance, α_1-adrenoceptor-mediated transactivation of EGFR and ERK1/2 activation which participate in smooth muscle cell growth (Zhang et al., 2004).

In hypertension, ERK1/2 activation levels are enhanced in hypertensive animal models such as SHR and (DOCA)-salt hypertensive rats (Kim et al., 1997; Kim et al., 2005b). Additionally, AngII-induced ERK1/2 activation is amplified in SHR VSMCs (Touyz et al., 2001; Kubo et al., 2002). Elevated levels of ERK1/2 activation can be involved in both vasoconstriction and proliferation of VSMC (Roberts, 2012). Furthermore, previous work shows that stretching of the arterial wall stimulates vessel contraction and enhances ERK1/2 activity (Oeckler et al., 2003). Moreover, ERK1/2 activates MLCK, enhances MLC phosphorylation and thus can induce VSMC contraction (Klemke et al., 1997). In addition, ERK1/2 signalling is implicated in proliferation of VSMC (Watts, 1998; Yamanaka et al., 2001). Jing et al., demonstrate that ERK1/2

activation is associated with hypertension-induced increases renal arterial wall thickness in SHR, which can be ameliorated by inhibition of ERK1/2 phosphorylation (Jing et al., 2011). $G_{\alpha q}$ coupled vasoconstrictors such as AngII and ET1 have been reported as promoter of proliferation of VSMC via the ERK1/2 signalling pathway (Zhao et al., 2005; Ljuca and Drevensek, 2010). These studies highlight the important role of the MAPK/ERK signalling pathway in hypertension and hypertensive vascular remodelling.

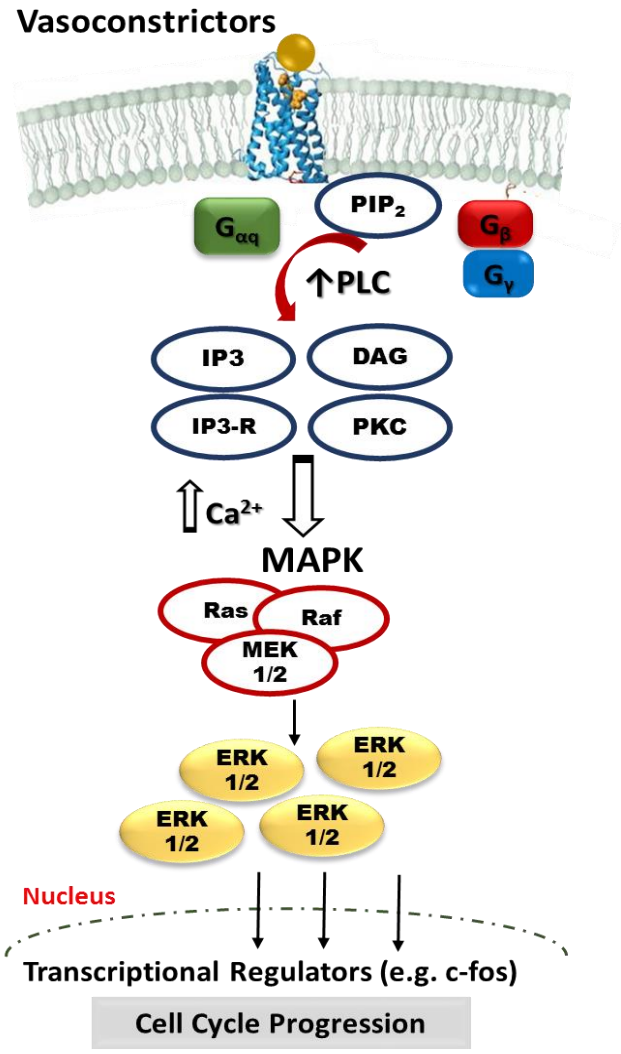


Figure 1.8 Schematic diagram showing the role of the GPCR on MAPK/ERK signalling cascade activation.

Activation of vasoconstrictor GPCRs results in generation of the second messengers DAG and IP_3, which increase $[Ca^{2+}]_i$ resulting in Ras/Raf/MEK cascade activation, which regulates a variety of nuclear genes to stimulate cell cycle progression. PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptors; PKC, protein kinase C.

1.3.3.2. $G_{\alpha q}$ modulates PI3K/Akt signalling pathway

Phosphoinositide 3-kinase (PI3K) is an enzyme that has multiple catalytic and regulatory subunits, and belongs to a family of enzymes that can be classified based on their lipid substrate specificity into three classes; class I PI3K including class IA PI3K and class IB PI3K, class II PI3K and class III PI3K. They form heterodimeric complexes with several adaptor proteins that link different upstream and downstream signalling cascades (Vanhaesebroeck et al., 1997). One major PI3K effector is Akt, also known as protein kinase B (PKB) which is a serine/threonine kinase. There are currently three Akt isoforms; Akt1 (PKB_{α}), Akt2 (PKB_{β}) and Akt3 (PKB_{γ}). Akt2 distribution is limited to insulin-responsive tissues while Akt1 and Akt3 are ubiquitously expressed (Bellacosa et al., 2004). Akt enzymes have two phosphorylation sites, Thr 308 located in the activation loop and S473 in a C-terminal motif (**Figure 1.9**). Class I PI3Ks are acutely activated by tyrosine kinase receptors and/or GPCRs, leading to generation of the phosphatidylinositol-3,4,5-trisphosphate (PIP_3) (Vanhaesebroeck et al., 2001; Guillermet-Guibert et al., 2008). PIP_3 binds to the PH domain of Akt, enabling its membrane recruitment and subsequent phosphorylation by 3' phosphoinositide dependent-kinase 1 (PDK1) at the Thr 308 residue. PDK1 is also activated by PIP_3 (Alessi et al., 1997). To terminate PI3K-PIP_3-PDK1-Akt signalling, PIP_3 is dephosphorylated by PTEN (Leslie and Downes, 2004). Once phosphorylated Akt activates diverse downstream effectors such as protein kinases and adaptor proteins that mediate signalling cascades controlling cell cycle progression and proliferation (Bellacosa et al., 2004). The Akt proliferative effect is mediate by phosphorylation of glycogen synthase kinase 3 (GSK3) and thus the inhibition of GSK3 activity, a regulator for various downstream targets (Beurel et al., 2015). Moreover, Akt can stimulate cell proliferation via regulation of the cell-cycle. Akt signalling increases cyclin D1, increases c-myc transcription and prevents cell cycle arrest, thus promoting cell survival and cell cycle progression (**Figure 1.10**) (Chang et al., 2003; Liang and Slingerland, 2003). Moreover, Akt signalling is known to participate in the pathogenesis of vascular remodelling through proliferation and migration of VSMC (Fernández-Hernando et al., 2009). Furthermore, the activation of many different $G_{\alpha q}$ coupled receptors has been linked to the activation of PI3K-Akt and subsequently promotion of cell growth (Murga et al., 1998; Takahashi et al., 1999; Ushio-Fukai et al., 1999). Previously published studies highlight the involvement of the Akt-GSK3β pathway in proliferation of VSMC (Park et al., 2003), increasing

proliferation and survival of VSMCs and the proliferation of adventitial fibroblasts associated with arterial injury-induced medial thickening (Stabile et al., 2003; Havelka and Kibbe, 2011). These such evidence emphasises the important role of PI3K/Akt signalling in proliferation of VSMC.

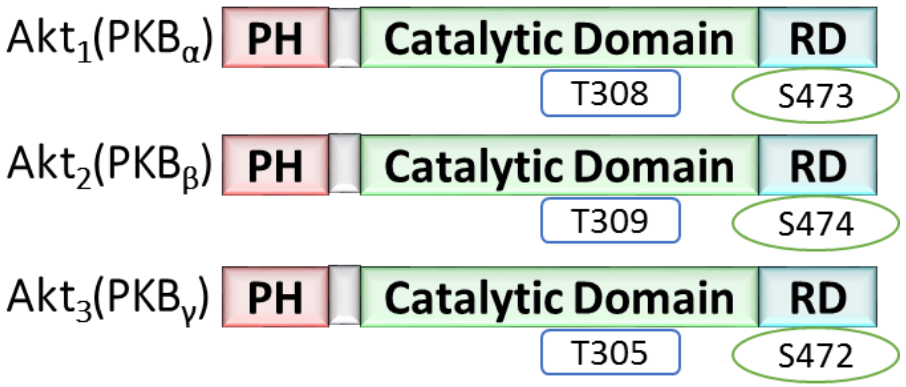


Figure 1.9 Sturctures of different Akt isoforms showing their activation sites.

All the Akt isoforms are composed of a catalytic (kinase) domain in the central zone of the molecule, a PH domain (pleckstrin homology) may acts as a PIP_3 binding site and a RD (regulatory domain) is located at the C-terminal. Threonine and serine phosphorylation sites are located in the activation loop of the kinase domain and in the RD domain. Adapted from Martelli et al., 2012.

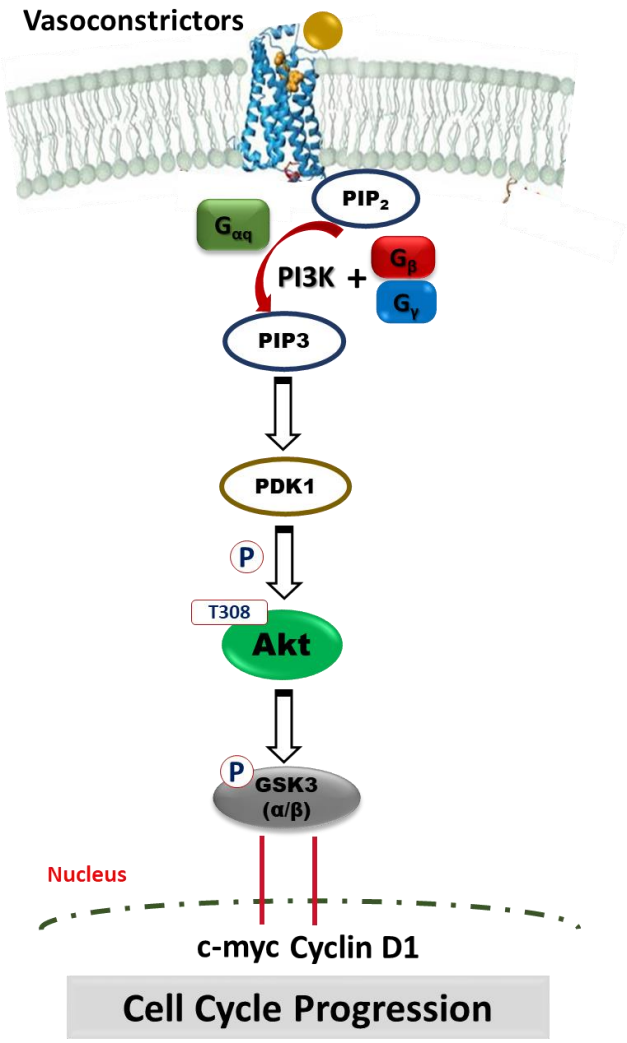


Figure 1.10 Schematic diagram showing the effect of GPCR activation on the PI3K/Akt signalling cascade.

Activation of vasoconstrictor GPCRs results in activation of PI3K which converts PIP_2 to PIP_3. PIP_3 activates PDK1, which phosphorylates Akt at T308. Activated Akt phosphorylates and inhibits GSK3(α/β) activity, resulting in the stimulation of cell cycle progression, survival and proliferation. PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PI3K, phosphoinositide 3-kinase; PDK1, phosphoinositide dependent-kinase 1; Akt, protein kinase B; GSK3(α/β), glycogen synthase kinase 3.

1.3.4. GPCR regulation

1.3.4.1. GPCR signalling termination

Regulation of GPCR activity and signalling pathways is tightly regulated by processes termed desensitization which can be described as the loss of response as a consequence of repeated or prolonged agonist stimulation (Hausdorff et al., 1990). GPCR desensitization can be defined as the physical uncoupling of G proteins from their associate receptors resulting in a decrease in their ability to activate and initiate intracellular signalling cascades (Penela et al., 2003). Receptor desensitization is thought to provide a protective mechanism to prevent prolonged or inappropriate signalling (Lodowski et al., 2003; Brinks and Eckhart, 2010). Receptor desensitization is initiated by GPCR phosphorylation which can be mediated by two major proteins kinases classes. The first class is G protein coupled receptor kinases (GRKs) which phosphorylate agonist-bound active GPCRs mediating homologous receptor desensitization (Willets et al., 2003). Homologous desensitization is triggered when a ligand bind to its cognate receptor and only the ligand occupied receptor will undergo a decreased capacity to signal (Kelly et al., 2008). Secondly, GPCRs can also be phosphorylated by second messenger kinases such as PKA and PKC, which are able to phosphorylate receptors that possess appropriate PKA or PKC motifs, irrespective of whether the GPCR is occupied by agonist, thus producing heterologous desensitization (Stadel et al., 1983; Hausdorff et al., 1990; Willets et al., 2003). Heterologous GPCR desensitization, is a more general process that can affect the responsiveness of both agonist-occupied or unoccupied GPCRs (Kelly et al., 2008). Receptor phosphorylation subsequently increases the affinity of GPCRs for arrestin proteins, whose binding physically prevents further GPCR/G protein interactions and therefore lead to a cessation of G protein signalling (Brinks and Eckhart, 2010).

1.3.4.2. Role of GRKs and arrestins in GPCR desensitization

The GRK family consist of seven serine/threonine kinases that belong to the AGC kinase family that are activated by (active) ligand-occupied GPCRs (Homan and Tesmer, 2014). GRKs can be functionally divided into three subcategories: the visual GRKs including GRK1 (rhodopsin kinase) and GRK7 (iodopsin kinase); the non-visual GRKs including β-adrenergic receptor kinase, containing GRK2 (β-ARK1) and GRK3 (β-ARK2) and; the GRK4 sub-group, which includes GRK4, GRK5 and GRK6 (Willets et al., 2003). At the tissue level, GRK1 and GRK7 are predominantly expressed in the retina and are involved in photoreceptor regulation. GRK4 has a limited tissue distribution and

is mainly found in testes, the kidney and some areas of the brain, while GRKs 2, 3, 5 and 6 are ubiquitously expressed. Therefore, most GPCRs are potentially regulated by only four GRKs, 2, 3, 5, or 6 (Premont et al., 1996; Pugh and Lamb, 2000; Premont and Gainetdinov, 2007). Structurally, GRKs are multi-domain proteins with a shared central serine/threonine protein kinase catalytic domain (~270 residues), an N-terminal domain (~185 residues) which is thought to be important for receptor recognition and contains a regulator of G protein signalling (RGS) homology domain (RH) (~120 residues) and finally a variable-length C-terminal domain (~105-230 residues) (Penela et al., 2010b; Homan and Tesmer, 2014) (**Figure 1.11**). The C-terminal domain structure is the determining factor for GRK membrane localization. Indeed, GRKs 1 and 7 contain prenylation motifs and are constitutively attached to the plasma membrane. GRKs 4 and 6 are palmitoylated which enables their membrane attachment, whereas the C-terminus of GRK5 contains positive lipid-binding sites which interact with the negative phospholipid membrane. Conversely, GRK2 and GRK3 are cytoplasmic proteins which are recruited to ligand bound GPCRs at the plasma membrane. Agonist-dependent membrane translocation of GRK2 and GRK3 is mediated by several structural components including the C-terminal pleckstrin homology (PH) domain which contains binding sites for the membrane phospholipid PIP_2 and $G_{\beta\gamma}$ dimer subunits (Penela et al., 2003; Ribas et al., 2007; Homan and Tesmer, 2014). In addition, a second $G_{\beta\gamma}$ binding site has been mapped in the N-terminal region of GRK2 (Eichmann et al., 2003). Furthermore, both kinases contain N-terminal RH domains, which bind to active, GTP bound $G_{\alpha q}$. This binding can subsequently inhibit GPCR/$G_{\alpha q}$ interactions to terminate G protein signalling (Tesmer et al., 2005).

Arrestins are a family of multifunctional adaptor proteins with only four different isoforms. The visual isoforms (arrestin1 and arrestin4) are expressed only in the retina, while the widely expressed somatic isoforms arrestin2 (β-arrestin1) and arrestin3 (β-arrestin2) are involved in the regulation of most GPCRs (Premont and Gainetdinov, 2007; Evron et al., 2012). Structurally, arrestins consist of two curved-shaped β-sandwiches, labelled as N and C domains (**Figure 1.11**) (Scheerer and Sommer, 2017). Arrestin2 and arrestin3 have 78% matching amino acid sequence homology with differences in the C-domain. Although arrestin2 and 3 are thought mainly to reside in the

cytoplasm, both have a nuclear localization signal located at the N-domain, and as such can be translocated into the nucleus (Wang et al., 2003; Hoeppner et al., 2012). Additionally, arrestin3 has a nuclear export signal located at C-domain that enables its shuttling to the cytoplasm (Wang et al., 2003).

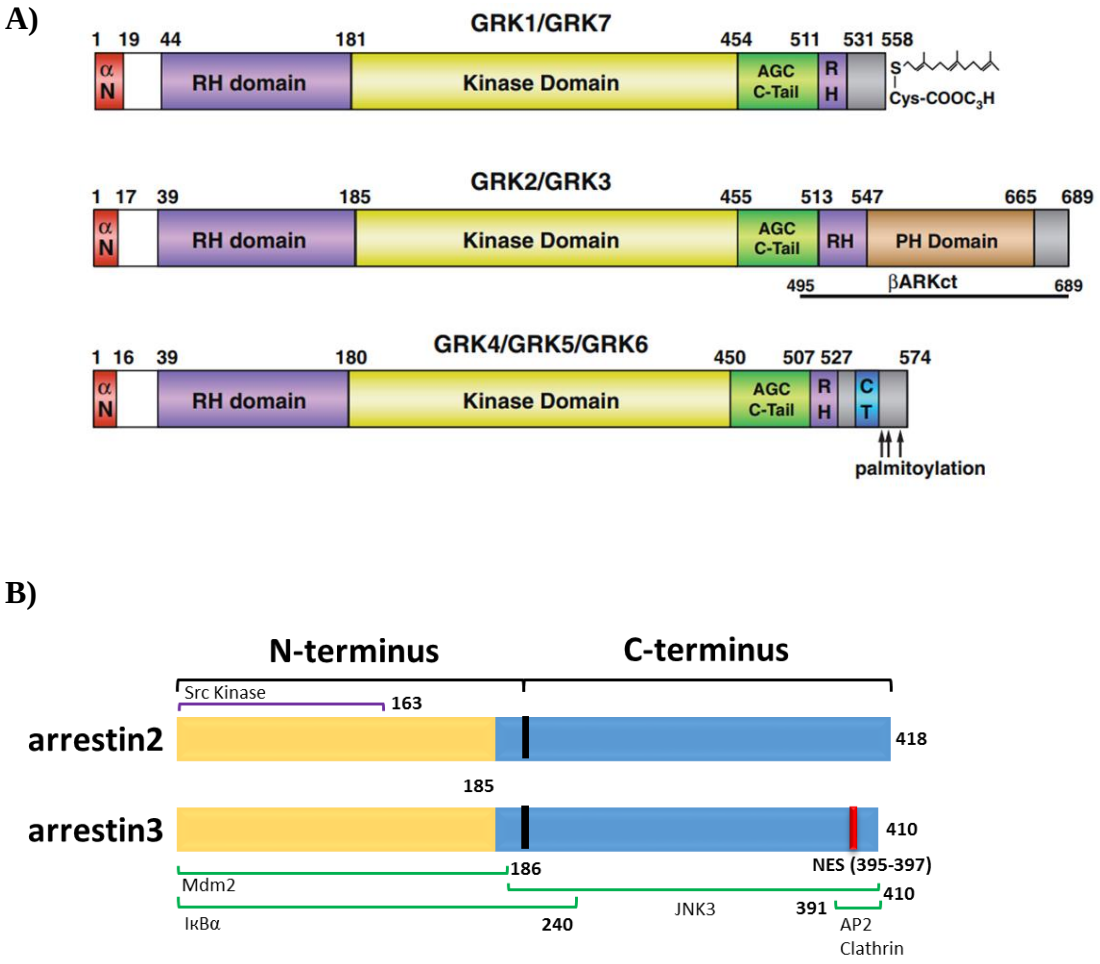


Figure 1.11 Basic structures for the GRK subfamilies and arrestins.

A) Basic multiple domain structure for GRKs contain an N-terminal regulator of G-protein signalling (RGS) domain (red) and a central serine/threonine kinase catalytic domain (yellow). The major differences between GRK members are within the C-terminal domains (green). The C-terminal is prenylated in the visual GRK subfamily and contains palmitoylation sites in the GRK4 subfamily. In GRK2 and GRK3, C-terminus contains a pleckstrin homology (PH) domain with binding sites for $G_{\beta\gamma}$ and PIP_2 (Homan and Tesmer, 2014). B) Arrestins structural domains contain two major domains: the N-terminus domain and C-terminus domain. The N domain is implicated in nuclear localization (yellow) of both arrestins and the nuclear export signal (NES) (red) is in arrestin3 only. Binding sites such as the binding site for Src, Mdm2, JNK3 and IκBα are shown as purple and green lines. Modified from Ma and Pei, 2007; Sharma and Parameswaran, 2015.

Agonist binding and/or GPCR activation induces GRK recruitment and subsequent phosphorylation of serine/threonine moieties situated in the third intracellular loop or C-terminal tail of the receptor (Benovic et al., 1991; Lefkowitz, 1993). This results in the creation of specific phosphorylation patterns called phosphorylation barcoding, which has been proposed as the determinant of arrestin recruitment (Penela et al., 2010b). GPCR phosphorylation consequently increases the affinity of GPCRs for arrestin proteins, promoting their recruitment and creating a low-affinity pre-complex with the receptor (Attramadal et al., 1992; Scheerer and Sommer, 2017). Phosphorylated GPCRs induce conformational changes in the arrestin structure that allow the second binding step, i.e. tight arrestin-GPCR binding, which creates a high-affinity complex (Scheerer and Sommer, 2017). The physical association of GPCR and arrestin prevents further GPCR/G-protein interaction, terminating intracellular G protein-dependent signalling. Additionally, phosphorylated GPCR-arrestin complexes are targeted for clathrin-mediated endocytosis, a process which initiates the recycling of GPCRs to the plasma membrane and their re-sensitization. Based on the strength of the GPCR-arrestin interaction, receptors undergo either recycling (weak interaction; class A GPCR), or sustained internalization into endosomes (strong interaction; class B GPCR), or degradation (Oakley et al., 2000; Kelly et al., 2008; Penela et al., 2010b). Furthermore, arrestins function as ligand-regulated adaptor scaffolds which enable the formation of signalling complexes by recruiting proteins to activated GPCRs (Luttrell and Miller, 2013).

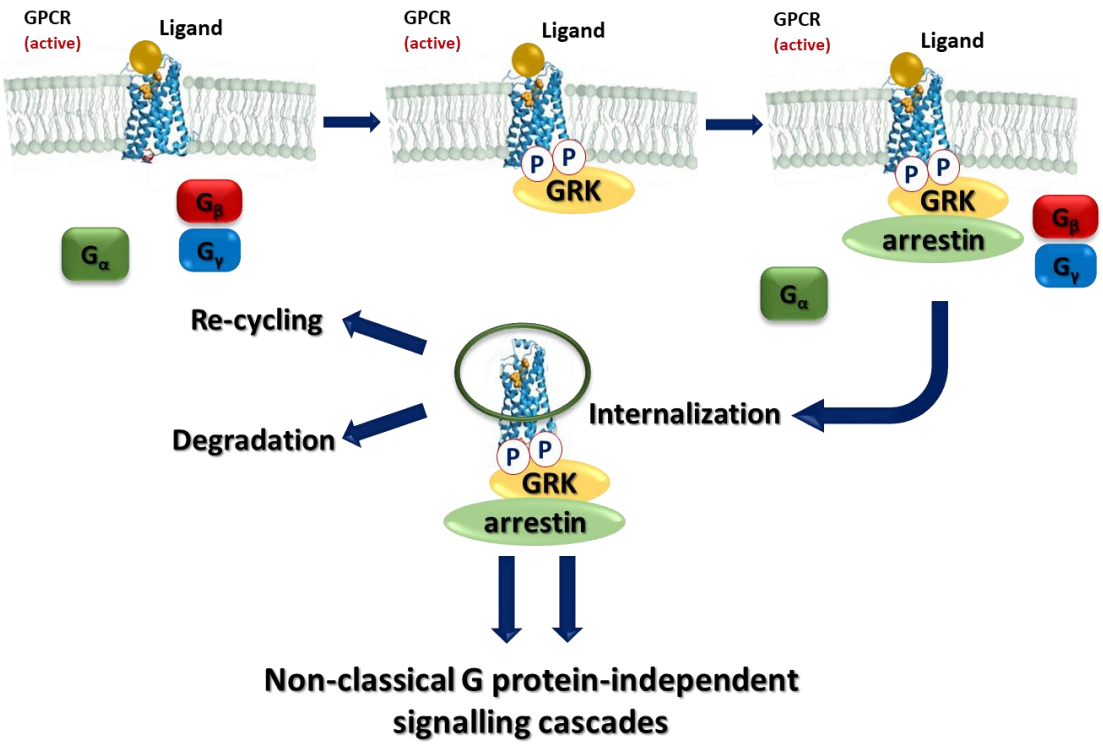


Figure 1.12 GPCR regulation by GRKs and arrestins.

Upon GPCR activation and G protein activation, agonist-occupied GPCRs are subsequently phosphorylated by GRKs in the third intracellular loop or C-terminal. Receptor phosphorylation increases the affinity for arrestin recruitment. This enables arrestin binding to the GPCR, which results in termination of G protein signalling, receptor desensitization, internalization, and initiation of G protein-independent signalling.

1.3.4.3. Multiple functions of GRK2 beyond GPCR desensitization

The role of GRKs and arrestins in GPCR function are not limited to receptor desensitization, and previous evidence indicates that both GRKs and arrestins are capable of interacting with a diversity of cellular proteins. This implicated in signal transduction in a G protein-independent manner that is also called non-classical or non-canonical GPCR signalling. This could be involved in sustained GPCR effects (Shenoy and Lefkowitz, 2011, Penela et al., 2010a). As GRK2 a multiple domain protein (total of ~689 residues, ~80 kDa), GRK2 is capable of having several forms of protein-protein interactions. As mentioned in *Section 1.3.4.2*, GRK2 binds directly to $G_{\alpha q}$ subunits via its N-terminal RH domain and can also bind to $G_{\beta\gamma}$ dimers through its C terminal PH domain (Willets et al., 2004; Tesmer et al., 2005; Homan and Tesmer, 2014). Furthermore, GRK2 can regulate the activity of some tyrosine kinase receptors such as the EGF, IGF-1, and PDGF receptors (Hildreth et al., 2004; Hupfeld and Olefsky, 2007; Chen et al., 2008). GRK2 can also phosphorylate non-receptor substrates such as R-Smad, resulting in the prevention of R-Smad complex nuclear translocation (Ho et al., 2005a). Moreover, GRK2 can modulate cellular responses in a phosphorylation-independent process, acting as a scaffold for a diverse number of proteins implicated in signalling pathways and trafficking (Penela et al., 2010b). Indeed, previous reports have shown that GRK2 can be associated directly with PI3K, clathrin, MEK and caveolin-1 (Shiina et al., 2001; Naga Prasad et al., 2002a; Schutzer et al., 2005; Jimenez-Sainz et al., 2006). Multiple phosphorylation-dependent and/or independent GRK2 interactions suggest that GRK2 might act as an effector contributing to the regulation of various cellular signal transduction processes. Furthermore, alterations in GRK2 expression and activity are implicated in several pathological conditions, which implies that these GRK2 alterations could participate in disease onset or progression (Ribas et al., 2007; Penela et al., 2010b). For example, changes in GRK2 expression and/or activity have been reported in several diseases such as opioid dependence, heart failure, ovarian malignancies, Alzheimer's disease, multiple sclerosis and rheumatoid arthritis (Lombardi et al., 2001; King et al., 2003; Ferrer-Alcon et al., 2004; Iaccarino et al., 2005; Vroon et al., 2005; Obrenovich et al., 2009; Rengo et al., 2014). Interestingly, hypertension is associated with elevated levels of GRK2 expression in arterial smooth muscle (Gros et al., 1997; Gros et al., 2000; Eckhart et al., 2002; Cohn et al., 2008; Cohn et al., 2009; Willets et al., 2015), which suggests that GRK2 may play a role in the development of this disease. Therefore,

the potential mechanisms by which GRK2 participates in hypertension and vascular remodelling will be discussed in the following section.

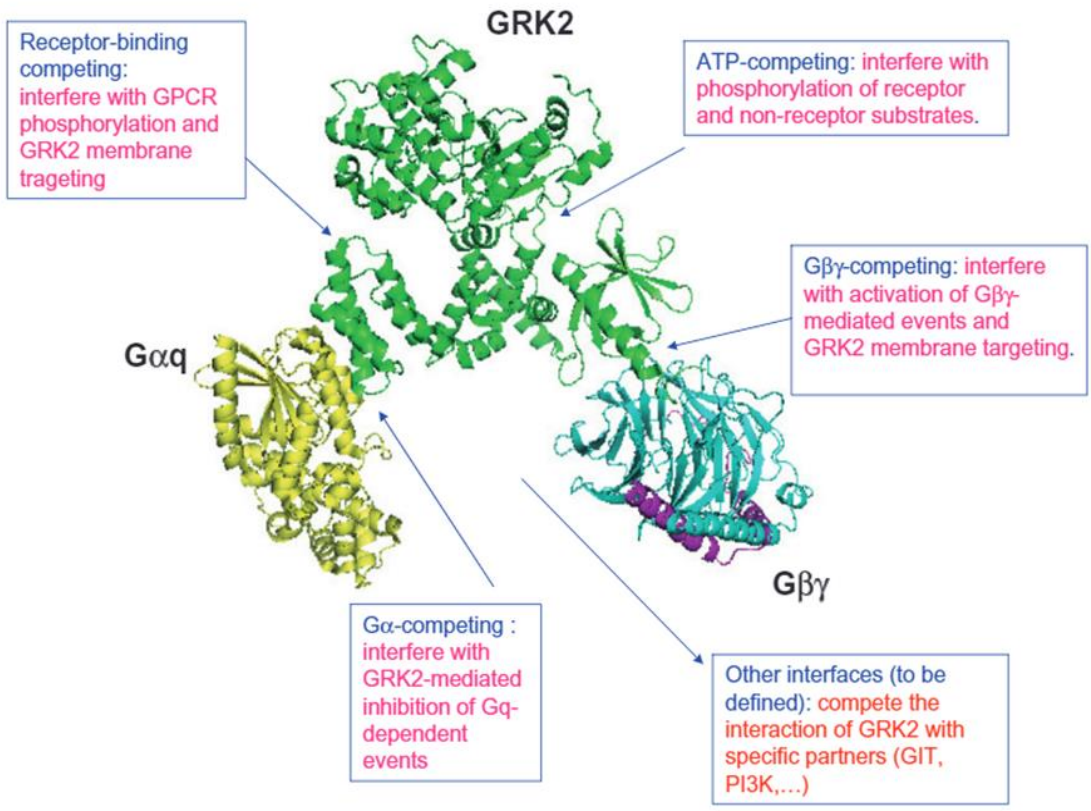


Figure 1.13 Structure of GRK2 with $G_{\beta\gamma}$ and $G_{\alpha q}$ interaction.

Different GRK2 interactions with several signalling proteins may participate in various cellular functions. Crystal structure showing $G_{\beta\gamma}$ interaction with the PH domain, $G_{\alpha q}$ interaction with the RH domain and ATP-binding site located in the catalytic domain Tesmer et al., 2005; Penela et al., 2010b.

1.3.4.4. What is known about the roles that GRK2 plays in hypertension and vascular remodelling?

In VSMCs, continuous exposure to elevated circulating levels of vasoconstrictors such as ET1, AngII or UTP increases signalling through their cognate GPCRs (ET_A, AT_1 and $P2Y_2$), which couple to $G_{\alpha q}$ signalling that plays a major role in hypertension pathogenesis (Harris et al., 2007). Increased $G_{\alpha q}$ signalling elevates intracellular Ca^{2+} concentrations, leading to increased VSMCs contraction, increasing peripheral vascular resistance, and also contributes to vascular remodelling (Grassi, 2006; Harris et al., 2008; Lymperopoulos and Bathgate, 2013). Existing evidence suggests that GRK2 is an important regulator of the sensitivity and responsiveness of these receptors in VSMCs, acting to prevent prolonged or inappropriate $G_{\alpha q}$ signalling. Indeed, previously published studies confirm that GRK2 is the key negative regulator for ET_A and $P2Y_2$ receptor mediated PLC activity in VSMCs (Morris et al., 2010; Morris et al., 2011). Moreover, GRK2 also regulates AT_1 activity and has been shown to phosphorylate the AT_1 expressed in human embryonic kidney 293 (HEK293) cells and in cardiomyocytes (Oppermann et al., 1996; Rajagopal et al., 2006; Violin et al., 2006). Furthermore, Rainbow et al., 2018, reported that inhibition of GRK2 activity in VSMCs decreases the desensitization process of AngII and UTP-stimulated PLC-mediated arterial contractions, thus confirming GRK2 as the major regulator of vasoconstrictor GPCRs function in whole arteries (Rainbow et al., 2018). Therefore, GRK2 may show central roles in preventing the increased $G_{\alpha q}$ signalling observed during hypertension. Certainly, recent studies show that GRK2 expression is enhanced in hypertensive animal models (Gros et al., 2000; Eckhart et al., 2002; Cohn et al., 2008; Willets et al., 2015), as well as in hypertensive patients (Gros Robert, 1997; Cohn et al., 2009). Although it is not clear if the changes in GRK2 expression are a causal factor or a consequence of hypertension, it has been shown that enhanced vascular tone in hypertension can act as a promoter for GRK2 expression in aorta smooth muscle (Ramos-Ruiz et al., 2000). Moreover, $G_{\alpha q}$ mediated vasoconstriction increased GRK2-promotor activity in HEK293 cells (Ramos-Ruiz et al., 2000), and $G_{\alpha q}$-mediated activation of MAPK/ERK increased endogenous GRK2 protein expression in primary cardiomyocyte cells (Theilade et al., 2002; Theilade et al., 2005). Interestingly, GRK2 has been reported to inhibit endothelial NO production, highlighting another mechanism by which increased GRK2 expression could play a pathophysiological role in the development of hypertension (Feldman, 2002). Additionally, GRK2 is reported to mediate the desensitization of β-adrenoceptors, which

play a leading role in mediating vasodilation. Therefore, increased GRK2 expression may impair vasodilation in hypertension, which possibly contributes to the shifting in the balance between vasoconstrictors and vasodilators processes toward more vasoconstriction resulting in elevation of BP (Gros et al., 2000).

At the level of vascular remodelling, GRK2 overexpression in VSMC resulted in a 30% increase in vascular wall thickness (Eckhart et al., 2002), suggesting a link between GRK2 expression levels and agonist-induced VSMC proliferation (Ramos-Ruiz et al., 2000). Although little is known about the molecular mechanisms by which GRK2 may regulate VSMC proliferation, there is some evidence showing that GRK2 plays an important role in cell proliferation. Penela et al., reported that cyclin-dependent kinase 2 (CDK2) phosphorylates GRK2 and consequently interacts with the prolyl-isomerase promotes (Pin1), to promote cell cycle progression. This interaction results in temporary down-regulation in GRK2 expression in the G_2 phase, which is important for normal cell cycle progression (Penela et al., 2010a). Moreover, GRK2 knockout results in embryonic lethal-hypoplasia of ventricular myocardium (Jaber et al., 1996). Collectively these findings suggest that GRK2 plays a key role in cell growth and development in the cardiovascular system. Many research articles describe the possible molecular mechanism by which vasoconstrictors can induce VSMC proliferation (Komuro et al., 1988; Hirata et al., 1989; Muthalif et al., 1996; Wen et al., 1996; Qin et al., 2004; Lee et al., 2006; Ljuca and Drevensek, 2010). However, whether GRK2 (the main kinase regulator of vascular contractile GPCRs) over-expression in hypertension is involved in vasoconstrictor induced VSMC proliferation remains to be established.

Overall, hypertension-induced vascular remodelling is the main contributor to end-organ damage. It is characterized by an increase in vascular wall thickness and a reduction in lumen diameter, which is strongly associated with many cardiovascular diseases. Proliferation of VSMCs is one of the important changes during the development of hypertensive vascular remodelling. Hypertension is connected with increased circulating levels of many vasoconstrictors that leads to continuous activation of $G_{\alpha q}$ signalling, enhanced VSMCs contraction, and contributes to VSMC proliferation via activation of various signalling pathways. In addition, hypertension is associated with elevated levels of GRK2 in arterial smooth muscle cells, which is known to negatively regulate $G_{\alpha q}$ signalling. However, the roles of GRK2 protein in VSMC proliferation, associated with hypertensive vascular remodelling have not been extensively studied. This thesis aims to

examine the potential effects of GRK2 in vasoconstrictor-induced VSMC proliferation and remodelling that are associated with hypertension.

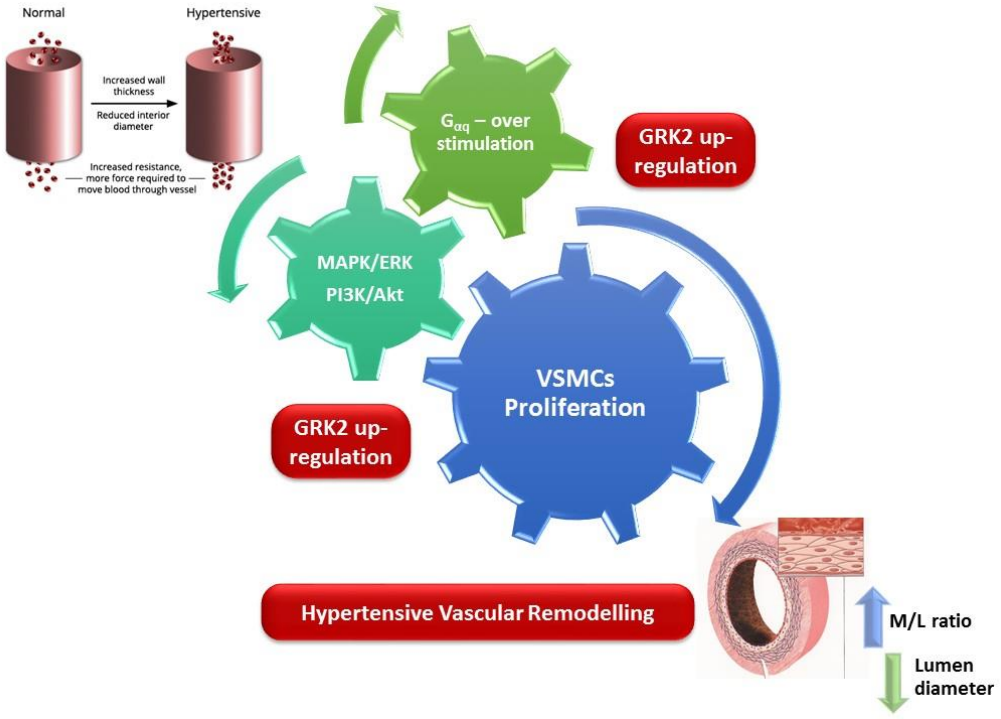


Figure 1.14 Proposed relationship between GRK2 and vasoconstrictor GPCRs and proliferation of VSMC.

Hypertension is associated with increased circulatory levels of vasoconstrictors, which mediate their vasoconstriction ability via $G_{\alpha q}$ stimulation, increased $G_{\alpha q}$-related activation of MAPK/ERK and PI3K/Akt signal transduction that promote proliferation of VSMC. Consequently, over-stimulation of vasoconstrictor GPCR signalling leads to increased media/lumen ratios and reduced vessel inner diameter. As GRK2 is the main negative regulator of vasoconstrictor GPCR signalling it is possible that GRK2 plays a major role in hypertension-induced vascular remodelling.

1.4. Aims and objectives

1.4.1. Aims

To investigate the roles that GRK2 plays in the control and regulation of vasoconstrictor-stimulated proliferation of VSMC. Furthermore, to characterise the possible molecular mechanisms underlying GRK2 regulation proliferation of VSMC, in particular to identify the effect of GRK2 on $G_{\alpha q}$-mediated MAPK/ERK and PI3K/Akt signalling pathways.

1.4.2. Objectives

1.4.2.1. Is GRK2 essential for vasoconstrictor-mediated VSMC proliferation?

As hypertension is associated with increased circulatory levels of vasoconstrictors, initially the proliferative effect of vasoconstrictors (AngII, ET1, V, NA and UTP) on VSMCs will be investigated. Next, the requirement for GRK2 expression and/or its catalytic activity in vasoconstrictor-induced VSMC proliferation will be determined. To achieve this the effects of vasoconstrictors on VSMC proliferation will be determined using standard [^{3}H]-thymidine incorporation assays. Also, the effect of these vasoconstrictors on the proliferative marker (Ki67) activation using immunocytochemistry technique will be investigated. After that, similar experiments will be conducted following specific inhibition of GRK2 kinase activity using compound 101 (COMP101), a small molecule inhibitor (Lowe et al., 2015), and following depletion of GRK2 protein expression using siRNA techniques (Nash et al., 2018) to determine whether GRK2 expression and/or its catalytic activity regulate vasoconstrictor-stimulated VSMC proliferation.

1.4.2.2. What are the molecular mechanisms underlying GRK2 in regulation of cell proliferation?

Vasoconstrictors can activate several different signalling pathways that are linked to cell survival and proliferation including PI3K/Akt and MAPK/ERK (Takahashi et al., 1999; Zhao et al., 2005). Standard western blotting techniques will be applied to study changes in the phosphorylated (active forms) of MAPKs in order to identify the possible pathways that are involved in the vasoconstrictor-mediated VSMC proliferation. GRK2 expression levels/activity will be altered (using the techniques outline above) to determine whether this protein regulates pro-survival signalling pathways in VSMCs.

Chapter Two

2. Materials and Methods

2.1. Materials

2.1.1. Materials for primary cell preparations, growth medium and supplements, peptides and inhibitors

Angiotensin II (AngII), uridine-triphosphate (UTP), noradrenaline (NA), platelet-derived growth factor-BB human (PDGF-BB), bovine serum albumin (BSA), digestive enzyme (papain) and anti-oxidant (dithiothreitol; DTT) were obtained from ***Sigma Aldrich, UK.*** Dulbecco's phosphate buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM-GlutaMAX) as well as their supplements including foetal calf serum (FCS), penicillin/ streptomycin (P/S), amphotericin B, and trypsin-EDTA were supplied from ***Gibco Life Technologies, UK.*** Endothelin-1 (ET1), vasopressin (V), paroxetine maleate, fluoxetine hydrochloride and compound 101 were purchased from ***Tocris Bioscience, UK.*** PD98059 were purchased from ***Cell Signaling Technology, USA.*** LY294002 was purchased from ***Cambridge Bioscience LTD, UK.*** Flou-4-AM was purchased from ***Life Technologies, USA.*** Cell culture vessels including cell culture flasks (growth area: T25 and T175 cm^2) and filter acrodisc tuffryn (0.2µm; st.25mm) were obtained from ***VWR International LTD, UK*** and multi well plates (6 well and 24 well) were from ***Greiner Bio-one LTD, UK.*** Tissue culture sterile polystyrene, non-pyrogenic individual wrap pipettes (0.1, 5, 10 and 25 ml) and filter pipette tips (10, 200 and 1000 µl) were purchased from ***Fisher Scientific LTD, UK.***

2.1.2. Materials for cell proliferation assays

Radioactive [^{3}H]-thymidine (1mCi), Emulsifier-Safe™ and polyethylene liquid scintillation counting vials were provided by ***PerkinElmer, USA.*** ROCHE Cell Proliferation Kit I (MTT), trichloroacetic acid (TCA), potassium acetate, sodium-hydroxide (NaOH), diethyl ether, ethanol and dimethyl sulfoxide (DMSO) were purchased from ***Sigma Aldrich, UK.***

2.1.3. Materials for protein knockdown

Amaxa™ Basic Nuclecofector™ kit for primary smooth muscle cells was purchase from ***Lonza Bioscience, UK.*** Pre-designed silencers including a negative control siRNA, GRK2 'ARβBK1' siRNA (ID:200413), GRK5 siRNA (ID: s222052), GRK5 siRNA

(ID:s222053), GRK6 siRNA (ID: s133079), arrestin 2 'ARRβ1' siRNA (ID:s1623) and arrestin 3 'ARRβ2' siRNA (ID:s1626) were purchased from ***Invitrogen, Life Technologies, USA.***

2.1.4. Materials for immunoblotting

ProtoGel (30% acrylamide to bisacrylamide stabilized solution at 37.5:1 ratio) was obtained from ***Geneflow LTD, UK.*** TRIS-glycine-SDS running buffer (10X), pH 8.3 and Tween 20 were purchase from ***Fisher Scientific LTD, UK.*** Bradford reagent, Tris HCl, glycine, Ponceau S solution, methanol, sodium dodecyl sulfate (SDS), ammonium persulfate (APS) and N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) were obtained from ***Sigma Aldrich, UK.*** Chameleon^{TM} Duo pre-stained protein ladder and Odyssey blocking buffer were obtained from ***LI COR Biotechnology, USA.***

Gel casting accessories including casting stands, clamps, short plates, spacer plates, combs, extra thick blotting paper, nitrocellulose membrane (0.45 µm), electrophoresis chambers, semi-dry blotter and electrophoresis power supplies were obtained from ***Bio-Rad Laboratories Ltd, UK.***

All antibodies used in this experiments including anti-phospho-Akt (Thr308), anti p44/42 MAP kinase, anti phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), anti-GAPDH, anti-GRK2 (C-15), anti-GRK6, anti-GRK5, anti-β arrestin1/2 and the secondary antibodies anti-mouse IgG 'DyLight™ 800 Conjugate' and anti-rabbit IgG 'DyLight™ 680 Conjugate' were purchased from ***Cell Signaling Technology, USA.***

2.1.5. Materials for immunocytochemistry

Goat serum and Triton X100 were purchased from ***Sigma Aldrich, UK.*** Anti-Ki67 antibody was obtained from ***Abcam Limited, UK.*** In addition, the secondary antibody donkey anti-rabbit IgG (H+L) Alexa Fluor 488, was purchased from ***Fisher Scientific LTD, UK.*** The goat anti-rabbit IgG (H+L) Alexa Fluor 594 secondary antibody was purchase from ***Life Technologies, UK.*** Anti-fade mountain medium VECTASHIELD® with propidium iodide (PI) and VECTASHIELD® with 4′,6-diamidino-2-phenylindole (DAPI) were purchased from ***Vector Laboratories LTD, UK.*** Slide adhesion Polysine GRND and round coverslips (13mm) were obtained from ***VWR International LTD, UK.*** Coverslips no.1 (22x40mm) were obtained from ***Scientific Lab Supplies LTD, UK.***

2.1.6. Buffer and gel constituents

Table 2.1 Buffer and gel compositions.

Buffer	Composition
Low Ca^{2+} buffer (dissection buffer)	In mM: 5.4 KCl, 137 NaCl, 0.44 Na_2HPO_4, 0.42 NaH_2PO_4, 4 glucose, 6 mannitol, 10 HEPES, 1 $MgCl_2$ and 0.1 $CaCl_2$
Krebs buffer (for cell imaging)	In mM: 134 NaCl, 6 KCl, 1 $MgCl_2$, 10 glucose, 10 HEPES, 1.3 $CaCl_2$, pH 7.4
Krebs Buffer (for time-course stimulation)	In mM: 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $NaHCO_3$, 11.9 glucose, 10 HEPES and 1.3 $CaCl_2$; pH 7.4
1xSDS Lysis Buffer	2% SDS, 100µM Tris HCl, 10% glycerol, 0.1% bromophenol blue, 10% DTT, dH_2O, and 0.1% protease inhibitors; phenyl methane sulfonyl fluoride, leupeptin and benzamidine.
5x Loading Buffer	8% SDS, 50mM Tris HCl, 10% glycerol, 5% β-mercaptoethanol and 0.005% bromophenol blue
10% Running Gel	7.9 ml dH_2O, 6.7 ml acrylamide, 5 ml Tris (1.5M), 200µl SDS (10% *w/v*), 200µl APS (10% *w/v*) and 8µl TEMED
Stacking Gel	5.5 ml dH_2O, 1.3 ml acrylamide, 1 ml Tris (1M), 80µl SDS (10% *w/v*), 80µl APS (10% *w/v*) and 8µl TEMED
Blotting Buffer	Tris-base 48 mM, glycine 39 mM, SDS 0.037% and methanol (20% *v/v*)
Tris Buffered Saline with Tween (TBST)	20 mM Tris HCl, 150 mM NaCl with Tween 20 (0.05 % *v/v*); pH 7.5
5% non-fat dry milk	non-fat dry milk (5% *w/v*) in TBST
4% Paraformaldehyde	2 g paraformaldehyde in 20 ml Na_2HPO_4 (0.2M), 5 ml NaH_2PO_4 (0.2M) and 25 ml of dH_2O
2% Phosphate Buffered Saline with Triton-X100 (PBST)	PBS and Triton-X 100 (0.2% *v/v*)
10% goat serum PBST	Goat serum in PBST (10% *v/v*)

2.2. Methods

2.2.1. Preparation and culturing of rat aortic smooth muscle (RASM) cells

2.2.1.1. Rat aorta enzymatic digestion and cells preparation

Primary RASM from adult male Wistar rats aortas were prepared by enzymatic digestion as previously described (Xu et al., 2009). All procedures were carried out in a tissue culture hood using standard sterile tissue culture techniques. Adult male Wistar rat was euthanatized by cervical dislocation and then, placed in the supine position. Sterile forceps and scissors were used to open the chest and the thoracic aorta was isolated and placed in a culture dish containing sterile ice-cold, low Ca^{2+} buffer (described in **Table 2.1**). After removal of blood, fat and surrounding tissue, the artery was placed in another cell culture dish containing fresh ice-cold low Ca^{2+} buffer (**Figure 2.1**). Next, the aorta was cut into small pieces (2mm thickness) and transferred into pre-warmed (37˚C) enzyme mix [contain papain (1.05 µg/µl) and dithiothreitol (0.9 µg/µl) dissolved in bovine serum albumin (BSA) [(0.1% *w/v*) in low Ca^{2+} buffer] and incubated in a water bath maintained at 37°C for 60 min. After that, aortic tissues were washed several times with BSA in low Ca^{2+} buffer, followed by several washes with low Ca^{2+} buffer to remove all remaining enzyme and prevent further digestion of the tissue. The tissue was then gently triturated with a wide-bore pipette into 3 ml of low Ca^{2+} buffer to release the cells from the tissue. The cells suspension was then transferred into a cell culture flask (T25) and 7 ml of Dulbecco's Modified Eagle Medium (DMEM-GlutaMAX) supplemented with penicillin/streptomycin (P/S) (1% *v/v*) and foetal calf serum (FCS) (10% *v/v*) added. The cells were maintained at 37˚C with 5% CO_2 in a humidified air.

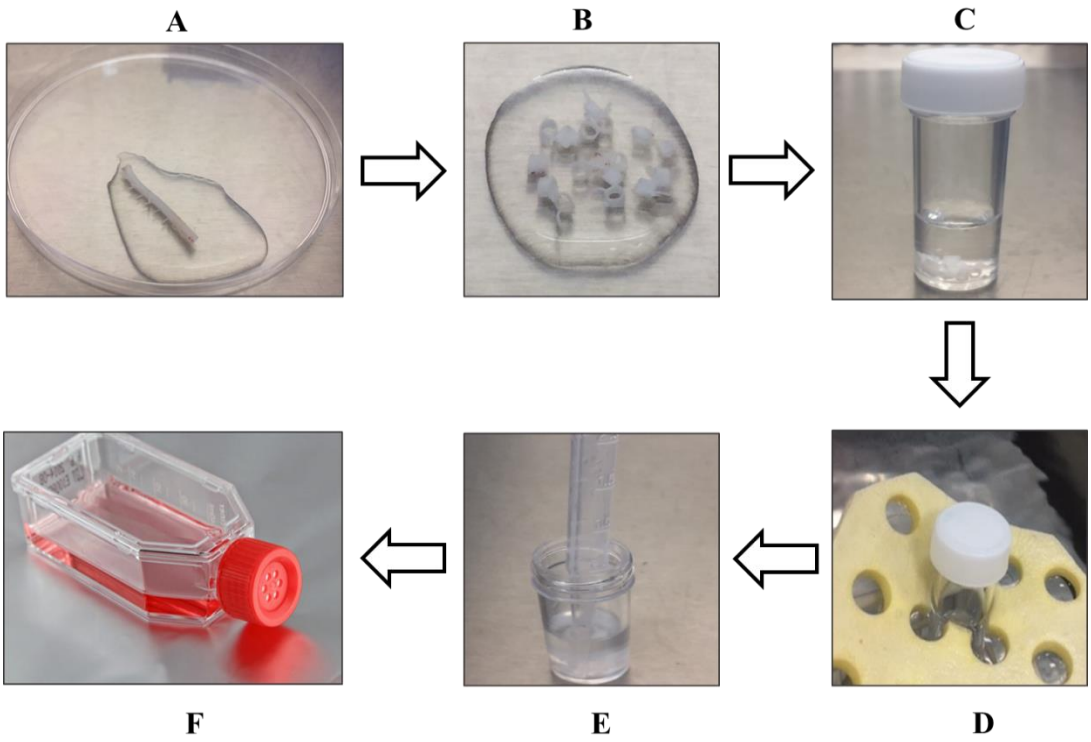


Figure 2.1 Rat aorta enzymatic digestion and cell preparation.

Thoracic aorta (A) was cut into small pieces (B) and transferred into pre-warmed (37˚C) enzyme mix (C). Tissue was then incubated in a water bath at 37°C for 60 min. (D). After that, tissue was triturated with a wide-bore pipette in 3ml of low Ca^{2+} buffer to release the cells from the tissue (E). The cells suspension was then transferred into a cell culture flask (T25) and 7 ml of DMEM-GlutaMAX supplemented with P/S and FCS was added. The cells were maintained at 37˚C with 5% CO_2 in humidified air.

2.2.1.2. Subculture of RASM cells

On the second day, the cells were fed with fresh DMEM-GlutaMAX media supplemented with P/S and 10% FCS, and the media was replaced every 3-4 days. Once almost confluent (~80%), the cells were harvested and transferred to a T175 flask. After removing of the medium, the cells were washed with 6 ml phosphate buffer saline (PBS) to remove FCS, then incubated with 3 ml trypsin-EDTA (0.25%) at 37˚C with 5% CO_2 in humidified air for 1-2 min. The trypsinization process was terminated by addition of 7 ml of 10% FCS/DMEM-GlutaMAX medium. After that, the cell suspension were collected and spun down (1000 xg for 5 min). The supernatant was carefully removed. Cell pellets were re-suspended in 5 ml of 10% FCS/DMEM-GlutaMAX medium before being transferred into a cell culture flask (T175). Next, 30 ml of DMEM-GlutaMAX supplemented with P/S and 10% FCS were added. The cells were maintained at 37˚C with 5% CO_2 in humidified air. Only RASM cells from passages 1-5 were used for experiments.

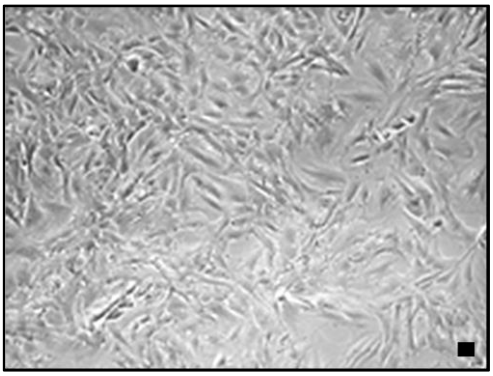


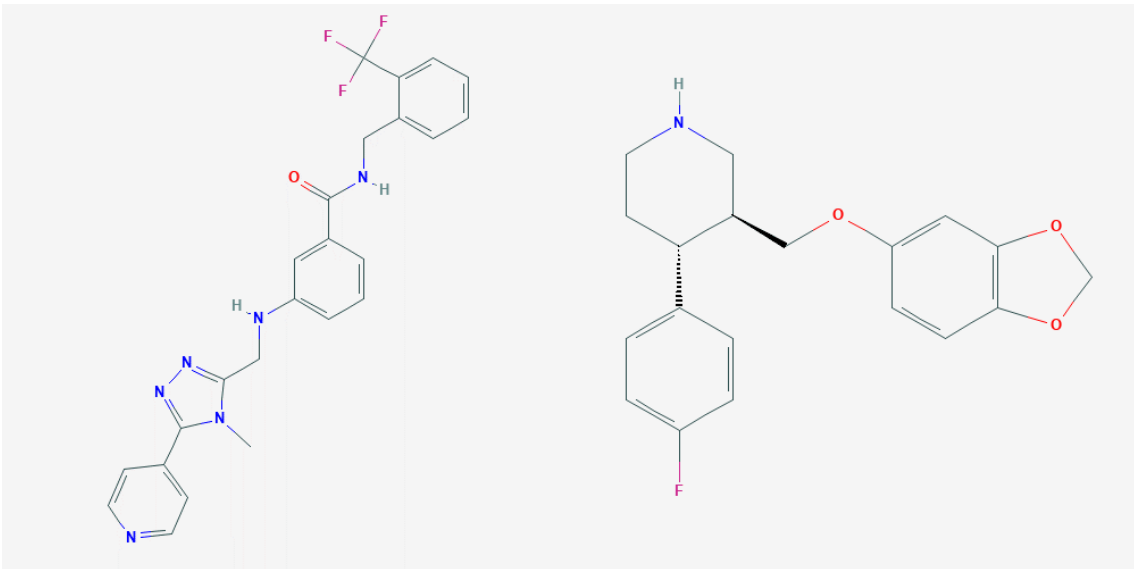
Figure 2.2 Rat aortic smooth muscle cells.

Representative phase-contrast images of primary RASM cells showing confluent cells using an inverted light microscope (objective 10x).

2.2.2. Concentrations of vasoconstrictors and inhibitors

Applied concentrations of vasoconstrictors for live cell Ca^{2+} imaging were previously characterised in our laboratory as maximum concentrations of AngII (100 nM); ET1 (100 nM) and UTP (100 µM) (Morris et al., 2010; Morris et al., 2011; Morris et al., 2012; Rainbow et al., 2018). Concentrations of vasoconstrictors used in the proliferation assays were indicated according to the published studies that used similar experimental methods in VSMC; AngII (100 nM) (Bokemeyer et al., 2000; Qin et al., 2004); ET1 (100 nM) (Ljuca and Drevensek, 2010); NA (1 µM) (Li et al., 2008); vasopressin (100 nM) (Asano et al., 1999) or; UTP (100 µM) (Willets et al., un-published). For immunoblotting experiments, the concentration of AngII (100 nM) was chosen to corresponding to published studies which used similar techniques to detect pERK (Eguchi et al., 2001; Qin et al., 2004) or pAkt (Schreiner et al., 2010) in VSMC. Moreover, previous experiments from our laboratory which conducted concentration-response curves confirmed that 50 nM of ET1 and 100 µM of UTP were able to produce maximal ERK phosphorylation in arterial smooth muscle cells (unpublished observations). The concentrations of the MEK1/2 inhibitor (20 µM), PD98059 and the PI3K inhibitor LY294002 (20 µM) were dictated by previous experimental evidence from our laboratory (Willets, 2009; Morris et al., 2012) and from previous studies by other groups (Zhao et al., 2001; Liu et al., 2010a; Fang et al., 2018). The GRK2 inhibitor Compound 101 was used at concentration of 30 µM, which was previously defined as a suitable concentration to completely block GRK2 activity (Lowe et al., 2015) and in our hands was able to completely block GRK2 mediated desensitization of $P2Y_2$ receptor activity in arterial smooth muscle cells (Rainbow et al., 2018). The choice of paroxetine concentration (5 µM) was determined as the minimal concentration which completely blocked GRK2 mediated desensitization of both histamine H_1 in uterine smooth muscle cells, and $P2Y_2$ receptor desensitization in arterial smooth muscle, whilst having no effect upon the desensitization of GPCRs whose inactivation did not require GRK2 cells (Rainbow et al., 2018). As fluoxetine was included as a structurally dissimilar SSRI control to eliminate off-target effects, it was used at the same concentration as paroxetine. The chemical structures for GRK2 inhibitors COMP101 and paroxetine as well as fluoxetine are show in (**Figure 2.3**).

A) COMP101 **B) Paroxetine**



C) Fluoxetine

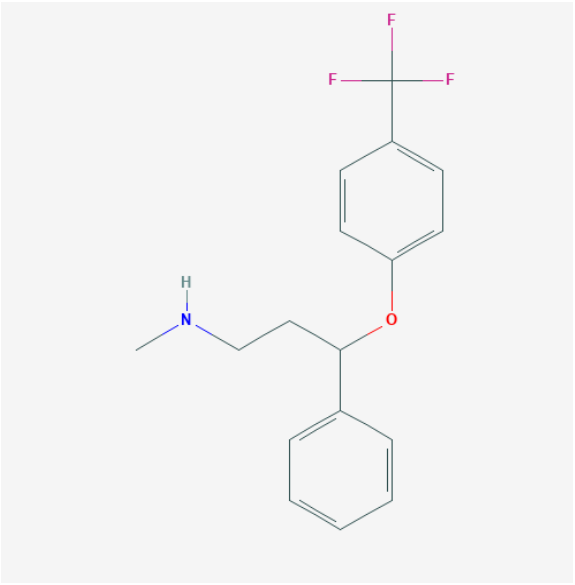


Figure 2.3 Chemical structures of COMP101, Paroxetine and Fluoxetine.

A) Chemical structures of small molecule inhibitor of GRK2; CMPD101 which discovered by *Takeda Pharmaceutical Company* (Ikeda et al., 2007). B) Paroxetine, SSRI, with GRK2 inhibitory effect (Thal et al., 2012). C) Fluoxetine, SSRI, without GRK2 inhibitory effect. Structures adapted from National Center for Biotechnology Information, PubChem Database.

2.2.3. Single cell confocal imaging

Primary RASM cells were prepared as mentioned in *Section 2.1.1* and plated onto 25mm round glass coverslips in 6 well plates and maintained at 37˚C and 5% CO_2 for 3-4 days. Before imaging, cells were loaded with the Ca^{2+} sensitive dye Flou-4 AM (4 µM) for 60 min in the dark, at room temperature. After loading, cells were maintained at 37˚C using a peltier unit, and constantly perfused with oxygenated Krebs buffer (**Table 2.1**). Real-time images were taken using an Olympus FV500 laser scanning confocal IX70 inverted microscope (oil immersion objective x60). AT_1 receptor desensitization was performed by subjecting RASM cells to challenge with AngII (100 nM, 30 s) (R1) and stimulating again 5 min later (R2). A similar desensitization protocol was used for ET_A receptor, where RASM cells were challenge ET1 (50 nM, 30 s) (R1), washed for 5 min, and then re-challenged with same concentration of ET1 (R2). To induce $P2Y_2$ receptor desensitization, UTP was added via the perfusion line for the following time periods; UTP (1 µM, termed R1) for 30 s, UTP (100 µM) for 1 min, and finally UTP (1 µM termed R2) for 30 s with 5 min washing between agonist additions. To assess the effect of GRK2 inhibitors, the cells were pre-incubated with paroxetine (5 µM) or fluoxetine (5 µM) for 1 h together with Fluo-4-AM. RASM was then subjected to the desensitization protocol outlined above. The changes in the intracellular Ca^{2+} level are characterized as the ratio of the fluorescence emission (F) over the initial basal fluorescence (F_0) [F/F_0]. Receptor desensitization was determined as the relative percentage change of R2 to R1.

2.2.4. Small interfering RNA (siRNA) knockdown of endogenous GRKs levels in RASM cells

GRKs expression was specifically depleted in RASM cells by nucleofection with a previously characterised anti-GRKs siRNA (Morris et al., 2010; Nash et al., 2018). RASM cells (~80% confluence) were harvested and used for siRNA transfection. The medium was discarded and the cells were washed with 10 ml PBS to remove FCS, then incubated with 5 ml trypsin-EDTA (0.25%) at 37˚C with 5% CO_2 in humidified air for 1-2 min. Trypsinization was stopped by addition of 15 ml of 10% FCS/DMEM-GlutaMAX medium and all the suspension transferred to a 30 ml universal tube before centrifugation (1000 xg for 5 min) to recover the cells. After removal of the supernatant, the cell pellet was re-suspended in 10 ml of 10% FCS/DMEM-GlutaMAX medium. Then the cells were counted using a haemocytometer. The required volume which containing $2x10^6$ cell/reaction were spun down and the medium was removed. On the cells pellet, 100µl of

transfection reagent with 10 nM of either negative-control (NC) siRNA or anti GRK siRNA (**Table 2.2**) were added. After that, the mixture was transferred to an Amaxa cuvette and electroporated (program #D003) using an Amaxa Nucleofector device. Immediately, transfected cells were removed from the cuvette using a pipette and transferred to 500 µl pre-warmed 10% FCS/DMEM-GlutaMAX medium. The cells then maintained at 37°C for 10 min prior to seeding in an appropriate cell culture plate.

Table 2.2 Anti (target protein) siRNA sequences.

siRNA	**Sequence**	**Reference**
NC siRNA	non-target scramble sequence	
anti GRK2 siRNA	5'-GCAGGUACCUCCAGAUCUCtt-3'	(Morris et al., 2010)
anti GRK5 siRNA	5'-CAGUGGAAAUGGUUAGAAAtt-3′	(Nash et al., 2018)
anti GRK6 siRNA	5'-GACCAAGACUUCUACCAGAtt-3′	(Nash et al., 2018)
anti arrestin 2 siRNA	5′-GCCACUGACUCGGCUACAAtt-3′	(Morris et al., 2011)
anti arrestin 3 siRNA	5′-GCCUUCUGUGCCAAAUCUAtt-3′	(Morris et al., 2011)

2.2.5. Cell proliferation assays

2.2.5.1. [^{3}H]-thymidine incorporation assay

Proliferation of RASM cells was determined using a [^{3}H]-thymidine incorporation assay, which is a method for direct measurement of DNA synthesis, characterized by incorporation of radiolabelled thymidine into newly synthesized DNA during the S phase of the cell cycle. This method is suitable for quantifying cell proliferation in response to exogenous factors (Dawson and Young, 2001; Goncharova et al., 2006).

2.2.5.2. Determination of the appropriate RASM cell number for the [^{3}H]-thymidine incorporation assay

To determine the optimal cell number to be used in these experiments, RASM cells were seeded at different densities in 24-well plates ($1x10^4$, $2x10^4$, $3x10^4$, $4x10^4$, $5x10^4$ and $6x10^4$ cells/well) in DMEM supplemented with P/S and 10% FCS for 24 h, then starved for 24 h. After that, the cells were incubated with DMEM with or without 10% FCS for further 24 h. In the last 4 h, 0.5µCi/ml [^{3}H]-thymidine was added. Cells were then washed twice with 1 ml serum-free medium and incubated in ice-cold trichloroacetic acid (TCA) (5% *w/v*) for 1 h on ice. Precipitates were incubated with cold ethanol containing 200 µM potassium acetate for 5 min. After that, the precipitates were washed twice with cold ethanol/diethylether (3:1). Finally, the precipitates were solubilized in 500µl NaOH (0.5 M) for 10 min. The solution was transferred to scintillation tubes and 4 ml of Emulsifier was added. Finally, [^{3}H]-thymidine incorporation was quantified using liquid scintillation counting (Beckman Coulter LS6500 Multi-Purpose Scintillation Counter).

2.2.5.3. Stimulation of RASM cells' proliferation by different vasoconstrictors

RASM cells were seeded in 24-well plates ($5x10^4$cell/well) in DMEM supplemented with penicillin, streptomycin and 10% FCS for 24 h, then starved for 24 h. After that, cells were treated with FCS or agonists: AngII (100 nM); ET1 (100 nM); NA (1 µM); vasopressin (100 nM) or; UTP (100 µM) in DMEM supplemented with 1%, 5%, 10% FCS or serum-free. In addition, 0.5µCi/ml [^{3}H]-thymidine per well was added and re-incubated for further 24 h. After that, the procedure was completed as described above.

2.2.5.4. Inhibition of GRK2 catalytic activity prior to stimulation of RASM cells' proliferation by vasoconstrictors

Paroxetine and fluoxetine are selective serotonin reuptake inhibitors (SSRI) and previous reports indicate that paroxetine, but not the structurally distinct fluoxetine (used here as a control), can inhibit GRK2 activity (Thal et al., 2012). Furthermore, COMP101 is a small molecule that has previously been described as an inhibitor of GRK2 (Thal et al., 2011). To assess the effect of these putative GRK2 inhibitors on cell proliferation, serum-starved cells were pre-incubated with paroxetine (5 μM), fluoxetine (5 μM) or COMP101 (30 μM) for 45 min. After that, cells were treated with FCS ± agonist [AngII (100 nM), ET1 (100 nM) or NA (1 μM)] in DMEM supplemented with 10% FCS. Where required paroxetine (5 μM), fluoxetine (5 μM) or COMP101 (30 μM) were also included throughout the experiment. Finally, [^{3}H]-thymidine incorporation was determined as described above.

2.2.5.5. Depletion of GRK2 protein prior of stimulation of RASM cells' proliferation by vasoconstrictors

In order to determine whether GRK2 expression affects vasoconstrictor-driven RASM cells proliferation, GRK2 expression was specifically depleted in RASM cells by nucleofection (described in section 2.3) using a previously characterised anti-GRK2 siRNA (Morris et al., 2010). Then, RASM cells were seeded in 24 well plates (5 x 10^4 cells/well). 24 h post-nucleofection, cells were serum-starved for a further 24 h, prior to stimulation with the vasoconstrictors: AngII (100 nM), ET1 (100 nM) and NA (1 μM) or with PDGF (40 ng/ml) in 10% FCS medium for 24 h. After that, [^{3}H]-thymidine incorporation was determined as described above.

2.2.6. Non-radioactive cell proliferation assay (MTT assay)

The assay principle is based on measurements of the reduction in yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) to purple formazan crystals by metabolically active cells. An increase in cells' metabolic activity directly correlates to the amount of purple formazan crystals formed, which reflect the increase in the number of living cells (cell proliferation). RASM cells were cultured in a 96 well plate at a density of 1x10^3 cells/well. Cells were then serum-starved for 24 h prior to stimulation with 10% FCS or AngII (100 nM), ET1 (100 nM) or NA (1 μM) in DMEM supplemented with 10% FCS or serum-free for 21 h. Then, 0.5 mg/ml MTT per well was added and re-incubated for a further 4 h. After that, the purple

formazan crystals were solubilized in DMSO. Then, sample absorbance was spectrophotometrically quantified at λ=550-575 nm using an ELISA reader.

2.2.7. Immunoblotting

2.2.7.1. RASM cells' time-course stimulation and sample preparations

Immunoblotting analysis was performed as described previously (Mahmood and Yang, 2012). RASM cells were plated into 6 well plates and incubated for 48 h, then, serum-starved for a further 24 h. After that, the cells were washed two times with pre-warmed 1 ml Krebs buffer (**Table 2.1**) and incubated with 2 ml Krebs buffer at 37˚C for 15 min. RASM were stimulated for different time periods (0 'basal', 5, 10, 15, 30, and 60 min) with AngII (100 nM), ET1 (50 nM) or UTP (100 µM). When the time reached zero, the plate was transferred to ice and all buffers removed. Cell lysates were prepared by addition of 1xSDS lysis buffer [2% SDS, 100µM Tris HCL, 10% glycerol, 0.1% bromophenol blue, 10% DTT, dH_2O, and 0.1% protease inhibitors 'phenyl methane sulfonyl fluoride, leupeptin and benzamidine']. Cell lysate samples were collected into labelled Eppendorf tubes and stored at -20ºC. Prior to use, the sample was sonicated using an Ultrasonicator and the proteins were denatured by incubating the samples at 95 ºC for 5 min.

To assess the effect of GRK2 inhibitor on cell signalling, serum-starved cells were pre-incubated with either COMP101 (30µM) or DMSO (vehicle control) for 30 min. After that, cells were stimulated for different time periods (0 'basal', 5, 10, 15, 30, and 60 min) with AngII (100 nM), ET1 (50 nM) or UTP (100 µM). COMP101 (30 µM) and DMSO were included throughout the experiment. Cells were then lysed as described above. Furthermore, to determine whether GRK2 expression affects vasoconstrictor signalling, GRK2 was specifically depleted in RASM cells by nucleofection (described in *Section 2.3*) using a previously characterised anti-GRK2 siRNA (Morris et al., 2010). Then, either GRK2 siRNA or NC siRNA transfected cells were seeded in 6 well plates (1 x 10^6 cells/well). 24 h post-nucleofection, cells were serum-starved for a further 24 h prior to stimulate with agonist at different time points as mentioned above. Additionally, GRK2 was over-expressed by transfection of RASM cells by nucleofection (described in section 2.3) using 2µg of eGFP-GRK2 construct, or eGFP plasmid as a control. Then, either eGPF-GRK2 or eGFP transfected cells were seeded into 6 well plates (1 x 10^6 cells/well). 96 h post-nucleofection, cells were serum-starved for 24 h prior to stimulation with agonist and assayed as described above.

2.2.7.2. Sample preparation for GRK2 protein expression change during cell cycle phases

Another method of sample preparation was performed to investigate GRK2 protein expression changes during G_0/G_1 and G_2/M phases of the cell cycle. RASM cells were plated into 6 well plates and incubated for 48 h, then serum-starved for a further 24 h. After that, the cells were synchronized at G_0/G_1, by serum-starvation for a further 24 h. In addition, RASM cells also incubated with the cell cycle inhibitor nocodazol (1µM) for 24 h to stop the cell cycle at the G_2/M phase. Next, cells were washed twice with PBS before lysis with 100µl lysis buffer [8200µl dH_2O, 200µl EDTA, 400µl NaCl (5M), 200µl Tris HCl (1M), 1000µl Triton X100 (10% *v/v*) and 10µl of each protease inhibitors (phenyl methane sulfonyl fluoride, leupeptin and benzamidine)]. The total protein amount in each sample was quantified using a Bradford Protein Assay.

2.2.7.3. Bradford Protein Assay

Initially, eight serial dilutions of BSA standards were prepared with a protein concentration range [2-0.064 µg/µl] as described in **Table 2.3.** After that, each unknown protein sample was diluted with 0.1M NaOH (dilution factor 8X). Aliquots of each standard and unknown protein samples (20µl) were mixed in a 1.5 ml cuvette with 1 ml of Bradford protein reagent. Protein standards were assayed in duplicate while unknown protein samples in triplicate. After 15 min incubation at room temperature, the absorbance of protein standards and unknown protein samples were recorded spectrophotometrically at a wavelength of λ=595nm. A protein standard curve was plotted (**Figure 2.4**) and unknown protein concentrations were interpolated from the curve (using GraphPad Prism v7). After that, samples were prepared for immunoblotting by mixing 40 µg of protein with 5x loading buffer (**Table 2.1**).

Table 2.3 BSA serial dilution.

	Concentration	Serial Dilution in 0.1M NaOH
1	**2 µg/µl**	2 µg/µl BSA in NaOH
2	**1 µg/µl**	300µl 2 µg/µl BSA + 300µl NaOH
3	**0.5 µg/µl**	300µl 1 µg/µl BSA + 300µl NaOH
4	**0.25 µg/µl**	300µl 0.5 µg/µl BSA + 300µl NaOH
5	**0.125 µg/µl**	300µl 0.25 µg/µl BSA + 300µl NaOH
6	**0.064 µg/µl**	300µl 0.125 µg/µl BSA + 300µl NaOH
7	**0.032 µg/µl**	300µl 0.064 µg/µl BSA + 300µl NaOH
8	**0.016 µg/µl**	300µl 0.032 µg/µl BSA + 300µl NaOH

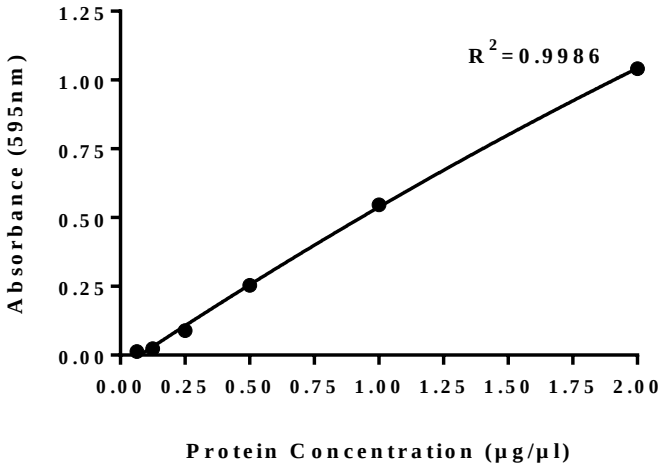


Figure 2.4 Bradford protein assay standard curve.

The standard curve was generated to calculate unknown sample protein concentrations (µg/µl). R^2 value of this curve was 0.9986, where y values are absorbance and x values are protein concentration. In each reaction, 20 µl of each BSA concentration and unknown samples were mixed with 1ml of Bradford reagent and incubated for 15 min at room temperature. Absorbance was measured at 595 nm.

2.2.7.4. Protein separation using gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared (for two gels) 10% running gel as described in (**Table 2.1**) and poured into two 1.5mm-spacer glass plates. Then, 1ml of isopropanol was added on the top of the gel to remove any air bubbles. Once the running gels had set, isopropanol was discarded and stacking gels were prepared as described in (**Table 2.1**) and poured on the top of the running gel. To produce wells, a 10 well comb was inserted in the stacking gel. After setting of the stacking gel, the glass plates were removed and fixed into a gel chamber and placed into a gel tank. After filling of the gel tank with 1x Tris-glycine-SDS running buffer, the combs were carefully removed. 5 µl of protein ladder (Chameleon^{TM} Duo pre stained protein ladder) was loaded in the first lane of the gel followed by 40 µl of each cell lysate or equivalent amount to 40 µg protein in the rest of the lanes depending upon which lysis method had been used to prepare the samples. Protein separation were performed at 140 volts for 100 min using a Bio-Rad Power-Supply.

2.2.7.5. Protein transfer using semi-dry blotting system

Separated proteins were transferred onto the nitrocellulose membranes using a semi-dry transfer system. At the same size as the running gel, two extra thick blotting papers were cut, along with 0.45µm nitrocellulose membrane and immersed in blotting buffer (**Table 2.1**) for 5 min. On a semi-dry blotter, running gels were placed on the nitrocellulose membrane and located between two extra thick blotting papers in the form of a sandwich. After rolling out all air bubbles, the semi-dry blotter lid was attached and protein transfer performed at 21 volts for 30 min. Protein transfer efficiency was verified by staining of the nitrocellulose membrane with Ponceau S staining solution for 5 min (**Figure 2.5**). After staining, the membranes were washed with TBST (**Table 2.1**) 3x5 min to remove all stain residues.

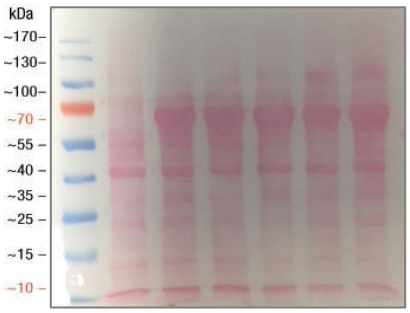


Figure 2.5 Ponceau S staining for nitrocellulose membranes.

RASM cells lysate (40µl/ lane) was separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau S stain for 1 min. Ponceau S, a negative stain, which binds to the positively charged amino groups of the proteins. It also binds non-covalently to non-polar regions in the protein. After that, the membrane was de-stained by 3x washing with TBST.

2.2.7.6. Immunoblotting with primary and secondary antibodies

Non-specific binding sites on the nitrocellulose membranes were blocked by incubation of the membrane in (5% *w/v*) non-fat dry milk in TBST or by Odyssey blocking buffer on a rocking platform for 1 h. After blocking, the membranes were washed with TBST for 2 min prior to incubation with primary antibodies (**Table 2.4**) with rocking overnight at 4°C. The next day, the membranes were washed with TBST three times, 5 min each. Then membranes were incubated away from light with fluorescence-tagged secondary antibodies (**Table 2.4**) for 1 h. Finally, the secondary antibodies were washed with TBST, two times for 15 min each.

Table 2.4 Primary and secondary antibody dilutions.

Antibody	Dilution
anti- phospho-Akt (Thr308)	(1:1000) / BSA (5% *w/v*)
anti-p44/42 MAPK (tERK1/2)	(1:2000) / BSA (5% *w/v*)
anti-phospho-p44/42 MAPK (pERK1/2) (Thr202/Tyr204)	(1:3000) / BSA (5% *w/v*)
anti-GAPDH	(1:3000) / BSA (5% *w/v*)
anti-GRK2 (C-15)	(1:1000) / non-fat dry milk (5% *w/v*)
anti-GRK6	(1:1000) / non-fat dry milk (5% *w/v*)
anti-GRK5	(1:1000) / non-fat dry milk (5% *w/v*)
anti-phospho-GSK3(α/β) (Ser21/9)	(1:1000) / BSA (5% *w/v*)
anti-β arrestin 1/2	(1:1000) / non-fat dry milk (5% *w/v*)
anti-mouse IgG 'DyLight™ 800 Conjugate'	(1:15000) / TBST
anti-rabbit IgG 'DyLight™ 680 Conjugate'	(1:15000) / TBST

2.2.7.7. Band detection and quantification

Band near-infrared fluorescence (NIR) fluorescent signals were detected using the Odyssey® CLx Imaging System and the protein of interest was identified by protein size according to the protein ladder used. Detected protein bands were analysed by Image J (1.51) software. The phospho-p44/42 MAP kinase bands were normalized relative to total p44/42 MAP kinase protein level and phospho-Akt were normalized to GAPDH.

2.2.8. Immunocytochemistry

2.2.8.1. Coverslip preparation and cell stimulation with agonist

RASM cells were seeded on glass coverslips (13mm) in 24-well plates ($2x10^4$ cells/well) in DMEM supplemented with P/S and 10% FCS for 24 h. Cells were then washed twice with PBS and serum-starved for 24 h. After that, cells were stimulated with 10% FCS or the agonists: AngII (100 nM), ET1 (100 nM) or UTP (100 µM) in serum-free DMEM for a further 24 h. To assess the effect of GRK2 inhibitors on cell proliferation, serum-starved cells were pre-incubated with paroxetine (5 µM), fluoxetine (5 µM) or COMP101 (30 µM) for 45 min. After that, cells were treated with 10% FCS or one of the agonists in serum-free DMEM for a further 24 h. DMSO, paroxetine (5 µM), fluoxetine (5 µM) or COMP101 (30 µM) were included throughout the experiment.

In order to determine whether GRK expression affected vasoconstrictor-stimulation of proliferative marker Ki67 expression, endogenous GRK expression was depleted in RASM cells by nucleofection as described in *Section 2.2.3* using anti-GRK2 siRNA, anti-GRK5 siRNA or anti-GRK6 siRNA. Then, RASM cells were cultured on 13 mm glass coverslips (2×10^3 cells/well). One day later, cells were serum-starved for a further 24 h prior to stimulation with the vasoconstrictors for 24 h. Finally, the immunocytochemistry protocol was performed as described above.

2.2.8.2. Cell fixation and antibodies incubation

After removal of the culture media, cells were fixed using freshly prepared 4% paraformaldehyde (**Table 2.1**) for 10 min at room temperature. After that, the cells were washed 3 times with PBS, before being permeabilized with PBST [PBS/Triton-X 100 (0.2% *v/v*)] for 5 min. Next, non-specific binding sites were blocked with 10% goat serum/PBST for 5 min. Cells were incubated overnight at 4°C with anti-proliferative marker Ki67 (1:400) in 10% goat serum PBST. On the next day the cells were washed twice with PBS and once with PBST. The cells were incubated away from light with a secondary anti-rabbit IgG Alexa Fluor 488 (1:400) for 40 min at room temperature. After washing with PBS, the cells incubated (permeabilized) with PBST for 10 min.

2.2.8.3. Preparation of the slides, imaging and quantification

Each coverslip was inverted onto a microscope slide and mounted using VECTASHIELD® antifade Mounting Medium with propidium iodide (PI) as a nuclear counterstain. After that, colourless nail varnish was used to seal the coverslips onto slides and prevent sample dehydration. Slides were stored at room temperature overnight protected from light. On the next day, immunofluorescence pictures were captured using an Olympus FV500 laser scanning confocal IX70 inverted microscope (oil immersion objective x60). For each experimental condition, a total of ten images were taken from separate fields of 2 coverslips for four independent cell preparations. All images shown for Ki67 for the control groups *vs.* COMP101, paroxetine and fluoxetine pre-treated groups or negative control siRNA *vs.* GRKs siRNA knockdown cells were acquired with the same microscope settings.

Ki67 immunofluorescence was quantified by two methods. Firstly, the percentage of active nuclei was calculated. This was performed by manual counting of the active nuclei **(n)** and the number of the total nuclei **(N)** in each field. After that, the percentage of active nuclei was calculated by using this equation **[(n/N) x 100]**. Alternatively, the mean of nuclear fluorescence intensity of Ki67 the immunofluorescence was assessed in the nuclei of all cells using Image J software (National Institutes of Health, Bethesda, MD).

2.2.9. Statistical analysis

All experiments were repeated ≥ 3 times from different cells preparations. Statistical comparisons were applied by unpaired Student's t-test to compare the means of two groups (Kim, 2015). For parametric multiple data sets, one-way or two-way analysis of variance (ANOVA) was applied with appropriate *post hoc* testing (each test applied is outlined in the individual figure legends). The Tukey's *post hoc* test was used to compares every mean with every other mean, allows for the possibility of unequal sample sizes (Hazra and Gogtay, 2016). The Dunnett's *post hoc* test was used to compares every mean to a single control group mean (Dunnett, 1964). The Sidak's *post hoc* test, alternative to Bonferroni, which has more power assumes that each comparison is independent of the others (Abdi, 2007). This test was used to compares between two means at specific time points. For non-parametric data, Kruskall-Wallis tests were applied with Dunn's *post hoc* testing were used (Kruskal and Wallis, 1952; Dunn, 1964). Data are expressed as means ± SEM (standard error of the mean) for all experiments. The

results were considered statistically significant at a *P* value of <0.05. All statistical analyses were carried out using GraphPad Prism, version 7 (San Diego, CA, USA).

Chapter Three

3. Identification and characterisation of optimal techniques to measure vasoconstrictor-stimulated RASM cell proliferation

3.1. Introduction

A major characteristic of VSMC is the ability to switch phenotypes, i.e. from a contractile 'quiescent' to a synthetic 'activated' phenotype, in response to various stimuli. Phenotypic switching results in downregulation of contractile markers, an upregulation of proliferative genes, increased migration capability and increased synthesis of extra cellular matrix components (Owens et al., 2004), all of which contribute to the vascular remodelling process. Although hypertension is a multifactorial disease, one major contributing factor is an associated rise in the levels of circulating vasoconstrictors, which consequently increases the activity of their cognate GPCRs, and over-stimulates $G_{\alpha q}$ mediated PLC-DAG-IP_3 signalling (Bazan et al., 1992; Schiffrin, 1995; Bader et al., 2001). Changes in vasoconstrictor homeostasis not only increases $[Ca^{2+}]_i$ and amplifies VSMCs contractile responses (Kosch et al., 2001), but augments the downstream signalling pathways that mediate VSMC proliferation (Schiffrin, 1995; Mehta and Griendling, 2007). Indeed, over the years numerous studies have confirmed AngII and ET1 as pro-proliferative agents that contribute significantly to induce proliferation of VSMC (Watanabe et al., 2001; Daou and Srivastava, 2004; Qin et al., 2004; Huang et al., 2011), and their effects can be blocked using pharmacological antagonists (Li et al., 1996a; Mabrouk et al., 2001).

A wide variety of assays have been utilised to study the effects of vasoconstrictors on proliferation of VSMC. Many of these assays measure either DNA replication or cellular metabolic activity. Indeed, proliferation of VSMC is commonly evaluated using the [^{3}H]-thymidine incorporation assay, which quantifies radioactive thymidine incorporated into newly synthesised DNA during the S-phase of the cell cycle (Dawson and Young, 2001; Goncharova et al., 2006). In addition, the expression of proteins linked to the cell replication process, such as Ki67 and proliferating cell associated nuclear antigen (PCNA) have also been used as indicators for proliferation of VSMC (Clarke et al., 2006; Wang et al., 2009; Wu and Lu, 2013). The expression of Ki67 and PCNA is cell cycle-dependent and both are usually only detectable during the active phases of cell

growth (Jurikova et al., 2016). Ki67 antigen is a large protein (~345 kDa) that has a short half-life of ~1 h, whose expression is virtually undetectable in the resting G_0 phase, but is markedly up-regulated in active cell cycle phases, with maximal expression found during the M phase (Gerdes et al., 1984; Zacchetti et al., 2003). Conversely, PCNA is a small protein component subunit (~36 kDa) of DNA polymerase-δ, and is important for DNA replication and repair. Unlike Ki67, PCNA has a relatively long half-life 8-20 h and its expression can be detected in early G_0, peaks in G_1 and S phases and diminishes in the G_2 and M phases (Prelich et al., 1987; Zacchetti et al., 2003). Alternatively, the ability of mitochondria to metabolise the tetrazolium compound 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), has been successfully applied to assesses cell growth, cell viability and proliferation, and is based on measurements of the reduction of tetrazolium MTT in the mitochondria of metabolically active cells (Tantini et al., 2005). Since a wide variety of different assays have previously been utilised to study cellular proliferation the aim of this chapter was to assess and develop reproducible, reliable proliferation assays to study the effects of different vasoconstrictors [AngII, ET1, noradrenaline (NA), vasopressin (V) and UTP] that activate $G_{\alpha q}$ coupled receptors on proliferation of VSMC.

3.2. Aim

To assess the effectiveness and reliability of existing proliferation assays and identify the most appropriate to study the effects of vasoconstrictors on growth of VSMC.

3.3. Results

3.1.1. Characterization of RASM cells

Vascular smooth muscle cells are one of the major vascular components, providing structural integrity to blood vessels and playing an important functional contractile role. In addition, VSMC are able to respond to different stimuli which alters their contractile, migratory and proliferative properties (Owens et al., 2004; Adhikari et al., 2015). It has commonly been assumed that VSMC play a critical role in various cardiovascular diseases, therefore they are central to cardiovascular research (Ray et al., 2001). Accordingly, primary culture of VSMC provides an appropriate tool to study vascular smooth muscle cell proliferation. Although our laboratory uses a long-established method for the isolation of RASM, I wished to confirm that my isolation techniques were indeed producing pure arterial smooth muscle cell cultures.

Primary RASM cells were isolated from rat aortae by enzymatic digestion as described in Methods (*Section 2.2.1*). Three to four days after isolation, primary RASM cells were identified under an inverted phase contrast light microscope. RASM cells became approximately 90% confluent within about two weeks (**Figure 3.1 A**). Confluent RASM cell showed an elongated spindle shape, characteristic of smooth muscle cell cultures (Adhikari et al., 2015).

In order to confirm that the enzymatic digestion isolation technique produced a pure population of RASM cells, I examined the presence of specific smooth muscle markers in RASM cells cultures. RASM cells were characterized by fluorescence immunocytochemistry with antibodies against smooth muscle cell markers such as smooth muscle alpha actin (SM α-actin) and calponin (Rensen et al., 2007). RASM cells obtained by enzymatic digestion showed a high degree of purity. Immunocytochemistry images confirmed that the vast majority of the cells (>95%) showed positive SM α-actin and calponin staining (**Figure 3.1 B**). Furthermore, immunoblotting was carried out on RASM whole cell lysates, and clear bands of SM α-actin and calponin were observed (**Figure 3.1 C**). Collectively, these results confirmed that our isolation technique routinely produced pure populations of RASM.

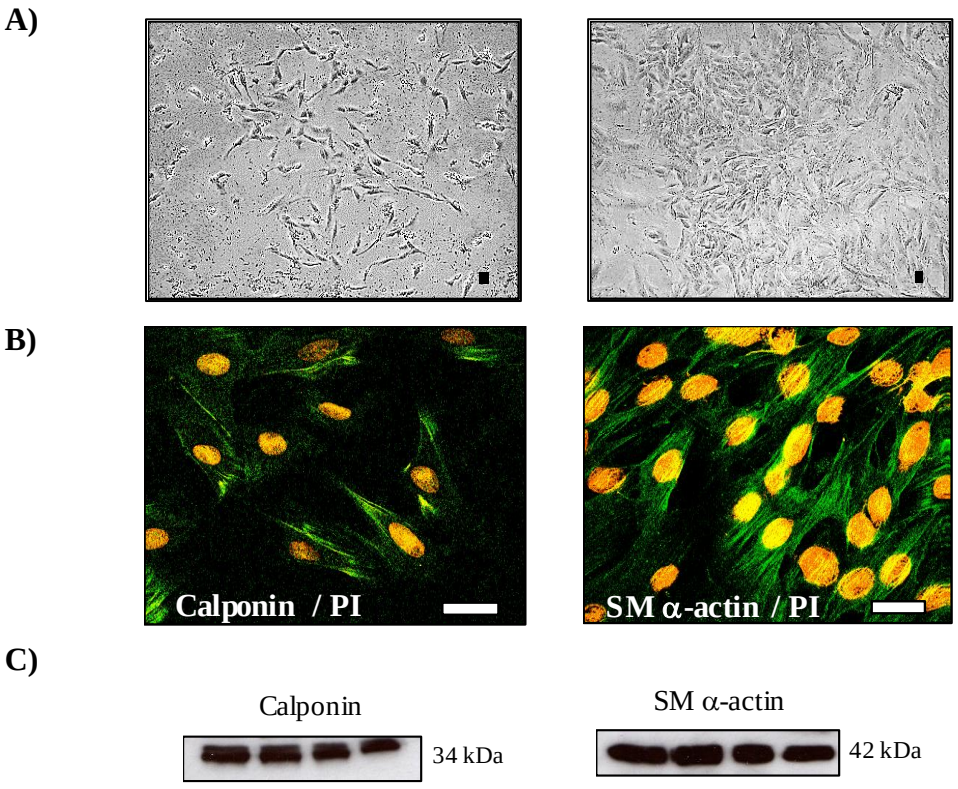


Figure 3.1 Morphological and immunofluorescence characterization of RASM cells.

A) Representative phase-contrast images of RASM cells 3 days after isolation (left panel) and two weeks later showing confluent cells (right panel) using an inverted light microscope (objective 10x) (Scale bar is 40µm). B) Representative fluorescent immunocytochemistry staining for SM α-actin and calponin in RASM cells show pure cell populations. Images were taken using Nikon C1Si laser scanning confocal inverted microscope (oil immersion objective 60x) (Scale bar is 180µm). C) Immunoblotting of RASM whole cell lysates from different cell preparations show strong immunoreactive bands for calponin (left panel) and SM α-actin (right panel).

3.3.2. RASM cells AT_1, ET_A and $P2Y_2$ receptor activation and desensitization

Vasoconstrictors such as AngII, ET_1 and UTP induce contraction of VSMC via activation of their cognate GPCRs. Several previous studies confirmed that AT_1, ET_A and $P2Y_2$ receptors are expressed in VSMC (White et al., 1993; Freeman and Tallant, 1994; Wang et al., 2002). In order to confirm that AT_1, ET_A and $P2Y_2$ receptors are expressed endogenously in our RASM cells preparations and respond to vasoconstrictors, I examined whether AngII, ET1 and UTP could induce changes in intracellular Ca^{2+} $[Ca^{2+}]_i$ signalling. To further confirm the presence of the correct receptor populations, the desensitization profiles of each agonist were determined and compared to previous findings using our standard (R1/R2) GPCR desensitization protocol (Morris et al., 2010; Morris et al., 2011; Morris et al., 2012). In resting cells, the $[Ca^{2+}]_i$ concentration is low. When the cells are excited by activation of $G_{\alpha q}$-coupled GPCRs with agonist, this results in rapid increase in $[Ca^{2+}]_i$ via PLC activation. The change in $[Ca^{2+}]_i$ between the resting phase and excitation phase of the cells was measured using Fluo-4 with an excitation peak at 480 nm and emission peak at 525 nm. Fluo-4-AM is a cell permeable Ca^{2+} dye in acetoxymethyl ester form. Once inside the cell, cystolic esterases hydrolyse the ester, leaving free Fluo-4 which efficiently binds the Ca^{2+} inside the cell (Liu et al., 2010c).

AT_1 receptor desensitization was performed by subjecting RASM cells to challenge with AngII (100 nM, 30 s) (R1) and stimulating again 5 min later (R2) which was decreased by ~45% compared with the R1 response (**Figure 3.2 A**). A similar desensitization protocol was used for ET_A receptor, where RASM cells were challenge ET1 (50 nM, 30 s) (R1), washed for 5 min, and then re-challenged with same concentration of ET1 (R2). This resulted in a reproducible reduction in the R2 response of ~80% (**Figure 3.2 B**). In agreement to our previously published data in mesenteric arterial smooth muscle (Morris et al., 2010), the ET_A receptor antagonist BQ123 blocked the ET1-induced Ca^{2+} signalling (data not shown), indicating that ET_A receptors are the predominant mediator of ET1 responses in RASM cells.

Our lab previously identified that $P2Y_2$ receptor as the predominant mediator of UTP-stimulated Ca^{2+} signalling in mesenteric arterial smooth muscle (Morris et al., 2011). Furthermore, data showed that application of repeated maximal concentrations of

UTP failed to produce observable reductions in the R1/R2 ratio, which can be explained by the presence of a significant $P2Y_2$ receptor reserve expressed in RASM (Morris et al., 2011). To uncover $P2Y_2$ receptor desensitization, we applied a previously characterized $P2Y_2$ receptor desensitization protocol where RASM cells were challenged with an approximate EC_{50} concentration of UTP (1 μM) for 5 min before (R1) and 5 min after (R2) the addition of a maximal UTP challenge (R_{max};100 μM) (Morris et al., 2011). As shown in **Figure 3.2 C**, R2 responses were reduced (by ~70%) following R_{max} exposure compared with the R1 response. (**Figure 3.2 C**). Cumulative data show that the remaining receptor response (R1/R2 ratio) was ~45%, ~20% and ~30% for AT_1, ET_A and $P2Y_2$ receptors respectively (**Figure 3.2 D**). These results provide good evidence that AT_1, ET_A and $P2Y_2$ receptors are expressed in RASM cells and respond well to AngII, ET1 and UTP to produce changes in Ca^{2+} signalling, which is a prelude to smooth muscle contraction.

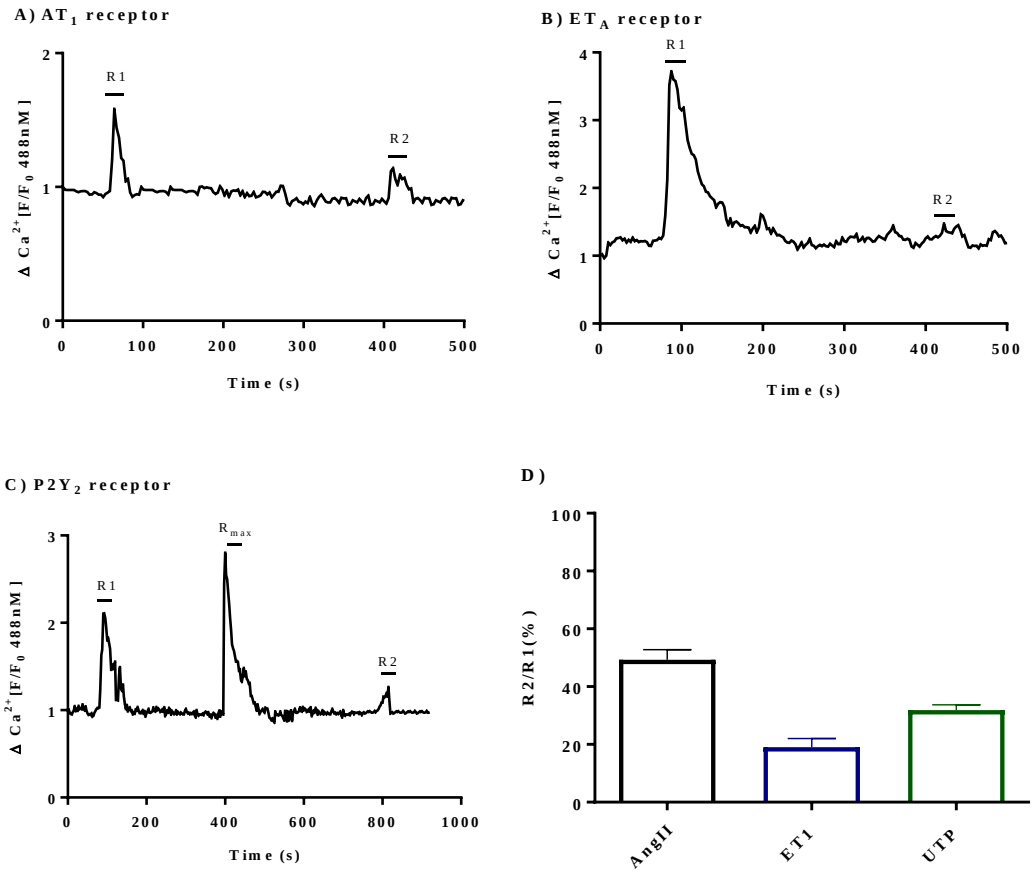


Figure 3.2 AT_1, ET_A and $P2Y_2$ receptor activation and desensitization in RASM cells.

Representative traces from single cells showing activation and desensitization of AT_1 receptor (A), ET_A receptor (B) and $P2Y_2$ receptor (C) $[Ca^{2+}]_i$ signals following AngII, ET1 or UTP challenge, respectively. Real-time images were taken using an Olympus FV500 laser-scanning confocal IX70 inverted microscope (oil immersion objective 60x) to track $[Ca^{2+}]_i$. Receptor desensitization was determined as the percentage reduction of the R2/R1 ratio. (D) Cumulative data show the degree of AT_1, ET_A and $P2Y_2$ receptor desensitization as means ± SEM from 14–16 cells prepared from three different animal preparations.

3.3.3. Determination of optimal VSMC cell number for the [^{3}H]-thymidine incorporation assay

Previous research shows the proliferative effect of contractile agonists in vascular smooth muscle cells (Hirata et al., 1989; Harper et al., 1998; Qin et al., 2004). Thus, I wished to develop a suitable, reproducible proliferation assay to measure this effect. The [^{3}H]-thymidine incorporation assay is a commonly applicable experiment for the evaluation of smooth muscle cells proliferation rate in response to exogenous factors. The [^{3}H]-thymidine incorporation technique is based on the direct quantification of radiolabelled thymidine in newly synthesized DNA (Dawson and Young, 2001; Goncharova et al., 2006). To begin this process, I needed to determine the optimal RASM cell number to generate the largest signal to noise ratio. In order to determine the suitable cell density to be used in the assay, RASM were seeded in 24 well plates at different cell numbers ($1x10^4$, $2x10^4$, $3x10^4$, $4x10^4$, $5x10^4$ and $6x10^4$ cell/well). After cell growth was arrested following 24 h serum-starvation, RASM cells were either stimulated with medium containing 10% FCS, or serum-free DMEM for 24 h. Assays were then completed as described in Methods (*Section 2.2.5*). As shown in **Figure 3.3**, there was a proportional relationship between cell number and the increase in cell proliferation. The degree of cell proliferation plateaued at around $5x10^4$ cells per well, while the basal level of thymidine incorporation continued to increase as the plating density increased. Therefore, to provide an optimal signal to noise ratio, all further experiments were conducted using $5x10^4$ cells per well.

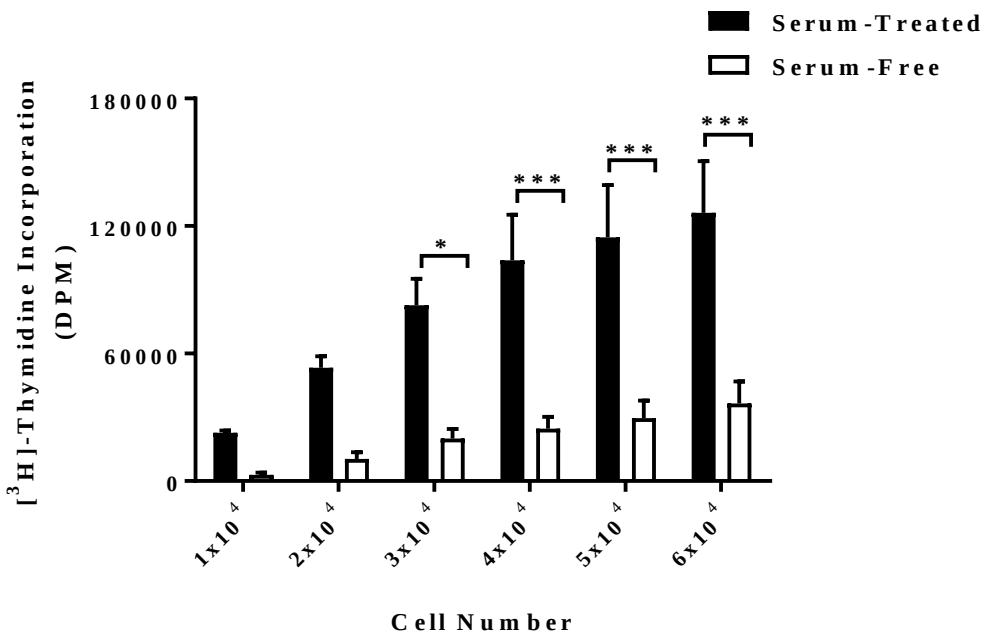


Figure 3.3 Determination of the optimal plating density for the [^{3}H]-thymidine incorporation assay.

RASM cells were plated into 24-well plates at the following seeding densities 1x10^4, 2x10^4, 3x10^4, 4x10^4, 5x10^4 and 6x10^4 cells/well. After 24 h serum-starvation, RASM were incubated with DMEM medium with or without 10% FCS. [^{3}H]-thymidine incorporation assays were conducted as described in the Methods (*Section 2.2.4*). Data are shown as means ± SEM for 4 replicates from 4 different animal preparations. Statistically significant changes compared to serum-free incubated cells are shown as; *P<0.05, ***P<0.001 (two-way ANOVA, Sidak's *post-hoc* test).

3.3.4. Comparative effects of 4 h or 24 h [^{3}H]-thymidine pulsing on vasoconstrictor-induced VSMC proliferation in serum-free DMEM

After determination of the optimal RASM cells density, I next assessed [^{3}H]-thymidine incorporation in RASM cells in response to different vasoconstrictors. Some previously published protocols reported incubation of agonist-stimulated cells with [^{3}H]-thymidine for 24 h (Goncharova et al., 2006), while others studies labelled cells with [^{3}H]-thymidine in the last 2 h (Ediger and Toews, 2000) or last 4 h (Hirata et al., 1989) before lysis of the cells. Thus, to determine the appropriate time required for [^{3}H]-thymidine labelling and to maximise the level of [^{3}H]-thymidine incorporation in response to vasoconstrictor in RASM cells, [^{3}H]-thymidine incorporation assays were conducted using two different [^{3}H]-thymidine labelling periods.

Early passage (one or two) RASM cells were grown in 24-well plates and then serum-starved for 24 h to synchronize cells in the G_0/G_1 phase of the cell cycle. To determine whether vasoconstrictors promoted cell proliferation, RASM cells were stimulated with different vasoconstrictor [AngII (100 nM), ET1 (100 nM), NA (1 μM), V (100 nM) or UTP (100 μM)] in serum-free DMEM for 24 h. RASM cells were either incubated with [^{3}H]-thymidine in the last 4 h or for 24 h, before cells were processed as described in the Methods (*Section 2.2.5*). **Figure 3.4 A and B** reveal that stimulation with 10% FCS resulted in a marked increase in RASM proliferation in comparison to serum-free conditions. Although, stimulation of serum-free incubated cells with different vasoconstrictors with 4 h or 24 h [^{3}H]-thymidine incorporation did not detectibly increase the proliferation of RASM cells, 24 h [^{3}H]-thymidine incubation showed greater levels of [^{3}H]-thymidine incorporation than 4 h.

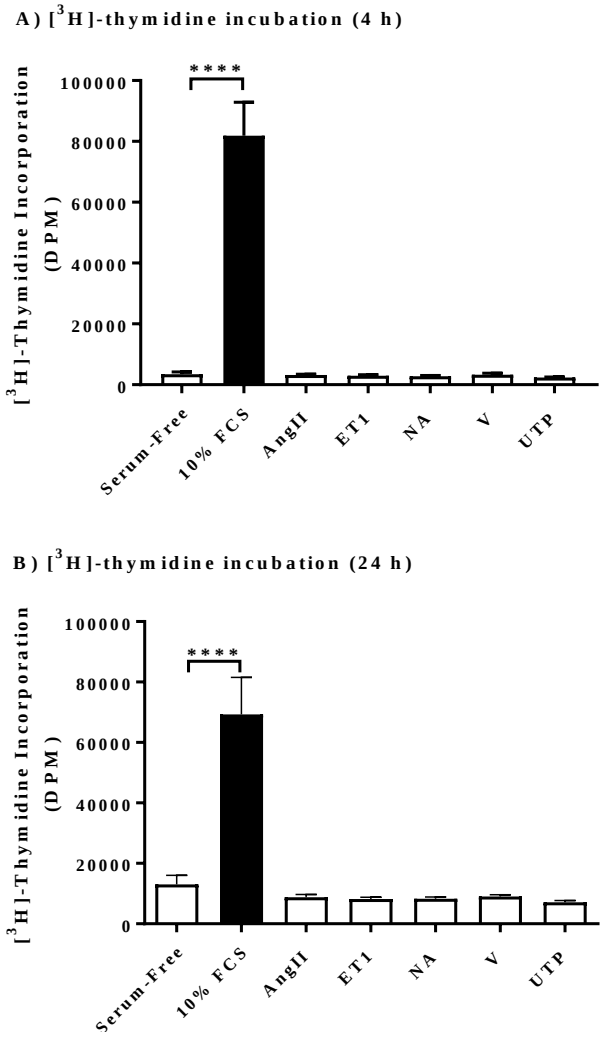


Figure 3.4 Comparative effects of 4 h or 24 h [^{3}H]-thymidine pulsing on vasoconstrictor-induced VSMC proliferation.

Serum-starved RASM cells were stimulated with 10% FCS alone, or vasoconstrictors [AngII (100 nM), ET1 (100 nM), NA (1 µM), V (100 nM) or UTP (100 µM)] in serum-free DMEM medium. [^{3}H]-thymidine was added either for the last 4 h (A) or throughout the 24 h time period (B). Data represent means ± SEM for 4 replicates for each experimental condition from 4 different cell preparations. Statistically significant changes compared to serum-free control unstimulated cells are shown as; ****P<0.0001 (one-way ANOVA, Dunnett's *post-hoc* test).

3.3.5. Comparison of single and multiple applications of vasoconstrictors on VSMC proliferation in serum-free DMEM

The findings in the above sections showed that stimulation of RASM cells with different vasoconstrictors in serum-free DMEM for 24 h did not show a measurable change in RASM cell proliferation. Most of these vasoconstrictors are peptides or labile compounds and one might expect them to rapidly degrade which may be why we were unable to measure their effects upon cell growth. To counteract this potential problem, I stimulated RASM three times within the 24 h period with different vasoconstrictors [AngII (100 nM), ET1 (100 nM), NA (1µM) or UTP (100 µM)] in serum-free DMEM medium, with [^{3}H]-thymidine included for 24 h. Again the [^{3}H]-thymidine incorporation assay was conducted as described in the Methods (*Section 2.2.5*). **Figures 3.5 A and B** reveal that stimulation with 10% FCS resulted in a marked increase in the proliferation of RASM in comparison to control cells, while stimulation of serum-free cells with different vasoconstrictors, either once or three times did not significantly affect the proliferation of RASM cells. Therefore, multiple addition of vasoconstrictors failed to induce RASM proliferation suggesting that the lack of effect was not due to ligand degradation.

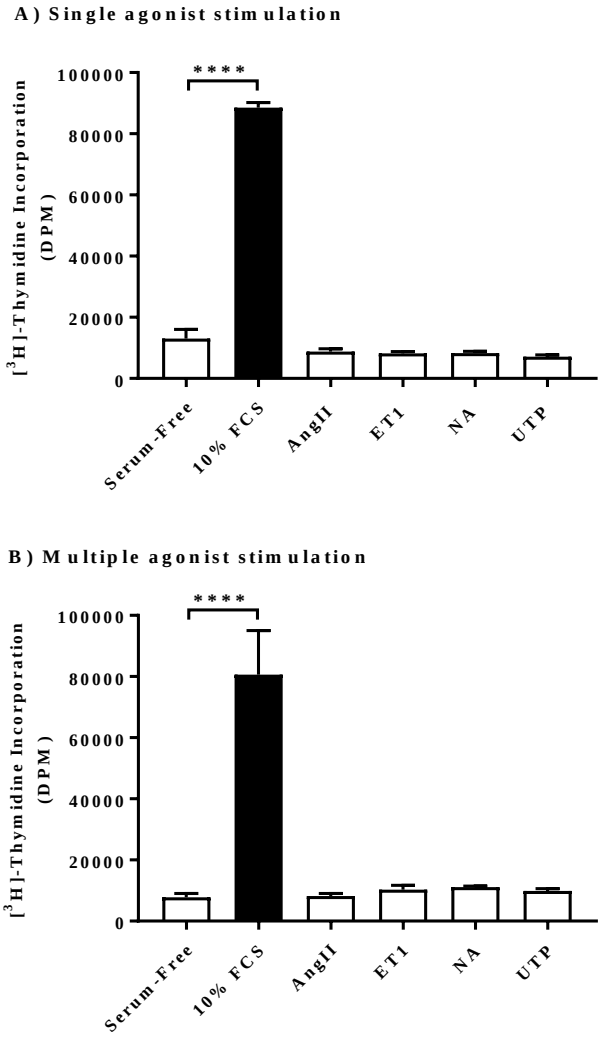


Figure 3.5 Comparison of single and multiple applications of vasoconstrictors on VSMC proliferation.

RASM cells were subjected to either a single or multiple stimulations with vasoconstrictors [AngII (100 nM), ET1 (100 nM), NA (1 μM) or UTP (100 μM)] in serum-free DMEM with [^{3}H]-thymidine for 24 h. Single (A) or multiple (3x) (B) stimulation of RASM cells with vasoconstrictor did not produce a measurable increase in [^{3}H]-thymidine incorporation when compared to controls. Data represent means ± SEM for 4 replicates for each experimental condition from 4 different cells preparations. Statistically significant changes compared to serum-free un-stimulated cells are shown as; ****P<0.0001 (one-way ANOVA, Dunnett's *post-hoc* test).

3.3.6. Effects of different FCS concentrations on vasoconstrictor-induced VSMC proliferation

It is well established that hypertensive vascular remodelling is a cumulative process, which manifests over a long time (Mulvany, 1999). In addition, as pervious research confirmed that AngII (Qin et al., 2004), ET1 (Hirata et al., 1989), vasopressin (Alonso et al., 2009) and UTP (Harper et al., 1998) induced VSMC proliferation, I conducted further experiments to determine the optimal [^{3}H]-thymidine incorporation assay experimental conditions for vasoconstrictors in RASM cells. Our data in the previous section revealed that it was not possible to show a measurable response to vasoconstrictors and RASM cell proliferation in serum-free medium. Therefore, to hopefully uncover the anticipated effects of vasoconstrictors on RASM growth, I included various concentration of FCS to initiate RASM cell growth, and examine the cumulative effects of serum and vasoconstrictors. Growth-arrested RASM cells were stimulated with vasoconstrictors [AngII (100 nM), ET1 (100 nM), NA (1µM), V (100 nM) or UTP (100 µM)] in the presence of increasing amounts (1, 5 or 10 %) of FCS prior to assessing proliferation using the [^{3}H]-thymidine incorporation assay. As shown in **Figure 3.6 B and C**, the presence of 1% or 5% FCS, none of the vasoconstrictors increased [^{3}H]-thymidine incorporation above that observed in the control non-treated cells. However, when the level of FCS was increased to 10%, AngII (P<0.05), ET1 (P<0.01) and NA (P<0.01) produced significant increases in the level of [^{3}H]-thymidine incorporation, when compared to 10 % FCS alone (**Figure 3.6 D**). In contrast UTP failed to stimulate [^{3}H]-thymidine incorporation under any conditions.

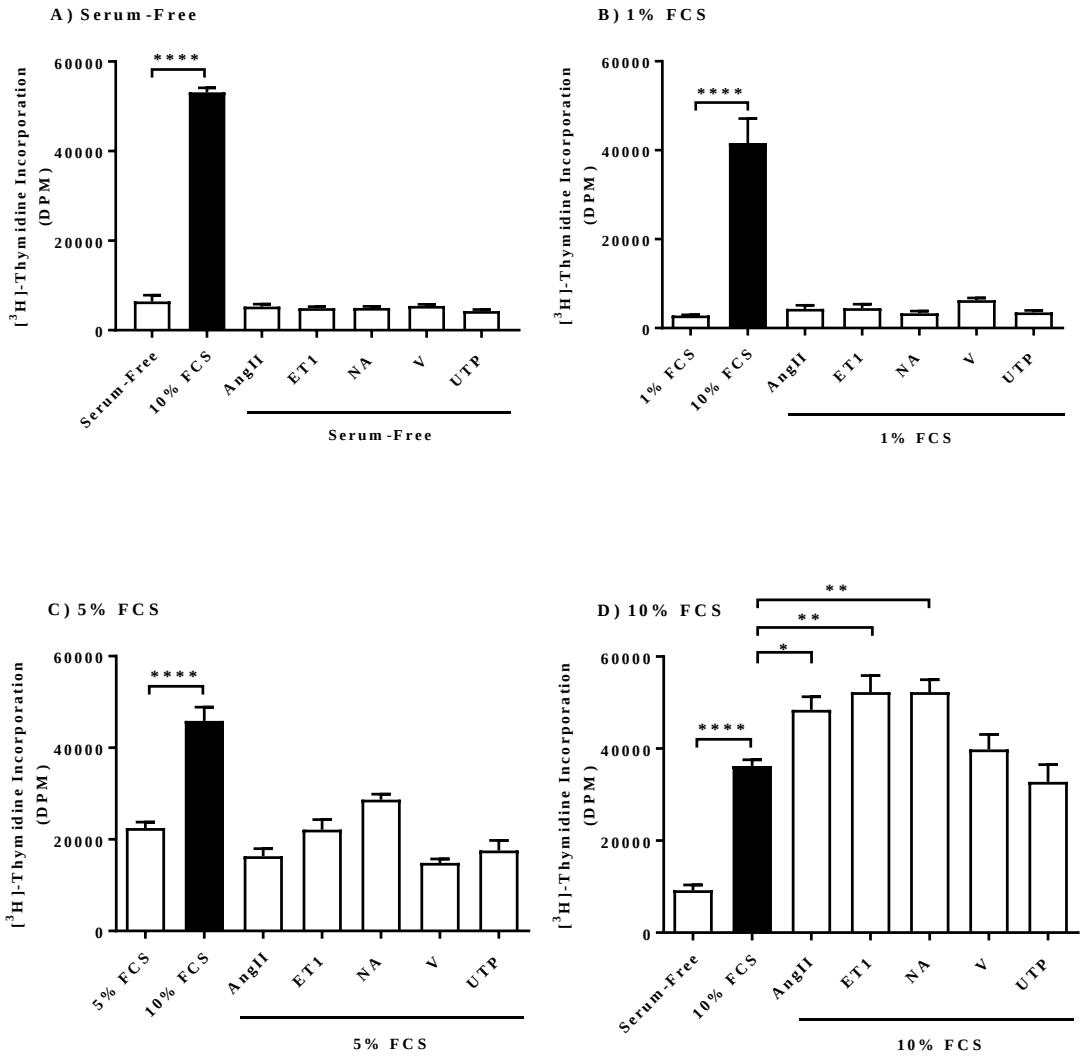


Figure 3.6 Effect of different FCS concentrations on vasoconstrictor-induced VSMC proliferation.

Stimulation of RASM cells with 10% FCS resulted in a significant increase in cell proliferation in comparison to serum-free (A), 1% FCS (B), 5% FCS (C) and serum-free (D). Stimulation of RASM cells with AngII, ET1 or NA in the presence of 10% FCS resulted in a significant increase in the cell proliferation in comparison to 10% FCS treated cells (D). Data shown are means ± SEM for 4 replicates for each experimental condition from 4 different cells preparations. Statistically significant changes are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$ versus serum-free, 1% FCS, 5% FCS or 10% FCS (one-way ANOVA, Dunnett's *post-hoc* test).

3.3.7. Effect of different vasoconstrictors on the induction of VSMC proliferation using MTT assay

In order to confirm the thymidine assay findings, I identified additional cell proliferation assays and attempted to optimise them to measure the proliferative effects of vasoconstrictors on RASM. The MTT assay is a method that examines another index of cell growth, cell viability and proliferation, and is based on measurements of the reduction of tetrazolium MTT in the mitochondria of metabolically active cells (Tantini et al., 2005). Serum-starved RASM cells were stimulated either once or three times with the agonists including AngII (100 nM), ET1 (100 nM) or NA (1 µM) in DMEM medium with 10% FCS or in serum-free conditions. MTT reduction assays were then completed as described in Methods (*Section 2.2.6*). Sample absorbance values are generally accepted as reflections of RASM cell viability and number. My data revealed that using a single stimulation, FCS (10%) treatment significantly enhanced MTT reduction when compared to non-treated serum-starved RASM cells (**Figure 3.7 A and B**). However, no change was detected with vasoconstrictor-stimulated cells in serum-free medium (**Figure 3.7 A**) or in 10% FCS supplemented medium (**Figure 3.7 B**). Moreover, in studies where vasoconstrictors were added multiple times within the 24 h period, only NA was able to induce an increase in MTT reduction when added with or without serum (**Figure 3.7 A and B**). In contrast, even when multiple additions of AngII or ET1 were applied, this had little effect upon MTT reduction and cell growth (**Figure 3.7 C and D**). These results indicate that we were unable to detect reliable changes in cell growth using the MTT assay in our RASM cells.

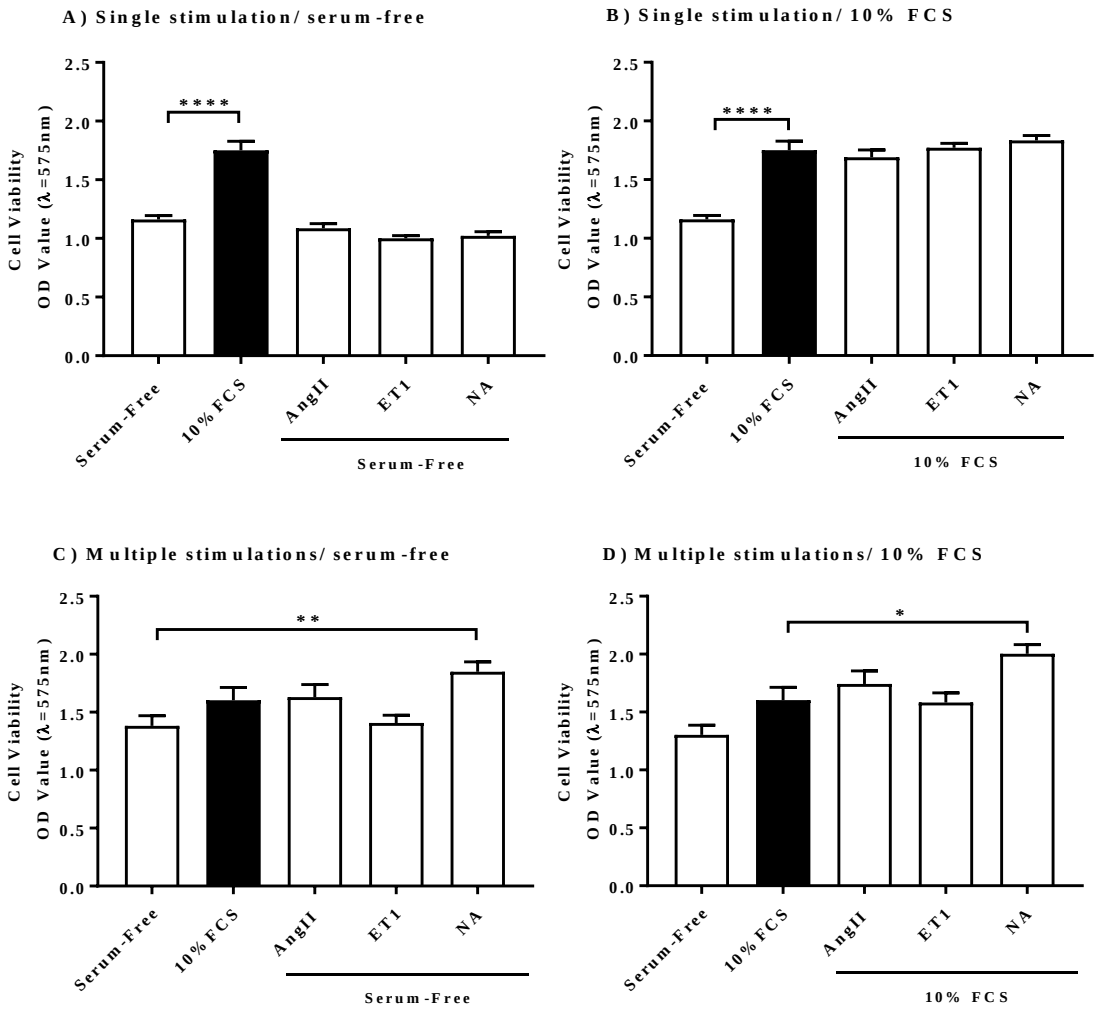


Figure 3.7 Effect of different vasoconstrictors on the induction of VSMC proliferation using the MTT assay.

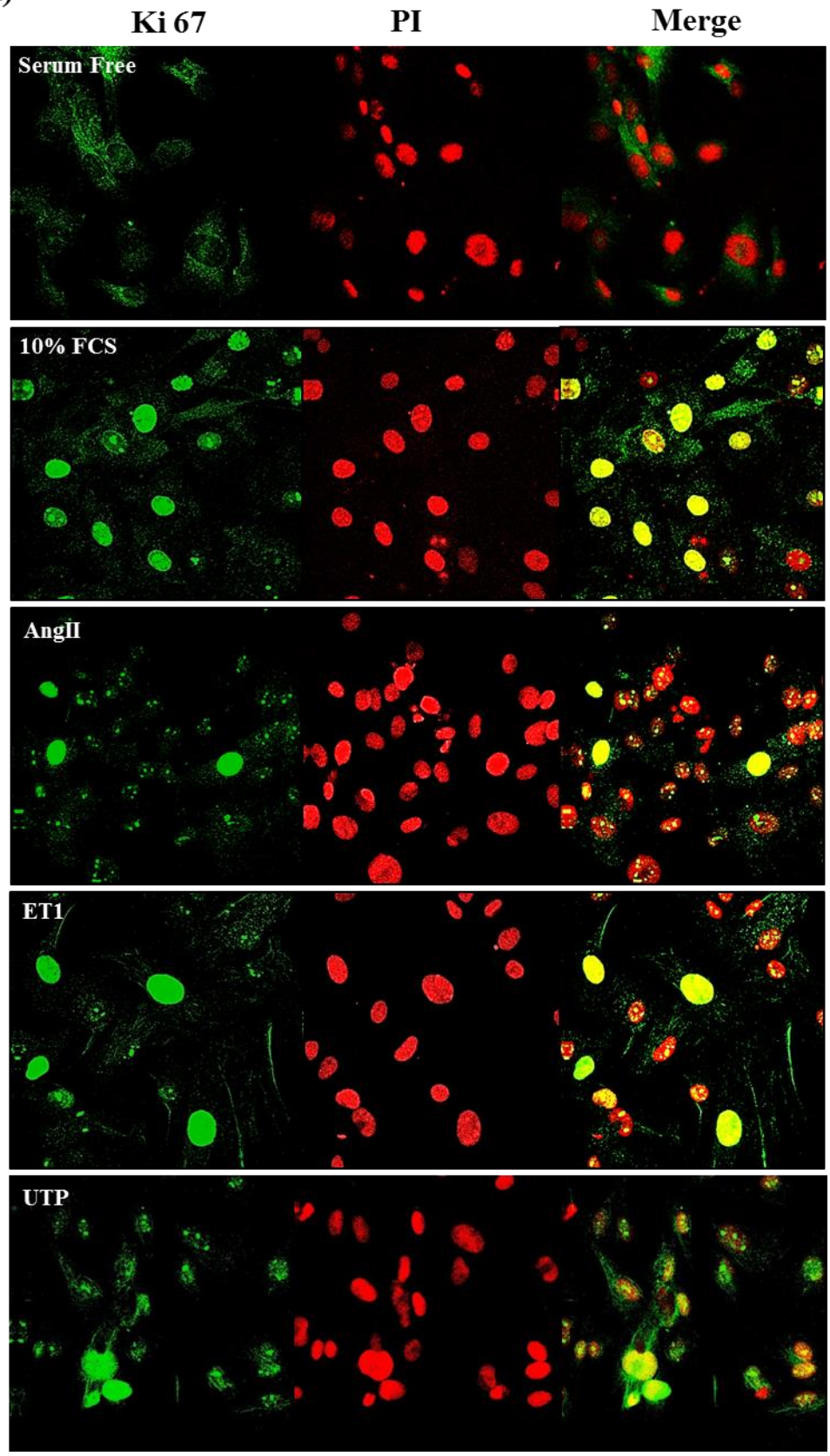
Serum-starved RASM cells were stimulated with AngII (100 nM), ET1 (100 nM) or NA (1 μM) in 10% FCS-containing DMEM or serum-free DMEM. The effects of a single stimulation with vasoconstrictor are shown in either serum-free (A) or 10% FCS-supplemented DMEM (B). The effects of three times stimulations with vasoconstrictor in serum-free (C) or 10% FCS-supplemented DMEM (D) are shown. Data shown are 4 replicates for 4 independent experiments, each using RASM cells prepared from a different animal. Bar graphs are representative of means ± SEM. Statistically significant changes are shown as $^{****}P<0.0001$, $^{**}P<0.01$, $^{*}P<0.05$ (one-way ANOVA, Dunnett's multiple comparison *post-hoc* test).

3.3.8. Effect of different vasoconstrictors on proliferative marker (Ki67)

The nuclear antigen Ki67 has been extensively used as a marker for cell proliferation (Li et al., 2015). Ki67 expression is typically un-detectable in resting G_0 phase but is intensely up-regulated in active cell cycle phases (Gerdes et al., 1984). Although the determination of Ki67 expression has been used extensively to study cancerous cell proliferation, previous studies have used expression of Ki67 as a marker for proliferation of VSMC (Clarke et al., 2006; Wang et al., 2009). Therefore, I evaluated the effects of vasoconstrictors on the proliferative marker, Ki67 expression to assess its suitability to measure the proliferative effects of vasoconstrictors on the growth of RASM. RASM cells were stimulated with 10% FCS or the agonists: AngII (100 nM), ET1 (100 nM) or UTP (100 µM) in serum-free DMEM. Ki67 expression was assessed as described in the Methods (*Section 2.2.8*). As shown in **Figure 3.8 A**, immunofluorescence immunocytochemistry images show that Ki67 was un-detectible in growth-arrested, synchronized cells (i.e. serum-free DMEM incubated cells). However, Ki67 expression was easily detectable in cells stimulated with 10% FCS, or in cells stimulated with AngII, ET1 or UTP in serum-free DMEM. To quantify the changes in Ki67 expression, Ki67 immunofluorescence was measured as mean nuclear fluorescence intensity (MFI) using Image J (**Figure 3.8 B**). Ki67 expression was significantly increased in cells stimulated with 10% FCS versus serum-starved cells. Similarly, MFI was significantly increased in AngII- or ET1-stimulated cells in comparison to control.

A commonly used Ki67 estimation method is through the calculation of the Ki67 index which reflects the percentage of Ki67 active nuclei in immunoreactive cells (Klöppel and La Rosa, 2018). Therefore, a second set of analyses was performed to calculate the percentage of Ki67 active nuclei. This was performed by manual counting of active nuclei **(n)** and the number of total nuclei **(N)** in each field. After that the percentage of active nuclei was calculated by using of these equation **[(n/N) x 100]**. As shown in **Figure 3.8 C**, approximately 50% of the cells stimulated with 10% FCS showed Ki67 active nuclei in comparison to ~5% in serum-starved cells. Stimulation of RASM cells with AngII, ET1 or UTP resulted in ~20%, 40% and 25% activation, respectively. Using either of the two methods of analysis, it can be seen that stimulation of RASM cells with AngII or ET1 resulted in an increase in Ki67 expression while UTP showed a non-significant increase in Ki67 expression in RASM cells.

A)



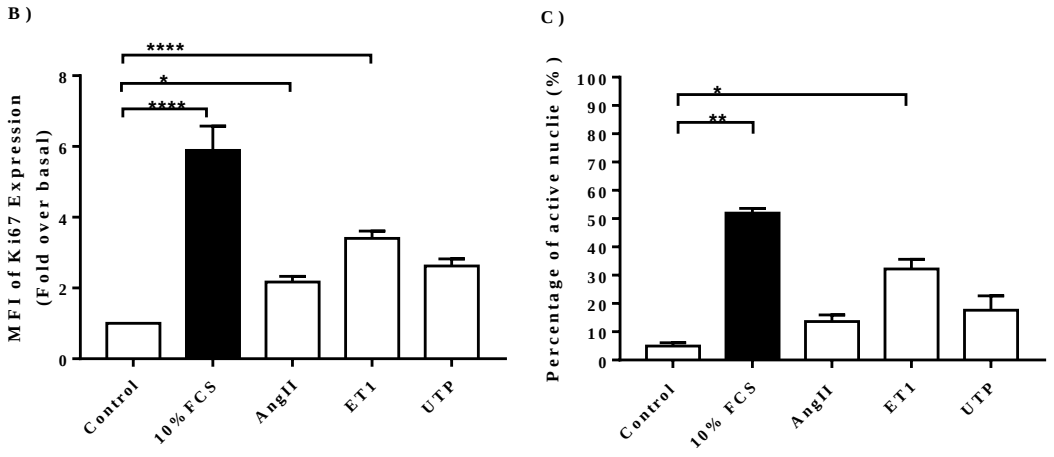


Figure 3.8 Effect of different vasoconstrictors on the proliferative marker Ki67.

Synchronized RASM cells were stimulated with AngII (100 nM), ET1 (100 nM) or UTP (100 μM) in serum-free DMEM. RASM cells were fixed and incubated with anti Ki67. A) Representative images for Ki67 expression in response to 10% FCS, AngII, ET1 or UTP. B) The activity of the proliferative marker Ki67 was measured as mean of nuclear fluorescence intensity using Image J. In addition, the activity of proliferative marker Ki67 was measured as a percentage of the number of active nuclei (C). Bar graphs are representative of means ± SEM. Statistically significant changes are shown as $^{*}P<0.05$; $^{**}P<0.01$; $^{****}P<0.0001$ versus serum-free incubated cells (Kruskal-Wallis, Dunn's *post-hoc* test).

3.3.9. Effect of different vasoconstrictors on proliferative marker (PCNA)

Proliferating cell nuclear antigen (PCNA) is a proliferative marker whose expression is very low in quiescent cells and markedly elevated during the S and G_2 phases of the cell cycle (Jurikova et al., 2016). In addition, PCNA has been extensively used in cancer diagnosis and to monitor prognosis (Naryzhny and Lee, 2007), and has been previous used to investigate changes in VSMC growth and proliferation (Wu and Lu, 2013). Therefore, I assessed its usefulness in RASM to detect vasoconstrictor-stimulated cell proliferation. Serum-starved RASM cells were stimulated either by 10% FCS or the agonists including AngII (100 nM) or ET1 (100 nM) in serum-free DMEM. After that, the immunoblotting procedure completed as described in Method (*Section 2.2.6*). Total cell lysates were analysed by western blotting with anti-PCNA antibody and anti-GAPDH antibody as a loading control. Immunoblotting results illustrate that stimulation of RASM with 10% FCS, AngII or ET1 showed higher PCNA expression in comparison to control, unstimulated cells (**Figure 3.9**).

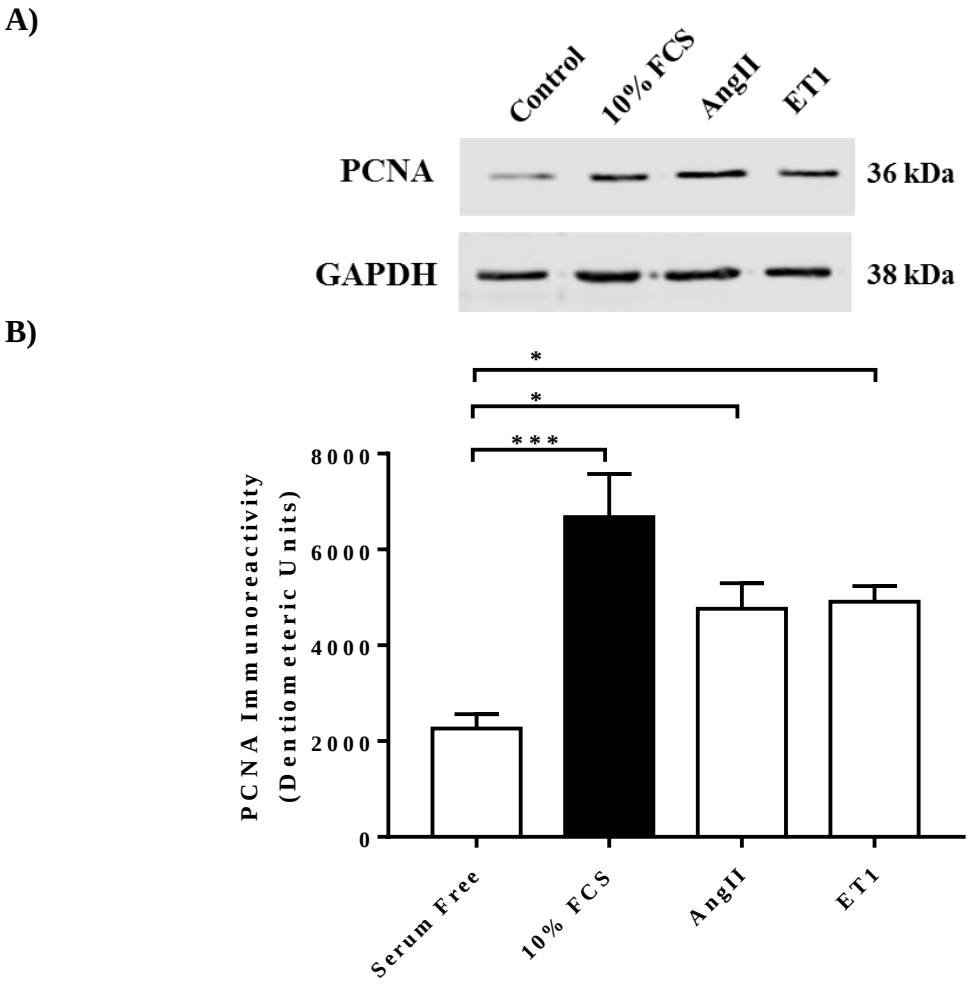


Figure 3.9 Effect of different vasoconstrictors on expression of the proliferative marker PCNA.

Confluent serum-starved RASM cells were stimulated with AngII (100 nM), ET1 (100 nM) and UTP (100 μM) in serum-free DMEM 24 h. Total cell lysates were analysed by western blotting with anti-PCNA and anti-GAPDH antibodies. Data are shown for 8 independent experiments, each using RASM cells prepared from a different animal. Bar graph is representative of mean ± SEM of densitometric analyses of PCNA band by Image J. Statistically significant changes are shown as $^{*}P<0.05$; $^{***}P<0.001$ versus serum-free (control) cells (one-way ANOVA, Dunnett's multiple comparison *post-hoc* test).

3.4. Discussion

Abnormal proliferation of VSMC plays an important role in vascular diseases (Rudijanto, 2007). Indeed, VSMC proliferation and migration are pathologic hallmarks of hypertensive vascular remodelling, atherosclerosis and restenosis after coronary angioplasty (Hahn et al., 1993). Previous evidence suggests that GPCRs have a fundamental role in the pathogenesis of cardiovascular diseases (Premont and Gainetdinov, 2007; Brinks and Eckhart, 2010). As hypertension is associated with elevated levels of circulatory vasoconstrictors (Harris et al., 2008), the current chapter assessed the effects of vasoconstrictors on VSMC proliferation in isolated RASM cells. AngII, ET1 and UTP are potent vasoconstrictors which are known to induce arterial smooth muscle cell contraction through their binding to plasma membrane GPCRs; AT_1, ET_A and $P2Y_2$ receptors respectively, all of which are known to couple to $G_{\alpha q}$ (Paul et al., 2006; Huang et al., 2011; Morris et al., 2011). $G_{\alpha q}$ activation generates second messengers, elevates $[Ca^{2+}]_i$ and consequently induces vasoconstriction (Wynne et al., 2009). Since the $[Ca^{2+}]_i$ is essential in the contractile process of VSMCs, initially, the endogenous expression of functioning AT_1, ET_A and $P2Y_2$ receptors in an isolated RASM cells preparations and their response to vasoconstrictors was confirmed. This was performed by determining vasoconstrictor-mediated changes in the $[Ca^{2+}]_i$ using standard GPCR desensitization protocols (Morris et al., 2010; Morris et al., 2011; Morris et al., 2012). The effects of AngII/AT_1, ET1/ET_A and UTP/$P2Y_2$ provoked PLC/Ca^{2+} signalling in isolated RASM cells were a rapid and transient increase in $[Ca^{2+}]_i$. The rapid increase in $[Ca^{2+}]_i$ is mediated via $G_{\alpha q}$-PLC-IP_3 activation. IP_3 acts on IP_3Rs in the sarcoplasmic reticulum (SR) (a major Ca^{2+} source) resulting in Ca^{2+} release into the cytoplasm through the ryanodine receptor (RyR) (Lesh et al., 1998; Hill-Eubanks et al., 2011). Another mechanism by which $[Ca^{2+}]_i$ is raised by DAG which causes activation of PKC. PKC influences vascular Ca^{2+} channels activity such as voltage-dependent L-type Ca^{2+} channels triggering Ca^{2+} influx (Jackson, 2000; Ghosh et al., 2017). Normally, GPCRs are subject to desensitization and loss of receptor responsiveness following repeated agonist stimulation (Hausdorff et al., 1990). The effects of AngII/AT_1 and ET1/ET_A provoked Ca^{2+} signalling in RASM cells revealed about 45% and 80% reduction in the R2/R1 ratio respectively, showing AT_1 and ET_A receptor desensitization. These results are consistent with previous reports which showed that AT_1 receptors (Hein et al., 1997) and ET_A receptor (Morris et al., 2010) are subject to rapid desensitization and internalization. In 2011, our lab identified that $P2Y_2$ receptor is the predominant mediator

of UTP-stimulated Ca^{2+} signalling in mesenteric arterial smooth muscle. Moreover, they revealed that application of repeated maximum dose of UTP did not produce $P2Y_2$ receptor desensitization, which suggests the presence of a large $P2Y_2$ receptor reserve in isolated RASM cells (Morris et al., 2011). Therefore, to uncover $P2Y_2$ receptor desensitization, a previously utilised receptor desensitization protocol [R1/R_{max}/R2] was applied (Morris et al., 2011). As expected, Ca^{2+} signals showed increased in $[Ca^{2+}]_i$ reflecting amplification of the signals in the UTP-stimulated $P2Y_2$ receptor-PLC signalling pathway. Re-challenging $P2Y_2$ receptors (after R_{max} exposure) resulted in a ~70% reduction in Ca^{2+} signals indicating an extensive $P2Y_2$ receptors desensitization. These results are consistent with our lab previous findings, where UTP stimulation caused the desensitization of both $P2Y_2$ receptors and reduced arterial contraction to repeat stimulation in mesenteric smooth muscle cells (Morris et al., 2011; Rainbow et al., 2018). Overall, consistent with the literature, AT_1, ET_A and $P2Y_2$ receptors are expressed in primary culture of enzymatically dissociated RASM cells and respond to the vasoconstrictor ligands (Gunther et al., 1982; Komuro et al., 1988; Govindan et al., 2010). Additionally, immunocytochemistry and immunoblotting data showed positive SM α-actin and calponin, confirming that they display some of the same characteristics as contractile smooth muscle cells. Therefore, I confirm that my isolation techniques were indeed producing pure arterial smooth muscle cultures, the cells expressed vasoconstrictor GPCRs and their activation profiles were similar to those expected and similar to what our lab have previously produced. Accordingly, primary cultures of RASM cells provide an appropriate tool to study vascular smooth muscle cell proliferation.

There are many different proliferation assays used for evaluation of cell growth. Cultured primary VSMC, as the passage passed, have been reported to undergo phenotype modifications which are characterized by reduced expression of smooth muscle cell contractile markers such as α-actin and acquired the characteristics of active proliferative cells in a passage-dependent manner (Gabbiani et al., 1984; Barja et al., 1986; Chang et al., 2014). The loss of VSMCs' contractile function and enhanced growth responsiveness has made it difficult to examine the proliferative effect of the weak mitogens i.e. vasoconstrictors, therefore, all proliferation experiments were conducted on early (≤ 2) passage cultured cells. The proliferative effect of different vasoconstrictors on VSMCs were initially evaluated by the [^{3}H]-thymidine incorporation assay. VSMCs were

stimulated by the vasoconstrictors; AngII, ET1, NA, V or UTP. Firstly, [^{3}H]-thymidine incorporation experiments were undertaken in serum-starved cells. However, in the absence of serum it was not possible to observe measureable changes in cell growth. This finding is consistent with that of Grainger et al. (1994) who reported that AngII and ET1 did not stimulate DNA synthesis of VSMC in serum-free culture conditions (Grainger et al., 1994). Stimulation of quiescent VSMC with serum results in rapid changes in the c-fos, c-myc and actin mRNA levels, indicating possible participation of these genes in the onset of VSMC proliferation (Kindy and Sonenshein, 1986). Evidence indicates that the vasoactive peptides AngII and ET1 also stimulate c-fos, c-myc and c-jun in VSMC (Komuro et al., 1988; Naftilan et al., 1989b; Naftilan et al., 1990). Accordingly, vasoactive agents that modify growth genes could be implicated in the regulation of smooth muscle cell proliferation. There are several possible explanations for our finding i.e. undetectable proliferative effect of vasoconstrictors in serum-deprived culture. A probable explanation for this might be the inability of [^{3}H]-thymidine to incorporate in VSMC within 4 h. A number of published protocols incubated stimulated cells with [^{3}H]-thymidine for different time periods such as 2 h, 3 h, 4 h and 24 h (Hirata et al., 1989; Ediger and Toews, 2000; Goncharova et al., 2006; Walcher et al., 2006). Thus, [^{3}H]-thymidine incorporation assays were conducted using two different [^{3}H]-thymidine labelling periods of 4 h and 24 h. The latter one (24 h), [^{3}H]-thymidine incubation showed improved levels of [^{3}H]-thymidine incorporation in RASM cells compared to 4 h, even though stimulation of serum-free incubated cells with different vasoconstrictors in both time periods did not increase RASM cell proliferation. These results seem to be consistent with other studies which measure the uptake of [^{3}H]-thymidine in VSMC for 24 h (Lu et al., 2003; Kim et al., 2005c). Another possible explanation for the undetectable changes in cell growth in response to vasoconstrictors could be the instability of vasoconstrictors. For instance, AngII displays rapid and extensive degradation in high density cell cultures. Indeed Bunkenburg et al. (1992) show that 76% of AngII degraded after just 1 h incubation at 37°C in serum-free medium, and after 24 h incubation only 1% of AngII remained (Bunkenburg et al., 1992). In an attempt to combat the instability of peptides such as AngII and ET1, repeated applications of vasoconstrictors were applied. However, in the absence of serum, multiple stimulations of RASM cells by vasoconstrictors failed to promote cell growth, indicating that the inability to produce detectable vasoconstrictor-promoted cell growth was unrelated to their instability. As most vasoconstrictors are weak proliferative agents that take many

years to promote VSMC growth and vascular remodelling, it is conceivable that they might struggle to counteract the effects of serum starvation after 24 h. To overcome this problem, the influence of vasoconstrictors on the proliferation of VSMC was studied in the absence or presence of different concentrations of FCS. VSMC were stimulated with agonists in the presence of 1, 5 or 10% FCS containing medium. The vasoconstrictors had no effect on the proliferation of VSMC in serum-free medium, 1% FCS or 5% FCS. However, stimulation of VSMC with agonist in 10% FCS medium revealed that AngII, ET1 and NA stimulated cell proliferation. I found that stimulation of isolated RASM cells with agonist in 10% FCS medium gave the most reproducible results. In agreement with this protocol, Campbell-Bos et al., studied the proliferative effects (via cell counting) of AngII and vasopressin on human arterial smooth muscle cells in 10% FCS medium, and they reported that the proliferative effect of the vasoconstrictors could be modulated by serum components (Campbell-Boswell and Robertson, 1981). Furthermore, Bunkenburg et al. (1992) reported that AngII required serum-containing factors to increase [^{3}H]-thymidine incorporation and VSMC proliferation (Bunkenburg et al., 1992). A possible explanation for the apparent proliferative effect of vasoconstrictors in the presence of FCS is the probability of the slow mitogenic action of vasoconstrictors and in this case, 10% FCS can accelerate their effects on DNA synthesis. Several reports have shown that vasoactive GPCR agonists promote delayed mitogenic effects in VSMC (Weber et al., 1994; Molloy et al., 1996; Pawlowski et al., 1997). Zhang et al. (2010), showed that AngII induced VSMC proliferation in 1% FCS medium after four days, when the cells were re-stimulated every 24 h (Zhang et al., 2010). Furthermore, delayed proliferative responses might be correlated with the production of some substance that has mitogenic activity. For instance, vasoactive agonists such as AngII, ET1, and α-thrombin stimulate VSMC proliferation indirectly through regulation of epiregulin (a potent mitogen delivered by VSMC) that may contribute to VSMC proliferation and vascular remodelling (Taylor et al., 1999). Additionally, this could explain why hypertensive vascular wall thickness manifests over a long time period (Mulvany, 1999). One of the limitations with inclusion of 10% FCS is the potential difficulty to specify if the proliferative effect is due to vasoconstrictors alone, or as result of multiple interactions between the vasoconstrictor and FCS components.

Our results may be explained by the fact that abnormal VSMC proliferation develops under certain pathological conditions such as hypertension, atherosclerosis and

restenosis after angioplasty (De Mey, 1995; Rudijanto, 2007; Marx et al., 2011). Consistent with the literature, our laboratory previously demonstrated that the proliferative potential of smooth muscle cells derived from SHR is greater than control cells (Tanner et al., 2003; Li et al., 2010). Also, stimulation of aortic SHR smooth muscle cells with AngII or ET1 significantly induced [^{3}H]-thymidine incorporation and cell proliferation in serum-free culture (Willets et al., un-published). Moreover, hypertension-associated structural wall changes, such as increased media layer thickness, have been observed in small resistance arteries especially mesenteric vessels (Rizzoni et al., 1994; Dickhout and Lee, 1997). It is possible that our results would be different if we used VSMCs obtained from smaller resistance arteries, such as mesenteric or femoral arteries. However, I choose to use aorta for our cell preparations because it produced a reproducible good yield of cells. In conclusion, the [^{3}H]-thymidine incorporation assay was optimized to examine the proliferative effect of vasoconstrictors on an isolated RASM cells. Optimum experimental conditions are using early (≤ 2) passaged cultured cells, seeding $5x10^4$ cells per well, single stimulation with vasoconstrictor in 10% FCS DMEM medium and [^{3}H]-thymidine incubation for 24 h (**Figure 3.10**).

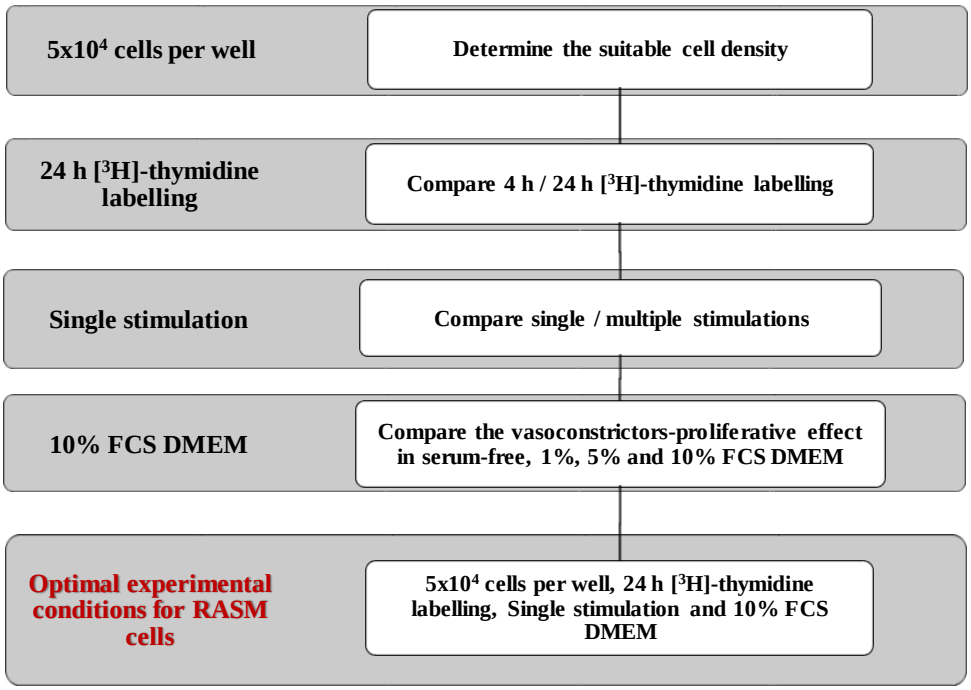


Figure 3.10 Flow chart showing the optimization of the [^{3}H]-thymidine incorporation assay.

Different experimental conditions used to improve [^{3}H]-thymidine incorporation in RASM cells. Optimum experimental conditions to study the effect of vasoconstrictor on RASM cells proliferation are seeding 5x10^4 cells per well, 24 h [^{3}H]-thymidine incubation, single stimulation with vasoconstrictor and incubated in 10% FCS DMEM.

Although the proliferative effect of AngII, ET1 and NA on VSMC proliferation has been confirmed, the exact mechanisms by which they induce DNA synthesis and proliferation in VSMCs is a subject of debate. My finding have shown that AngII stimulates VSMC proliferation which are consistent with data observed in earlier studies (Hahn et al., 1993; Qin et al., 2004; Yu et al., 2018). Proliferative effects of AngII mediated via AT_1 receptor could be blocked by AT_1 antagonists such as losartan or valsartan (Zhang et al., 2010; Liao et al., 2012; Valente et al., 2012). AngII has been reported to induced cell proliferation through the production of reactive oxygen species (ROS) and subsequent activation of ERK1/2 and p38 MAPK (Yaghini et al., 2010) and the stimulation of ET1 gene expression (Hong et al., 2004), induction of ET1 biosynthesis and production (Sung et al., 1994) in VSMC which may contribute to cell proliferation.

Furthermore, AngII plays an important role in cell cycle regulating particularly by increasing CDK2 activity (Kubo et al., 2000) and down-regulating Mfn2 expression (Liao et al., 2012) to induce VSMC proliferation. In contrast, several reports showed that AngII alone was not mitogenic, but did have the ability to induce VSMC proliferation in response to other growth factors such as PDGF and EGF in SHR-derived cells (Bunkenburg et al., 1992; Stouffer and Owens, 1992). Moreover, AngII has been reported to promote cellular protein synthesis in VSMC, which resulted in cellular hypertrophy, but not hyperplasia (Geisterfer et al., 1988). This combined with increased expression of growth factor genes including c-fos, c-myc, and c-jun and increased PDGF-AA mRNA secretion (Kawahara et al., 1988; Naftilan et al., 1989a; Naftilan et al., 1990). Therefore, it seems highly likely that AngII has multiple actions which combine to stimulate cellular growth. Indeed, Gibbons et al. (1992) revealed that AngII has a bifunctional effect on VSMC growth, i.e. activates proliferative and anti-proliferative pathways. The anti-proliferative effect occurrs through enhancing TGF-β1 production, which counteracts the growth stimulating effects of AngII, with cell hypertrophy being the resultant effect (Gibbons et al., 1992). Furthermore, they reported that the anti-proliferative effect of AngII could be inactivated under certain pathological conditions (Gibbons et al., 1992). This may explain why AngII induced [^{3}H]-thymidine incorporation and cell proliferation in the present of 10% FCS in our experiments.

My data showed that ET1 mediated mitogenesis and [^{3}H]-thymidine incorporation. This is in line previous studies that demonstrate ET1 to have proliferative effects on VSMC (Komuro et al., 1988; Hirata et al., 1989; Ohlstein et al., 1992; Ljuca and Drevensek, 2010). Evidence indicates that ET1-stimulated growth in VSMC is mediated through its effect on ET_A receptors in VSMC, which could be inhibited by BQ123, a selective ET_A receptor antagonist (Ohlstein et al., 1992). Additionally, ET1 results reflect other work that also found that ET1 stimulated DNA synthesis, cell number and increased cyclin D1 expression in arterial smooth muscle cells. Elevated levels of cyclin D1 consequently accelerate the G_0/G_1 to S phase transition in the cell cycle. Moreover, ET1 is a powerful co-mitogen for other growth factors such as PDGF and EGF (Quelle et al., 1993; Hafizi et al., 1999; Wedgwood et al., 2001). ET1 exerts a potentiating effect on DNA synthesis in pulmonary arterial smooth muscle cells through stimulation of early response genes (c-fos and c-myc) and thereby promotes the transition from G_0 to G_1 phase of the cell cycle (Komuro et al., 1988; Janakidevi et al., 1992; Biasin et al., 2014).

Moreover, stimulation of human pulmonary arterial smooth muscle cells with FCS resulted in release of endogenous ET1, which might be responsible for the observed serum-induced cell proliferation (Wort et al., 2001). As there are currently limited published data describing the effects of NA on VSMC proliferation, it is difficult here to compare my findings with other work on the same cell type. Recent data show that NA can induce PCNA expression, cell cycle progression and proliferation via interaction with the α_{1D}-adrenoceptor in pulmonary arterial smooth muscle cells (Liu et al., 2017; Luo et al., 2018), which mirrors our findings in VSMC. Furthermore, our findings support the results obtained in human osteoblasts, cardiac fibroblasts and human corneal epithelial cells in which it was shown that NA stimulates cell proliferation (Murphy et al., 1998; Leicht et al., 2003; Kodama and Togari, 2013).

Although UTP (Harper et al., 1998) and vasopressin (Alonso et al., 2009) have been shown to induce a proliferative response, in this study they did not stimulate VSMC proliferation. In agreement with our findings, vasopressin can inhibit human arterial smooth muscle cell proliferation (Campbell-Boswell and Robertson, 1981). It has been suggested that vasopressin exerts diverse actions on VSMC proliferation. Vasopressin increased the proliferation of adult rat aortic smooth muscle cell although it markedly attenuated the proliferation of the A10 cell line that is derived from foetal rat aorta (Nagano et al., 2000). In accordance with the present results, studies have demonstrated that UTP has an anti-proliferative effect on human vascular smooth muscle cells (White et al., 2000). Moreover, previous studies reported that the effect of extracellular nucleotides on $P2Y_2$ receptors causes cell cycle arrest, and anti-proliferative effects in human oesophageal carcinoma cells (Maaser et al., 2002) and in colorectal cancer cells (Hopfner et al., 2001). At present the explanation for these contradictory responses remains unclear. However, a possible explanation for these results is that the anti-proliferative effect of UTP might be due to its effect on other receptors. It is known that $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$ are expressed in VSMC (Erlinge and Burnstock, 2008). Another possible reason is that the anti-proliferative effect might be related to the ability of UTP to activate alternative signaling pathways. For instance, Murthy et al., reported that $P2Y_2$ receptors coupled to PLC-β1 via $G_{\alpha q/11}$ and to PLC-β3 via $G_{\beta\gamma i3}$ (Murthy and Makhlouf, 1998). As previous studies indicate that PLC-β3 can act as negative regulator of endothelial cell proliferation (Bhattacharya et al., 2009), this might suggest that UTP could activate this pathway in VSMCs and prevent cell growth.

To confirm our [^{3}H]-thymidine incorporation assay finding, I used an alternative proliferation assay, which did not just measure DNA synthesis. Thus, I used the MTT assay, which measures mitochondrial activity and thus cellular metabolism. Since growth requires energy then generally greater metabolic rate should reflect more cells and therefore an increase in cellular growth. Unfortunately, with an exception to NA stimulation, the MTT assay were unable to detect reliable changes in RASM cell number/growth in serum-free or with 10% FCS containing medium. Even though previous work showed that NA stimulated MTT reduction and promotes pulmonary arterial smooth muscle cells viability through activation of α_{1D}- adrenoceptor (Liu et al., 2017), it has also been reported that using the MTT assay to examine human airway smooth muscle cells proliferation might not be an accurate way under certain conditions (Zhang and Cox, 1996). This is particularly true when the mitogenic agent can influence other enzymes and factors that accelerate MTT reduction in addition to their effect in increasing cell proliferation (Zhang and Cox, 1996). For example, NA is likely to stimulate mitochondrial metabolism through activation of β-adrenoceptor (Case et al., 2016), therefore the reported effects might not be related to cellular proliferation.

Even though, the [^{3}H]-thymidine incorporation assay was a potentially useful method to evaluate the proliferative effect of vasoconstrictors, the requirement for serum inclusion to display the proliferative effects of vasoconstrictors is open to criticism. Therefore, another technique is required to assess cell proliferation without the need for serum. I have established the mitogenic potential of AngII, ET1 on RASM cells by investigating their effect on commonly used proliferative markers in serum-free culture. Detection of proliferating smooth muscle cells was performed by determining the expression of nuclear antigen Ki67 and PCNA (Jurikova et al., 2016). In agreement with our [^{3}H]-thymidine incorporation assay findings, AngII and ET1 induced activation of Ki67 in serum-deprived cultures confirming their mitogenic potency. Numerous reports have used expression of Ki67 as a marker for VSMC proliferation in response to other stimuli such as visfatin (Wang et al., 2009), during atherosclerotic plaque development (Clarke et al., 2006) or after arterial injury (Aoyagi et al., 1995). Currently, there are no published data describing the effects of vasoconstrictors on Ki67 expression in VSMC, thus, it is difficult here to compare the data in this thesis with other work on smooth muscle cells. However, my findings broadly support the work of other studies, albeit in different cell backgrounds, which correlate vasoconstrictor treatment to increased Ki67

expression. AngII has been reported to induce Ki67 expression in human umbilical vein endothelial cells, which could be blocked by the AT_1 receptor antagonist candesartan (Herr et al., 2008). Moreover, ET1 increased Ki67 expression and cell proliferation in rat cortical astrocyte cells (Gadea et al., 2008). Although there is no reported relationship between UTP level and Ki67 expression, Qiu et al. (2018) identified a negative correlation between $P2Y_2$ receptor expression, Ki67 index and the histological grade of breast cancer, which suggested that the $P2Y_2$ receptor may contribute to tumour cell migration and invasion, but not proliferation (Qiu et al., 2018). In agreement with data presented in this thesis, recent evidence revealed that AngII stimulated PCNA expression in the A7r5 rat VSMC line (Yu et al., 2018), and both AngII and ET1 promote PCNA expression in primary cultures of VSMC (Li et al., 2013; Zhang et al., 2016).

In summary, it has been shown in this chapter that isolated RASM cells are pure aortic smooth muscle cells cultures that express functional vasoconstrictor GPCRs. Furthermore, this chapter has assessed the suitability and reproducibility of a number of different proliferation assays. I have shown that optimized [^{3}H]-thymidine incorporation assay is effective, however, there are certain drawbacks associated with the addition of serum to uncover the effects of vasoconstrictors. I have also shown that Ki67 and PCNA assays are sensitive enough to observe proliferative effects of vasoconstrictors alone in serum-free conditions. On the other hand, the MTT assay did not show any changes on cell growth and was not a suitable assay to use. Since different assays have been assessed and the reproducible and reliable proliferation assays were developed to study the effects of vasoconstrictors on VSMC growth, the next chapter describes the effect of GRK2 on vasoconstrictor-induced proliferation of VSMC.

Chapter Four

4. Impact of GRK2 on cell proliferation

4.1. Introduction

G protein coupled receptor kinase 2 (GRK2) is a member of the GRK family and is a serine/threonine protein kinase able to phosphorylate and desensitize the active configuration of GPCRs (described earlier in *Section 1.3.4.2*). The classical role of GRK2 has been explained as GRK2-mediated GPCR desensitization (Brinks and Eckhart, 2010). It has been believed that the traditional function of GRK2 is as part of a protective mechanism for the cell against overstimulation, however, this mechanism is impaired with the progression of certain diseases such as heart failure and hypertension (Choi et al., 1997; Cohn et al., 2009). It is now well defined that its role is not limited to receptor regulation. Indeed, GRK2 is able to interact with, regulate, or be regulated by a variety of non-receptor substrates (*Section 1.3.4.3*), (Willets et al., 2003; Tesmer et al., 2005; Homan and Tesmer, 2014). GRK2 has high physiological importance, as alterations in GRK2 expression and/or kinase activity implicated in several pathological conditions (Ribas et al., 2007; Penela et al., 2010b). Moreover, GRK2 depletion or inhibition differentially changes the GPCR-mediated vasoconstrictor effects (Rainbow et al., 2018), indicating that changes in GRK2 levels or catalytic activity might mediate important effects in hypertension pathophysiology.

It has been reported that hypertension is correlated with elevated GRK2 expression (Cohn et al., 2008; Cohn et al., 2009; Willets et al., 2015). Although the exact mechanism by which hypertension is associated with increased GRK2 expression is not yet fully understood, a complex interaction of diverse GPCR and non-GPCR signal transduction pathways appears to be able to modulate GRK2 expression in vascular cells (Ramos-Ruiz et al., 2000; Eckhart et al., 2002; Penela et al., 2010b). For example, continuous $G_{\alpha q}$ activation enhances GRK2 promoter transcriptional activity and increases GRK2 expression in VSMCs (Ramos-Ruiz et al., 2000). Additionally, TGF-β has been reported as a regulator for GRK2 expression in VSMCs, enhancing both mRNA expression and protein levels (Guo et al., 2009). Together with multiple phosphorylation-dependent (such as phosphorylation of R-Smad) and/or independent GRK2 interactions (such as direct interaction with MEK and caveolin-1) (Shiina et al., 2001; Naga Prasad et al.,

2002a; Schutzer et al., 2005; Jimenez-Sainz et al., 2006), we propose that GRK2 might act as an effector, contributing to the regulation of VSMC proliferation. Changes in GRK2 expression or its catalytic activity might mediate important effects in hypertensive vascular remodelling. Here, in Chapter Four, the previously characterised proliferation assays (*Chapter 3, Section 3.4*) were utilised to determine whether GRK2 plays a role in vasoconstrictor-stimulated cell growth.

4.2. Aim

To objectively measure and assess the effect of GRK2 expression and its catalytic activity on vasoconstrictor-induced RASM cell proliferation.

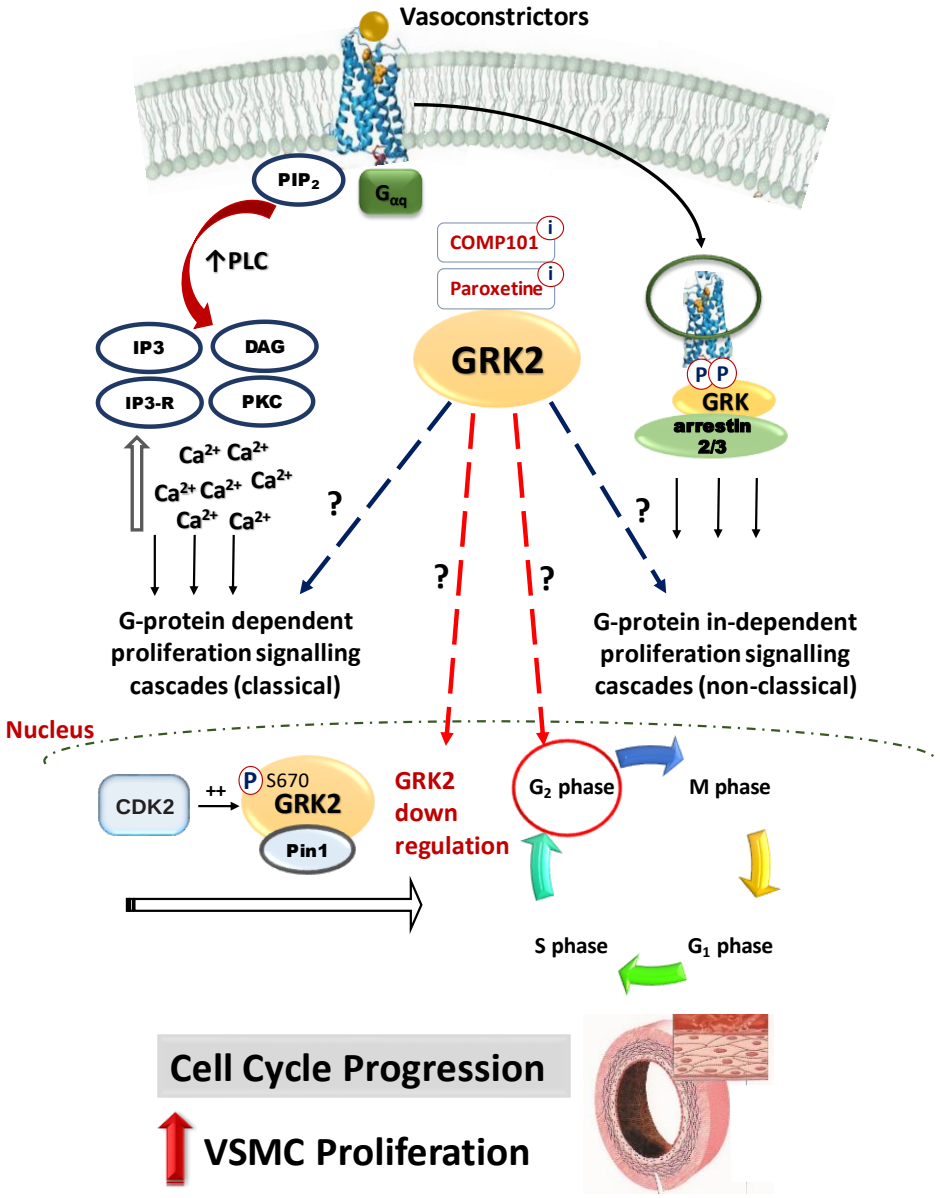


Figure 4.1 Diagram for the proposed effects of altered GRK2 expression and activity on proliferation of VSMC.

Hypertension associated with elevated GRK2 expression (Gros et al., 2000). Proposed effect of GRK2 on cell proliferation might be through its effect on vasoconstrictor-stimulated $G_{\alpha q}$-dependent or independent mediated different signalling pathways induced cell proliferation. Another proposed effect via regulation of cell cycle progression; down-regulated GRK2 expression during cell cycle progression (Penela et al., 2010a), could mediate the proliferation of VSMC.

4.3. Results

4.3.1. Paroxetine inhibits UTP-induced $P2Y_2$ desensitization in RASM cells

siRNA techniques have been previously utilised to suppress GRK2 expression and thus identify its role in the regulation of $P2Y_2$ and ET_A receptor signalling (Morris et al., 2010; Morris et al., 2011). These findings identified GRK2 as the key kinase that induces receptor desensitization and regulates Ca^{2+} signalling mediated by these receptors in VSMC (Morris et al., 2010; Morris et al., 2011). In addition, the SSRI paroxetine has been identified as a GRK2 inhibitor (Thal et al., 2011), and more recent evidence from our laboratory shows that paroxetine can inhibit GRK2-mediated desensitization of $P2Y_2$ and AT_1 receptor induced contractions in isolated mesenteric smooth muscle cells and arteries (Rainbow et al., 2018). Therefore, as GRK2 is the key kinase in the desensitization of $P2Y_2$ receptor signalling (Morris et al., 2012) in RASM cells, I determined the effectiveness of paroxetine to inhibit GRK2-mediated $P2Y_2$ receptor desensitization in RASM cells.

RASM cells were pre-incubated with either paroxetine (5 μM) or fluoxetine (5 μM) which is a SSRI that does not inhibit GRK2 activity and thus acts as a control for off-target (non-GRK2) effects (Thal et al., 2011). A standard, previously characterized desensitization protocol was used to induce $P2Y_2$ receptor desensitization, where RASM cells were challenged with an approximate EC_{50} concentration of UTP (1 μM) for 5 min before (R1) and 5 min after (R2) the addition of a maximal UTP challenge (R_{max};100 μM) (Morris et al., 2011). As shown in **Figure 4.2 A**, comparison of the R2/R1 ratio indicated that in control (DMSO treated cells) the ratio was 30% indicating a level of 70% $P2Y_2$ receptor desensitization. In addition, pre-incubation with paroxetine resulted in a significant increase in the R2/R1 ratio showing reduced $P2Y_2$ receptor desensitization, possibly through inhibition of GRK2 activity. Pre-incubation of RASM with fluoxetine had no effect upon $P2Y_2$ receptor desensitization. These results revealed that paroxetine, but not fluoxetine, attenuates UTP-induced $P2Y_2$ receptor desensitization likely via inhibition of GRK2.

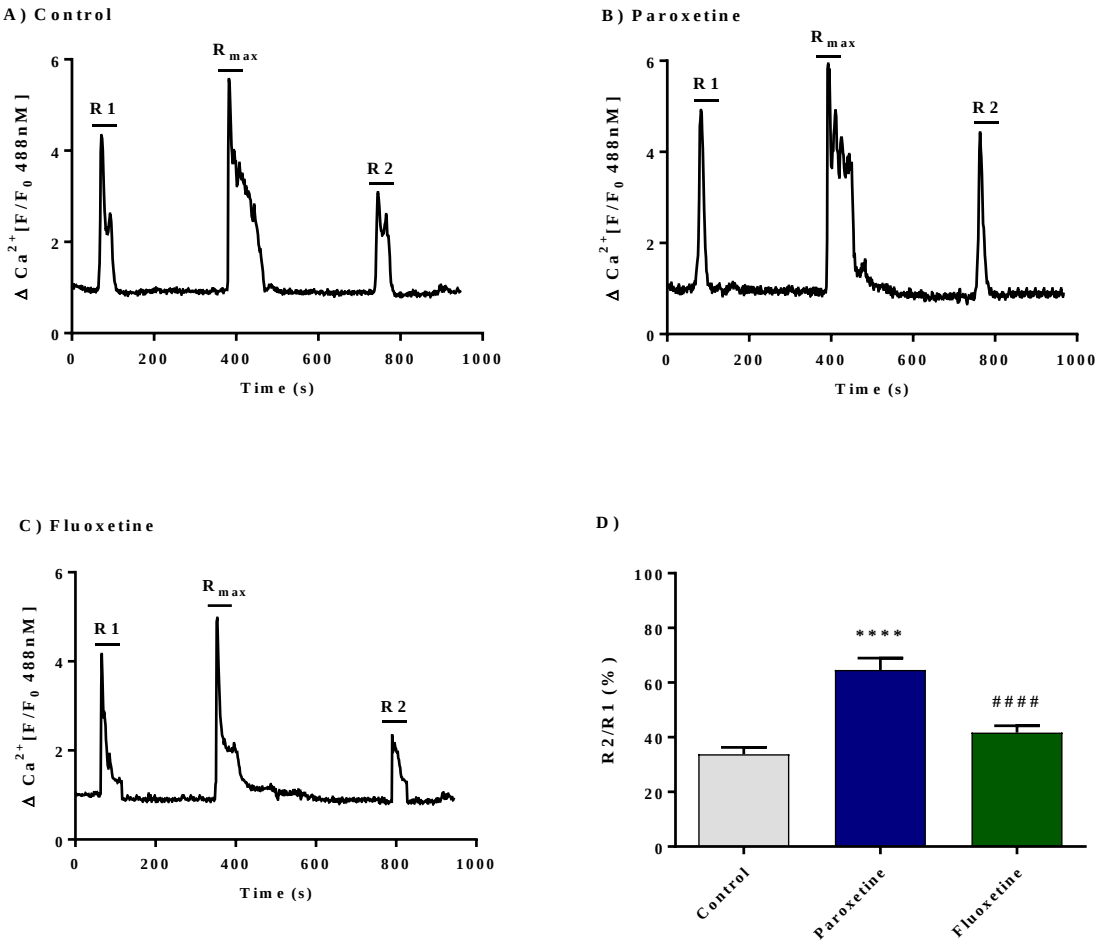


Figure 4.2 Paroxetine inhibits UTP-induced $P2Y_2$ receptor desensitization.

RASM cells pre-incubated with (5 μM) paroxetine (B) or (5 μM) fluoxetine (C) for 1 h before being subjected to standard R1/R_{max}/R2 (UTP) desensitization protocol. Representative traces from single cells show $P2Y_2$ receptor Ca^{2+} signal desensitization following UTP challenge in vehicle control (A), paroxetine (B) or fluoxetine (C) pre-incubated cells. Real-time images were taken using an Olympus FV500 laser-scanning confocal IX70 inverted microscope (oil immersion objective 60x) to track $[Ca^{2+}]_i$. (D) Cumulative data indicate the degree of $P2Y_2$ receptor desensitization (means ± SEM from 14–16 cells prepared from three different animals). $^{****}P<0.0001$ in comparison to control; $^{\#\#\#\#}P<0.0001$ in comparison to paroxetine (one-way ANOVA, Tukey's multiple comparison *post-hoc* test).

4.3.2. Paroxetine inhibits vasoconstrictor-induced VSMC proliferation

As the previous results (*Section 4.3.1*) and our published work (Rainbow et al., 2018) indicate that paroxetine inhibits GRK2 mediated $P2Y_2$ and AT_1 receptor desensitization, paroxetine was subsequently used to inhibit GRK2 function and determine whether GRK2 activity affected vasoconstrictor-induced RASM cell proliferation. Serum-starved RASM cells were pre-incubated with (5 µM) paroxetine or (5 µM) fluoxetine for 30 min, before addition of vasoconstrictors [AngII (100 nM), ET1 (100 nM) or NA (1µM)] in 10% FCS (DMSO was used as the vehicle control). The effects of paroxetine or fluoxetine on 10% FCS and different vasoconstrictor-induced RASM cell proliferation are shown in **Figure 4.3**. In agreement with previous data (*Section 3.3.6*), addition of the vasoconstrictors AngII, ET1 or NA increased the level of [^{3}H]-thymidine incorporation above that seen with 10% FCS alone. Treatment of RASM cells with paroxetine resulted in a significant reduction in [^{3}H]-thymidine incorporation/cell proliferation rate induced by 10% FCS, AngII, ET1 and NA compared to control cells (**Figure 4.3**). Whereas, fluoxetine had no effect on [^{3}H]-thymidine incorporation/cell proliferation induced by any of the stimulants. These results suggest that paroxetine, but not fluoxetine, inhibits vasoconstrictor-induced RASM cell proliferation, highlighting a potential role for GRK2 in cellular proliferation.

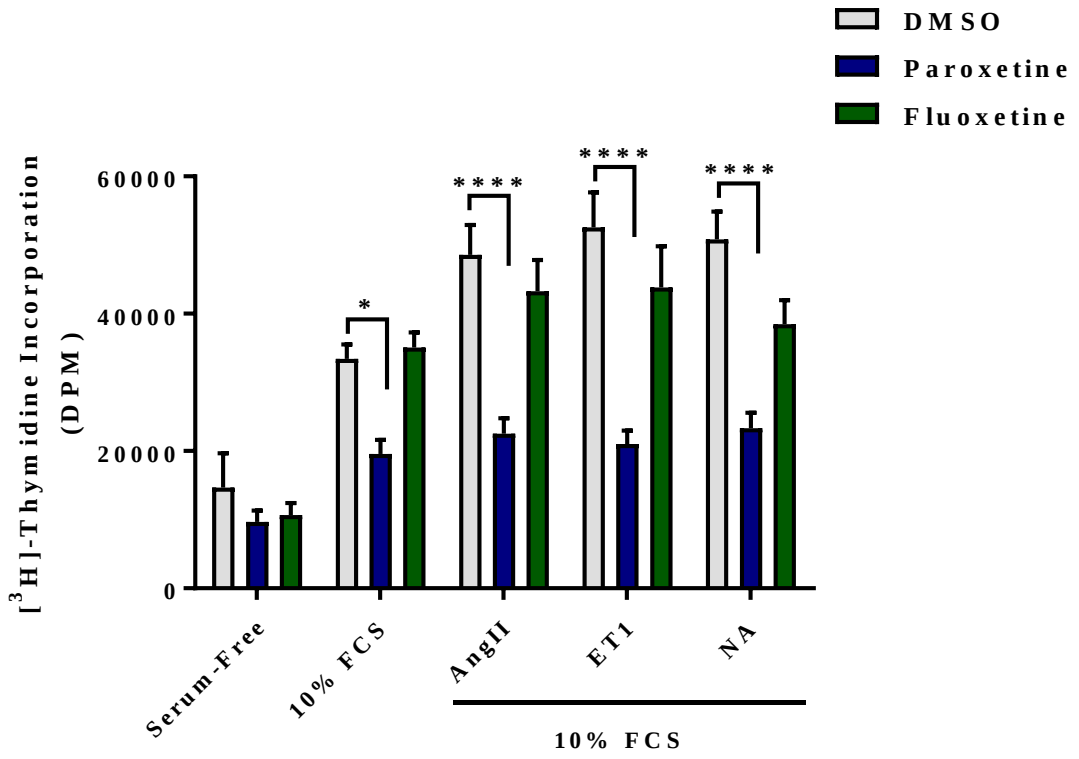


Figure 4.3 Paroxetine inhibit vasoconstrictor-induced VSMC proliferation.

Serum-starved RASM cells were pre-treated with paroxetine (5 μM) or fluoxetine (5 μM) for 30 min, prior to stimulation with vasoconstrictors [AngII (100 nM), ET1 (100 nM) or NA (1μM)] in 10% FCS containing paroxetine (5 μM) or fluoxetine (5 μM) for 24 h. Paroxetine significantly inhibited 10% FCS, AngII, ET1 or NA induced RASM cells proliferation. Data represent means ± SEM for 4 replicates for each experimental condition from 4 different cells preparations. Statistically significant changes compared to serum-free control unstimulated cells are shown as; *P<0.05, ****P<0.0001 (two-way ANOVA, Tukey's *post-hoc* test).

4.3.3. The small molecule GRK2 inhibitor COMP101 attenuated vasoconstrictor-induced VSMC proliferation

To further confirm whether inhibiting GRK2 activity could prevent vasoconstrictor-driven RASM cell proliferation, the effect of an alternative GRK2 small-molecule inhibitor COMP101 (Thal et al., 2011) on agonist-stimulated [^{3}H]-thymidine incorporation was tested. Serum-starved RASM cells were pre-incubated with COMP101 (30 μM) for 30 min prior to vasoconstrictor stimulation [AngII (100 nM), ET1 (100 nM) or NA (1μM)]. As shown in **Figure 4.4**, in the presence of DMSO, the profile of vasoconstrictor-stimulated [^{3}H]-thymidine incorporation was similar to that observed in previous experiments (*Section 3.3.6 and 4.3.2*), where AngII, ET1 or NA stimulation produced higher levels of [^{3}H]-thymidine incorporation than that seen with 10% FCS alone. In contrast, treatment of RASM cells with COMP101 significantly attenuated ET1 and NA induced RASM cells proliferation when compared to control, but had no effect upon FCS induced cell proliferation. These results confirmed that inhibition of GRK2 catalytic activity attenuated vasoconstrictor-induced [^{3}H]-thymidine incorporation.

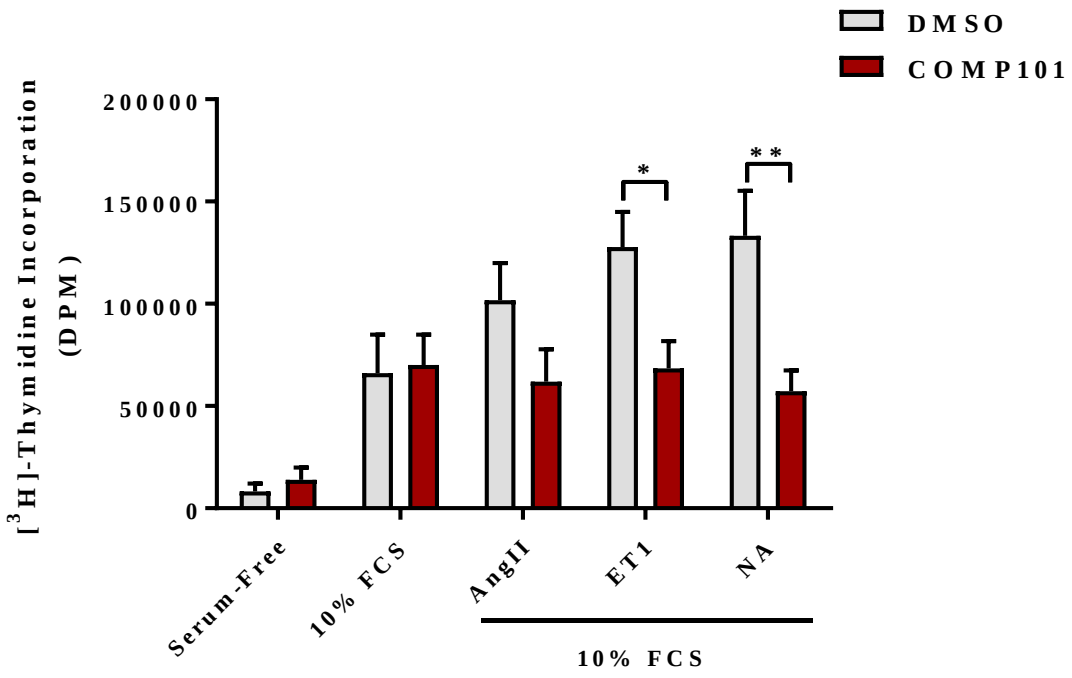


Figure 4.4 The small molecule GRK2 inhibitor COMP101 attenuated vasoconstrictor-induced VSMC proliferation.

Serum-starved RASM cells were pre-treated with COMP101 (30 μM) for 30 min, prior to stimulation with the vasoconstrictors [AngII (100 nM), ET1 (100 nM) or NA (1μM)] in 10% FCS containing COMP101 (30 μM) for 24 h. Data show that COMP101 significantly inhibited ET1 or NA induced RASM cells proliferation. Data represent means ± SEM for 4 replicates for each experimental condition from 4 different cells preparations. Statistically significant changes compared to serum-free control unstimulated cells are shown as; **P*<0.05, ***P*<0.01 (two-way ANOVA, Sidak's *post-hoc* test).

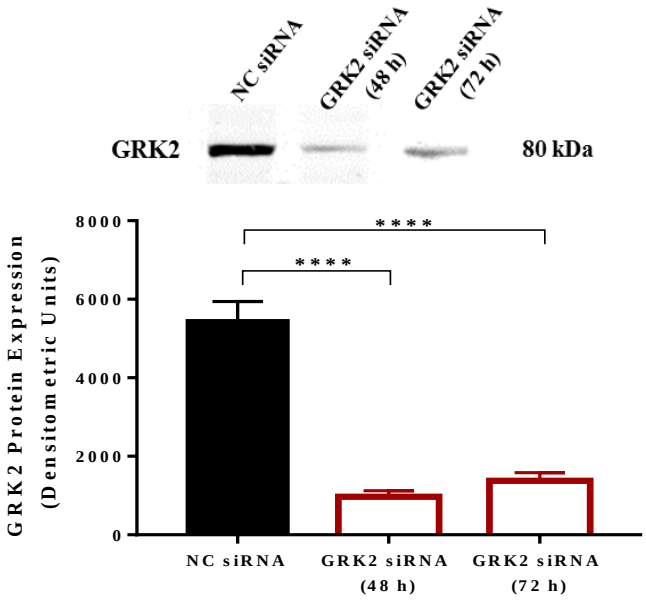
4.3.4. siRNA-induced GRK2 knockdown attenuates vasoconstrictor-induced VSMC proliferation

As the previous data (*Section 4.2.2. and 4.2.3*) indicated that GRK2 activity is implicated in vasoconstrictor-induced RASM proliferation, I next wanted to determine whether the presence of GRK2 in addition to its kinase functionality was important in mediating RASM proliferation. Here GRK2 expression was depleted using standard siRNA techniques (Morris et al., 2010) and the effects on vasoconstrictor-driven RASM cell proliferation was determined using the [^{3}H]-thymidine incorporation assay. Briefly, GRK2 expression was selectively depleted in RASM cells using nucleofection with a previously characterised anti-GRK2 siRNA (Morris et al., 2010), as described in the Methods (*Section 2.2.4*). Twenty four hours post-nucleofection, cells were serum-starved for a further 24 h, prior to stimulation with the vasoconstrictors AngII (100 nM), ET1 (100 nM), or NA (1 μM) in 10% FCS medium. Numerous different agonists can induce RASM cell growth including PDGF which dose so by activating the tyrosine kinase-coupled PDGF receptor (Kim et al., 2015). Interestingly, PDGF signalling has previously been reported to be regulated by GRK2 in VSMCs (Wu et al., 2005). Therefore, to further investigate whether the effects of GRK2 inhibition were selective for GPCR-driven proliferation, RASM growth was also stimulated with PDGF (40 ng/ml) in the presence and absence of GRK2 siRNA.

Immunoblotting showed that treatment of RASM cells with anti-GRK2 siRNA produced a marked reduction ~80% (48 h) and ~70% (72 h) in GRK2 protein expression post-transfection (more validation in *Section 4.3.6*) confirming the effectiveness of the anti-GRK2 siRNA construct (**Figure 4.5 A**). Moreover, the profile of vasoconstrictor-stimulated [^{3}H]-thymidine incorporation in negative control (NC) siRNA transfected cells was equivalent to that detected in non-transfected cells (*Section 3.3.6, 4.3.2 and 4.3.3*).

As expected, inclusion of AngII, ET1 or NA resulted in more [^{3}H]-thymidine incorporation than that seen with 10% FCS alone (**Figure 4.5 B**). In contrast, depletion of GRK2 significantly attenuated 10% FCS, AngII, ET1 and NA induced [^{3}H]-thymidine incorporation in RASM cells, but had no effect upon PDGF-induced cell proliferation (**Figure 4.5 B**). These data strongly suggest that GRK2 expression is essential to facilitate vasoconstrictor-induced RASM cells proliferation.

A)



B)

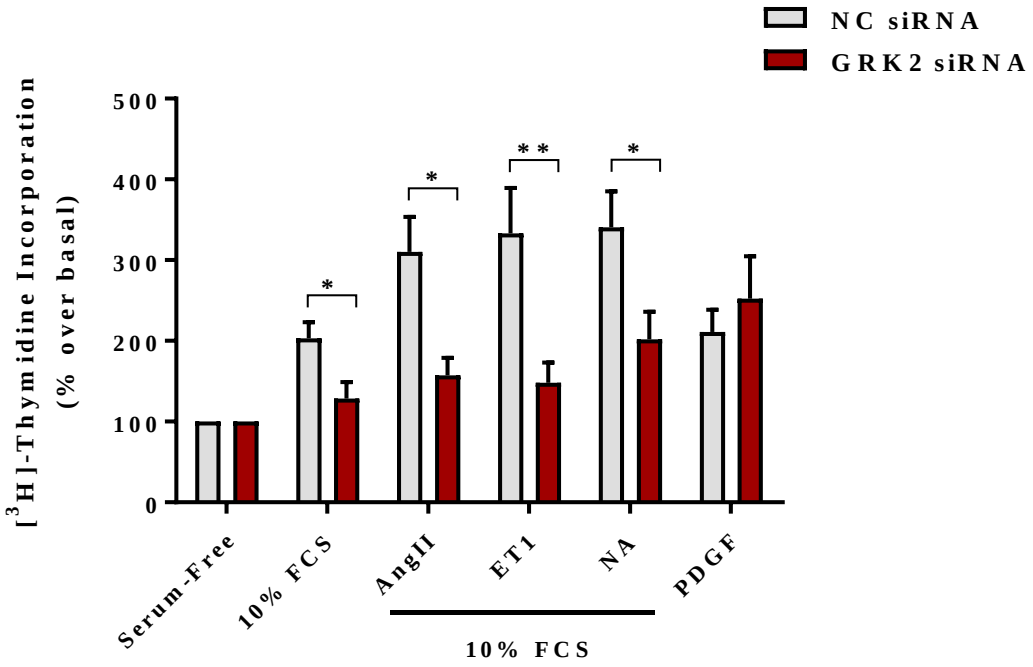


Figure 4.5 GRK2 siRNA knockdown attenuates vasoconstrictor-induced VSMC proliferation.

(A) GRK2 expression was specifically depleted by nucleofection with anti-GRK2 siRNA (10 nM), leading to a reduction in GRK2 expression of ~80% after 48 h, and ~70% after 72 h transfection. Cumulative data are represented as means ± SEM. (B) Serum-starved NC or anti-GRK2 siRNA treated RASM were stimulated with AngII

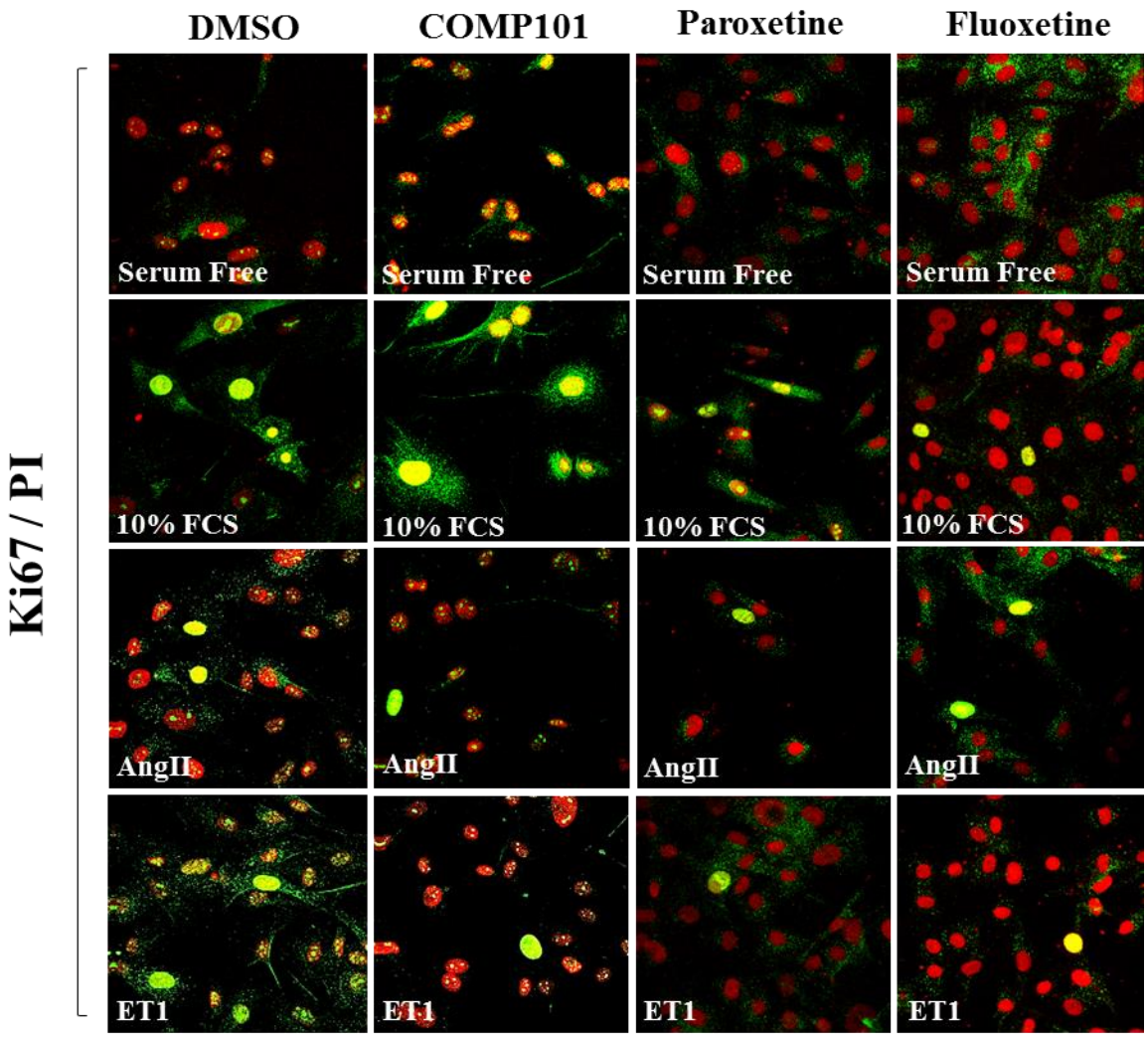
(100 nM), ET1 (100 nM), NA (1 µM) in 10% FCS or with PDGF (40 ng/ml) for 24 h. Cell proliferation was measured using the [^{3}H]-thymidine incorporation assay. GRK2 siRNA significantly inhibited 10% FCS, AngII, ET1 and NA induced [^{3}H]-thymidine incorporation. Data shown are 4 replicates for 4 independent experiments, each using RASM cells prepared from a different animal. Cumulative data are shown as means ± SEM. Statistically significant changes compared to respective agonist-stimulated values in NC siRNA nucleofected cells are shown as; *P<0.05, **P<0.01 (one-way ANOVA, Dunnett's *post-hoc* test (A) and Kruskal-Wallis, Dunn's post-hoc test (B)).

4.3.5. What role does GRK2 catalytic activity play in vasoconstrictor-induced Ki67 expression?

Previous findings in *Sections 4.3.2 and 4.3.3* showed that paroxetine and COMP101 markedly inhibited vasoconstrictor-induced [^{3}H]-thymidine incorporation in RASM cells. To confirm and expand on these data, the effects of GRK2 inhibitors on an alternative measure of cell proliferation (Ki67 expression) was conducted. RASM cells were pre-incubated with COMP101 (30 μM), paroxetine (5 μM) or fluoxetine (5 μM) for 30 min prior to stimulation with 10% FCS or the agonists: AngII (100 nM) or ET1 (100 nM) in serum-free DMEM containing the required concentration of inhibitor. Procedures were then completed as described in Methods (*Section 2.2.8*). In agreement with previous findings (*Section 3.3.8*), immunofluorescent immunocytochemistry images of control cells (DMSO-incubated cells) showed that Ki67 expression was un-detectable in growth arrested cells (i.e. serum-free DMEM incubated cells). In contrast, when RASM cells were incubated with 10% FCS, AngII or ET1, a marked increase in Ki67 expression was observed (**Figure 4.6 A**).

When COMP101- or paroxetine-treated RASM cells were stimulated with 10% FCS, a significant reduction in the expression of Ki67 was detected (**Figure 4.6 B**). Likewise, COMP101 caused a significant reduction in AngII- or ET1-stimulated Ki67 expression (**Figure 4.6 C and D**). These results are similar to those of [^{3}H]-thymidine incorporation assay (*Section 4.3.3*). Another important finding was treatment of RASM cells with paroxetine significantly inhibited ET1 induced Ki67 expression (**Figure 4.6 D**). Surprisingly, this inhibition of Ki67 expression was also replicated to a lesser extent by fluoxetine (**Figure 4.6 D**). On other hand, when RASM cells were stimulated with AngII in the presence of paroxetine or fluoxetine, no significant difference in the expression of Ki67 was detected (**Figure 4.6 C**).

A)


DMSO
COMP101
Paroxetine
Fluoxetine
Ki67 / PI
Serum Free
Serum Free
Serum Free
Serum Free
10% FCS
10% FCS
10% FCS
10% FCS
AngII
AngII
AngII
AngII
ET1
ET1
ET1
ET1

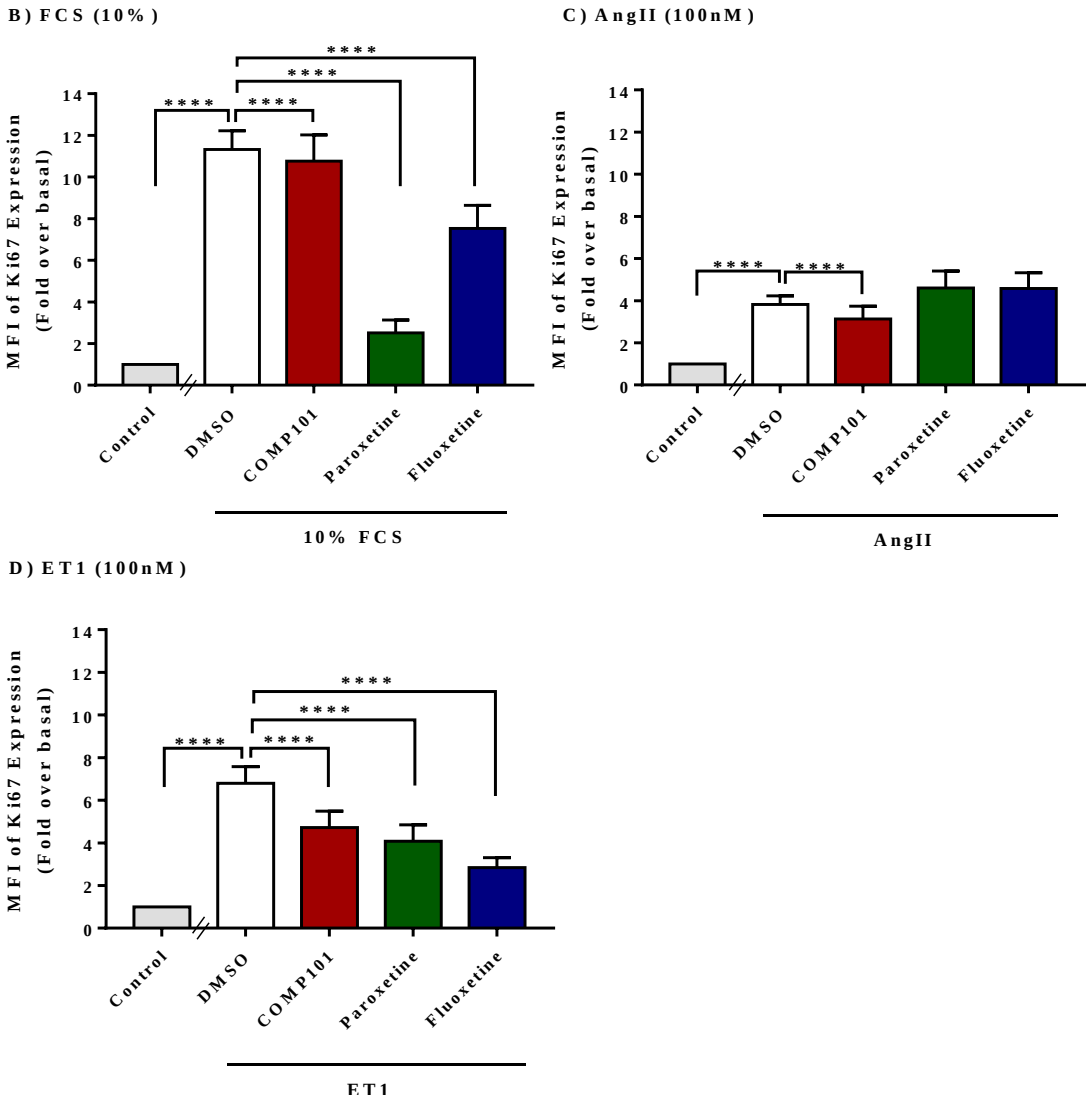


Figure 4.6 GRK2 inhibitors prevent Ki67 expression.

Growth-arrested RASM cells were pre-incubated with COMP101 (30 µM), paroxetine (5 µM) or fluoxetine (5 µM) for 30 min prior to stimulation with 10% FCS, AngII (100 nM) or ET1 (100 nM). After 24 h, RASM cells were fixed and Ki67 expression determined using immunocytochemistry. A) Representative immunofluorescence images for Ki67 expression in control, COMP101, paroxetine and fluoxetine treated cells. The expression of the proliferative marker Ki67 was measured as fold over basal for mean of nuclear fluorescence intensity using Image J (N ≥ 500 cells). Cumulative data (means ± SEM) show that all inhibitors significantly decreased 10% FCS (B) and ET1 (D) induced Ki67 expression in comparison to control. However, only COMP101 significantly decrease AngII induced Ki67

expression (C). Statistically significant changes are shown as $^{****}P<0.0001$ versus control cells (Kruskal-Wallis, Dunn's *post-hoc* test).

4.3.6. What roles do other GRKs play in vasoconstrictor-induced Ki67 expression?

Cultured aortic smooth muscle cells not only express GRK2 but also GRK5 and GRK6 (Ishizaka et al., 1997; Morris et al., 2010). Interestingly, data from other cell backgrounds suggests a positive correlation exists between individual GRK isoform expression and Ki67 expression (Li, 2013; Nogues et al., 2016; Jiang et al., 2018). Therefore, the effects of altering GRK2, GRK5 and GRK6 expression on RASM cell growth were examined using Ki67 expression as a marker of cell proliferation.

GRK2, GRK5 and GRK6 expression were selectively depleted in RASM cells using previously characterised anti-GRK2 (Morris et al., 2010), anti-GRK5 or anti-GRK6 siRNAs (Nash et al., 2018), via the nucleofection technique described in the main Methods (*Section 2.2.4*). To determine the effectiveness of the individual siRNA constructs in depleting targeted GRKs cells were lysed 48 h after nucleofection and probed for GRK expression using selective anti-GRK2, anti-GRK5 or anti-GRK6 antibodies. Immunoblotting data revealed that transfection of RASM cells with anti-GRK2, anti-GRK5 and anti-GRK6 siRNAs caused ≥70% knockdown of the targeted kinase in comparison to NC siRNA transfected cells (**Figure 4.7 B, C and D**). Furthermore, knockdown of each targeted GRK was specific, i.e. without affecting the expression of other non-targeted GRKs expression (**Figure 4.7 A**). To further confirm siRNA transfection efficiency, GRK expression was visualised using immunocytochemistry techniques, which showed reduced GRK2, GRK5 and GRK6 expression in ~70%-80% of transfected cells with corresponding anti-GRK siRNAs (**Figure 4.8**).

After confirming the selectivity of individual GRK siRNAs, the effects of depleting individual endogenous GRK expression on the ability of vasoconstrictors to activate Ki67 expression was examined next. In these experiments, growth-arrested cells (48 h post-nucleofection) were stimulated with 10% FCS or AngII (100 nM) or ET1 (100 nM) in serum-free DMEM. Levels of Ki67 expression were assessed as described in the Methods (*Section 2.2.7*). Immunofluorescent immunocytochemistry images of control cells (NC siRNA transfected cells) showed FCS, AngII and ET1 increased levels Ki67 expression above the baseline (**Figure 4.9 B, C and D**), indicating that transfection of RASM with NC siRNA produced similar results to those obtained in non-transfected cells (*Section 3.3.8*). In agreement with previous data (*Section 4.2.4*), where GRK2 knockdown considerably inhibited the effect of vasoconstrictor-induced [^{3}H]-thymidine

incorporation, depletion of GRK2 resulted in a significant reduction in ET1 induced Ki67 expression (**Figure 4.9 D**). Likewise, depletion of GRK6 resulted in a significant reduction (P<0.0001) in 10% FCS, AngII and ET1 induced Ki67 expression (**Figure 4.9 B, C and D**). However, GRK5 knockdown markedly increased Ki67 expression in 10% FCS, AngII and ET1 treated cells (**Figure 4.9 B, C and D**).

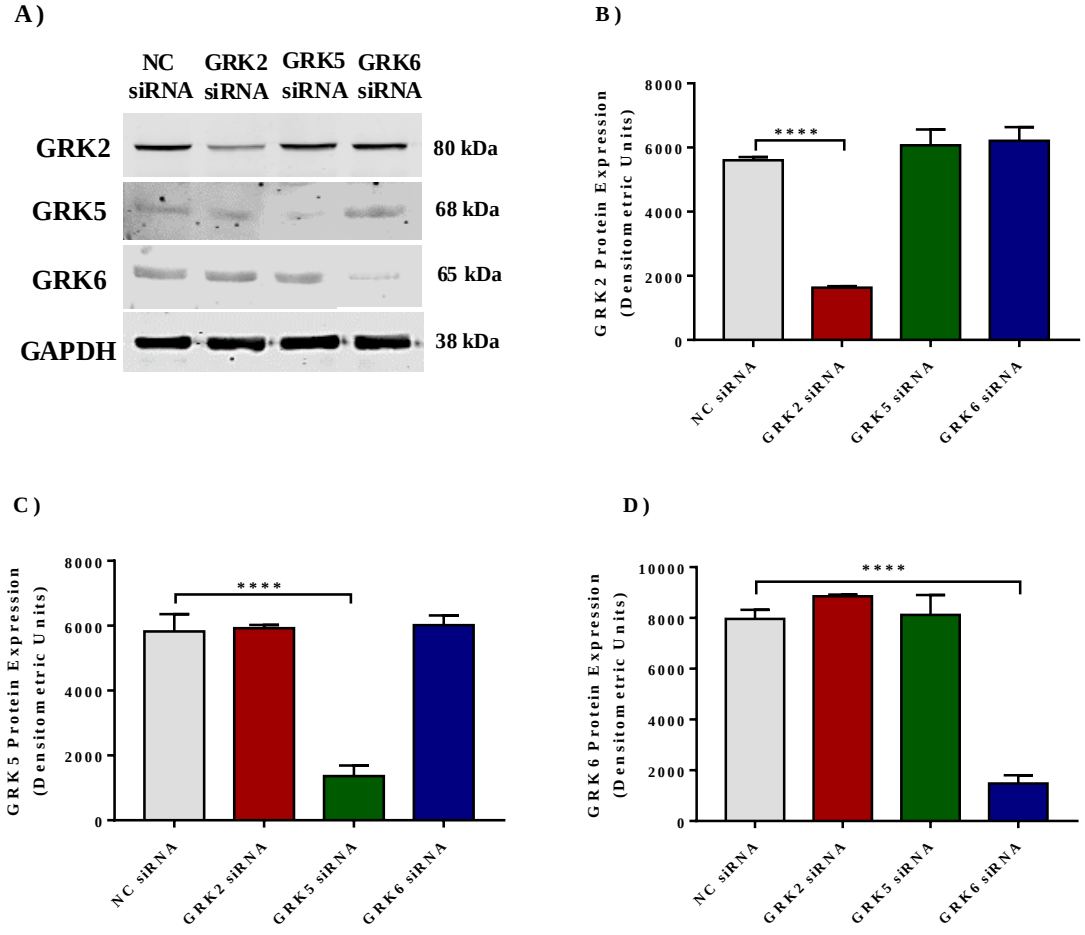
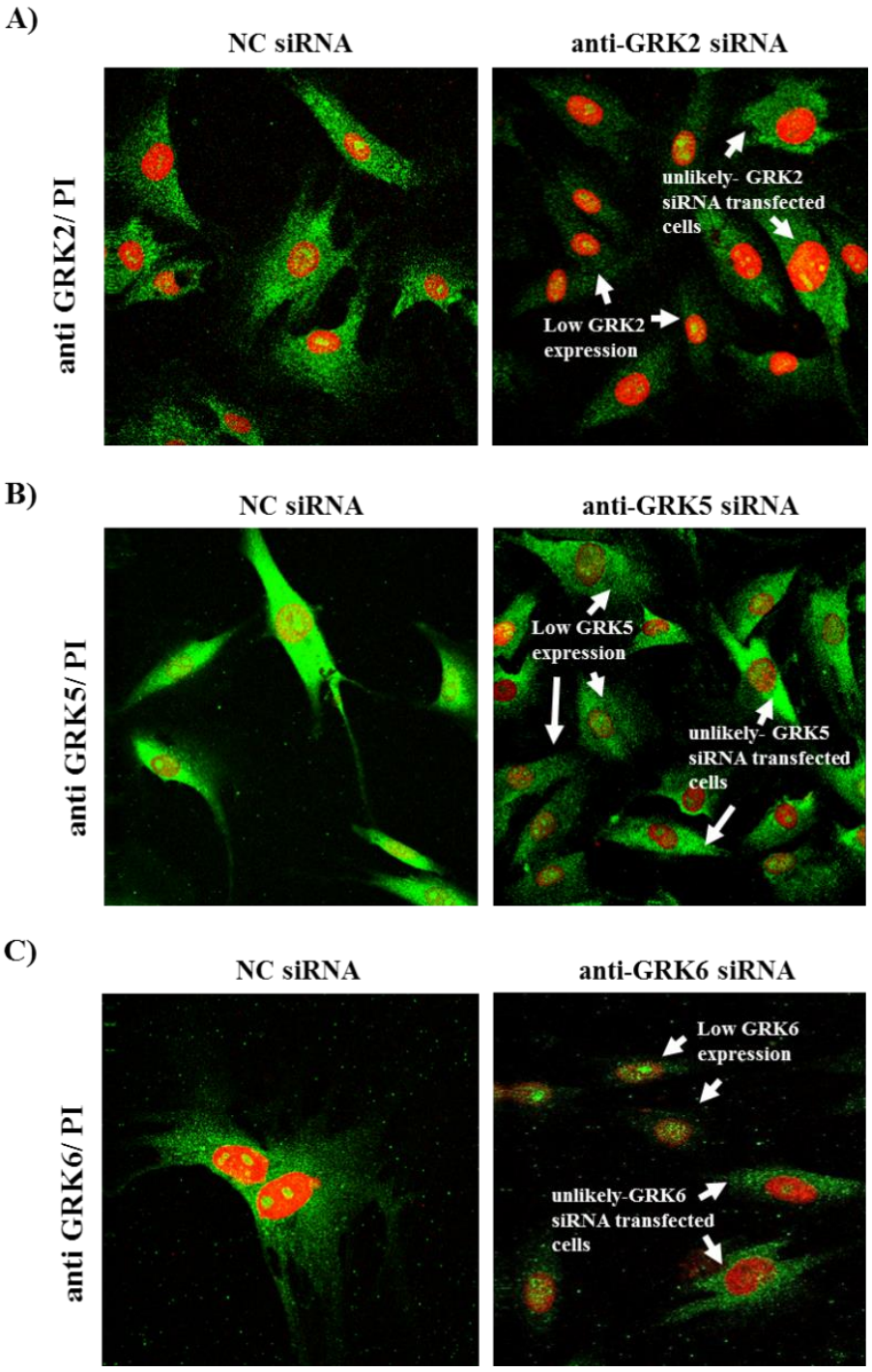


Figure 4.7 Specific GRK siRNAs markedly deplete endogenous GRK2, GRK5 and GRK6 in RASM cells.

Representative immunoblots showing relative GRK protein expression in RASM (A) treated with 10 nM of anti-GRK2 (lane 2), anti-GRK5 (lane 3), anti-GRK6 (lane 4) or NC (lane 1) siRNAs using the Lonza nucleofector system. Cumulative densitometric data showed a marked reduction in GRK2 (B), GRK6 (C) and GRK5 (D) protein expression compared to NC siRNA treated cells. Data show mean+SEM from four cell preparations. ****P<0.0001 *versus* NC siRNA transfected cells (one-way ANOVA, Dunnett's *post-hoc* test).


A)
NC siRNA
anti-GRK2 siRNA
anti GRK2/ PI
unlikely- GRK2 siRNA transfected cells
Low GRK2 expression
B)
NC siRNA
anti-GRK5 siRNA
anti GRK5/ PI
Low GRK5 expression
unlikely- GRK5 siRNA transfected cells
C)
NC siRNA
anti-GRK6 siRNA
anti GRK6/ PI
Low GRK6 expression
unlikely-GRK6 siRNA transfected cells

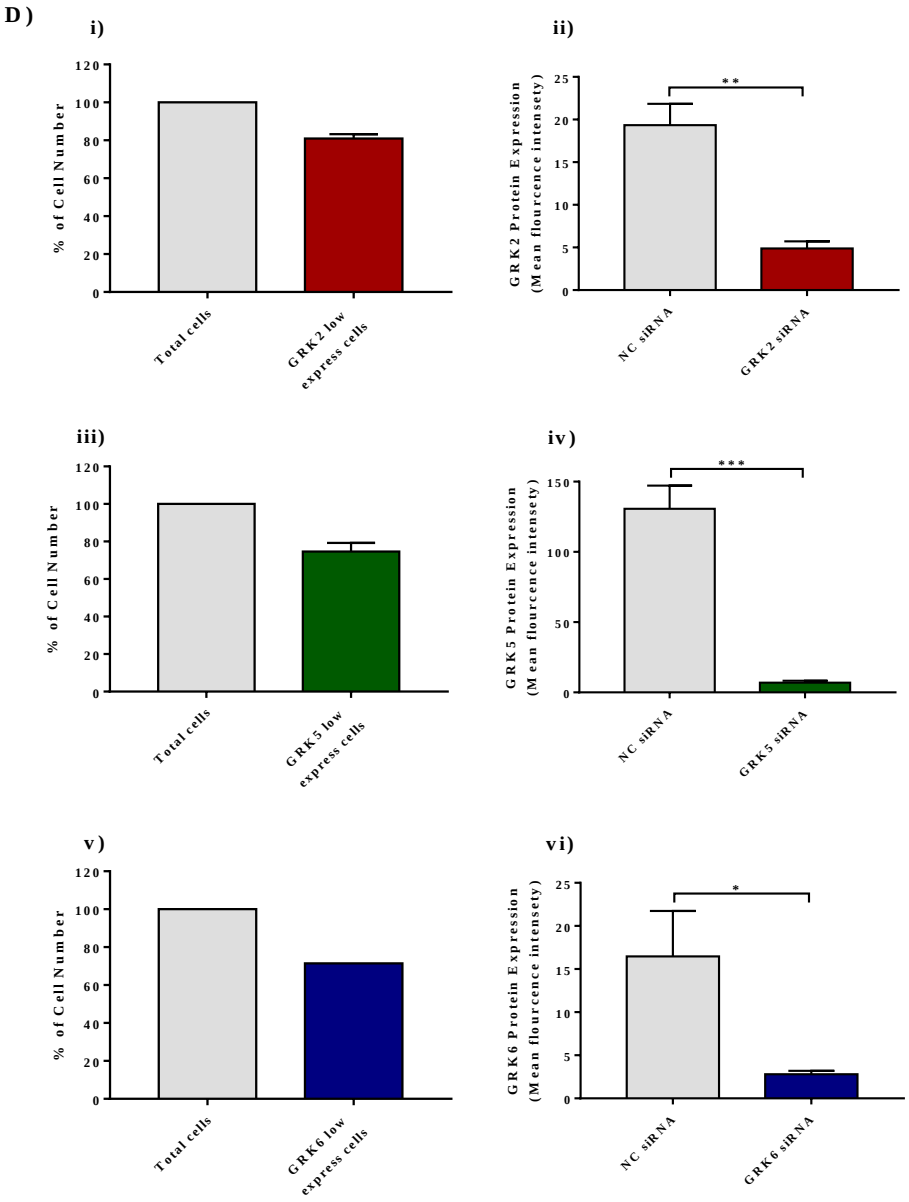
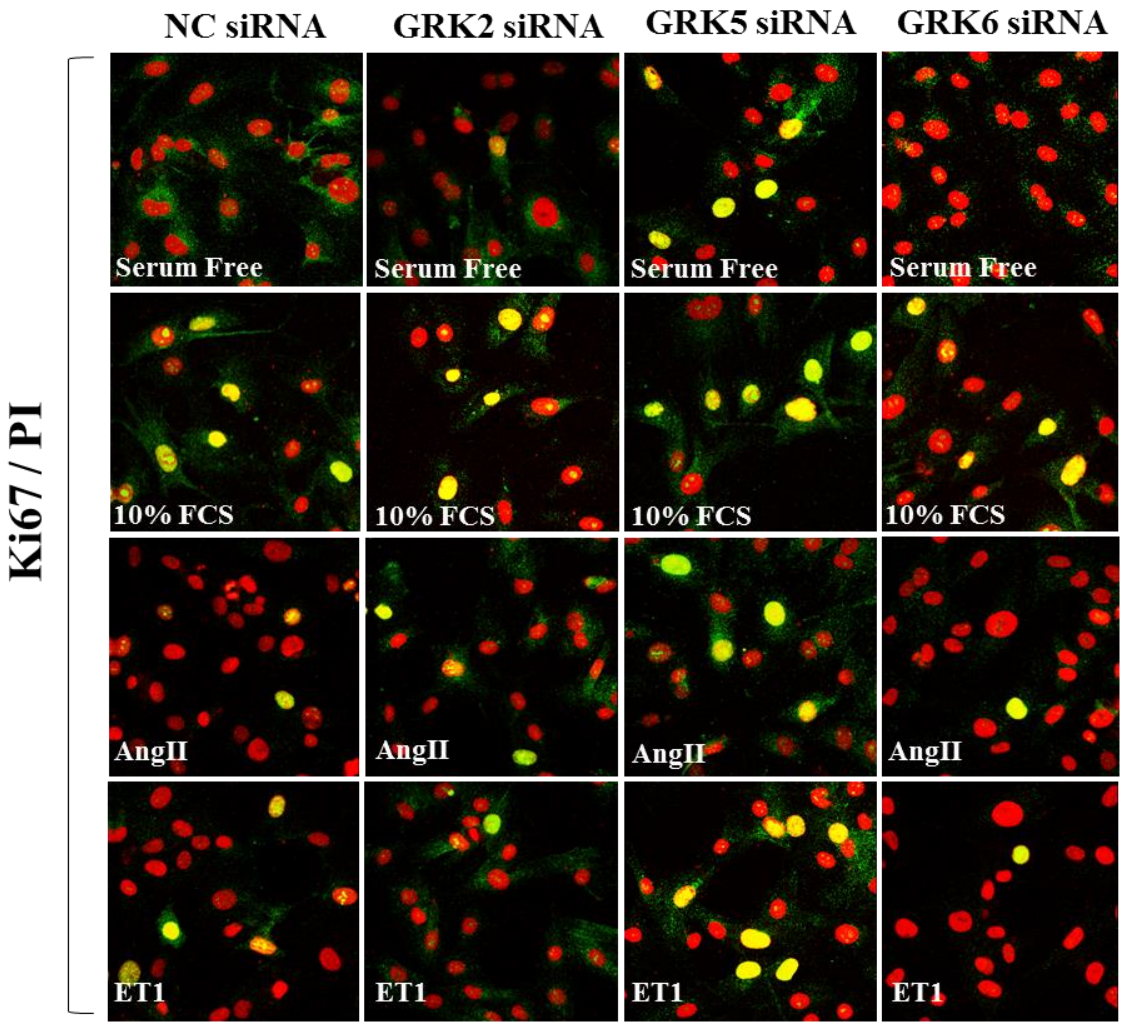


Figure 4.8 Effects of siRNA-mediated suppression of GRK levels in RASM cells.

Representative immunocytochemistry images showing relative GRK protein expression in RASM treated with 10 nM of anti-GRK2, anti-GRK5, anti-GRK6 or NC siRNAs using the Lonza nucleofector system. Representative immunocytochemistry images of GRK2 (A), GRK5 (B) and GRK6 (C) display a transfection efficiency of ~70-80% for each siRNA construct. Cumulative data showed a marked reduction in GRK2 (Di-ii), GRK5 (Diii-iv) and GRK6 (Dv-vi) protein expression compared to NC siRNA treated cells. Data show mean+SEM from four cells. *P<0.05; **P<0.01; ***P<0.001 *versus* NC siRNA transfected cells (unpaired t-test).

A)


NC siRNA
GRK2 siRNA
GRK5 siRNA
GRK6 siRNA
Ki67 / PI
Serum Free
10% FCS
AngII
ET1

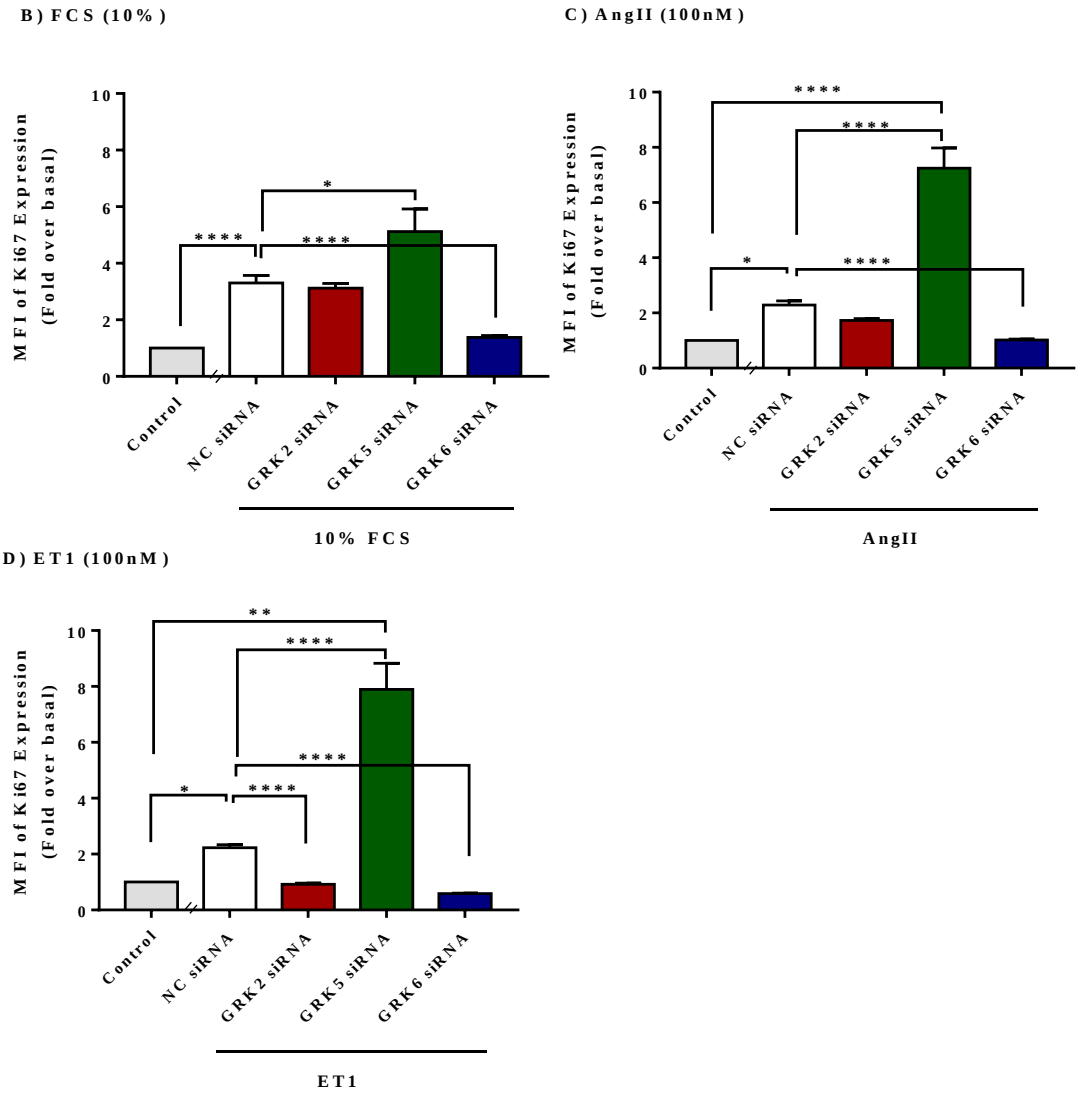


Figure 4.9 Depletion of endogenous GRK2, GRK5 and GRK6 affects on Ki67 expression.

RASM cells were nucleofected with (10 nM) anti-GRK2 siRNA, anti-GRK5 siRNA, anti-GRK6 or NC siRNAs. 24 h later, cells were serum-starved and after another 24 h, stimulated with 10%FCS, AngII (100 nM) or ET1 (100 nM). A) Representative immunofluorescence immunocytochemistry images for Ki67 expression in anti-GRK2 siRNA, anti-GRK5 siRNA, anti-GRK6 or NC siRNAs treated cells. The expression of the proliferative marker Ki67 was measured as fold over basal for mean of nuclear fluorescence intensity using Image J (N ≥ 500 cells). Cumulative data (means ± SEM) indicate that GRK2 depletion significantly decreased ET1 induced Ki67 expression in comparison to NC (D). Knockdown of GRK6 significantly decreased 10% FCS, AngII

and ET1 induced Ki67 expression (B, C and D), whereas GRK5 depletion significantly increased Ki67 expression in 10% FCS, AngII or ET1 treated cells (B, C and D). Statistically significant changes are shown as $^{*}P<0.05$, $^{****}P<0.0001$ (Kruskal-Wallis, Dunn's *post-hoc* test).

4.3.7. Effect of COMP101 on vasoconstrictor-induced PCNA expression

Previous findings (*Section 4.3.3 and 4.3.6*) indicated that the small molecule GRK2 inhibitor COMP101 inhibited vasoconstrictor-induced [^{3}H]-thymidine incorporation and Ki67 expression in RASM cells. Actually, further confirmation was carried out by testing GRK2 catalytic activity on the expression of PCNA proliferative marker. RASM cells were pre-incubated with COMP101 (30 μM) for 30 min prior to stimulation with 10% FCS, AngII (100 nM) or ET1 (100 nM) in serum-free DMEM contain 30 μM COMP101. Procedures were then completed as described in the Methods (*Section 2.2.7*). Total cell lysates were analysed by western blot with an anti-PCNA antibody, and an anti-GAPDH antibody as a loading control. Consistent with previous findings (*Section 3.3.9*), immunoblotting results illustrate that stimulation of RASM with 10% FCS, AngII or ET1 showed higher PCNA expression in comparison to control unstimulated cells (**Figure 4.10**). Furthermore, inclusion of COMP101 did not inhibit agonist-stimulated changes in PCNA expression (**Figure 4.10**).

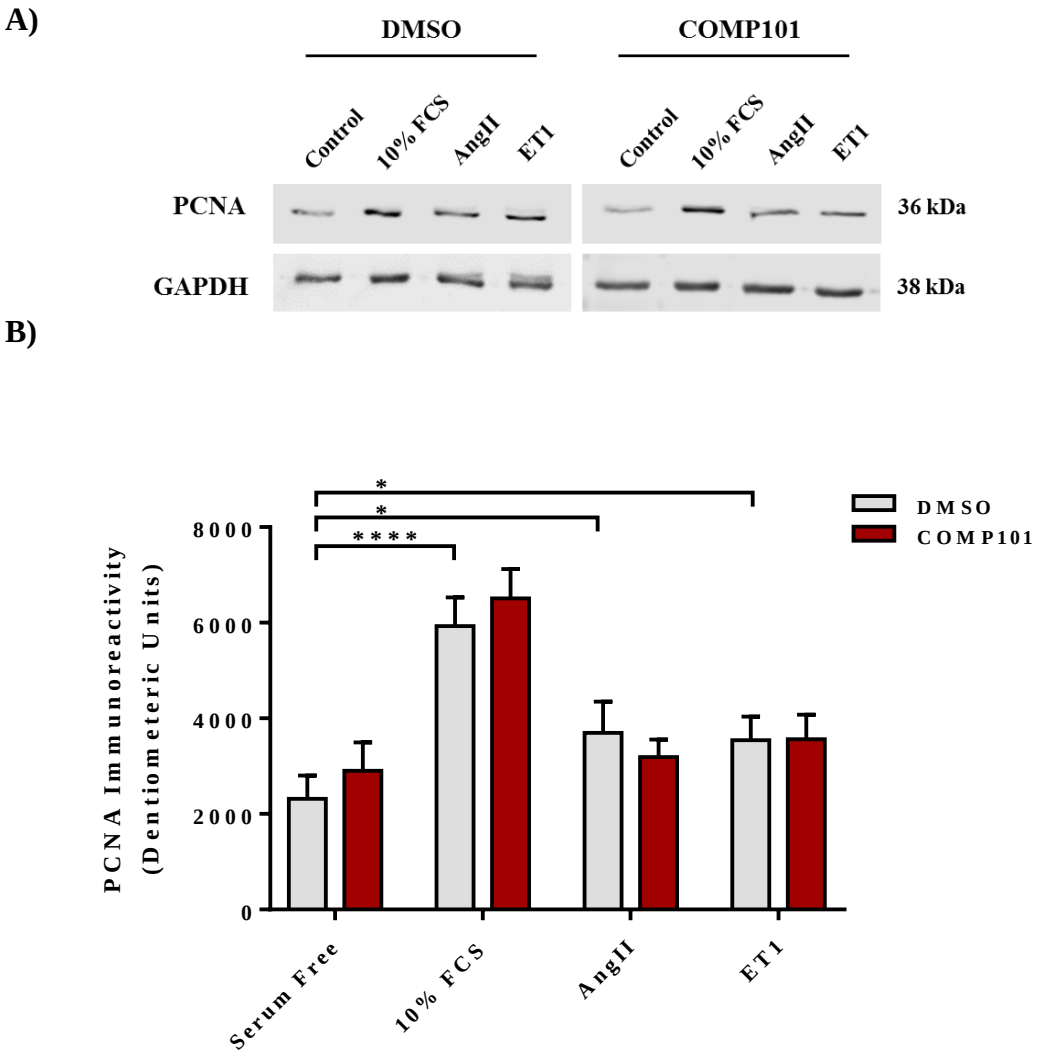


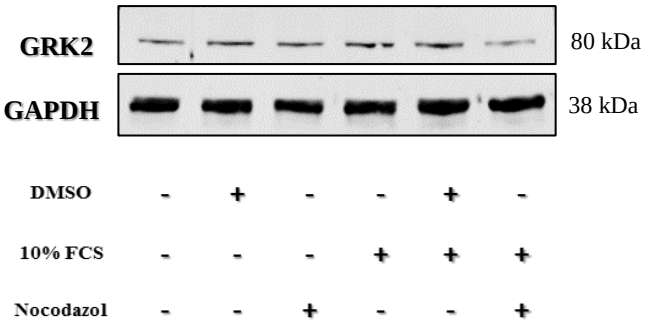
Figure 4.10 Effect of COMP101 on vasoconstrictor-induced PCNA expression.

After 24 h serum-starvation, cells were pre-incubated with COMP101 (30 µM) or vehicle control (DMSO) for 30 min, before stimulation with 10% FCS, AngII (100 nM) or ET1 (100 nM) for a further 24 h. Total cell lysates were analysed by western blot with anti-PCNA and anti-GAPDH antibodies. Stimulation of RASM with 10% FCS, AngII or ET1 resulted in a marked increase in PCNA expression. Inhibition of GRK2 kinase activity with COMP101 had no effect upon changes in PCNA level expression. Data are shown for 8 independent experiments, each using RASM cells prepared from a different animal. Bar graph is representative for mean ± SEM of densitometric analyses of PCNA band by Image J. Statistically significant changes for the control are shown as $^{*}P<0.05$, $^{****}P<0.0001$ (one-way ANOVA, Dunnett's *post-hoc* test).

4.3.8. GRK2 expression is downregulated during cell cycle progression

According to Penela et.al, (2010), a study which described the complex GRK2 interactions during the cell cycle progression, CDK2 can phosphorylate GRK2 at S670, resulting in a temporary suppression of GRK2 expression during the G_2 phase of the cell cycle. Indeed, it was postulated that this reduction of GRK2 expression is important for normal cycle progression (Penela et al., 2010a). To investigate whether a similar reduction in GRK2 protein expression during G_0/G_1 and G_2/M phases of the cell cycle is observed in RASM, GRK2 expression levels across the cell cycle were examined. In order to synchronize RASM cells at G_0/G_1, RASM cells were incubated in serum-free medium for 24 h. In addition, RASM cells were also incubated with the cell cycle inhibitor nocodazol (1 µM) for 24 h to arrest the cell cycle at the G_2/M phase. After 24 h, total cell lysates were blotted for GRK2 expression, and GRK2 expression was significantly reduced at the G_2/M phase and a non-significant reduction was seen at G_0/G_1 phase (**Figure 4.11**). To further investigate the relationship between GRK2 expression and cell cycle progression, the profile of GRK2 expression during the cell cycle was examined. Here RASM cell growth was synchronized by serum-starvation for 24 h, and cells were then released for senescence by stimulation with 10% FCS. Cells were lysed every 2 h after addition of serum and GRK2 expression determined by immunoblotting. As shown in **Figure 4.12**, little change in the expression of GRK2 was observed over the time course of the experiment, with the exception of a slight (non-significant) decrease in GRK2 protein expression 10-12 h after release from senescence. Also, a non-significant increase in GRK2 protein expression 16 h after release from senescence.

A)



B)

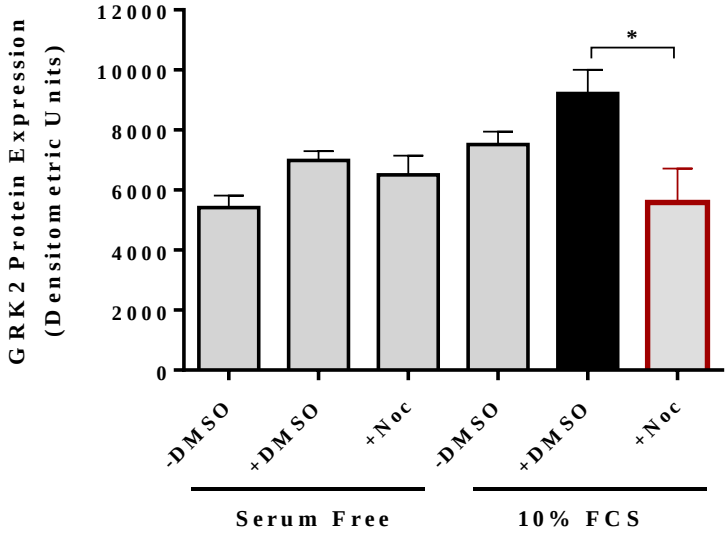
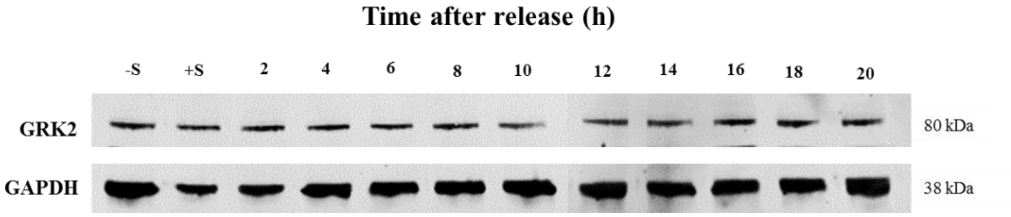


Figure 4.11 GRK2 protein expression is downregulated during G_2/M phase of the cell cycle.

RASM cells were synchronized at G_0/G_1 phase by serum-starvation and at G_2/M phase with nocodazol (1µM) for 24 h. Total lysates were analysed by western blot with anti GRK2 and anti GAPDH antibodies. A) Representative immunoblotting for RASM cells at G_0/G and at G_2/M phases. B) Cumulative data show that GRK2 protein expression significantly decreased at G_2/M phase. Data are shown for 6 independent experiments, each using RASM cells prepared from a different animal. Bar graph is representative of densitometric analyses of bands by Image J and normalized to loading control. Statistically significant changes compared to DMSO incubated cells shown as: $^{*}P<0.05$ (one-way ANOVA, Tukey's *post-hoc* test).

A)



B)

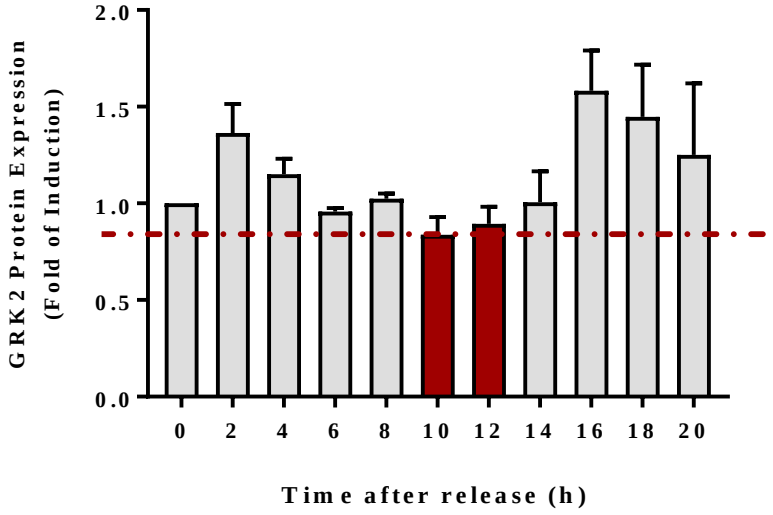


Figure 4.12 GRK2 protein expression level changes after releas of cells from serum-starvation.

Growth-arrested RASM cells were stimulated with 10% FCS and cells were then harvested at 2 h intervals. Total cell lysates were analysed by western blot with anti-GRK2 and anti-GAPDH antibodies. A) Representative immunoblots show GRK2 expression up to 20 h after cell cycle release. B) Cumulative data show as mean ± SEM for 3 independent experiments, each using RASM cells prepared from a different animal. Bar graph is representative of densitometric analyses of bands by Image J and normalized to loading control (Kruskal-Wallis, Dunn's *post-hoc* test).

Table 4.1 Summary of the data for the effect of GRKs on cell proliferation.

	[^{3}H]-thymidine incorporation				Ki67 expression					
	Paroxetine	**Fluoxetine**	**COMP101**	**GRK2 siRNA**	**Paroxetine**	**Fluoxetine**	**COMP101**	**GRK2 siRNA**	**GRK5 siRNA**	**GRK6 siRNA**
Basal	↔	↔	↔	↔	↔	↔	↔	↔	↑	↔
FCS	↓	↔	↔	↓	↓	↓	↓	↔	↑	↓
AngII	↓	↔	↔	↓	↔	↔	↓	↔	↑	↓
ET1	↓	↔	↓	↓	↓	↓	↓	↓	↑	↓
NA	↓	↔	↓	↓	N/A	N/A	N/A	N/A	N/A	N/A
PDGF	N/A	N/A	N/A	↔	N/A	N/A	N/A	N/A	N/A	N/A

Symbol: ↔ no change, ↓ significant decrease, ↑ significant increase and N/A not available

4.4. Discussion

Prior evidence suggests that GPCRs have a fundamental role in controlling cardiovascular disease (Premont and Gainetdinov, 2007; Brinks and Eckhart, 2010). Although GRK2 is a key GPCR regulator and terminator for G protein-dependent signalling, its role has expanded to include regulation of other G protein-independent signalling pathways (Reiter and Lefkowitz, 2006; Brinks and Eckhart, 2010; Evron et al., 2012). Indeed, mounting evidence highlights GRK2 as the sole negative regulator of α_{1D}-adrenoceptor (Mayor et al., 1998), ET_A (Morris et al., 2010), $P2Y_2$ (Morris et al., 2011) and possibly AT_1 (Olivares-Reyes et al., 2001) receptors, all of which are known to couple to $G_{\alpha q}$ and induce vasoconstriction (Harris et al., 2008; Hill-Eubanks et al., 2011; Lymperopoulos and Bathgate, 2013). Some previous evidence suggests that activation of $G_{\alpha q}$ signalling pathways increases GRK2 expression and activity in cardiovascular cells (Chuang et al., 1995; Ramos-Ruiz et al., 2000). Furthermore, it has been reported that GRK2 interacts with and phosphorylates many target proteins which are important in cell proliferation, in a G protein-independent manner (Reiter and Lefkowitz, 2006). For example, GRK2 can interact directly with PI3K (Naga Prasad et al., 2002a), ERK (Robinson and Pitcher, 2013) and Smad proteins (Ho et al., 2005b). Collectively these findings suggest that GRK2 may function not only as a negative regulator of GPCR signalling but has extended functions to regulate different downstream signalling pathways that mediate pathological processes in hypertensive vascular remodelling (Kamal et al., 2012). Together with elevated GRK2 levels and exaggerated GRK2 activity during hypertension (Gros Robert, 1997; Gros et al., 2000; Eckhart et al., 2002; Cohn et al., 2008; Cohn et al., 2009), we hypothesized that GRK2 would display a significant effect on vasoconstrictor-induced VSMC proliferation. Therefore, the current chapter investigated the effects of GRK2 expression and its catalytic activity on vasoconstrictor induced VSMC proliferation. Since, AngII, ET1 and NA are stimulators of cell proliferation via their respective $G_{\alpha q}$ coupled GPCRs (reviewed in *Section 3.4*), these agonists were utilised to stimulate proliferation and to determine whether GRK2 plays a role in vasoconstrictor-induced proliferation of VSMC.

Although little is known about the molecular mechanisms by which GRK2 may regulate VSMC proliferation, there is some evidence indicating that GRK2 plays a fundamental role in cell proliferation. A strong relationship between GRK2 and cell cycle

progression has been previously reported (Penela et al., 2010a; Wei et al., 2013). For example, in breast cancer cells GRK2 is involved in complex cellular processes in cell cycle progression, where cyclin-dependent kinase 2 (CDK2) mediates GRK2 phosphorylation, promoting its interaction with the Pin1. This interaction contributes to temporary GRK2 down-regulation at the G_2 phase, which is important for normal cell cycle progression (Penela et al., 2010a). Moreover, reduced GRK2 protein cellular expression increases cyclin A, cyclin B and cyclin E expression to produce cell cycle arrest (Wei et al., 2013). In this regard, it has been described that GRK2 expression is required for cell cycle checkpoints and it regulates the cell cycle in numerous ways (Jiang et al., 2009; Penela et al., 2010b). In the cardiovascular system, it has been reported that GRK2 activity is increased in idiopathic dilated cardiomyopathy patients (Ungerer et al., 1993). Likewise, in the spontaneously hypertensive heart failure rat model (SHHF) left ventricular hypertrophy is associated with elevated GRK2 expression and activity, which is believed to be a key factor in the progression of heart failure (Anderson et al., 1999). Moreover, it has been reported that GRK2 knockout results in embryonic lethality-hypoplasia of ventricular myocardium (Jaber et al., 1996). In addition, GRK2 overexpression in VSMC results in increased arterial blood pressure and cell hypertrophy (Eckhart et al., 2002). Collectively, these findings indicate that GRK2 participates in cell growth and proliferation in the cardiovascular system.

Building from the idea that GRK2 plays an essential role in cell proliferation, I investigated the effects of pharmacological inhibition of GRK2 by paroxetine or COMP101 on vasoconstrictor-induced VSMC proliferation. Both paroxetine and fluoxetine are SSRIs, and previous data indicate that paroxetine, but not fluoxetine, can bind directly to GRK2 to inhibit kinase activity (Thal et al., 2012). Here treatment of VSMC with paroxetine resulted in inhibition of vasoconstrictor-induced cell proliferation. Our results show that paroxetine (not fluoxetine) inhibits AngII, ET1, and NA induced [^{3}H]-thymidine incorporation. These findings were further confirmed by Ki67 data which show that paroxetine attenuates ET1-induced Ki67 expression. Numerous previous studies have investigated the effects of paroxetine on cell growth in different cell types, but there are no published data examining the effect of paroxetine on VSMC proliferation (Sari and Zhou, 2003; Chou et al., 2007; Qiu et al., 2007; Schuster et al., 2007). For example, Qiu et al. demonstrated that paroxetine reverses the corticosterone-induced suppressive effect on hippocampal cell proliferation (Qiu et al.,

2007). In contrast, Sari et al, reported that paroxetine inhibited 5-HT induced foetal heart cell proliferation (Sari and Zhou, 2003). Moreover, paroxetine-induced apoptosis in human MG63 osteosarcoma cells (Chou et al., 2007) and inhibited [^{3}H]-thymidine incorporation and induced apoptosis in B-, T-lymphoid cells and in myeloid cell lines (Schuster et al., 2007). Although these studies utilised paroxetine as a 5-HT uptake inhibitor, our findings revealed that the anti-proliferative effect of paroxetine on AngII, ET1 and NA induced VSMC proliferation is possibly mediated via GRK2 inhibition. This idea was further supported by GRK2 depletion data which showed the same findings. In agreement with our findings, Schumacher et al, (2015) reported that paroxetine-mediated GRK2 inhibition *in vivo* reduced ventricular remodelling in mouse via an SSRI-independent process, and this effect was specific to paroxetine in contrast to fluoxetine (Schumacher et al., 2015). Our suggestion for the anti-proliferative effects of paroxetine as a GRK2 inhibitor is supported by our $[Ca^{2+}]_i$ imaging data. Our results revealed that treatment of VSMCs with paroxetine inhibited UTP-induced $P2Y_2$ receptor desensitization, a process known to be regulated by GRK2 (Morris et al., 2011), whilst inclusion of fluoxetine had no effect. This also accords with our earlier observations, which confirmed that paroxetine functions as a selective GRK2 inhibitor for UTP-mediated $P2Y_2$ receptor desensitization in mesenteric arteries (Rainbow et al., 2018). Additionally, paroxetine inhibits GRK2-mediated β_2-adrenoceptor phosphorylation, arrestin recruitment and receptor desensitization (Guo et al., 2017).

Although strong evidence suggests that paroxetine inhibits GRK2 activity (Thal et al., 2011; Rainbow et al., 2018), it is important to consider that SSRIs are designed to inhibit serotonin transporter (SERT) activity, which will enhance serotonin (5-HT) bioavailability at the synaptic cleft (Leonard, 1995). Even though previous reports show that SERT is expressed in human coronary arterial smooth muscle cells (Baskar et al., 2015), there is no published evidence confirming SERT expression in aortic smooth muscle cells *in vitro*. Moreover, although 5-HT receptors (such as the 5-HT_{2A} receptor) are expressed in rat aorta (Watts et al., 2001), it is unlikely that such receptors would be activated *in vitro* because the lack of agonist (5-HT) in our experiments. Also, as 5-HT is a neurotransmitter released from presynaptic nerve terminals (Fink and Gothert, 2007), whether cultured smooth muscle cells are able to synthesise and release 5-HT *in vitro* is unknown. Therefore, the observed effects of SSRIs on VSMC may be unrelated to inhibition of 5-HT uptake and a subsequent increase in 5-HT bioavailability. Collectively,

these data strongly suggest that paroxetine prevents vasoconstrictor-stimulated VSMC growth by inhibiting GRK2 activity and not through blockade of 5-HT uptake.

To further confirm that inhibition of GRK2 attenuates VSMC proliferation, we used another putative GRK2 inhibitor, COMP101 to suppress GRK2 activity (Lowe et al., 2015). Similarly, treatment of the VSMCs with COMP101 resulted in inhibition of ET1 and NA induced [^{3}H]-thymidine incorporation. Furthermore, COMP101 attenuates AngII and ET1 induced Ki67 expression. These data indicate that pharmacologic inhibition of GRK2 by paroxetine or COMP101 attenuates vasoconstrictor-induced VSMC proliferation.

Although [^{3}H]-thymidine incorporation data did not show any effect for fluoxetine on cell proliferation, one unanticipated finding was that fluoxetine attenuates FCS and ET1 stimulated Ki67 expression. These results seem to be consistent with other research which found that long-term (chronic) fluoxetine treatment has a negative effect on Ki67 expression and decreases neurogenesis in the subventricular zone area in adult mouse brain (Ohira and Miyakawa, 2011). The reason for the anti-proliferative effects of fluoxetine is not clear but it may have something to do with direct inhibitory effects on cell cycle progression. Indeed, Stepulak et al. (2008) reported that fluoxetine reduces cell cycle regulating gene expression such as c-fos, c-jun, cyclin A, cyclin D1, p53 and p21 which resulted in a decrease in cell growth and proliferation of colon cancer and lung cancer cells (Stepulak et al., 2008). Moreover, inhibition of 5-HT transport by fluoxetine reduced human pulmonary arterial smooth muscle cell proliferation *in vitro*, implicating a SSRI-related mechanism in the regulation of growth (Eddahibi et al., 2006). Even though fluoxetine reduces FCS and ET1 promoted Ki67 expression, the observed effect seems to be un-related to GRK2 catalytic activity. This idea is supported by our [^{3}H]-thymidine incorporation finding, which revealed that fluoxetine had no effect on cell growth. Also, $[Ca^{2+}]_i$ imaging revealed that treatment of VSMC with fluoxetine did not affect UTP-induced $P2Y_2$ receptor desensitization, which is a GRK2-mediated mechanism (Morris et al., 2011; Rainbow et al., 2018). An explanation of these inconsistent findings for fluoxetine between the two different cell proliferation assessment methods is not clear. It might be that fluoxetine is not an appropriate control to be used in cell proliferation assays. In future investigations, it might be possible to use a different SSRI without any effect on cell cycle progression.

Further investigation to confirm the role of GRK2 in AngII, ET1 or NA induced cell growth have been undertaken in GRK2 depleted VSMCs cells. Consistent with our lab's previous findings (Nash et al., 2018), anti-GRK2 siRNA knockdown (~70-80%) targeted GRK2 expression without affecting other non-targeted GRKs expression. siRNA knockdown of GRK2 reduced VSMC proliferation and attenuated activation of Ki67. Therefore, the process appears to be GRK2-dependent. The present findings show that depletion of GRK2 reduced AngII, ET1 and NA induced [^{3}H]-thymidine incorporation. Consistent with the previous findings from our laboratory, which show ET1-stimulated VSMC proliferation is attenuated following genetic depletion of GRK2 (Willets et al., un-published). These results are further supported by research which reveals that AngII-induced increases in media thickness in GRK2$^{+/-}$ mice, indicating that partial deletion of GRK2 exerts a protective effect against AngII-related hypertensive vascular remodelling (Avendano et al., 2014). Another study demonstrated the effect of GRK2 expression on cardiac hypertrophy, showing that siRNA GRK2 knockdown is associated with a significant reduction in phenylephrine-induced hypertrophy in cardiac myoblasts (H9C2) cells (Sorriento et al., 2015). Moreover, inhibition of GRK2 activity decreases left ventricular hypertrophy in SHR (Sorriento et al., 2015). Likewise, depletion of GRK2 expression reduces the development of cardiac hypertrophy in GRK2 knockout mice (*in vivo*) (Schlegel et al., 2017). Furthermore, increased GRK2 expression in breast cancer patients correlates with increased Ki67 expression (Nogues et al., 2016). This idea is further supported by our previous laboratory findings which showed that GRK2 expression is up-regulated (doubled) in arterial smooth muscle cells of SHRs in comparison to normotensive (WKY) smooth muscle cells at the early onset of the hypertensive phenotype (Willets et al., 2015; and unpublished data). Elevated GRK2 expression in SHR is also associated with excessive MAPK/ERK signalling (Kim et al., 1997; Kubo et al., 2002; Roberts, 2012) and consequently enhanced cell proliferation (see *Chapter 5*). Collectively, these published data together with our findings, indicate a positive relationship between GRK2 expression/activity and cell proliferation.

On the contrary, it has been reported that GRK2 over-expression exerts a negative effect on [^{3}H]-thymidine incorporation and cell proliferation. Thus, over-expression of GRK2 attenuated agonist-induced proliferative signalling pathways in VSMC and reduced neointimal hyperplasia (Peppel et al., 2000; Peppel et al., 2002). A possible explanation for this might be due to the use of different species as the cells were isolated

from white rabbit aorta, and GRK2 was over-expressed by adenoviruses encoding GRK2. Moreover, smooth muscle cell proliferation was evoked with a combination of PDGF together with either ET1, thrombin agonist peptide or thromboxane A_2 resulting in activation of $G_{\alpha q}$ receptors and receptor tyrosine kinase at the same time. Even though ET_A receptors and PDGF receptors have been reported to be regulated by GRK2 (Wu et al., 2005; Morris et al., 2010), it seems that over-expression of GRK2 diminishes PDGF-related proliferative signalling pathways (powerful proliferative agonist) and thereby decreases cell proliferation.

Although my data show that depletion of GRK2 attenuates FCS-induced [^{3}H]-thymidine incorporation, the Ki67 data showed that siRNA GRK2 knockdown does not have an effect on FCS-induced Ki67 expression. This is consistent with our lab's previous findings that siRNA GRK2 knockdown does not affect FCS-induced [^{3}H]-thymidine incorporation in RASM cells (Willets et al., unpublished). Furthermore, inhibition of GRK2 catalytic activity by paroxetine attenuated FCS-stimulated [^{3}H]-thymidine incorporation. Moreover, both paroxetine and COMP101 attenuate Ki67 expression. It is clear from FCS data that both GRK2 expression and activity influence the FCS component. FCS comprises of a wide-verities of proteins content such as albumin, immunoglobulins, lipoproteins, growth factors, hormones and protease inhibitors (Pieper et al., 2003; Zheng et al., 2006; Issaq et al., 2007). Some components of FCS such as lysophosphatidic acid (LPA), can bind to and activate GPCRs (Riaz et al., 2016). Furthermore, FCS contains many growth factors that can activate tyrosine kinase receptors such as the transforming growth factor-β1 (TGF-β1) and the insulin-like growth factor (IGF) receptors (Hubbard and Miller, 2007). It has been reported that GRK2 regulates IGF receptor signalling (Zheng et al., 2012). Therefore, the observed effect of GRK2 depletion or inhibition on FCS-induced cell proliferation could be related to its effect on one or more of the receptor signalling pathways activated by components of FCS. Further investigation was undertaken to examine whether the inhibitory effects of GRK2 depletion were selective for GPCR-driven proliferation. Even though the PDGF receptor has been reported to be regulated by GRK2 (Wu et al., 2005), our results show that depletion of GRK2 level had no effect on PDGF-induced cell proliferation, which suggest that the growth promoting effects of GRK2 appear to be GPCR-specific and not a general effect.

It appears that GRK2 mediated proliferative effects are GPCR subtype specific i.e. AT_1, ET_A and α_{1D}-adrenoceptors. This could be explained by a process that is a receptor, G-protein, GRK2 and arrestins specific, i.e. the effect of GRK2 on cell proliferation is a result of specific barcoding. This barcode forms a complex interaction of active conformation (agonist-bound) receptor with GRK2, G-proteins as well as arrestins (Zhang et al., 2015). Further research should be undertaken to investigate whether GRK2 proliferative effects are specific to vasoconstrictor GPCR or not. Also, whether the observed GRK2-related proliferative effects are associated with a specific cell type (VSMC) or are a common feature facilitating cell growth and proliferation require further investigation. Taken together, the results suggest that GRK2 expression and kinase activity are required to mediate vasoconstrictor-stimulated VSMC proliferation. Thus, the increased expression of GRK2 observed in vessels from different models of hypertension (Gros et al., 2000; Eckhart et al., 2002) can be an underlying factor for increases in vascular wall thickness and therefore contributes to hypertensive vascular remodelling.

Another important question is what the effect of other GRKs which are expressed in smooth muscle cells on vasoconstrictor-stimulated cell proliferation? Prior studies have noted the GRK5 and GRK6 are express in cultured aortic smooth muscle cells (Ishizaka et al., 1997; Morris et al., 2010). Therefore, the effects of altering GRK5 and GRK6 expression on smooth muscle cell proliferation was examined using Ki67 expression as a marker of cell proliferation. In accordance with our lab's previous work (Nash et al., 2018), I have demonstrated that using anti-GRK5 and anti-GRK6 siRNA specifically knocked down (~70-80%) of each targeted GRK without affecting the expression of other non-targeted GRKs. Here selective depletion of GRK5 enhanced basal, FCS, AngII and ET1 induced Ki67 expression, which is consistent with previous laboratory findings which revealed that siRNA GRK5 knockdown increases FCS or agonist-induced [^{3}H]-thymidine incorporation in RASM cells (Willets et al., un-published). Collectively, these data indicate that GRK5 depletion has a general stimulatory effect on smooth muscle cell growth. This finding broadly supports the work of other studies linking GRK5 with cell cycle regulation and proliferation (Michal et al., 2012). GRK5 can phosphorylate various proteins that can interact with other proteins that regulate cell proliferation such as p53, IκBα, PDGF-β and HDAC5 (Wu et al., 2006; Martini et al., 2008; Chen et al., 2010; Islam et al., 2013). Indeed, in the carotid arterial wall, specific depletion of GRK5 in GRK5$^{-/-}$ mice enhances PDGF receptor

activation, NF-κB dependent gene expression and atherosclerosis development, indicating that GRK5 expression has an anti-atherogenic effect on the blood vessels (Wu et al., 2012). Furthermore, GRK5 can act as a class II HDAC kinase, targeting HDAC5 in cardiomyocyte nuclei, indicating that GRK5 plays an important role in pathological cardiac hypertrophy in a non-canonical GPCR-independent manner (Martini et al., 2008; Traynham et al., 2015). Additionally, our findings suggest that GRK5 has opposing effects to GRK2 on cell proliferation. In accordance with this idea, Metaye et al. (2002) indicate that GRK2 and GRK5 have an opposite effect on cancer cell growth regulation (Metaye et al., 2002). Interestingly, siRNA mediated knockdown of GRK6 markedly decreased FCS, AngII and ET1 induced Ki67 expression in VSMC, indicating that GRK6 depletion has a general inhibitory effect on VSMC proliferation. In contrast, no effect of GRK6 knockdown on [^{3}H]-thymidine incorporation was detected (Willets et al., un-published). The reason for these contradictory results is still unclear. However, in agreement with our Ki67 results, a positive correlation between GRK6 expression and Ki67 expression in hepatocellular carcinoma cells has been identified (Li, 2013). Similarly, increased GRK6 expression in papillary thyroid carcinoma cells directly stimulates cancer cell proliferation (Che et al., 2018). Whether the observed GRK5 and GRK6 effects are correlated to GPCR activation or a common feature on cell proliferation requires further investigation.

Numerous studies have illustrated the complex GRK2 interactions during cell cycle progression (Jiang et al., 2009; Penela et al., 2010a; Wei et al., 2013). GRK2 protein expression during G_0/G_1 and G_2/M phases of the cell cycle in VSMC were examined. VSMCs were synchronized at G_0/G_1 by serum-starvation for 24 h and cells were then released form cell cycle arrest by stimulation with 10% FCS. The GRK2 protein expression profile during the cell cycle showed a slight (non-significant) decrease in GRK2 protein expression 10-12 h after release from senescence. Cells were also synchronize at G_2/M phase by cell cycle inhibitor nocodazol, a G_2/M phase inhibitor. This induces cell cycle arrest at the G_2/M phase via its action as an inhibitor of microtubule polymerization (Blajeski et al., 2002). Our data revealed that GRK2 expression was reduced at the G_2/M phase of the cell cycle. These results seem to be consistent with other research which found GRK2 expression showed a temporary decrease during G_2 phase of the cell cycle (Penela et al., 2010a). At present, there are still many unanswered questions about the relationship between GRK2 expression and cell cycle progression.

Further research should be undertaken to investigate the effect of GRK2 protein expression and catalytic activity on each phase of cell cycle progression, cell-cycle checkpoints, cell cycle regulating genes and cyclin-dependent kinases (CDKs).

Overall, the data demonstrate that suppression of GRK2 expression or inhibition its catalytic activity effectively attenuates vasoconstrictor-mediated VSMC proliferation. Also, it appears that GRK2's ability to affect VSMC growth is GPCR specific. This indicates the potential connectivity of GRK2 with hypertension-induced changes in vascular wall thickness. Moreover, up-regulated GRK2 expression in VSMC in hypertension may drive the enhanced proliferation and increased wall thickening seen in vascular remodelling. Thus, pharmacological intervention of GRK2 will provides a novel concept in preventing pathological VSMC proliferation. Certainly, the picture is unclear regarding GRK2-related molecular mechanisms affecting proliferation of VSMC. As GRK2 levels are up-regulated in hypertension, it is important to identify GPCR-GRK2 related signalling cascades. In the chapters that follow, I present underlying mechanisms that may explain the role of GRK2 in vasoconstrictor stimulated proliferative signalling pathways.

Chapter Five

5. Do AngII and ET1 utilize MAPK/ERK signalling to mediate GRK2-dependent VSMC proliferation?

5.1. Introduction

Many research articles describe the possible molecular mechanisms by which vasoconstrictors can induce VSMC proliferation (Ljuca and Drevensek, 2010, Lee et al., 2006, Qin et al., 2004, Muthalif et al., 1996, Wen et al., 1996, Hirata et al., 1989, Komuro et al., 1988). The MAPK/ERK signalling pathway, which consists of a series of protein kinase cascades, is known to play important roles in the regulation of cellular proliferation (Zhang and Liu, 2002) and is one of the important intracellular signalling transduction cascades in VSMC growth and proliferation (Nelson et al., 1998, Bornfeldt et al., 1997). MAPK signalling functions as a regulator of gene expression in response to extracellular stimuli (Treisman, 1996) and previous findings report that AngII and ET1 promote VSMC proliferation via the MAPK/ERK signalling pathway (Huang et al., 2011, Zhao et al., 2005). AngII or ET1-stimulated Ras-Raf-MEK-ERK signalling occurs through many pathways including $G_{\alpha q}$ coupled receptor-induced PLC-mediated DAG-PKC and IP_3-Ca^{2+} activation or through tyrosine kinase 'EGFR' transactivation (Ohtsu et al., 2006, Schonwasser et al., 1998, Muthalif et al., 1996). All of these pathways subsequently phosphorylate p44/p42 MAPK (ERK1/2) at threonine 202 and tyrosine 204 (see *Section 1.3.3.1*) (Xu et al., 2001), to activate ERK1/2, which then translocates to the nucleus and acts as transcription factors mediating changes in gene expression that stimulate cell growth and proliferation (Zhang and Liu, 2002). Furthermore, enhanced ERK1/2 expression has been reported in hypertensive animal models (SHR) (Kim et al., 2005b, Kim et al., 1997) and ERK signalling is implicated in both vasoconstriction (Oeckler et al., 2003, Klemke et al., 1997) and proliferation of VSMC (Roberts, 2012, Yamanaka et al., 2001, Watts, 1998).

Increased GRK2 expression and altered functionality have been found in pathological conditions such as hypertension (Cohn et al., 2009, Cohn et al., 2008), where abnormal GRK2 expression or activity appears to contribute to disease progression by situation-specific molecular mechanisms. Together with multiple non-receptor substrates (Jimenez-Sainz et al., 2006, Schutzer et al., 2005, Naga Prasad et al., 2002, Shiina et al.,

2001), GRK2 can interact directly with ERK (Robinson and Pitcher, 2013). As hypertension is associated with elevated circulating levels of vasoconstrictors (Harris et al., 2008), persistent activation of the MAPK/ERK signalling pathways has been suggested to play a key role in hypertension-associated increased VSMC proliferation. Moreover, the data in Chapter 4 confirm that GRK2 expression and activity has a marked effect on vasoconstrictors-induced VSMC proliferation. Here, we hypothesize that GRK2 is involved in proliferator responses downstream of GPCRs stimulated by vasoconstrictors, suggesting that GRK2 has a role in regulating the mechanism of vasoconstrictor-stimulated MAPK/ERK signalling in VSMC. Although ERK signalling can be stimulated by AngII, ET1 and UTP (Morris et al., 2012), previous data in this thesis (*Chapter 3*) indicates that AngII and ET1 but not UTP are pro-proliferative agents in RASM, therefore I hypothesise that the ability and consequences of these agonists to activate ERK are different. Furthermore, despite the fact that GRK2 regulates AT_1, ET_A and $P2Y_2$ signalling, I hypothesise that GRK2 plays different roles in the regulation ERK signals produced by pro-proliferative and non-proliferative agonists. Therefore, the current chapter investigates the effects and the underlying mechanisms of GRK2 expression and activity on vasoconstrictors mediating MAPK/ERK activation and its links to VSMC proliferation.

5.2. Aim

To investigate the molecular mechanisms underlying GRK2 regulation of VSMC proliferation, specifically the possible relationship between GRK2 expression/catalytic activity and vasoconstrictor-stimulated ERK signalling.

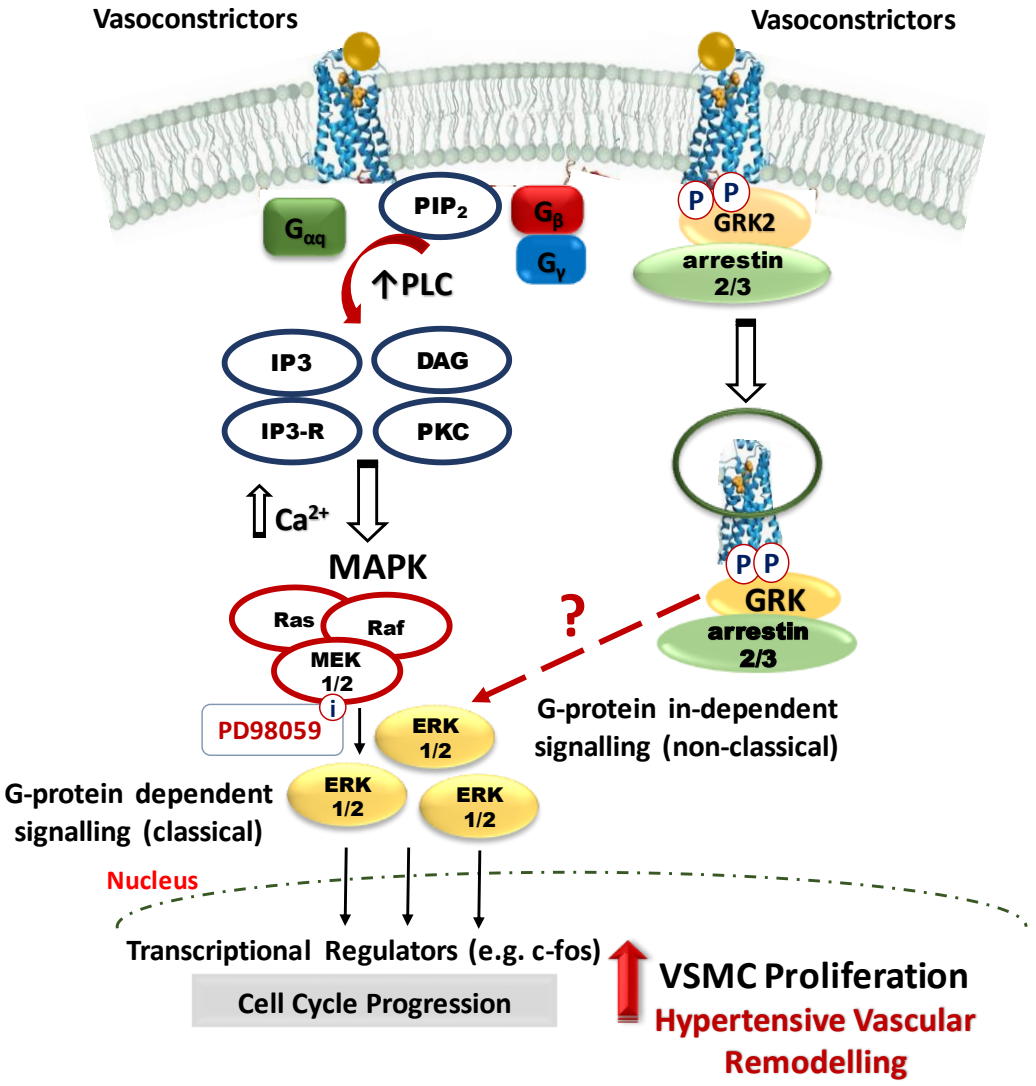


Figure 5.1 Schematic diagram showing the proposed G-protein dependent/independent pathways in the MAPK/ERK cascade activation by vasoconstrictors.

Activation of vasoconstrictor GPCRs results in generation of the second messengers DAG and IP_3, increases $[Ca^{2+}]_i$ and activates the Ras/Raf/MEK cascade, which regulates a variety of nuclear genes which stimulate cell cycle progression. Moreover, GRK2 can mediate direct activation of ERK1/2 (Robinson and Pitcher, 2013). PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptors; PKC, protein kinase C.

5.3. Results

5.3.1. Inhibition of ERK signalling attenuates vasoconstrictor-induced [^{3}H]-thymidine incorporation in RASM cells

MAPK signalling pathways, which consist of a series of protein kinase cascades, are known to play important roles in the regulation of cellular proliferation (Zhang and Liu, 2002). Many previously published studies show the importance of the ERK signalling pathway in AngII- and ET1-induced VSMC proliferation (Zhao et al., 2005; Ljuca and Drevensek, 2010). To examine whether the ERK pathway might be involved in the activation of VSMC proliferation induced by vasoconstrictors, PD98059, a potent and selective inhibitor of MEK1/2 [the kinase that phosphorylates ERK] which prevents ERK activation (Alessi et al., 1995), was used to study the effects of the ERK signalling pathway in vasoconstrictor-driven RASM cells proliferation using the [^{3}H]-thymidine incorporation assay. Briefly, serum starved RASM cells were pre-incubated with PD98059 (20 μM) for 30 min prior to stimulation with AngII (100 nM), ET1 (100 nM), NA (1 μM) or UTP (100 μM) in 10% FCS containing medium. Immunoblotting data show that treatment of RASM cells with the MEK1/2 inhibitor produced a marked reduction in ET1-induced ERK activation, confirming the effectiveness of PD98059 as a MEK1/2 inhibitor (**Figure 5.2 A**). Moreover, the prolife of vasoconstrictor-stimulated [^{3}H]-thymidine incorporation in control (DMSO) incubated cells was comparable to that detected previously (*Section 3.3.6, 4.3.2 and 4.3.3*). As expected, inclusion of AngII, ET1 or NA enhanced [^{3}H]-thymidine incorporation above that seen with 10% FCS alone (**Figure 5.2 B**). Inhibition of MEK1/2 attenuated 10% FCS, AngII, ET1 and NA induced [^{3}H]-thymidine incorporation in RASM cells (**Figure 5.2 B**). Moreover, the marked reduction of [^{3}H]-thymidine incorporation seen in UTP/FCS stimulated cells, is likely due to the inhibition of FCS stimulated proliferation (**Figure 5.2 B**). These data strongly suggest that the ERK signalling pathway is involved in vasoconstrictor-induced VSMC proliferation.

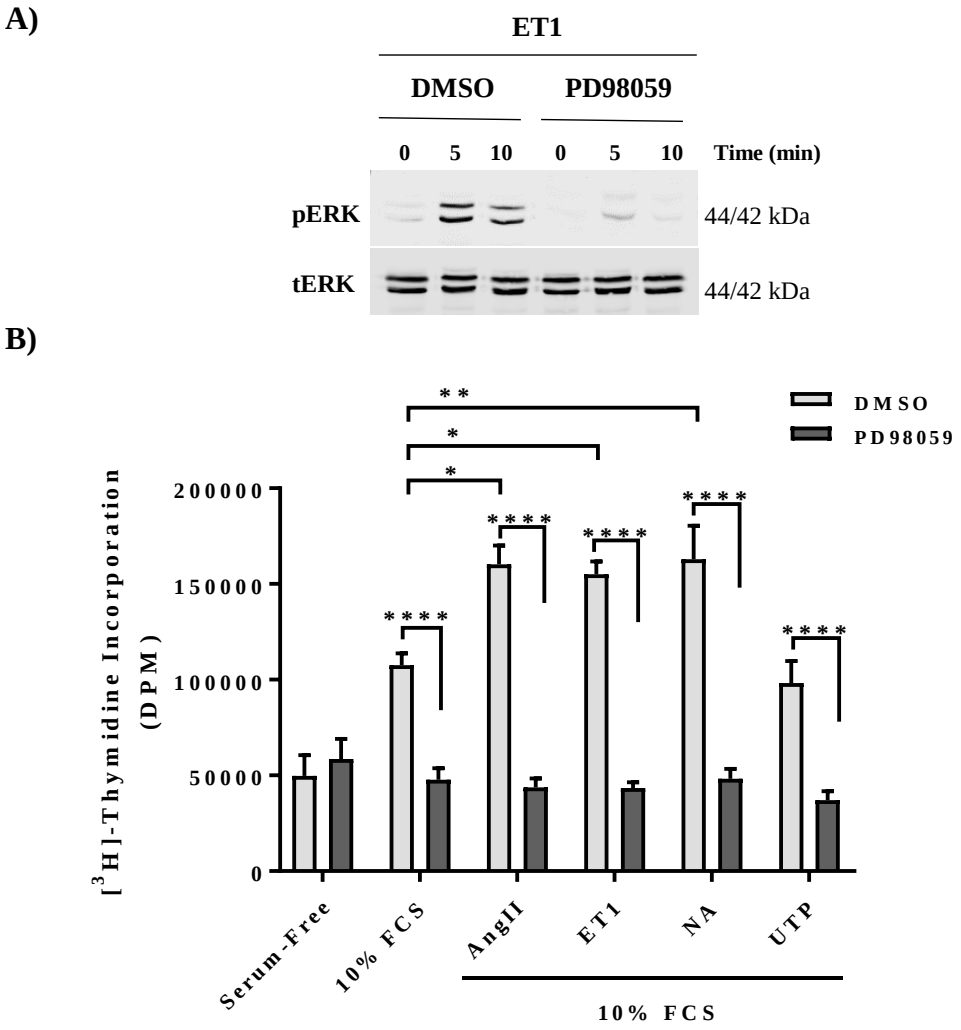


Figure 5.2 Inhibition of ERK signalling attenuates vasoconstrictor-induced VSMC proliferation.

A) Confluent serum-starved RASM cells were stimulated with ET1 (50 nM), with or without PD98059 (20 μM) pre-treatment (30 min), before being lysed and assayed for ERK phosphorylation as an index of ERK activation. The representative immunoblot shows that PD98059 pre-treatment markedly reduced ET1-stimulated ERK phosphorylation. B) Serum-starved RASM cells were pre-treated with vehicle control or PD98059 (20 μM) for 30 min, prior to stimulation with vasoconstrictors [AngII (100 nM), ET1 (100 nM), NA (1 μM) or UTP (100 μM) in 10% FCS]. PD98059 treatment inhibited 10% FCS, AngII, ET1, NA and UTP induced RASM cell proliferation. Data represent mean ± SEM for 4 replicates for each experimental condition from 4 different cell preparations. Statistically significant changes control cells *vs* PD98059 treated are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$ (two-way ANOVA, Sidak's *post-hoc* test).

5.3.2. GRK2 knockdown attenuates AngII- and ET1-induced ERK signalling activation

The [^{3}H]-thymidine incorporation results in the previous section (*Section 5.3.1*) revealed that ERK signalling is one important pathway involved in vasoconstrictor-stimulated VSMC proliferation. In addition GRK2 also plays a central role in vasoconstrictor-stimulated VSMC proliferation (*Section 4.3.4*), which raises the possibility that GRK2 may interact with the ERK signalling cascade to regulate its effects on growth. Therefore, the effects of GRK2 protein expression on vasoconstrictor-induced ERK signalling was investigated. Initially, the temporal-profiles of vasoconstrictor-stimulated ERK phosphorylation in GRK2 depleted RASM cells were examined. Briefly, GRK2 expression was suppressed using anti-GRK2 siRNA by nucleofection as described in Methods (*Section 2.2.4*). The efficiency of anti-GRK2 siRNA was previously confirmed (*Section 4.3.4 and 4.3.6*). After, 24 h transfection, cells were serum-starved for a further 24 h before being stimulated with AngII (100 nM), ET1 (50 nM) or UTP (100 µM) for up to 1 h. Total cell lysates were analysed by western blotting with an anti-pERK1/2 (Thr202/Tyr204) antibody and anti-tERK antibody as a loading control. Immunoblotting illustrated that stimulation of RASM with AngII, ET1 or UTP resulted in markedly increased ERK phosphorylation, which peaked at 5 min, followed by a gradual decline to a sustained level between 10 and 60 min. Quantitative analysis of the immunoblots revealed that depletion of GRK2 severely blunted the ERK temporal activation curve induced by AngII or ET1 in comparison to NC siRNA transfected cells (**Figure 5.3 A and B**). Conversely, knockdown of GRK2 enhanced UTP-stimulated ERK activation (**Figure 5.4**). These findings suggest that GRK2 expression is essential to facilitate AngII and ET1 induced ERK signalling in VSMC, whilst GRK2 negatively regulates UTP-mediated ERK signalling.

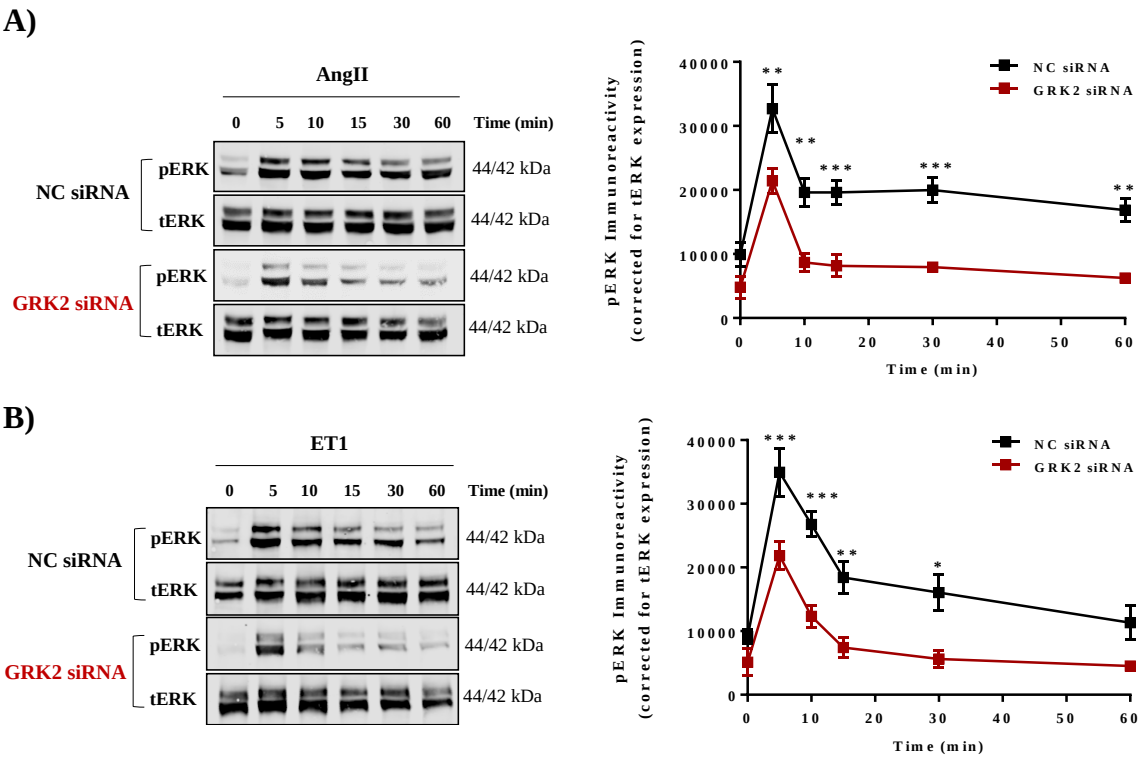


Figure 5.3 Depletion of GRK2 attenuates AngII- and ET1-induced ERK phosphorylation.

RASM cells were transfected with either NC or anti-GRK2 siRNA (10 nM). After 24 h cells were serum starved for a further 24 h before addition of AngII (100 nM) or ET1 (50 nM) for the indicated times. Cells were lysed and immunoblotted for pERK and tERK as described previously. Representative immunoblots and cumulative data show the effects of GRK2 knockdown on AngII (A) or ET1 (B) stimulated ERK phosphorylation. Line graphs (right panels) are densitometric analyses of pERK immunoreactivity by Image J and normalized to the loading control for 6 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in negative-control siRNA nucleofected cells are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (two-way ANOVA, Sidak's *post-hoc* test).

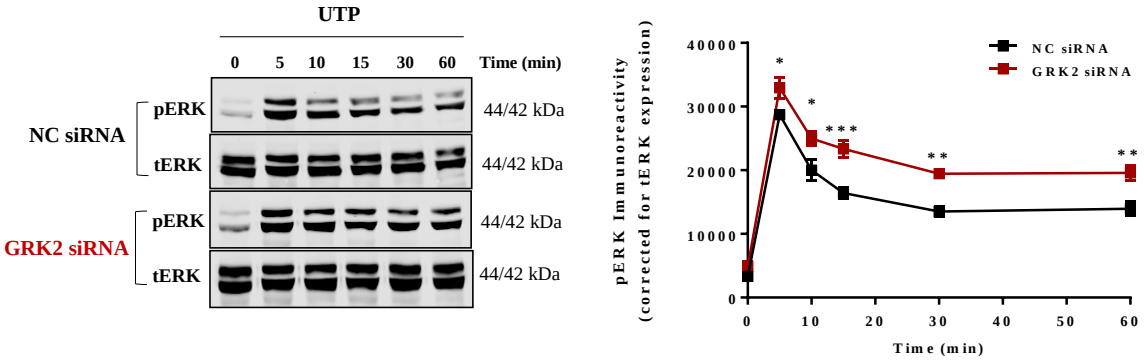


Figure 5.4 Depletion of GRK2 expression enhances UTP-induced ERK activation.

RASM cells were transfected with either NC or anti-GRK2 siRNA (10 nM). After 24 h cells were serum starved for a further 24 h before addition of UTP (100 µM) for the indicated times. Cells were lysed and immunoblotted for pERK and tERK as described previously. Representative immunoblots and cumulative data show the effects of GRK2 knockdown on UTP stimulated ERK phosphorylation. Line graphs (right panels) are densitometric analyses of pERK immunoreactivity by Image J and normalized to loading control for 4 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in control are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (two-way ANOVA, Sidak's *post-hoc* test).

5.3.3. COMP101 inhibits AngII- and ET1-induced MAPK/ERK signalling pathway activation

The data in *Section 4.3.3 and 4.3.5*, highlighted a requirement for GRK2 catalytic activity to enable vasoconstrictors-stimulated VSMC proliferation. Therefore, the effects of inhibiting GRK2 catalytic activity on vasoconstrictor-induced MAPK/ERK signalling was also investigated. In order to determine whether inhibiting GRK2 catalytic activity could prevent vasoconstrictor-driven MAPK/ERK signalling pathway, confluent overnight serum-starved RASM cells were pre-incubated for 30 min with COMP101 (30 μM) followed by stimulation at different time points (up to 60 min) with AngII (100 nM), ET1 (50 nM) or UTP (100 μM). Total cell lysates were analysed by western blot with anti-pERK antibody and anti-tERK antibody as a loading control. Immunoblotting results show that stimulation of RASM with AngII, ET1 or UTP result in a marked increase in ERK phosphorylation that peaked at 5 min followed by a gradual decline and then sustained pERK levels between 10 and 60 min. As shown in **Figure 5.5 A and B**, quantitative analysis of immunoblotting shows that inhibition of GRK2 catalytic activity results in a significant reduction in pERK activation curve induced by AngII or ET1 in comparison to DMSO incubated cells. On the other hand, UTP-induced ERK phosphorylation was enhanced and prolonged in the presence of COMP101 (**Figure 5.6**). This finding confirms that GRK2 catalytic activity is key to facilitating AngII and ET1-induced ERK signalling, yet negatively regulates UTP-stimulated ERK signalling in VSMC.

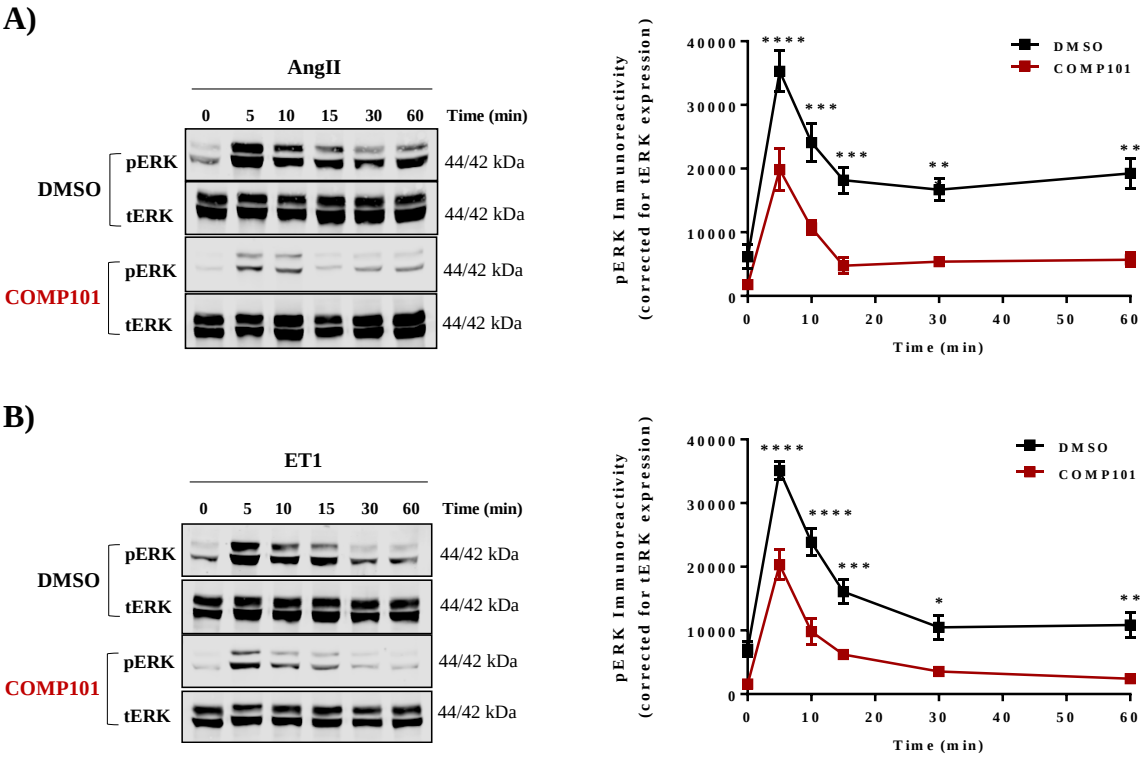


Figure 5.5 Inhibition of GRK2 attenuates AngII- and ET1-induced ERK phosphorylation.

Confluent, serum-starved RASM cells were pre-incubated with vehicle control or the GRK2 inhibitor COMP101 (30 µM) for 30 min before stimulation with AngII (100 nM) or ET1 (50 nM) for the indicated times. After agonist challenge cells were lysed and western blotted with anti-pERK and anti-tERK antibodies. Representative immunoblots (left panel) and cumulative data (right panels) show the effects of COMP101 on AngII (A) and ET1 (B) stimulated ERK phosphorylation. Cumulative data are representative of densitometric analyses of pERK immunoreactivity using Image J and normalized to loading control for 6 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM. Statistically significant changes compared to respective agonist-stimulated values in vehicle control (DMSO incubated cells) are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ (two-way ANOVA, Sidak's *post-hoc* test).

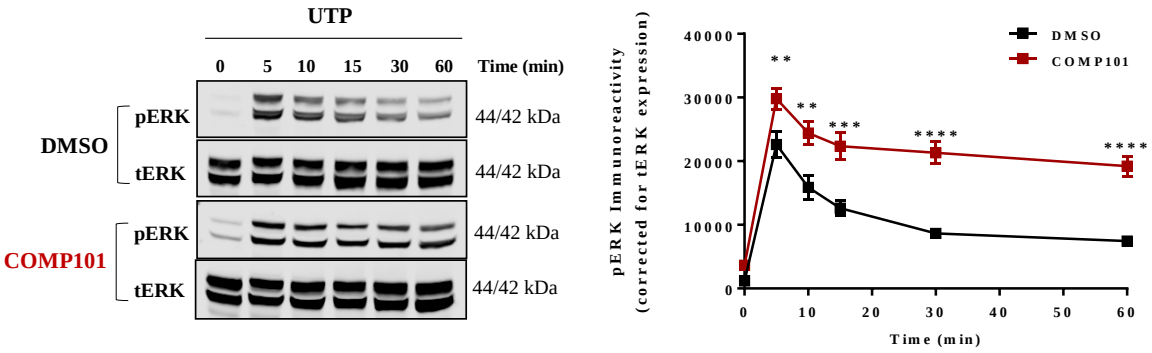


Figure 5.6 Inhibition of GRK2 catalytic activity enhances UTP-induced ERK activation.

Confluent, serum-starved RASM cells were pre-incubated with vehicle control or the GRK2 inhibitor COMP101 (30 μM) for 30 min before stimulation with UTP (100 μM) for the indicated times. After agonist challenge, cells were lysed and western blotted with anti-pERK and anti-tERK antibodies. Representative immunoblots (left panel) and cumulative data (right panels) show the effects of COMP101 on UTP-stimulated ERK phosphorylation. Cumulative data (mean ± SEM) are representative of densitometric analyses of pERK immunoreactivity using Image J and normalized to loading controls for 4 independent experiments, each using RASM cells prepared from a different animal. Statistically significant changes compared to respective agonist-stimulated values in control (DMSO incubated cells) are shown as; $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ (two-way ANOVA, Sidak's *post-hoc* test).

5.3.4. GRK2 over-expression enhanced AngII-induced ERK phosphorylation

Previous findings (*Sections 5.3.2 and 5.3.3*) showed that depletion of GRK2 expression or inhibiting its catalytic activity markedly inhibited AngII and ET1-induced ERK activation, suggesting a strong relationship between GRK2 and MAPK/ERK activation in RASM cells. To confirm and extend our findings, GRK2 was over-expressed in RASM cells using a previously characterised GFP tagged GRK2 construct (GFP-GRK2) via the nucleofection technique described in Methods (*Section 2.2.4*). To determine the effectiveness of the GFP-GRK2 construct in the up-regulation of GRK2 protein levels, RASM cells were lysed 96 h after nucleofection and probed for GRK2 expression using a selective anti-GRK2 antibody. Immunoblotting data revealed that transfection of RASM cells with GFP-GRK2 construct caused ≥70% over-expression of the targeted kinase in comparison to GFP-control transfected cells (**Figure 5.7 A**). To further confirm transfection efficiency, GFP-GRK2 transfected cells were visualised using confocal microscopy, which showed that GFP-GRK2 was expressed in ~60% of transfected cells (**Figure 5.7 B**). In an attempt to increase the transfection efficiency, cells were infected with an adenoviral GRK2 construct. The GRK2 adenovirus was previously prepared in our laboratory using the AdEasy kit. To assess the optimal infection conditions, RASM cells were infected with different amounts (2.5, 5 and 10µl) of GRK2 adenovirus stock. 48 h post infection, RASM cells were lysed and GRK2 expression was determined. Immunoblotting data revealed that transfection of RASM cells with 5 or 10 µl GRK2-adenovirus caused ~60% GRK2 over-expression in comparison to control-virus transfected cells (**Figure 5.8 A and B**). To estimate the number of cells expressing the adenoviral GRK2 construct, infected RASM were fixed and processed for immunocytochemical detection of GRK (as described in Methods). Comparison of control virus and GRK2 adenoviral infected cells showed high GRK2 expression in only ~50% (**Figure 5.8 B**). The degree of GRK2 overexpression was also quantitated using western blotting, which indicated about a three-fold higher level of GRK2 expression in cells infected with adenoviral GRK2 (**Figure 5.8 C**).

Next, the effects of GRK2 over-expression on AngII-induced ERK activation were investigated. Overnight serum-starved, GFP-GRK2 transfected or GRK2-adenovirus infected RASM cells were stimulated with AngII (100 nM) at different time points up to 60 min. Total cell lysates were analysed by western blot with anti-pERK antibody and anti-tERK antibody as a loading control. In agreement with previous data,

immunoblotting showed that stimulation of control RASM cells with AngII resulted in a marked increase in ERK phosphorylation that peaked at 5 min followed by a gradual decline and then sustained pERK protein between 10 and 60 min. Quantitative analysis of immunoblotting shows that ERK activation was enhanced in both GFP-GRK2 or GRK2-adenovirus over-expressed RASM cells (**Figure 5.9 A and B**). Although GRK2 over-expression was not evident in 100% of cells, these results indicate that GRK2 over-expression considerably enhances AngII-stimulated ERK signalling.

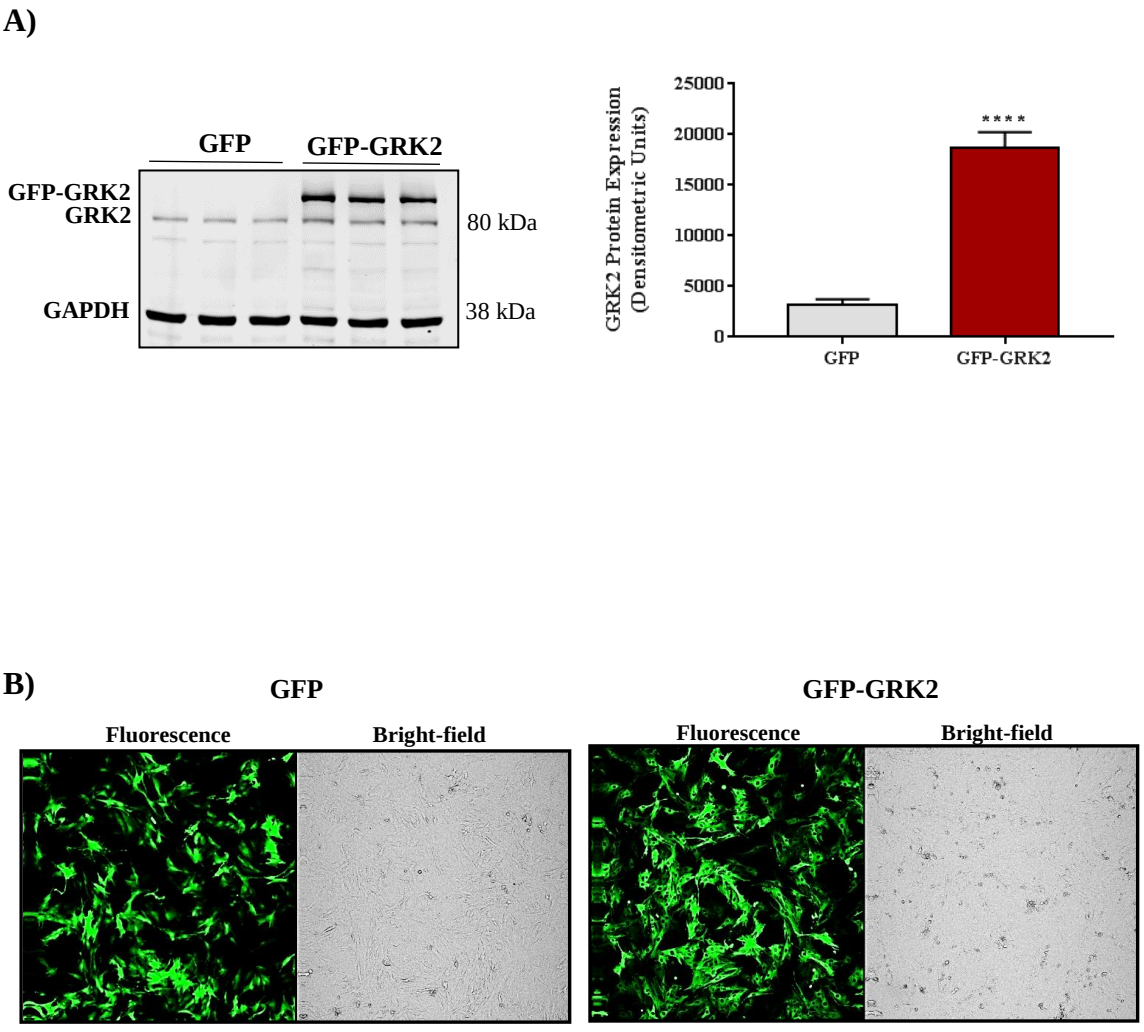


Figure 5.7 GFP-GRK2 mediated overexpression of GRK2 level in RASM cells.

(A) Representative immunoblots (left panel) showing relative GRK2 expression in RASM cells transfected with 2µg of GFP-GRK2 using Lonza nucleofection. Cumulative densitometric data (mean ± SEM from 4 different cells preperations) shows significantly increased GRK2 expression compared to GFP transfected cells (right panel), $^{****}P<0.0001$ GFP *vs* GFP-GRK2 transfected cells (unpaired t-test). (B) Confocal microscopy images of GFP (left panel) and GFP-GRK2 (right panel) transfected RASM cells 72 h post-transfection. Images were taken using an Olympus FV500 laser scanning confocal IX70 inverted microscope (objective x10).

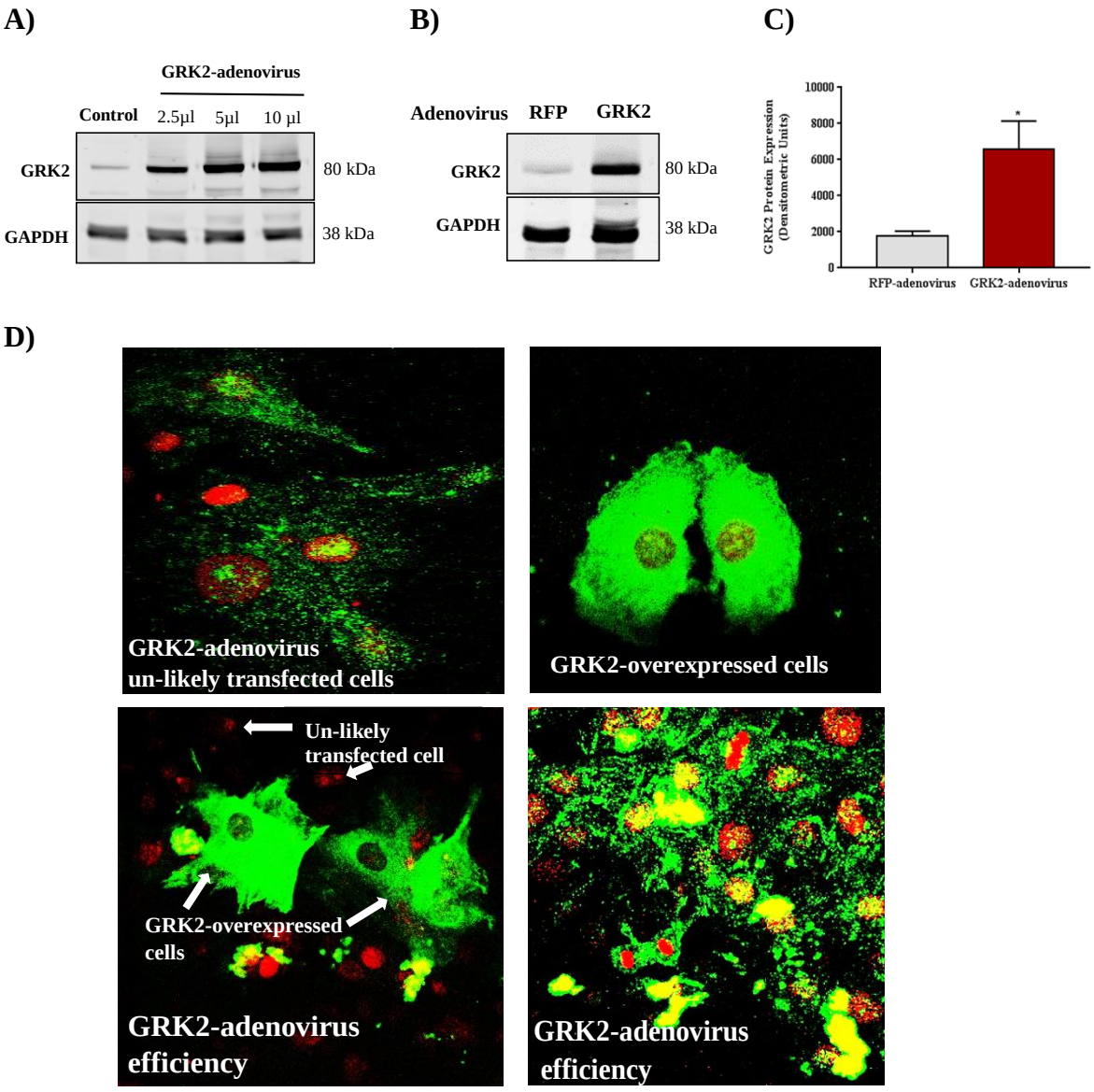


Figure 5.8 Assessment of GRK2 overexpression after infection of RASM cells with adenoviral GRK2.

Representative immunoblots (A and B) showing relative GRK expression in RASM cells treated with GRK2-adenovirus 48 h post-transfection. Cumulative densitometric data showed a large increase in GRK2 protein expression compared to control (C). (D) Representative immunocytochemistry images showing relative GRK protein expression in RASM treated with adenoviral GRK2, representative immunocytochemistry images for GRK2-overexpressed and un-likely-transfected cells (D; upper panels). Representative immunocytochemistry image (D; lower panels) display transfection efficiency (~50%) of the cells. Images were taken using an Olympus FV500 laser scanning confocal IX70

inverted microscope (oil immersion objective x60). Data show mean ± SEM from four different cells preparations. $^{*}P<0.05$ *vs* control cells (un-paired t-test).

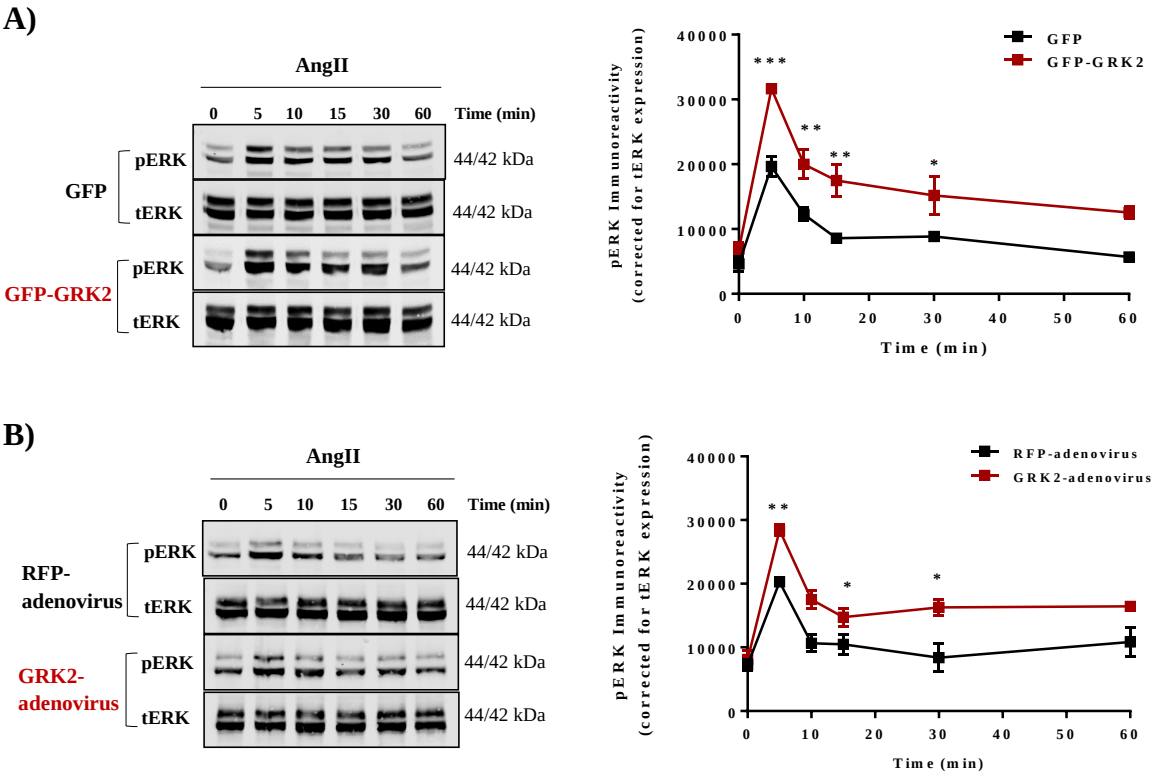


Figure 5.9 GRK2 over-expression enhances AngII-induced ERK phosphorylation.

Serum-starved cells transfected with GFP-GRK2 (A) or infected with GRK2-adenovirus (B) were stimulated with AngII (100 nM) for up to 60 min. Total lysates were analysed by western blot with anti-pERK and anti-tERK antibodies. Representative immunoblots (left panel) show the temporal prolife of AngII-stimulated ERK phosphorylation. Over-expression of GRK2 enhanced and prolonged pERK signalling induced by AngII. Line graphs (right panel) are representative of densitometric analyses of pERK band by Image J and normalized to loading control for 4 independent experiments, each using RASM cells prepared from different animals. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in control cells are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (two-way ANOVA, Sidak's *post-hoc* test).

5.3.5. Arrestin2 is essential for AngII-stimulated ERK signalling

Previous data (*Section 5.3.2, 5.3.4 and 5.3.3*) revealed that GRK2 protein expression and its catalytic activity are involved in AngII and ET1-stimulated ERK pathway activation. In addition, Morris et al. (2011), investigated the effects of arrestin2 and arrestin3 on ET1 and UTP induced ERK activation, highlighting a differential arrestin-dependent regulation of ET_A and $P2Y_2$ receptor-stimulated ERK signalling (Morris et al., 2012). However, the effect of arrestin2 and arrestin3 on AngII/AT_1 receptor stimulated ERK activation in VSMC was not investigated. To determine whether the presence of arrestin2 and arrestin3 are important in AngII mediating ERK activation, arrestin2 and arrestin3 expression were depleted using siRNA techniques, and the effects on AngII-driven ERK activation were determined using immunoblotting. Briefly, arrestin2 and arrestin3 expression were selectively depleted in RASM cells using nucleofection with a previously characterised anti-arrestin2 and anti-arrestin3 siRNA (Morris et al., 2012), as described in Methods (*Section 2.2.4*). Immunoblotting data show that treatment of RASM cells with anti-arrestin2 and anti-arrestin3 siRNA produced a marked reduction ~70% in arrestin2 and arrestin3 expression post-transfection, confirming the effectiveness of the anti-arrestin2 and anti-arrestin3 siRNAs (**Figure 5.10 B and C**). For further experiments, cell were left forty-eight hours post-nucleofection, prior to serum-starvation for a further 24 h and AngII stimulation. Total cell lysates were analysed by western blot with anti-pERK antibody and anti-tERK antibody as a loading control. AngII-stimulation of NC transfected RASM cells produced the expected temporal profile of ERK phosphorylation, with a peak 5 min followed by a plateau phase between 10 and 60 min (**Figure 5.11**). Depletion of arrestin2 expression attenuated both the peak and plateau phases of AngII-induced pERK signalling, whilst arrestin3 knockdown had no effect (**Figure 5.11 A-C**). These finding indicating that arrestin2 is essential for AngII-stimulated ERK signalling.

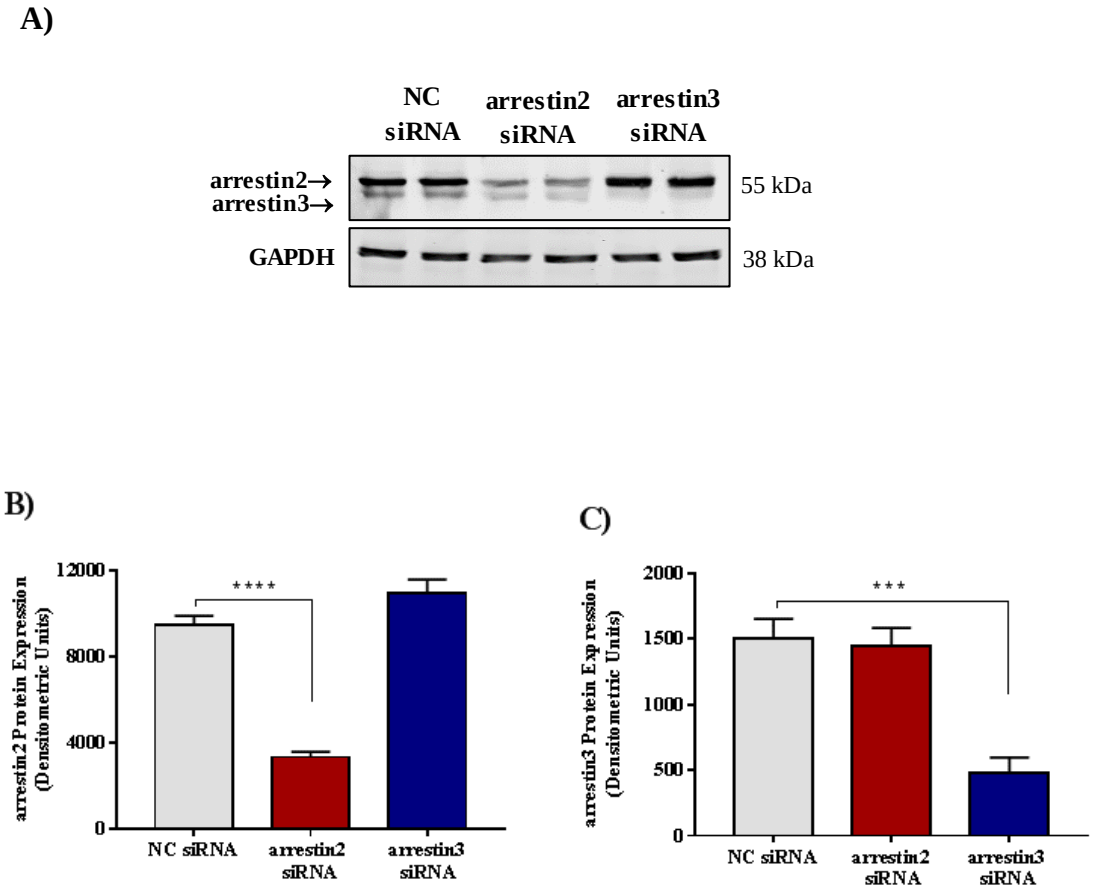


Figure 5.10 siRNA mediated suppression of arrestin2 and arrestin3 expression.

RASM cells were transfected with NC, anti-arrestin2 or anti-arrestin3 siRNAs (10 nM). After 72 h cells were lysed and arrestin expression determined by western blotting. A) Representative immunoblots show changes in arrestin2 and arrestin3 expression following siRNA treatment. Cumulative data (means ± SEM) are shown for the densitometric analyses of arrestin2 (B) and arrestin3 (C) bands using Image J for 4 independent experiments, each using RASM cells prepared from a different animal. Statistically significant changes *vs* NC siRNA nucleofected cells are shown as; $^{***}P<0.001$, $^{****}P<0.0001$ (one-way ANOVA, Dunnett's *post-hoc* test).

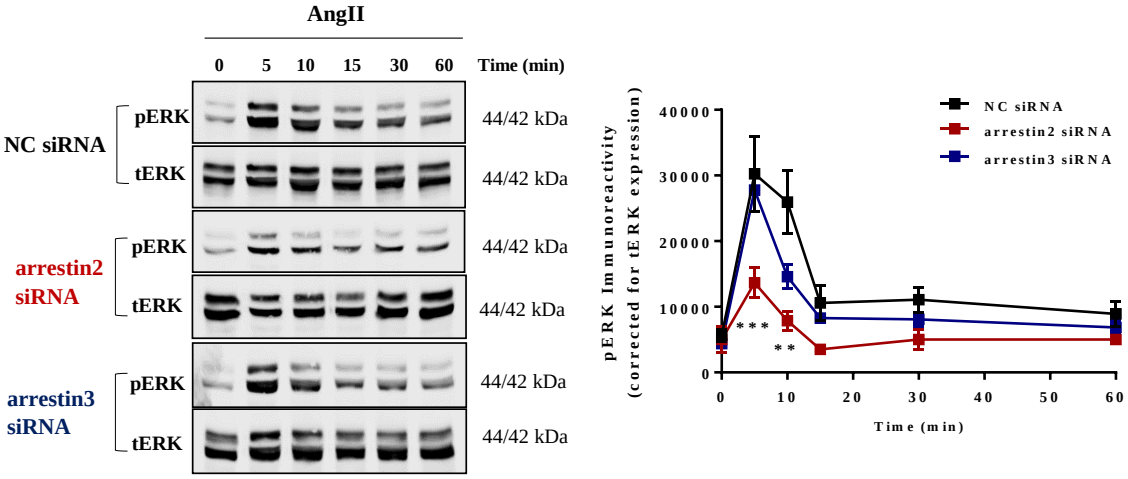


Figure 5.11 Depletion of arrestin2 attenutes AngII-stimulated ERK phosphorylation.

Serum-starved anti-arrestin2, anti-arrestin3 or NC siRNA treated RASM cells were stimulated with AngII (100 nM) for the indicated time periods. Total lysates were analysed by western blot with anti-pERK and anti-tERK antibodies. Representative immunoblots (left panel) show the temporal prolife of AngII-stimulated ERK phosphorylation. Cumulative data show the effects of arrestin2 or arrestin3 (right panel) depletion on AngII-induced ERK signalling. Data are representative of densitometric analyses of pERK bands by Image J and normalized to the loading control for 4 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in control cells are shown as; $^{**}P<0.01$, $^{***}P<0.001$ (two-way ANOVA, Sidak's *post-hoc* test).

5.3.6. Does GRK6 affect ET1-induced ERK activation?

Depletion of GRK6 attenuated the ability of vasoconstrictor to induce Ki67 expression (*Section 4.3.6*), suggesting that GRK6 may regulate VSMC growth. Since ERK signalling is also a key regulator of VSMC growth it is possible that GRK6 may regulate vasoconstrictor-stimulated ERK phosphorylation to mediate its effect on Ki67. In order to investigate this possibility the ability of ET1 to stimulate ERK phosphorylation was determined in cells transfected with anti-GRK6 siRNA using the previously described nucleofection technique (Nash et al., 2018), as described in Methods (*Section 2.2.4*). In comparison to NC transfected cells, transfection of RASM with anti-GRK6 siRNA resulted in an approximate 70% deletion of the target protein 48 h post-transfection in comparison to NC siRNA (**Figure 5.12 A**). For ERK studies, RASM cells were grown for 24 h in serum-containing media, then serum-starved for a further 24 h before being stimulated with ET1 (50 nM). Even though the ERK phosphorylation profile (plateau phase) was less pronounced in control cells, depletion of GRK6 had no effect upon the ERK activation curve (**Figure 5.12 B**) induced by ET1, indicating that GRK6 does not regulate ET1-stimulated ERK signalling and thus any GRK6-mediated effects on Ki67 expression are unlikely to involve ERK signalling.

A)

B)

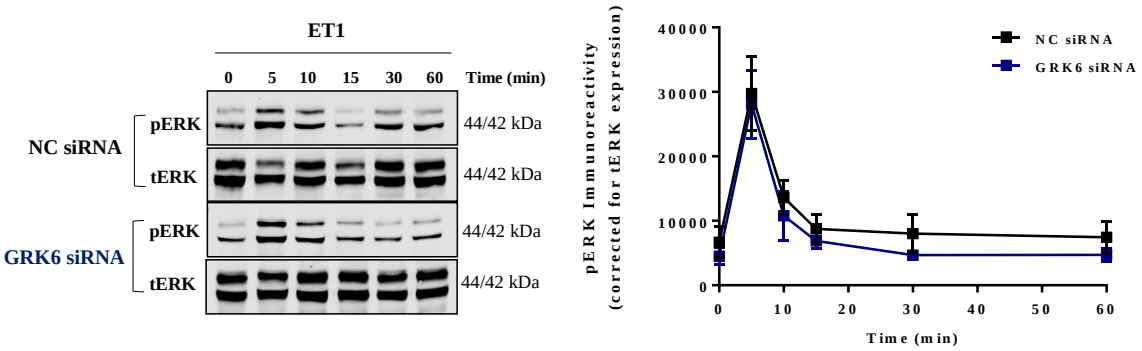


Figure 5.12 Depletion of GRK6 does not affect the time course of ET1-induced ERK phosphorylation.

A) Representative immunoblot and cumulative data show GRK6 expression after transfection of RASM cells with either NC or anti-GRK6 siRNAs (10 nM). Cumulative data show a marked reduction in GRK6 levels (A, right panel). Representative immunoblots (B, left panel) and cumulative data B, right panel) show the effects of GRK6 knockdown on the temporal prolife of ET1 (50 nM)-stimulated ERK signalling in RASM cells. Total lysates were analysed by western blot with anti-pERK and anti-tERK antibodies. Data (B, right panel) are representative of densitometric analyses of pERK bands by Image J and normalized to loading control for 4 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM. $^{**}P<0.01$ (un-paired t-test (A) and two-way ANOVA, Sidak's *post-hoc* test (B)).

5.3.7. What is the effect of COMP101 on vasoconstrictor-induced ERK signalling in VSMC from the spontaneously hypertensive rat (SHR)?

Previous sections (*Section 5.3.2 and 5.3.4*) shown that GRK2 protein expression is implicated in AngII- and ET1-stimulated ERK pathway activation. Several reports have shown that ERK signalling is chronically elevated in aortic cells derived from spontaneously hypertensive rats (SHR) (Kim et al., 1997; Roberts, 2012). Additionally, **Figure 5.13** provides data from our lab investigating GRK2 protein expression in WKY and SHR at different stages (6 weeks and 12 weeks old). Immunoblotting data shows that GRK2 expression was doubled in the arterial smooth muscle cells of SHR compared to WKY (control) normotensive animals. Moreover, this change only occurred at 12 weeks old when hypertension is established (Willets, un-published). Therefore, this model gives us an opportunity to assess ERK signalling in a model of hypertension which has elevated GRK2 expression. To know if this elevated GRK2 levels influence ERK activity in aortic smooth muscle cells of SHR, serum-starved aortic smooth muscle cells from 12 weeks old SHR or WKY rats were stimulated with AngII (100 nM), ET1 (50 nM) or UTP (100 µM). Samples were processed and blotted for pERK and tERK as described previously. Immunoblotting data illustrate that higher basal ERK activation profile in SHR aortic smooth muscle cells stimulated with AngII or ET1 in comparison to (control) WKY aortic smooth muscle cells (**Figure 5.14 C**) and (**Figure 5.15 C**). However, UTP-stimulated SHR aortic smooth muscle cells does not show change in ERK activation curve in comparison to WKY (**Figure 5.16 C**). After that, we examined if COMP101 (as GRK2 inhibitor) could reverse the enhanced ERK signal in the SHR aortic smooth muscle cells. In brief, serum starved aortic smooth muscle cells from 12 weeks old SHR or WKY rats were pre-incubated with COMP101 (30 µM) for 30 min followed by stimulation with AngII (100 nM), ET1 (50 nM) or UTP (100 µM). Samples were processed and blotted for pERK and tERK as described previously. In agreement to pervious finding (*Section 5.3.1.3*), inhibition of GRK2 catalytic activity with COMP101 result in a significant reduction in AngII-induced ERK activation (**Figure 5.14 D and E**). Similarly, treatment of RASM cells with COMP101 results in marked reduction in ET1-induced ERK activation in both WKY and SHR smooth muscle cells (**Figure 5.15 D and E**). Conversely, inhibition of GRK2 catalytic activity enhanced UTP-induced ERK activation curve in both WKY and SHR smooth muscle cells (**Figure 5.16 D and E**). These results confirm that elevated GRK2 expression underlies the enhanced AngII and ET1 stimulated ERK activity seen in SHR.

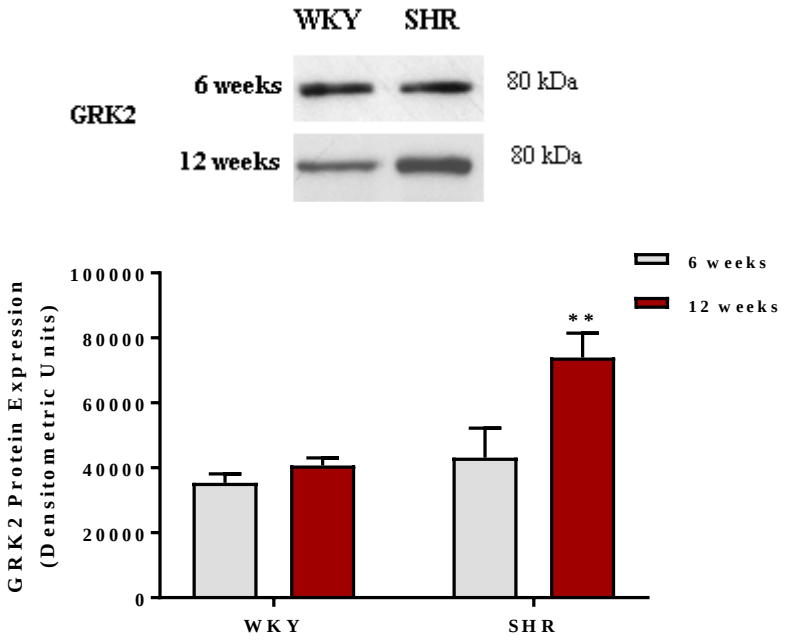


Figure 5.13 GRK2 expression is upregulated in 12 weeks old SHR.

RASM cells from WKY and SHR were lysed and GRK2 expression determined by western blotting. Representative immunoblot (A) and cumulative data (B) show the changes in GRK2 expression at 6 weeks and 12 weeks in WKY and SHR. Cumulative data (means ± SEM) are shown for the densitometric analyses of GRK2 bands using Image J for 4 independent experiments, each using RASM cells prepared from a different animal. Statistically significant changes *vs* WKY cells are shown as; $^{**}P<0.01$ (unpaired t-test) (Willets, unpublished).

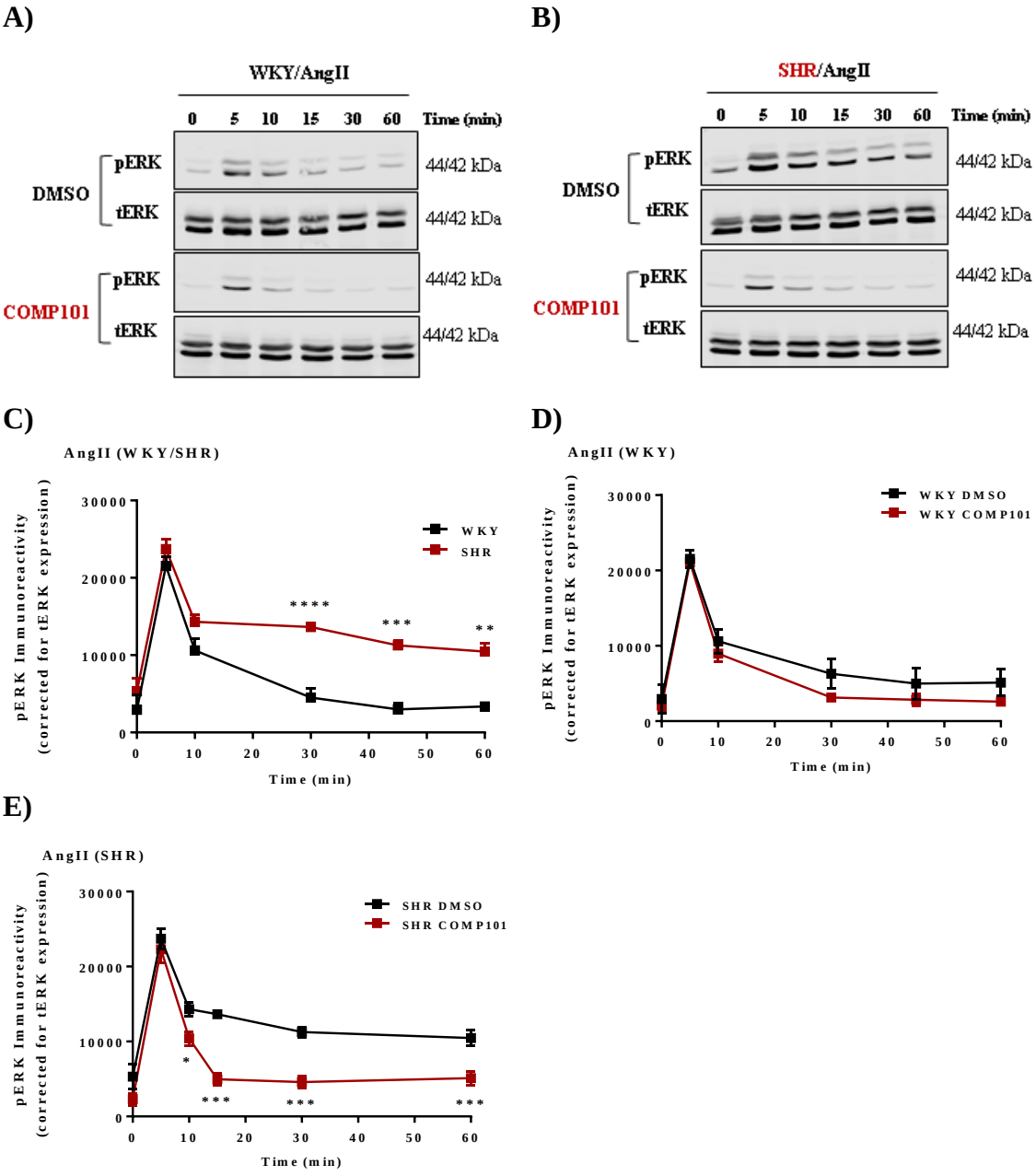


Figure 5.14 Inhibition of GRK2 catalytic activity attenuates AngII-induced ERK phosphorylation in SHR aortic smooth muscle cells.

Confluent serum-starved WKY or SHR RASM cells were pre-incubated with vehicle control or the GRK2 inhibitor COMP101 (30 µM) for 30 min, before stimulation with AngII (100 nM) for the indicated time periods. Total lysates were analysed by western blot with anti-pERK and anti-tERK antibodies. Representative immunoblots (A and B) show the temporal profile of AngII-stimulated ERK phosphorylation. Cumulative data show the comparative pERK profile in control SHR and WKY cells (C). The effects of COMP101 on AngII-induced ERK signalling are shown in (D) and (E). Data are

representative of densitometric analyses of pERK bands by Image J and normalized to the loading control for 5 independent experiments, each using cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to the respective agonist-stimulated values in control cells are shown as; $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ (two-way ANOVA, Sidak's *post-hoc* test).

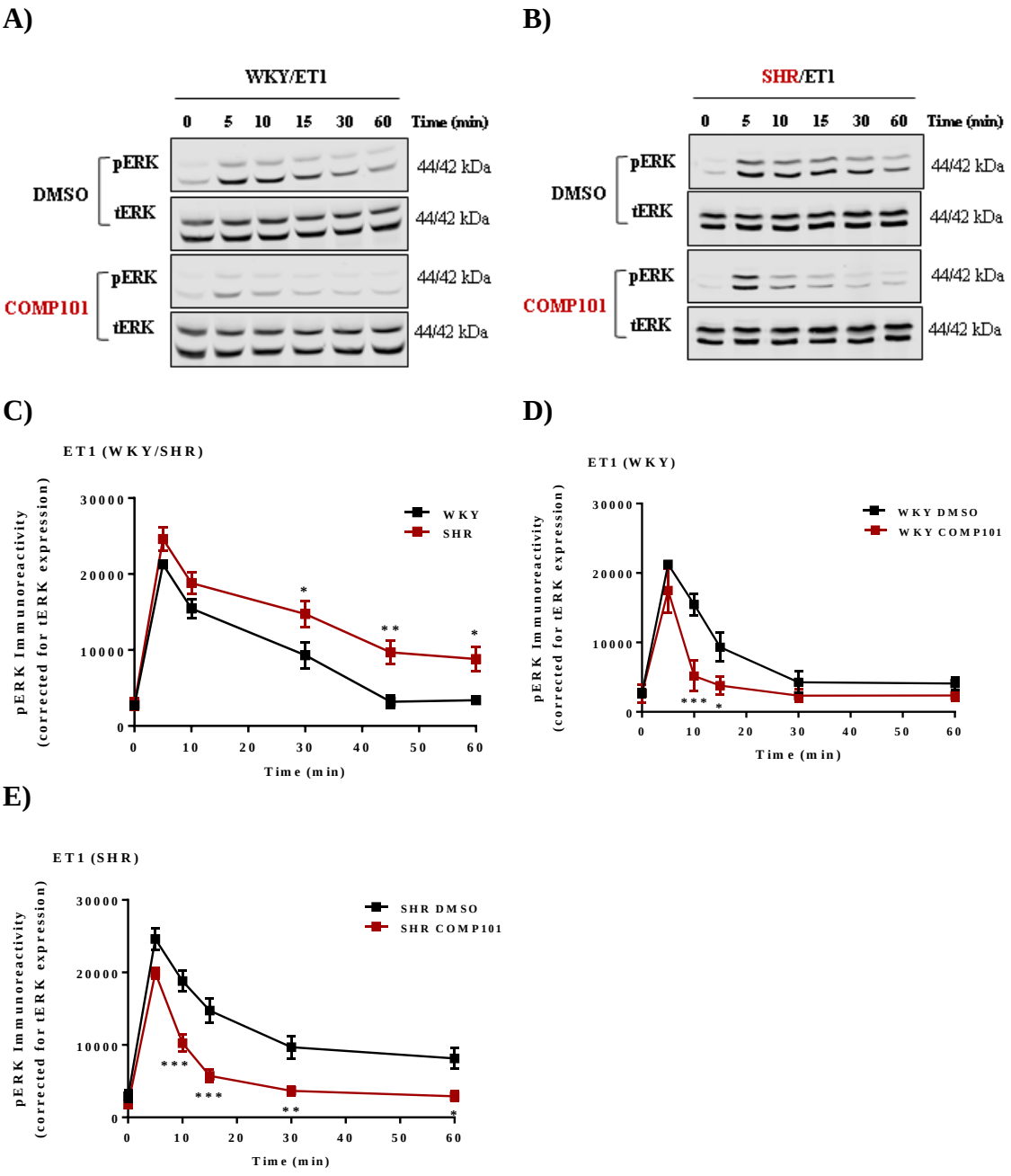


Figure 5.15 Inhibition of GRK2 catalytic activity decreases ET1-induced ERK phosphorylation in SHR aortic smooth muscle cells.

Confluent serum-starved WKY or SHR RASM cells were pre-incubated with vehicle control or the GRK2 inhibitor COMP101 (30 µM) for 30 min, before stimulation with ET1 (50 nM) for the indicated time periods. Total lysates were analysed by western blot with anti-pERK and anti-tERK antibodies. Representative immunoblots (A and B) show the temporal profile of ET1-stimulated ERK phosphorylation. Cumulative data (C) show the comparative pERK profiles in control SHR and WKY cells. The effects

of COMP101 on ET1-induced ERK signalling in WKY (D) and SHR (E) cells are shown. Data are representative of densitometric analyses of pERK bands by Image J and normalized to loading control for 5 independent experiments, each using cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in control cells are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (two-way ANOVA, Sidak's *post-hoc* test).

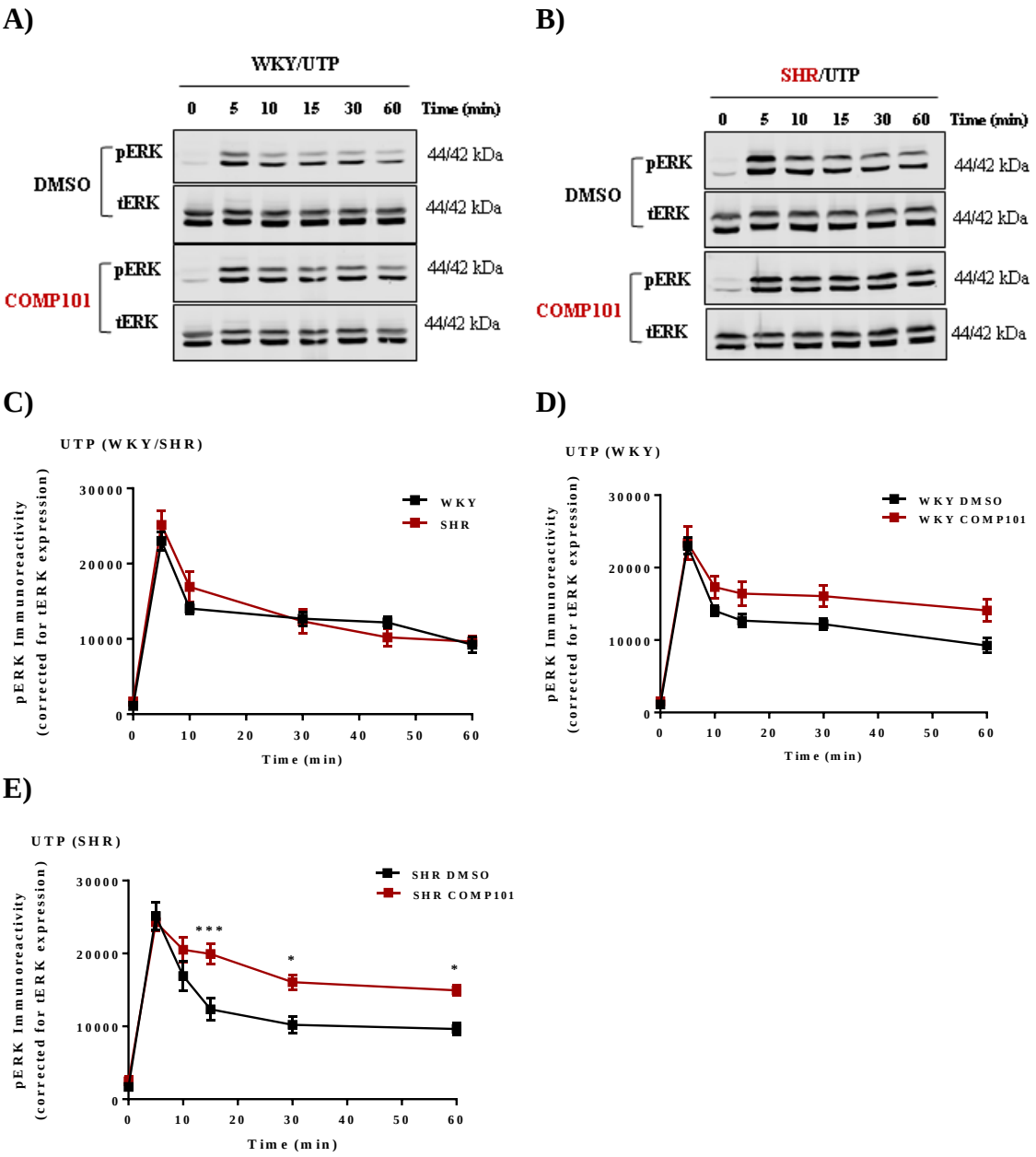


Figure 5.16 Inhibition of GRK2 catalytic activity enhances UTP-induced ERK phosphorylation in SHR aortic smooth muscle cells.

Confluent serum-starved WKY or SHR RASM cells were pre-incubated with vehicle control or the GRK2 inhibitor COMP101 (30 μM) for 30 min, before stimulation with UTP (100 μM) for the indicated time periods. Total lysates were analysed by western blot with anti-pERK and anti-tERK antibodies. Representative immunoblots (A and B) show the temporal profile of UTP-stimulated ERK phosphorylation. Cumulative data show the change in pERK profile in control SHR and WKY cells (C). The effects of COMP101 on UTP induced ERK signalling in WKY (D) and SHR (E) cells are shown.

Data are representative of densitometric analyses of pERK bands by Image J and normalized to loading control for ≥4 independent experiments, each using cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in control cells are shown as; $^{*}P<0.05$, $^{***}P<0.001$ (two-way ANOVA, Sidak's *post-hoc* test).

5.4. Discussion

Previous findings highlight the importance of the MAPK/ERK signalling pathway in VSMC proliferation (Wen et al., 1996; Bornfeldt et al., 1997; Nelson et al., 1998). VSMCs phenotype modulation from the contractile to a proliferative phenotype is associated with persistent MAPK/ERK signalling activation (Roy et al., 2001). Moreover, $G_{\alpha q}$ coupled vasoconstrictors such as AngII and ET1 promote VSMC proliferation via the ERK1/2 signalling pathway (Qin et al., 2004; Zhao et al., 2005; Ljuca and Drevensek, 2010). These studies were further confirmed by our [^{3}H]-thymidine incorporation assay data, which show that inhibition of MEK1/2 (the kinase that phosphorylates ERK) (Alessi et al., 1995) attenuates VSMC proliferation. In accordance with the present results, previous studies have demonstrated that inhibition of MEK1/2 attenuates ET1 and AngII dependent DNA synthesis in VSMC (Touyz et al., 1999; Chen et al., 2006). We report here that vasoconstrictors appear to exert their mitogenic effects through the activation of MEK1/2-ERK1/2 pathway to enhance DNA synthesis in cultured smooth muscle cells, suggesting that ERK signalling is involved in vasoconstrictor-induced VSMC proliferation, and may play an important role in the vascular wall structural changes seen in hypertension. Previous findings revealed that both GRK2 expression and its activity participated in VSMC growth (*Chapter 4*). Here, I propose that GRK2 plays an essential role in the MAPK/ERK signalling pathway linking to the proliferation process. This notion is supported by Ruiz et al., who reported that continuous $G_{\alpha q}$ activation enhances GRK2 expression in VSMC (Ramos-Ruiz et al., 2000). In addition, GRK2 expression is enhanced in hypertension (Eckhart et al., 2002; Willets et al., 2015). Although GRK2 is a critical regulator of vasoconstrictor's GPCR signalling (Kim et al., 2009; Morris et al., 2010; Morris et al., 2011), the data has revealed a non-classical effect that GRK2 plays in MAPK/ERK signalling. I have identified that both GRK2 expression and activity are required for AngII and ET1 induced MAPK/ERK signalling activation, but are not required for UTP induced MAPK/ERK signalling.

The data revealed that stimulation of RASM cells with AngII, ET1 or UTP resulted in significantly increased ERK phosphorylation, which peaked at 5 min, followed by a gradual decline to a sustained level between 10 and 60 min, which is in general agreement with earlier studies (Yoshizumi et al., 2001; Chen et al., 2009; Morris et al., 2012). GPCRs are capable of employing several diverse mechanisms to activate the MAPK/ERK

cascade (Pierce et al., 2001). Firstly, GPCRs can stimulate $G_{\alpha q}$ to activate PLC and PKC. PKC is able to phosphorylate various substrates, and initiates the MAPK/ERK signalling cascade (Zhao et al., 2005; Ljuca and Drevensek, 2010). Indeed, activated PKC is known to enhance ERK1/2 activity in VSMC, effects that can be blocked by PKC inhibition (Chen et al., 2009). Certainly, the PKC activator, phorbol 12-myristate 13-acetate (PMA) activates ERK, which can be blocked by inhibition of PKC (Wei et al., 2003). Additionally, the PKC inhibitor (Ro-31-8425) abolished AngII-induced early ERK activation (5 min), but had no effect on the later sustained phase (Ahn et al., 2004). The findings of Ca^{2+} imaging data (*Section 3.3.2*), which showed that the effects of AngII/AT_1 and ET1/ET_A triggered PLC/Ca^{2+} signalling as a rapid and transient increase in $[Ca^{2+}]_i$, suggest that the early peak of ERK phosphorylation could be related to PLC-Ca^{2+}-PKC activation. However, it is worth noting that AngII/AT_1 and ET1/ET_A mediated $[Ca^{2+}]_i$ signals are transient, and underpinned by the receptor desensitization. Indeed, AngII or ET1-mediate $[Ca^{2+}]_i$ signals return to basal within 3-5 minutes even in the continued presence of the agonist (Morris et al., 2010; Bernhem et al., 2017). Therefore, this suggests that AngII and/or ET1-stimulated prolonged (10-60 minutes) ERK activation is unlikely to be G protein-dependent. Interestingly, several alternative G protein-independent processes have been described by which GPCRs can mediate prolonged ERK signalling. For example, both AT_1 and ET_A receptors are known to utilise arrestins as molecular scaffolds to enable prolonged cytosolic ERK signalling (Luttrell et al., 2001; Lefkowitz and Shenoy, 2005; Morris et al., 2012). In addition, in RASM cells the sustained phase of UTP/$P2Y_2$ mediated ERK signal activation cannot be inhibited by PKC inhibitors, indicating that UTP/$P2Y_2$-mediated ERK activation is a G protein-independent, Src-EGFR dependent process (Morris et al., 2012). Thus, in RASM cells the prolonged ERK activation is probably mediated via a G protein-independent mechanism. In general, therefore, it seems that the observed pERK curve is biphasic, with an acute phase mediated through PLC-Ca^{2+}-PKC occurring within 2-5 minutes, and prolonged ERK activation sustained over one hour which likely modulates long-term responses, such as stimulation of cell proliferation. In support of this idea, previous studies have described that AngII produces a biphasic response in VSMC, where early pERK activity (within minutes) is induced via the G protein-dependent pathway, whereas late/sustained (within hours) activity is mainly mediated by G protein-independent mechanisms i.e. GRKs or arrestins (Griendling Kathy et al., 1997; Ahn et al., 2004; Lefkowitz and Shenoy, 2005). Collectively, these data suggest that the observed

pERK activation curve for AT_1, ET_A and $P2Y_2$ receptors in VSMC could result from a combination of $G_{\alpha q}$ dependent (rapid/transient) and independent (prolonged) mechanisms (**Figure 5.17**).

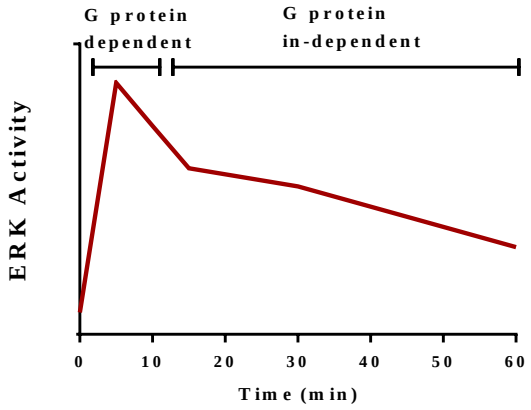


Figure 5.17 Hypothetical diagram for biphasic ERK activation by distinct G-protein-dependent/in-dependent pathways.

The rapid and transient ERK activation is probably a G protein-dependent mechanism, requires PKC activation and can be inhibited by a PKC inhibitor (Chen et al., 2009). In contrast, the sustained response possibly represents G protein-independent mediated ERK signalling through, for example, arrestin (Ahn et al., 2004).

Data in *Chapter 4* illustrated the involvement of both GRK2 expression and kinase activity in VSMC proliferation. Thus, GRK2 may play an essential role in MAPK/ERK signalling to promote cell proliferation. Despite previous work highlighting a negative regulatory role for GRK2 on AT_1 and ET_A receptor activity in VSMC (Kim et al., 2009; Morris et al., 2010), theoretically, manipulation of GRK2 expression/activity should increase GPCR activation and thus $G_{\alpha q}$ related signalling. However, depletion of GRK2 or inhibition of its catalytic activity markedly attenuated both the acute and sustained phases of ERK activation induced by AngII and ET1, suggesting that GRK2 participates in MAPK/ERK signalling activation in a G protein-independent manner. Contrastingly, as $P2Y_2$ receptor activation recruits GRK2 (Morris et al., 2011), GRK2 knockdown or inhibition enhanced both the acute and sustained phases of UTP induced ERK activation. Since suppression of GRK2 expression inhibits UTP-induced $P2Y_2$ receptor desensitization (Morris et al., 2011), elevated ERK signalling probably reflects the lack of $P2Y_2$ desensitization following GRK2 depletion or inhibition of its catalytic

activity. Indeed, deficiency of $P2Y_2$ desensitization triggered increased PLC-Ca^{2+}-PKC activation, which resulted in enhanced ERK signals in a G protein-dependent manner (Morris et al., 2012). This idea is consistent with earlier observations, which showed that UTP/$P2Y_2$-mediated ERK phosphorylation is enhanced following inhibition of receptor desensitization (via arrestin2 depletion) (Morris et al., 2012). Interestingly, GRK2 depletion/inhibition has been shown to downregulate AngII and/or ET1 (proliferative vasoconstrictors)-stimulated ERK signalling, yet this effect was not seen with UTP-stimulated ERK signalling (non-proliferative agonist). Thus, the effects of GRK2 on MAPK/ERK signalling seems to be ligand and receptor specific.

There are many possible mechanisms for the observed effect of GRK2 on the MAPK/ERK cascade. Indeed, a previous study has investigated the downstream effects of EGFR and has shown that GRK2 can regulate ERK signalling through direct interaction with Raf1, MEK1 and ERK2, suggesting that GRK2 acts as a scaffold protein for the MAPK/ERK cascade (Robinson and Pitcher, 2013). Further work is required to examine the direct interaction between GRK2 and MEK1/2 and ERK1/2 in response to AngII and ET1 in VSMC. There is, however, another possible mechanism, several published reports show that GPCR-stimulated ERK signalling can be mediated via RTK (such as EGFR and PDGFR) transactivation (Muthalif et al., 1996; Schonwasser et al., 1998; Heeneman et al., 2000; Ohtsu et al., 2006). Liu et al., revealed that AngII induced ERK signalling in smooth muscle cells of SHR through EGFR transaction, as the effect could be attenuated by an EGFR inhibitor [AG1478] (Liu et al., 2010b). Also, ET1 has been reported to induce ERK phosphorylation via EGFR transactivation in VSMC (Iwasaki et al., 1999) and in mesangial cells (Hua et al., 2003). This suggests that AngII and ET1 mitogenic responses can be mediated via a GPCR-RTK-ERK pathway. GPCRs induce EGFR transactivation via different mechanisms, for instance, AngII/AT_1 mediates EGFR transactivation via c-Src activation (Eguchi et al., 1999) or via ROS activation (Ushio-Fukai et al., 2001; Frank and Eguchi, 2003). After that, activated EGFR transmits its signal through phosphorylation of several tyrosine residues and formation of a complex with Shc, Sos and Grb2 leading to activation of the Ras-Raf-MEK-ERK cascade (Inagami and Eguchi, 2000). Moreover, GRK2 has been reported to mediate a regulatory role in EGF-stimulated ERK signalling (Gao et al., 2005). Additionally, GRK2 has been reported to regulate RTKs such as the PDGF receptor (Hildreth et al., 2004). Since GRK2 also has been reported as a key factor that mediates the cross-talk between GPCRs and

RTKs (Chen et al., 2008; Guo et al., 2009), the possible interference of GRK2 in both RTK and GPCR-induced ERK signalling cannot be ruled out and requires further investigation.

Prior studies have noted the importance of arrestins function as ligand-regulated adaptor scaffolds which facilitate downstream signalling from phosphorylated GPCRs (Luttrell and Miller, 2013). Several reports have shown that arrestins can act as scaffolds for ERK signalling (Luttrell et al., 2001; Ren et al., 2005). Moreover, arrestin2 directly interacts with MEK1 (Meng et al., 2009) indicating that arrestins play a role in ERK signalling. Therefore, it is important to ask, what is the role of arrestins in the GRK2-mediated ERK activation process? Morris et al. (2012) show that arrestin2 and arrestin3 regulate ERK activation by UTP/$P2Y_2$ and ET1/ET_A receptors respectively in RASM cells (Morris et al., 2012). However, the effect of arrestin2 and arrestin3 on AngII/AT_1 receptor stimulated ERK activation has not been previously studied in VSMC. Here, knockdown of arrestin2 attenuated both the peak and plateau phases of ERK activation, whereas arrestin3 knockdown had no effect. Thus it would appear that arrestin2, rather than arrestin3, mediates AngII-induced ERK signalling, although at present the exact mechanism is unclear. This outcome is contrary to that of Kim et al. (2005) who found that AngII-induced ERK activation was markedly enhanced following knockdown of GRK2 (Kim et al., 2005a). Moreover, the sustained ERK signalling is arrestin3-dependent not arrestin2-dependent (Ahn et al., 2004). This contradictory finding may be due to different cell backgrounds, i.e. HEK293 cells transfected with overexpressed exogenous AT_{1A}, compared to endogenously AT_1 receptors expressed in their native environment. Thus, the observed GRK2/arrestin2 effect on AngII-stimulated ERK signalling seems to be cell type specific. Additionally, previous work from our laboratory shows that ET1-stimulated ERK signalling was markedly reduced following arrestin3 depletion in RASM cells (Morris et al., 2012). In general, therefore, it seems possible that GRK2 mediated phosphorylation of AT_1 and ET_A is required to enable arrestin recruitment and thus promote sustained ERK signalling. Therefore, ERK signalling seems to be GRK2-arrestin2-dependent for AngII/AT_1 and GRK2-arrestin3-dependent in ET1/ET_A-stimulated cells. Further research is required to delineate the molecular mechanisms that arrestins play in GPCR/GRK2-mediated ERK signalling.

The findings in this chapter suggest a strong relationship between GRK2 and MAPK/ERK activation together with GRK2 up-regulation in hypertension, implying that

increased expression of GRK2 could be a key to enhancing MAPK/ERK signalling and thus increased VSMC proliferation. To further investigate this hypothesis two different ways to over-express GRK2 were utilised. The first technique was via the nucleofection using previously characterised GFP-tagged GRK2 construct (GFP-GRK2) which caused ≥70% over-expression in ~60% of the cells. It seems probably that these relatively low increase in expression due to the large size of GFP-GRK2 construct (~$33x10^3$ bp) which may reduce the transfection efficiency. In an attempt to increase the number of cells overexpressing GRK2, cells were infected with an adenoviral GRK2 construct. Unfortunately, the adenovirus induced GRK2 overexpression in ~50% of the cells only. The reason for the low infection rate was not clear. Despite the lower than expected transfection/infection rates, GRK2 overexpression still considerably enhance both the peak and plateau phases of ERK signalling induced by AngII. These results support the idea of a positive relationship between GRK2 and MAPK/ERK activation.

Interestingly prior studies have noted that GRK2 expression is increased in hypertensive animal models (Gros et al., 2000; Eckhart et al., 2002) and GRK2 expression is up-regulated in the arterial smooth muscle cells of the SHR with the onset of the hypertensive phenotype (Willets et al., 2015). Thus, we used aortic smooth muscle cells isolated from SHR to assess ERK signalling as a model of hypertension to assess the effects of GRK2 overexpression on vasoconstrictor-stimulated ERK signalling and cellular proliferation. Several reports have shown that ERK signalling is chronically elevated in aortic cells derived from SHR (Kim et al., 1997; Kubo et al., 2002; Roberts, 2012). Additionally, AngII-induced ERK activation is amplified in SHR VSMC (Touyz et al., 2001), which is known to contribute to hypertensive vascular remodelling in SHR (El Mabrouk et al., 2001). In agreement with published studies (Touyz et al., 2001; Zhu et al., 2015), our findings show that AngII and ET1 produce a higher ERK activation profile in SHR aortic smooth muscle cells, while UTP did not. Furthermore, inhibition of GRK2 catalytic activity markedly attenuated AngII/ET1-induced ERK activation and enhanced UTP-induced ERK activation in both WKY and SHR smooth muscle cells, indicating that the observed GRK2 effect on MAPK/ERK signalling is specific for AT_1 and /ET_A receptors, not the $P2Y_2$ receptor. Collectively these data strongly suggest that the elevated GRK2 level underlies the enhanced AngII and ET1 stimulated ERK activity seen in hypertension.

The ability of ERK to stimulate cell growth requires the nuclear translocation of ERK, where it subsequently promotes cell cycle progression (Brunet et al., 1999). Nuclear translocation of activated ERK occurs within 15 minutes (Yamamoto et al., 2006), a process mediated by passive diffusion, active transport, or by direct interaction with the nuclear pore complex (Adachi et al., 1999; Whitehurst et al., 2002). Upon translocation of activated ERK to the nucleus, it activates multiple transcription factors and ultimately stimulates cell proliferation (Mebratu and Tesfaigzi, 2009). Several published studies in different cell backgrounds reported that there is a strong correlation between sustained (but not transient) ERK signalling activation and its proliferative potential [i.e. induction of cell cycle progression] (Meloche et al., 1992; Cookonyx and McCormick, 1996; Yamamoto et al., 2006). Indeed, prolonged ERK activation stimulates transition from G_0/G_1 phase to S phase by promoting cyclin D1 expression (Weber et al., 1997; Roovers et al., 1999), downregulation of anti-proliferative genes such as AP-1 (Yamamoto et al., 2006), hyper-phosphorylation and stabilisation of c-fos (Murphy et al., 2002), and subsequently stimulating S phase entry. On other hand in HEK293 cells, AngII/AT_1 mediated nuclear translocated ERK signalling is a G protein-dependent mechanism (Ahn et al., 2004), and the arrestin-mediated scaffolding of ERK (sustained effect) has been shown has not translocated to the nucleus and be restricted to the cytosol (Luttrell et al., 2001; Aplin et al., 2007). Despite this, to develop a fuller picture, further research should be undertaken to investigate the AngII/AT_1 and ET1/ET_A mediated ERK signals and nuclear translocation in both transient and sustained phases of ERK activation in VSMC.

Findings from *Section 4.3.6* revealed that GRK6 depletion attenuated the ability of vasoconstrictors to induce Ki67 expression, suggesting that GRK6 may regulate VSMC proliferation. However, depletion of GRK6 had no effect upon ET1-promoted ERK activation indicating that GRK6 does not regulate ET1-stimulated ERK signalling. This result seems to be consistent with the fact that GRK6 had no effect on the regulation of ET_A receptor driven PLC/Ca^{2+} signalling in RASM (Morris et al., 2010). Thus the involvement of ERK signalling in GRK6-mediated effects on Ki67 expression is unexpected and at present unexplained.

In summary, both GRK2 expression and catalytic activity are required for transient and sustained ERK activation induced by AngII and ET1 in VSMCs. Moreover, our findings indicate a proportional relationship between GRK2 expression and ERK signal

activation in SHR smooth muscle cells. Therefore, up-regulated GRK2 expression in hypertension could be an underlying factor for long-term MAPK/ERK signalling cascade activation or potentiation. Although activation of AT_1, ET_A and $P2Y_2$ receptors recruits GRK2 (Kim et al., 2009; Morris et al., 2010; Morris et al., 2011), it is surprising that GRK2 only participates in proliferative vasoconstrictor (AngII and ET1) stimulated MAPK/ERK signalling and cell proliferation (**Figure 5.18**). The results demonstrate that GRK2 differentially alters ERK activation responses to different agonists, indicating that the role that GRK2 plays in hypertension is not limited to dysregulation of vasoconstrictor GPCR activation. Indeed, data from this chapter suggest that GRK2 may function as a scaffold protein for the MAPK/ERK cascade (GRK2-dependent), or alternatively mediate ERK signals through the recruitment of arrestins (GRK2-arrestin-dependent), or via mediating cross-talk with RTKs such as the EGFR (**Figure 5.19**).

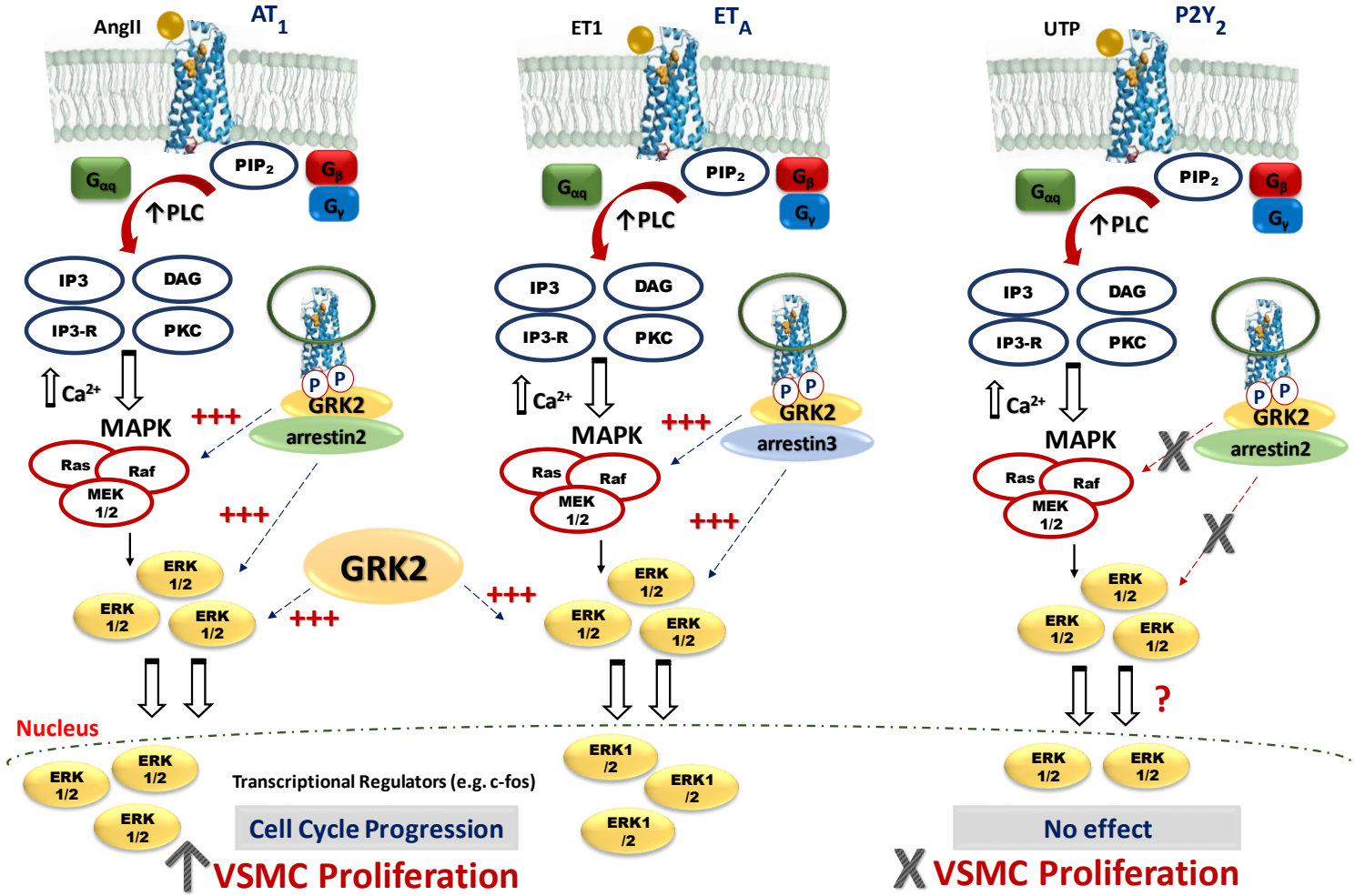


Figure 5.18 Schematic diagram showing the role of GRK2 on AT_1, ET_A and $P2Y_2$ receptor mediated MAPK/ERK cascade activation.

Activation of vasoconstrictor GPCRs results in generation of second messengers DAG and IP_3, which increase $[Ca^{2+}]_i$ in a G protein-dependent manner and mediate other G-protein independent pathways resulting in Ras/Raf/MEK cascade activation. Both GRK2 expression and catalytic activity are required for AngII and ET1 (not UTP) mediated ERK signal activation which may be via GPCR-GRK2-arrestins mechanism.

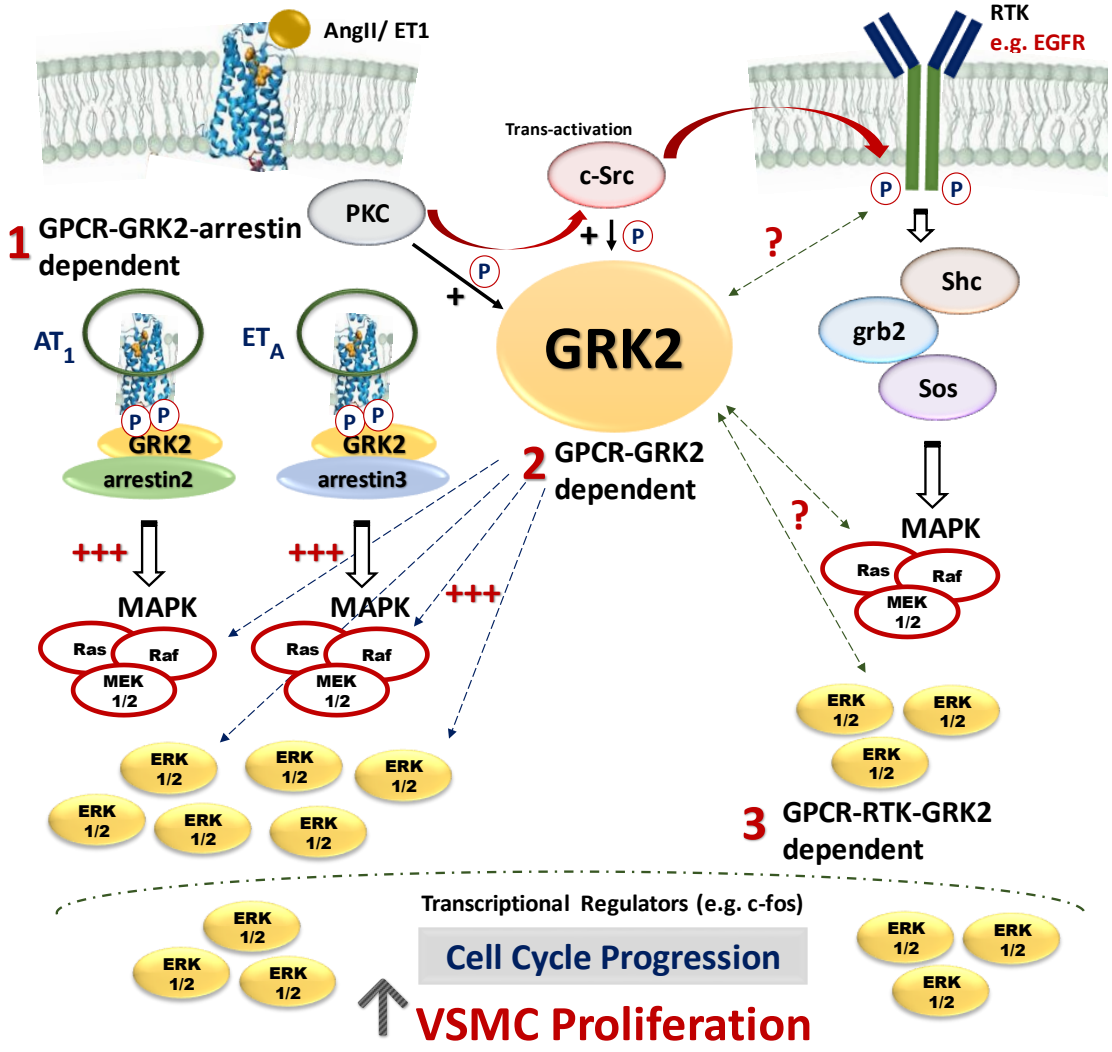


Figure 5.19 Summary of the possible mechanisms of GRK2 effects on ERK signalling pathway activation.

Suggested mechanisms by which GRK2 may affect GPCR-mediated ERK activation via a GPCR-GRK2-arrestin dependent manner **(1)** (Morris et al., 2012). As GRK2 can interact with Raf1, MEK1 or ERK2 (Robinson and Pitcher, 2013) suggesting that ERK activation could be GPCR-GRK2-dependent **(2)** or via transactivation of the EGFR (Inagami and Eguchi, 2000) mediating ERK activation in GPCR-RTK-GRK2 dependent mechanism **(3)**.

Chapter Six

6. Do AngII and ET1 employ the PI3K/Akt signalling pathway to mediate GRK2-dependent VSMC proliferation?

6.1. Introduction

PI3K/Akt signalling is one of the major signalling cascades that has been reported to be activated in cardiovascular diseases including hypertension and atherosclerosis (Carnevale and Lembo, 2012; Li et al., 2016). Moreover, PI3K/Akt signalling plays a critical role in regulating VSMC proliferation (Park et al., 2003; Chen et al., 2014), cell cycle progression (Braun-Dullaeus et al., 2001; Dugourd et al., 2003) and thus contributes to medial thickening and vascular remodelling (Stabile et al., 2003; Havelka and Kibbe, 2011). Importantly, the $G_{\alpha q}$ coupled receptors that interact with vasoconstrictors are known to activate PI3K/Akt signalling (Murga et al., 1998; Takahashi et al., 1999; Ushio-Fukai et al., 1999). PI3K is a lipid kinase that activates several downstream targets such as PIP_3 and PDK1, which subsequently promotes translocation of Akt (PKB) to the plasma membrane and phosphorylation of Akt at Thr 308 (see *Section 1.3.3.2*) (Alessi et al., 1997; Vanhaesebroeck et al., 2001; Guillermet-Guibert et al., 2008). Once phosphorylated, Akt acts as a multifunctional protein kinase, which activates several proteins involved in controlling cell cycle progression, cell growth and survival processes (Datta et al., 1999; Bellacosa et al., 2004). Akt can mediate its proliferative effects via phosphorylation of GSK3 (Beurel et al., 2015). There are two isoforms of GSK3, GSK3-α (~51 kDa) and GSK3-β (~47 kDa) which have a high degree of similarity in their catalytic domains, (Markou et al., 2008). Akt phosphorylates GSK3-α at Ser-21 and GSK3-β at Ser-9 residues resulting in inactivation of GSK3 (Beurel et al., 2015). Inactivation of GSK3 relieves its inhibitory effects on cyclin D1 and c-myc, resulting in increased their transcription (Chang et al., 2003; Liang and Slingerland, 2003), which subsequently promotes cell cycle progression and cell survival.

In addition to multiple non-receptor substrates (Shiina et al., 2001; Schutzer et al., 2005; Jimenez-Sainz et al., 2006), GRK2 can interact directly with PI3K (Naga Prasad et al., 2002b; Robinson and Pitcher, 2013) and Akt (Liu et al., 2005). Since hypertension is associated with the elevated circulatory levels of vasoconstrictors (Harris et al., 2008), continual activation of PI3K/Akt signalling pathways has been suggested to play a role

in hypertension-associated increases in VSMC cell proliferation and the development of vascular remodelling. Even though earlier findings (see *Chapter 4*) confirmed that GRK2 expression and activity have effects on vasoconstrictor-induced VSMC proliferation, whether GRK2 can affect changes in AngII and ET1-stimulated cell growth through modulation of the PI3K/Akt signalling pathway remains to be elucidated. Here, I hypothesize that GRK2 is involved in the proliferator responses downstream of GPCRs stimulated by AngII and ET1 and that PI3K/Akt signalling may be involve in VSMC proliferation. Furthermore, despite the fact that GRK2 regulates AT_1, ET_A and $P2Y_2$ signalling (Kim et al., 2009; Morris et al., 2010; Morris et al., 2011), I hypothesise that GRK2 plays distinctive roles in the regulation Akt of signals produced by pro-proliferative (AngII and ET1) and non-proliferative (UTP) agonists (see *Chapter 3*). Therefore, the current chapter investigates the roles of GRK2 and PI3K/Akt in vasoconstrictor-mediated VSMC proliferation.

6.2. Aim

To investigate the molecular mechanisms underlying GRK2 regulation of VSMC proliferation via examining the possible relationship between GRK2 expression/catalytic activity and vasoconstrictor-induced PI3K/Akt signalling.

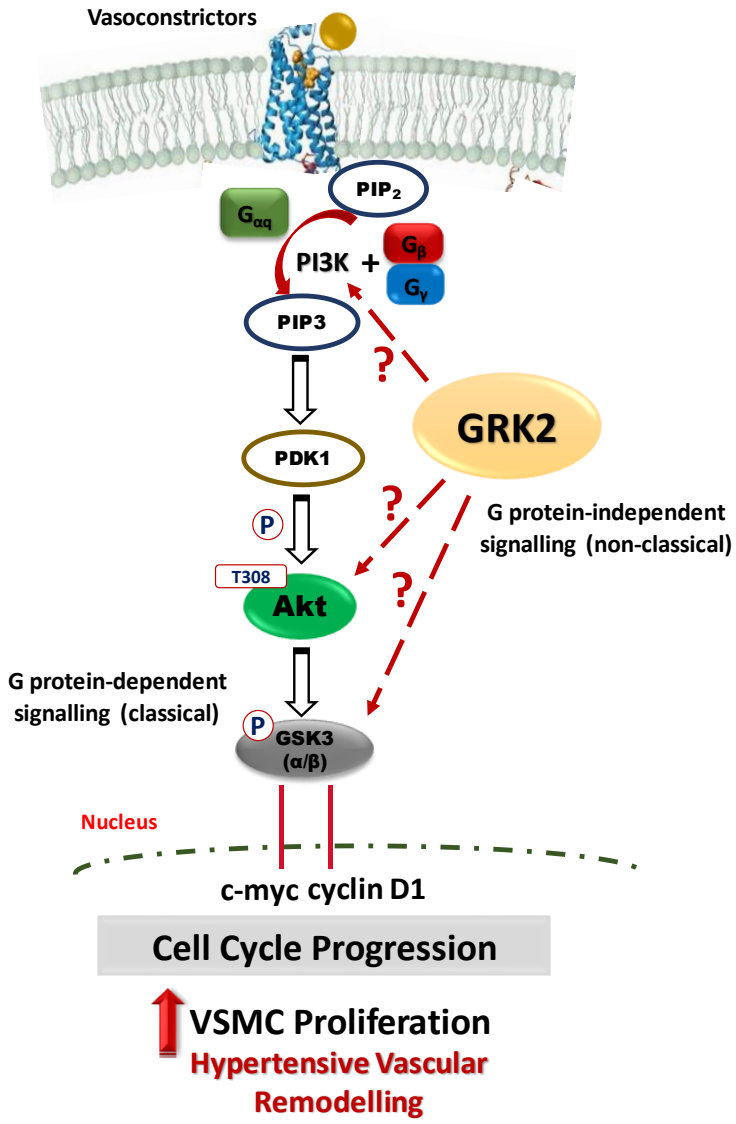


Figure 6.1 Schematic diagram showing GPCR-mediated activation of the PI3K/Akt signalling pathway.

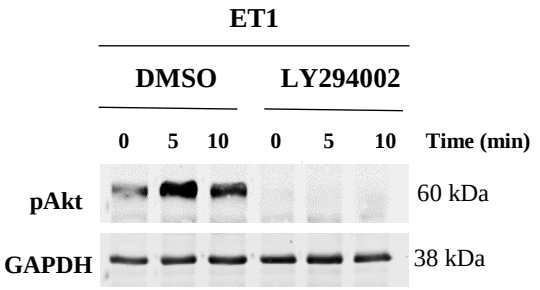
Upon GPCR activation, PI3K converts PIP_2 to PIP_3, resulting in PDK1 membrane translocation and Akt phosphorylation at Thr 308 residue. pAkt mediates phosphorylation of GSK3 (inhibition) and thus regulates cell-cycle progression. PI3K, phosphoinositide 3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate lipid; PDK1, 3' phosphoinositide dependent-kinase 1; GSK3, glycogen synthase kinase 3.

6.3. Results

6.3.1. Inhibition of the PI3K/Akt signalling pathway decreased vasoconstrictor-induced [^{3}H]-thymidine incorporation in RASM cells

The PI3K/Akt signalling pathway is one of the important cascades that is integrally linked to smooth muscle cell cycle progression and the promotion of cell proliferation (Stabile et al., 2003; Wang et al., 2018b). Furthermore, previously published studies show that PI3K/Akt signalling can be activated by AngII and ET1 in VSMC (Takahashi et al., 1999; Huang et al., 2011). In order to examine whether PI3K/Akt signalling was involved in vasoconstrictor-induced VSMC proliferation, RASM growth was examined using the [^{3}H]-thymidine incorporation assay in the presence or absence of LY294002; a potent PI3K inhibitor (Vlahos et al., 1994). Briefly, serum starved RASM cells were pre-incubated with LY294002 (20μM) for 30 min prior to stimulation with the vasoconstrictors AngII (100 nM), ET1 (100 nM), NA (1 μM) or UTP (100 μM) in 10% FCS medium. Immunoblotting of phospho-Akt (pAkt) showed that pre-treatment of RASM cells with the PI3K inhibitor abolished ET1-induced Akt activation, confirming the inhibitory effect of LY294002 (**Figure 6.2 A**). The prolife of vasoconstrictor-stimulated [^{3}H]-thymidine incorporation in vehicle control (DMSO) incubated cells was similar to that observed previously (*Section 3.3.6, 4.3.2 and 4.3.3*), which showed that stimulation of RASM cells with AngII, ET1 or NA resulted in greater [^{3}H]-thymidine incorporation than that seen with 10% FCS alone (**Figure 6.2 B**). However, inhibition of LY29002 significantly attenuated 10% FCS, AngII, ET1 and NA induced [^{3}H]-thymidine incorporation in RASM cells. Collectively, these data strongly suggest that inhibition of the PI3K/Akt signalling pathway attenuated VSMC proliferation.

A)



B)

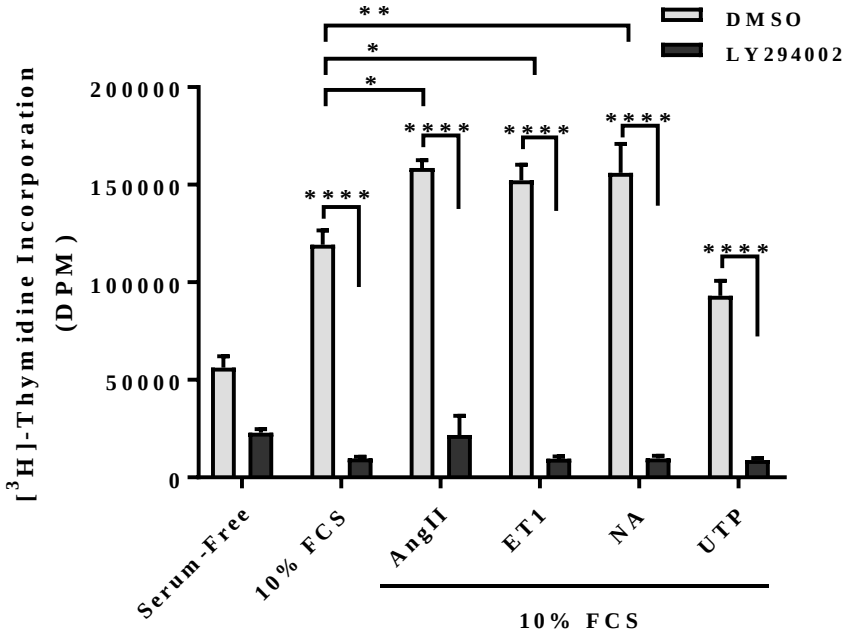


Figure 6.2 Inhibition of the PI3K/Akt signalling pathway attenuated vasoconstrictor-induced VSMC proliferation.

A) Confluent serum-starved RASM cells were stimulated with ET1 (50 nM), with or without LY2904002 (20 μM) pre-treatment (30 min), before lysis and immunoblotting for Akt phosphorylation. A representative immunoblot shows that LY2904002 pre-treatment blocked ET1-stimulated Akt phosphorylation. B) Serum-starved RASM cells were pre-treated with vehicle control or LY2904002 (20 μM) for 30 min, prior to stimulation with AngII (100 nM), ET1 (100 nM), NA (1 μM) or UTP (100 μM) in 10% FCS. LY2904002 treatment inhibited 10% FCS, AngII, ET1 and NA induced RASM cell proliferation. Data are represented as mean ± SEM for 4 replicates for each experimental condition from 4 different cell preparations. Statistically significant changes, control cells *vs* LY2904002 treated, are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$ (two-way ANOVA, Sidak's *post-hoc* test).

6.3.2. What is the effect of AngII and ET1 on PI3K downstream signalling?

The above findings (*Section 6.3.1*) indicated that inhibition of PI3K signalling by the PI3K inhibitor LY294002 had a global effect on cell growth. Therefore, in order to examine whether PI3K was involved in vasoconstrictor-driven VCMC proliferation, PI3K downstream substrates of Akt were examined. Serum-starved RASM cells were stimulated for up to an hour with AngII (100 nM) or ET1 (50 nM). Total cell lysates were then blotted for the phosphorylated Akt with GAPDH used as a loading control. As shown in **Figure 6.3**, stimulation of RASM cells with AngII or with ET1 induced phosphorylation of Akt that peaked at 5 min followed by gradual decline and sustained pAkt protein levels were observed between 10 and 60 min (**Figure 6.3 A and B**). The PI3K/Akt downstream substrate, GSK3-(α/β), was also investigated. Serum-starved RASM cells were stimulated for up to an hour with AngII (100 nM) or ET1 (50 nM). Total cell lysates were then blotted for the phosphorylated GSK3-α and β isoforms. GRK2 used as a loading control. As shown in **Figure 6.4**, stimulation of RASM cells with AngII or with ET1 induced robust phosphorylation of both GSK3-α and GSK3-β proteins (**Figure 6.4 A and B**). Pre-incubation with LY294002 (20 μM, for 30 min) showed that inhibition of PI3K attenuates AngII and ET1 induced GSK3-α isoform phosphorylation (**Figure 6.5 B and E**). Moreover, a marked reduction in AngII and ET1 induced GSK3-β phosphorylation was also observed (**Figure 6.5 C and F**). These results confirmed that AngII and ET1 activate PI3K/Akt downstream signalling which could be involved in vasoconstrictors-induced VCMC proliferation.

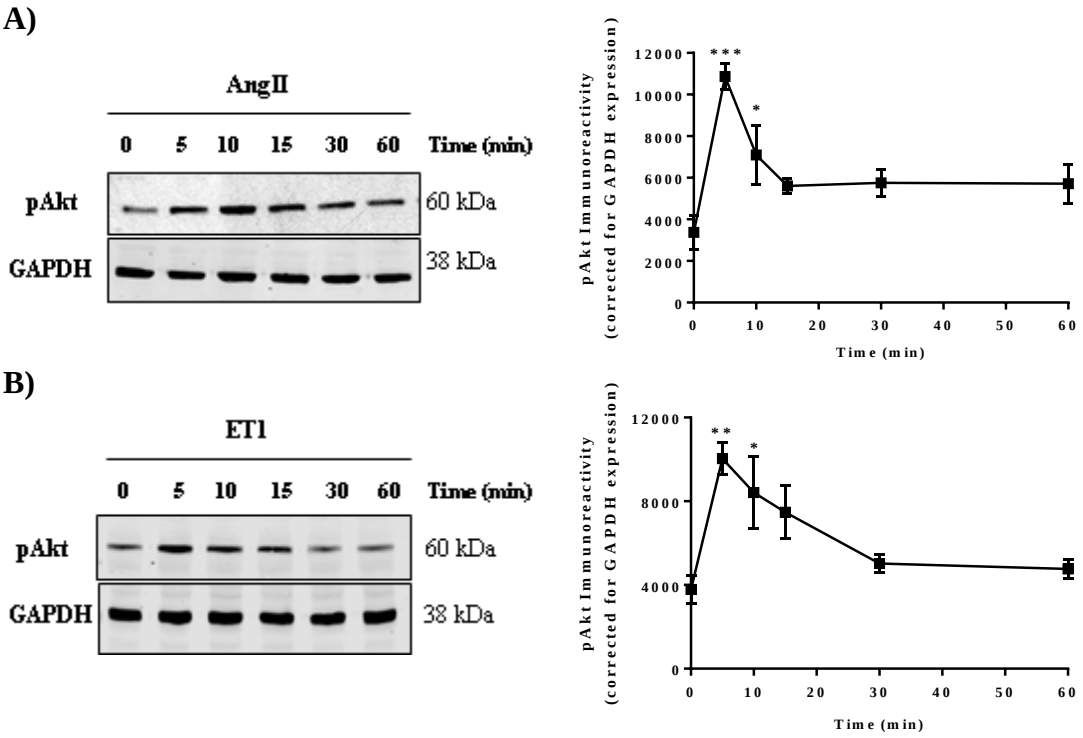


Figure 6.3 Temporal profile of AngII- and ET1-stimulated Akt phosphorylation in RASM cells.

Confluent serum-starved (24 h) RASM cells were treated with AngII (100 nM) or ET1 (50 nM) for the indicated times. RASM cells were then lysed and pAkt and GAPDH expression determined by western blotting. Representative immunoblots (left panel) and cumulative data (right panel) show that stimulation of RASM with AngII (A) or ET1 (B) resulted in Akt phosphorylation. Line graphs (right panel) are representative of densitometric analyses of the pAkt band by Image J and normalized to loading control for 4 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to values in control (basal) are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (one-way ANOVA, Dunnett's *post-hoc* test).

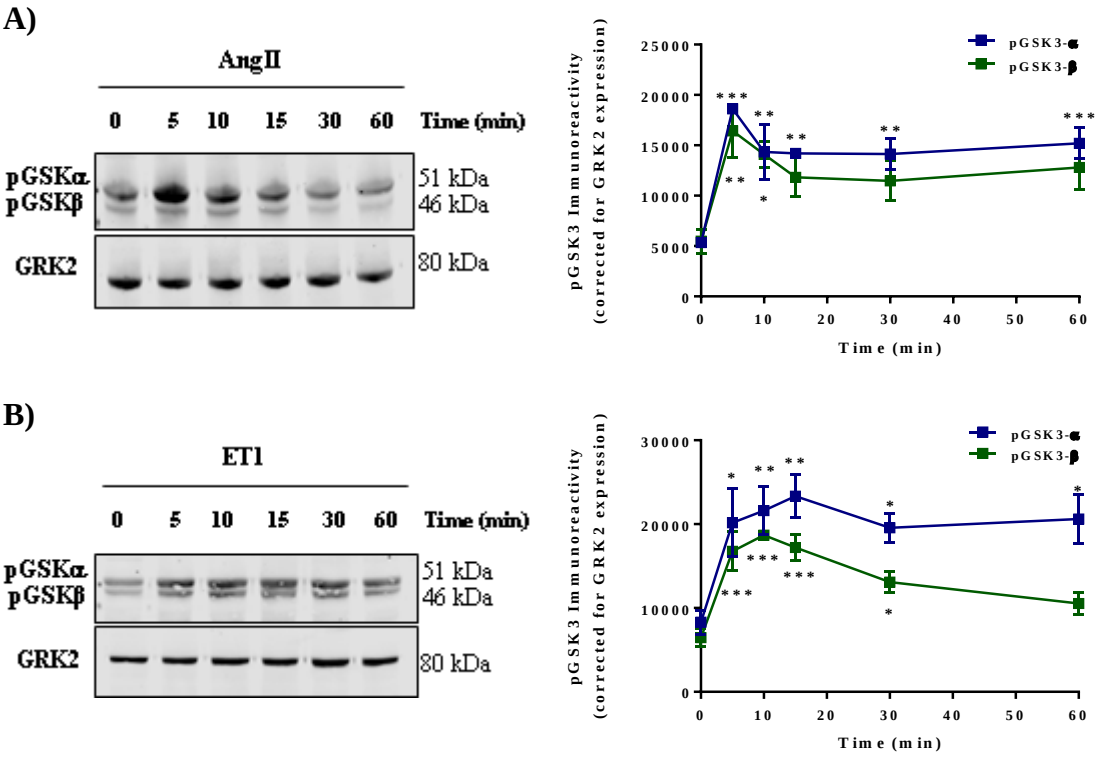


Figure 6.4 Temporal profile of AngII- and ET1-stimulated GSK3 phosphorylation in RASM cells.

Confluent serum-starved (24 h) RASM cells were treated with AngII (100 nM) or ET1 (50 nM) for the indicated times. RASM cells were then lysed and pGSK3-α, pGSK3-β and GRK2 expression determined by western blotting. Representative immunoblots (left panels) and cumulative data (right panels) show that stimulation of RASM cells with AngII (A) or ET1 (B) resulted in GSK3-α and GSK3-β phosphorylation. Line graphs (right panel) are representative of densitometric analyses of pGSK3-α and β isoform bands by Image J and normalized to loading control for 4 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to values in control (basal) are shown as; $^{*}P<0.05$, $^{**}P<0.01$ (one-way ANOVA, Dunnett's *post-hoc* test).

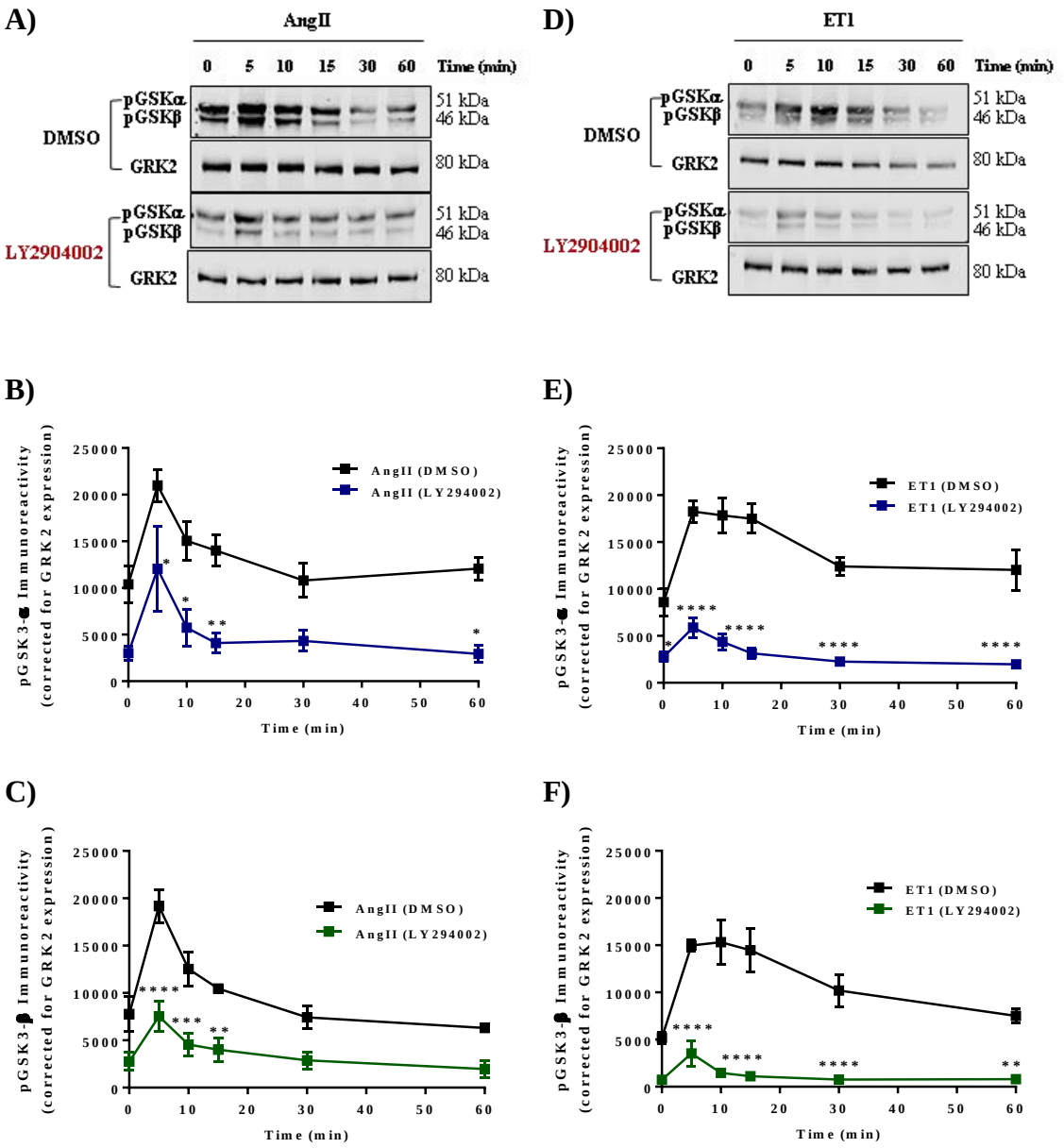


Figure 6.5 AngII and ET1 induce phosphorylation of GSK3-α and β isoforms through a PI3K-dependent mechanism.

Confluent serum-starved (24 h) RASM cells were pre-incubated with LY294002 (20 μM; 30 min) prior to stimulation with AngII (100 nM) or ET1 (50 nM) for the indicated times. RASM cells were then lysed and pGSK3-α, pGSK3-β and GRK2 expression determined. Representative immunoblots for AngII (A) and ET1 (D) and cumulative data for AngII (B and C) and ET1 (E and F) show the attenuation of the temporal profile of AngII and ET1-mediated GSK3-α and GSK3-β phosphorylation, respectively. Line graphs are representative of densitometric analyses of pGSK3-α and β isoform bands

by Image J and normalized to loading control for 4 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in (DMSO) control cells are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ (two-way ANOVA, Sidak's *post-hoc* test).

6.3.3. Suppression of GRK2 expression attenuates AngII- and ET1-induced Akt phosphorylation

Both PI3K (*Section 6.3.1* and *6.3.2*) and GRK2 (*Section 4.3.4*) appear to play vital roles to facilitate vasoconstrictor-stimulated RASM cell growth. Furthermore, GRK2 is reported to interact with PI3K (Naga Prasad et al., 2002a), which suggests that GRK2 could be regulating PI3K recruitment to AT_1 and ET_A receptor-induced Akt signalling in RASM cells. To examine this hypothesis, the effects of GRK2 protein expression on the temporal profiles of vasoconstrictor-induced PI3K/Akt signalling was determined. Briefly, GRK2 expression was depleted using anti GRK2 siRNA by nucleofection as described previously in Methods (*Section 2.2.4*). Serum-starved cells, 48 h post transfection, were stimulated with AngII (100 nM), ET1 (50 nM) or UTP (100 µM) for up to 60 min. Total cell lysates were analysed by western blot with anti-pAkt (T308) antibody and anti-GAPDH antibody as a loading control. Immunoblotting illustrated that stimulation of RASM with AngII, ET1 or UTP resulted in a marked increase in Akt phosphorylation that peaked at 5 min followed by gradual decline and sustained pAkt protein levels were observed between 10 and 60 min (**Figure 6.6 and Figure 6.7**). Quantitative analysis of the pAkt temporal profiles generated by AngII and ET1, revealed that depletion of GRK2 expression did not affect the peak phase (5 min), but resulted in a significant reduction in the prolonged phase response of Akt activation (**Figure 6.6 A and B**). In addition, depletion of GRK2 did not affect UTP-stimulated Akt phosphorylation (**Figure 6.7**). This finding highlights the importance of GRK2 expression in the sustained Akt activation induced by pro-proliferative vasoconstrictors (i.e. AngII and ET1).

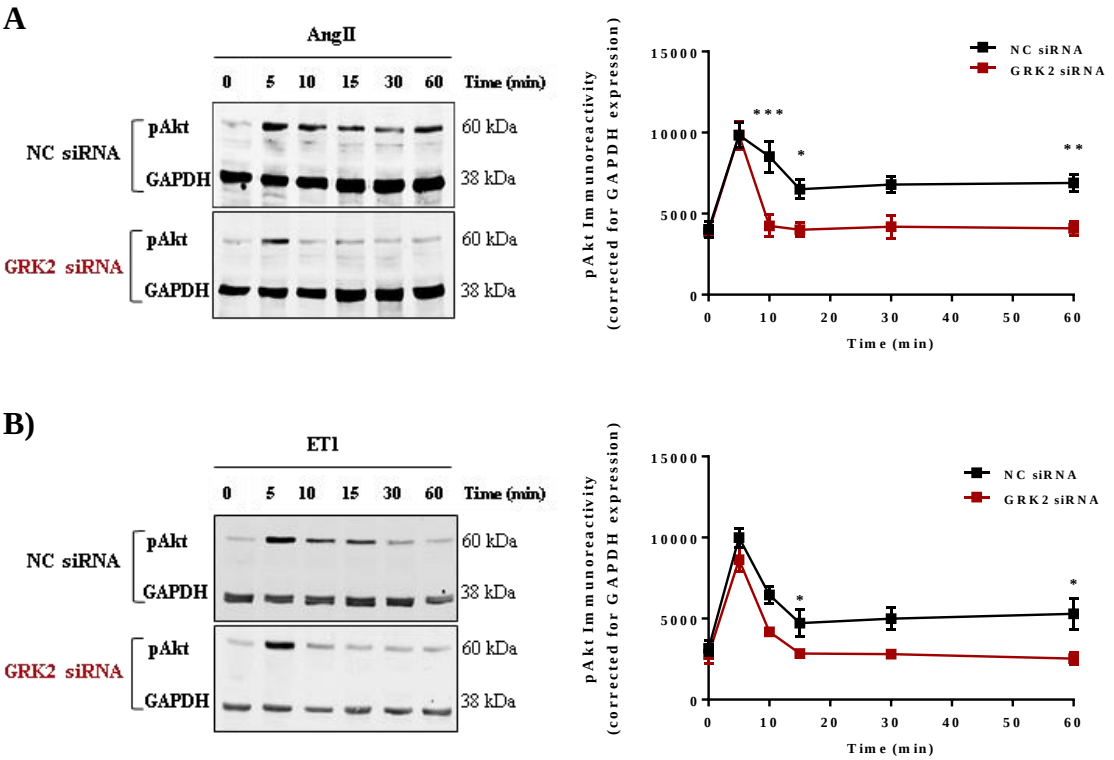


Figure 6.6 GRK2 siRNA attenuates AngII- and ET1-induced sustained phase of Akt phosphorylation.

RASM cells were transfected with either NC or anti-GRK2 siRNA (10 nM). After 24 h, cells were serum-starved for a further 24 h before addition of AngII (100 nM) or ET1 (50 nM) for the indicated times. Cells were lysed and immunoblotted for pAkt and GAPDH as described previously. Representative immunoblots (left panels) and cumulative data (right panels) show the effects of GRK2 knockdown on AngII (A) or ET1 (B) stimulated Akt phosphorylation. Line graphs (right panels) are densitometric analyses of pAkt immunoreactivity by Image J and normalized to loading control for ≥6 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in negative-control siRNA nucleofected cells are shown as; $^{*}P<0.05$, $^{***}P<0.001$ (two-way ANOVA, Sidak's *post-hoc* test).

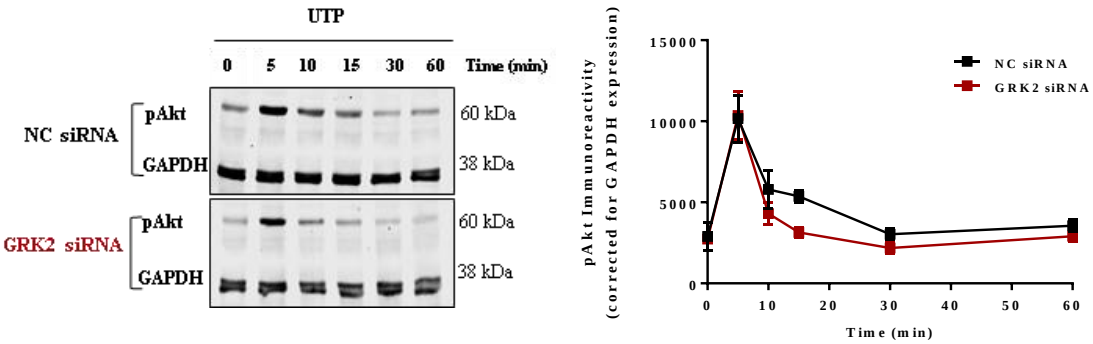


Figure 6.7 Depletion of GRK2 does not affect UTP-induced Akt activation.

RASM cells were transfected with either NC or anti-GRK2 siRNA (10 nM). After 24 h, cells were serum-starved for a further 24 h before addition of UTP (100 µM) for the indicated times. Cells were lysed and immunoblotted for pAkt and GAPDH as described previously. Representative immunoblots (left panel) and cumulative data show the effects of GRK2 knockdown on UTP-stimulated Akt phosphorylation. Line graphs (right panel) are densitometric analyses of pAkt immunoreactivity by Image J normalized to the loading control for ≥6 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM (two-way ANOVA, Sidak's *post-hoc* test).

6.3.4. Does GRK2 knockdown affect AngII- or ET1-induced GSK3 phosphorylation?

Since the expression of GRK2 appears to be vital to enable the sustained phase of AngII and ET1-stimulated Akt signalling (*Section 6.3.3*), and Akt is able to phosphorylate GSK3, it is likely that suppression of GRK2 expression may also affect agonist-induced GSK3 phosphorylation. To investigate this hypothesis, the profiles of AngII and ET1-stimulated GSK3-α and β phosphorylation in GRK2-depleted RASM cells were examined. Briefly, GRK2 expression was suppressed using anti GRK2 siRNA by nucleofection as described previously in Methods (*Section 2.2.4*), and assayed and immunoblotted for GSK3 phosphorylation as described earlier (*Section 6.3.2).* Stimulation of NC siRNA transfected cells with AngII or ET1 induced a similar pattern of GSK3-α and β phosphorylation as that observed in non-transfected cells (*Section 6.3.2*). Depletion of GRK2 level resulted in a significant reduction in AngII induced GSK3-α and GSK3-β phosphorylation (**Figure 6.8 A-C**). Furthermore, knockdown of GRK2 expression caused a significant reduction in ET1-induced GSK3-α and GSK3-β phosphorylation (**Figure 6.8 D-F**). These results confirm that GRK2 expression plays an important role in PI3K/Akt downstream signalling to GSK3.

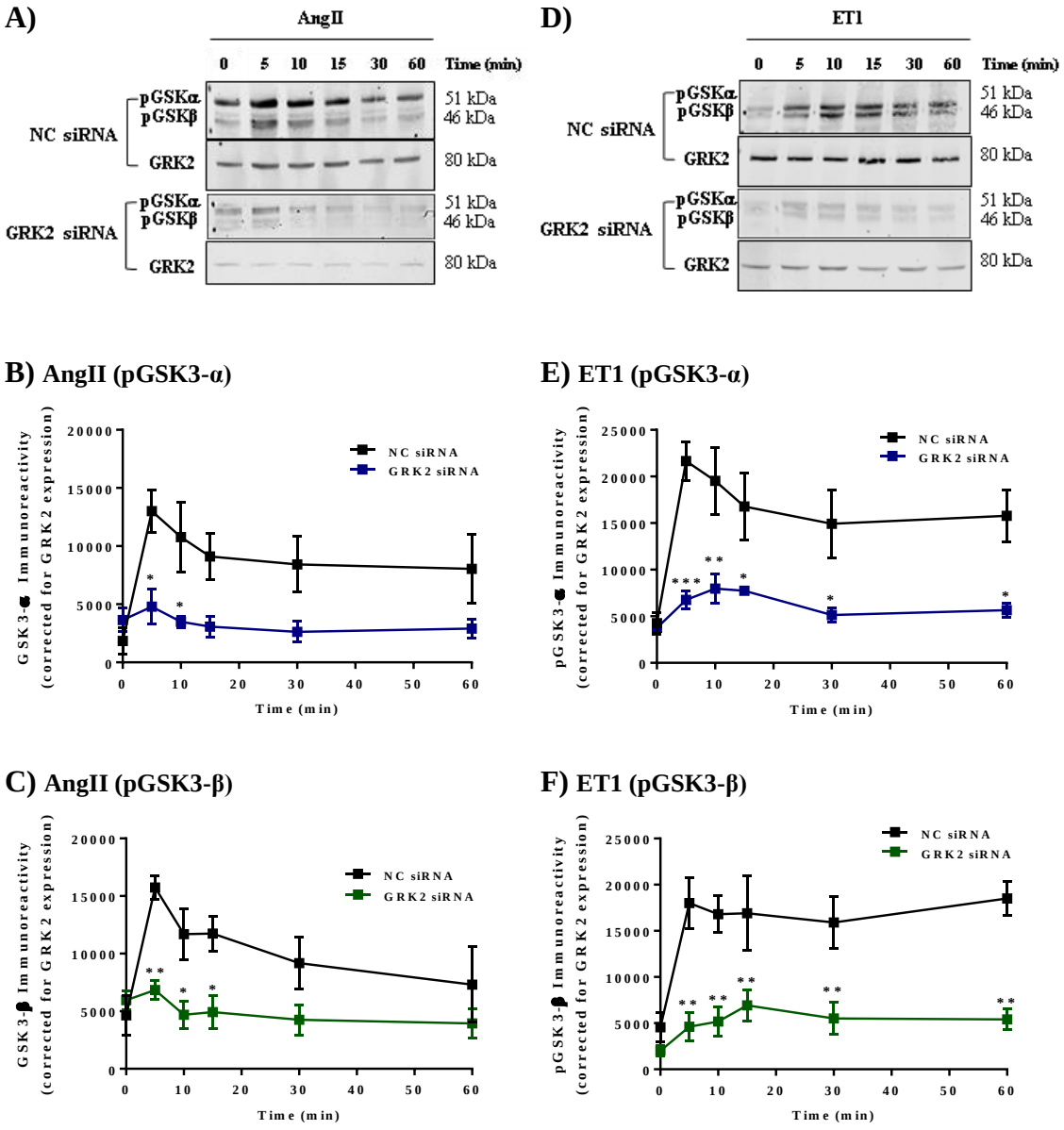


Figure 6.8 GRK2 depletion attenuated AngII- and ET1-induced GSK3 (α/β) phosphorylation.

RASM cells were transfected with either NC or anti-GRK2 siRNA (10 nM). After 24 h, cells were serum-starved for a further 24 h before addition of AngII (100 nM) or ET1 (50 nM) for the indicated times. Cells were lysed and immunoblotted for pGSK3-α, pGSK3-β and GRK2 expression. Representative immunoblots for AngII (A) and ET1 (D) and cumulative data for AngII (B and C) and ET1 (E and F) show the attenuation in the temporal profile of AngII and ET1-mediated GSK3-α and GSK3-β phosphorylation respectively. Line graphs are representative of densitometric analyses of pGSK3-α and β isoform bands by Image J and normalized to the loading control for

4 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in negative-control siRNA nucleofected cells are shown as; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (two-way ANOVA, Sidak's *post-hoc* test).

6.3.5. COMP101 does not affect AngII, ET1 or UTP induced PI3K/Akt activation

Initial data confirmed that inhibition of GRK2 catalytic activity was essential to enable vasoconstrictor-stimulated VSMC proliferation (*Section 4.3.3 and 4.3.5*), and was also vital for the prolonged phase of AngII and ET1-stimulated ERK signalling (*Section 5.3.3*). Therefore, to examine the possibility that GRK2 activity may also be required to facilitate vasoconstrictor induced PI3K/Akt signalling, confluent serum-starved RASM cells were pre-incubated with COMP101 (30 μM, 30 min) before stimulation with AngII (100 nM), ET1 (50 nM) or UTP (100 μM). Total cell lysates were analysed by western blot with an anti-pAkt antibody and anti-GAPDH antibody as a loading control. Stimulation of vehicle-treated (DMSO) RASM cells with AngII or ET1 induced a peak of Akt phosphorylation at 5 min, which declined to a more sustained plateau phase for up to 60 min (**Figure 6.9 A and B**) and was similar to previous findings in NC siRNA-treated cells (*Section 6.3.3*). Pre-treatment of RASM cells with COMP101 had no effect upon AngII, ET1 or UTP induced Akt phosphorylation (**Figure 6.9 A, B and C),** indicating that GRK2 catalytic activity does not affect PI3K/Akt signalling.

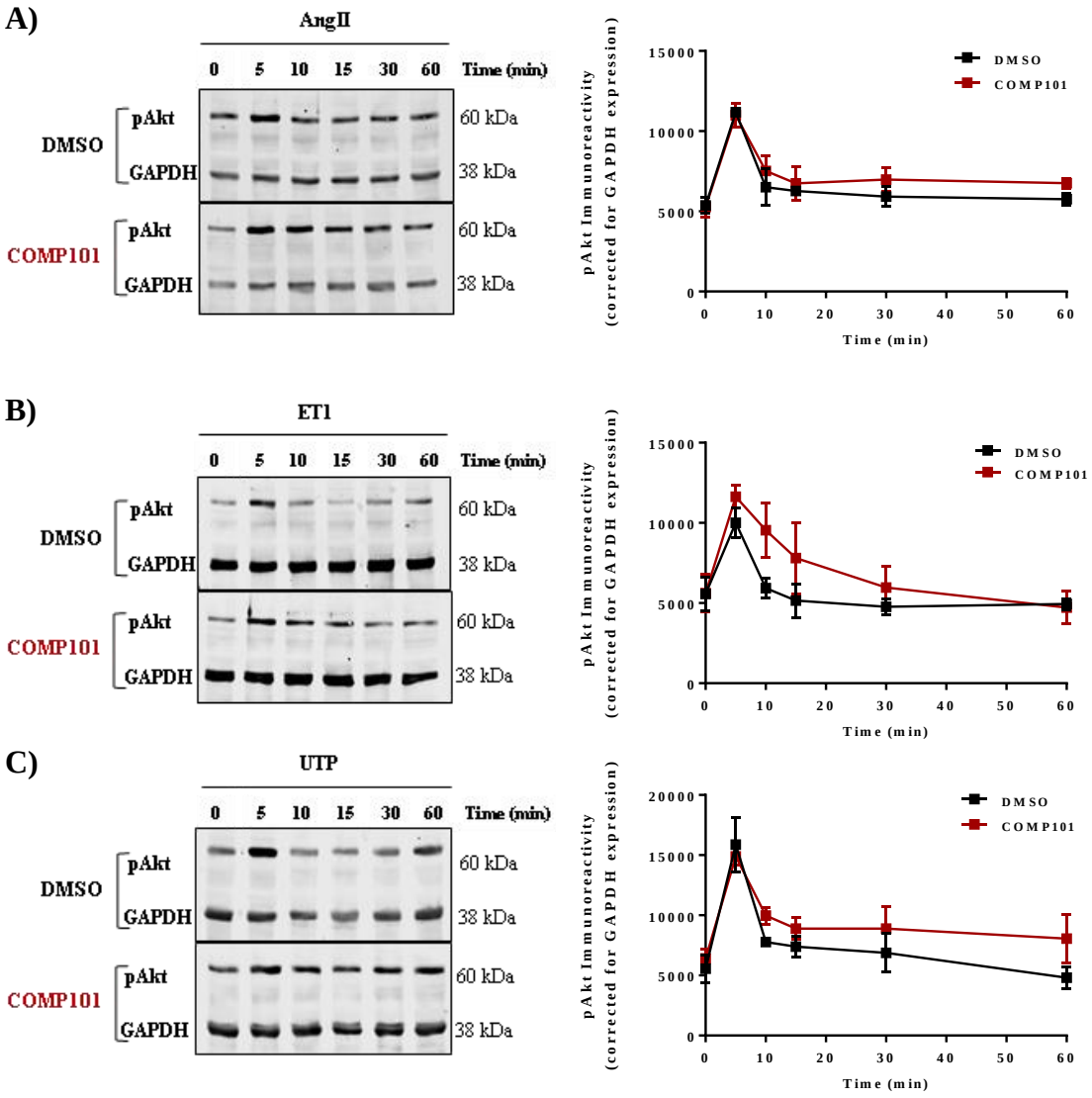


Figure 6.9 Inhibition of GRK2 activity does not affect AngII, ET1 or UTP induced Akt phosphorylation.

Confluent serum-starved RASM cells were pre-incubated with vehicle control or the GRK2 inhibitor COMP101 (30 μM) for 30 min, before stimulation with AngII (100 nM), ET1 (50 nM) or UTP (100 μM) for the indicated times. After agonist challenge, cells were lysed and western blotted with anti-pAkt and anti-GAPDH antibodies. Representative immunoblots (left panels) and cumulative data (right panels) show the effects of COMP101 on AngII (A), ET1 (B) and UTP (C) stimulated Akt phosphorylation. Cumulative data are representative of densitometric analyses of pAkt immunoreactivity using Image J and normalized to the loading control for ≥4

independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM (two-way ANOVA, Sidak's *post-hoc* test).

6.3.6. Does GRK6 affect ET1-induced Akt activation?

In order to investigate whether the effect of AngII and ET1 on pAkt is GRK2 specific, the effect of vasoconstrictor (ET1) on PI3K/Akt signalling was assessed in GRK6 depleted cells. Briefly, GRK6 expression was selectively depleted in RASM cells using nucleofection with a previously characterised anti-GRK6 siRNA (Nash et al., 2018), as described in Methods (*Section 2.2.4*). Twenty four hours post-nucleofection, cells were serum-starved for a further 24 h before being stimulated with ET1 (50 nM) and the temporal profile of Akt phosphorylation determined. Depletion of GRK6 had no effect upon ET1-stimulated Akt phosphorylation (**Figure 6.10**). These results indicate that GRK6 does not play a role in ET1-induced PI3K/Akt signalling.

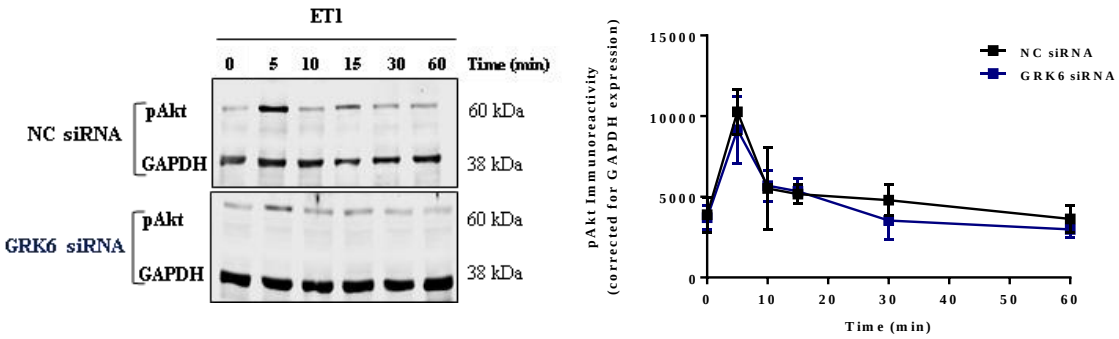


Figure 6.10 Depletion of GRK6 does not affect ET1-induced Akt activation.

RASM cells were transfected with either NC or anti-GRK6 siRNA (10 nM). After 24 h, cells were serum-starved for a further 24 h before stimulation with ET1 (50 nM) for the indicated times. Cells were lysed and immunoblotted for pAkt and GAPDH. Representative immunoblots (left panel) and cumulative data (right panel) show the effects of GRK6 knockdown on the temporal prolife of ET1-stimulated Akt signalling in RASM cells. Data (right panel) are representative of densitometric analyses of pAkt band by Image J and normalized to loading control for 4 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM (two-way ANOVA, Sidak's *post-hoc* test).

6.3.7. Are arrestin2 and/or arrestin3 essential for AngII-stimulated Akt activation?

A variety of studies suggest that arrestins can regulate AngII and ET1 stimulated Akt signalling (Cianfrocca et al., 2010; Kendall et al., 2014). Therefore, I next tested whether arrestin2 and arrestin3 are necessary for AngII-induced PI3K/Akt signalling. To determine whether arrestin2 and/or arrestin3 mediate AngII-induced Akt phosphorylation, arrestin2 and arrestin3 expression were depleted using previously characterised anti-arrestin2 and anti-arrestin3 siRNAs (Morris et al., 2012), as described in Methods (*Section 2.2.4*). Serum-starved RASM cells (72 h post-nucleofection) were stimulated with AngII (100 nM) for up to 60 min and total cell lysates were blotted for pAkt and GAPDH. Stimulation with AngII (**Figure 6.11**), caused an increase in Akt phosphorylation that peaked at 5 min followed by a gradual decline and sustained pAkt level over the 60 min time course. Depletion of arrestin2 or arrestin3 did not affect pAkt activation (**Figure 6.11**). These finding indicating that arrestin2 and arrestin3 had no effect AngII-stimulated PI3K/Akt signalling.

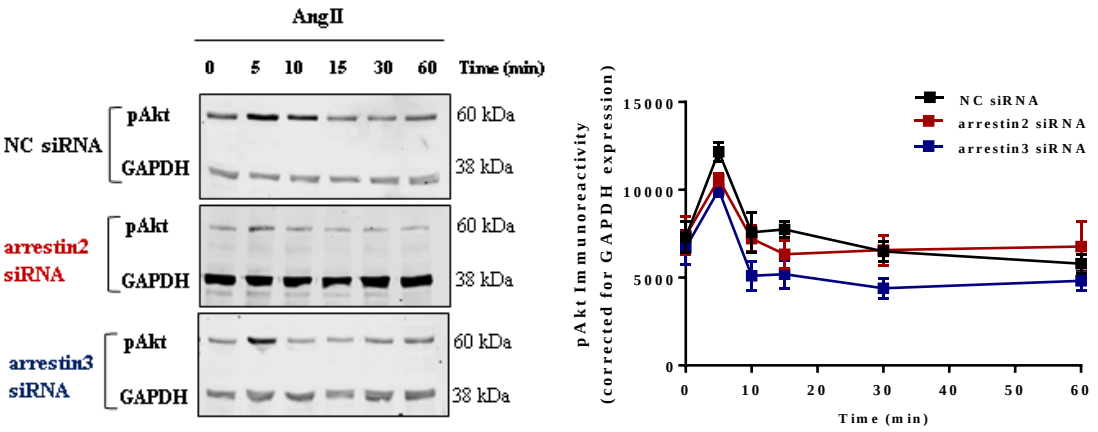


Figure 6.11 Effect of arrestin2 and arrestin3 on AngII-stimulated Akt activation.

Serum-starved anti-arrestin2, anti-arrestin3 or NC siRNA treated RASM cells were stimulated with AngII (100 nM) for the indicated time periods. Total lysates were analysed by western blot with anti-pAkt and anti-GAPDH antibodies. Representative immunoblots (left panel) show the temporal prolife of AngII-stimulated Akt phosphorylation. Cumulative data show the effects of arrestin2 or arrestin3 (right panel) depletion on AngII-induced Akt signalling. Data are representative of densitometric analyses of pAkt bands by Image J and normalized to the loading control for 4 independent experiments, each using RASM cells prepared from a different animal. Data are shown as mean ± SEM, statistically significant changes compared to respective agonist-stimulated values in control cells are shown as; $^{**}P<0.01$, $^{***}P<0.001$ (two-way ANOVA, Sidak's *post-hoc* test).

6.4. Discussion

Prior studies have noted that the PI3K/Akt signalling pathway is implicated in VSMC proliferation and cardiovascular diseases (Muto et al., 2007; Liu et al., 2010a). The involvement of PI3K/Akt in cardiovascular disease was suggested from studies in which enhanced PI3K ($p110_{\gamma}$ and $p110_{\beta}$) activity and expression were observed in aortae of hypertensive rats (Northcott Carrie et al., 2002). Furthermore, PI3K has been reported to mediate VSMC proliferation and arterial wall thickening after balloon injury (Shigematsu et al., 2000). In addition, $PI3K_{\gamma}$ deficiency protects against AngII-mediated mesenteric arterial wall structural remodelling in $PI3K_{\gamma}^{-/-}$ mice (Vecchione et al., 2005), and inhibition of PI3K significantly reduced VSMC proliferation and neointimal formation in the mouse femoral artery injury model (Fang et al., 2018). Based on published findings, the PI3K downstream mediator Akt acts as a potent promotor of cell survival and proliferation (Fernandez-Hernando et al., 2009). Indeed, enhanced Akt activity is also associated with AngII-induced hypertension (Ljuca et al., 2001). Moreover, inhibition of Akt signalling with a dominant negative Akt mutant (AA-Akt) effectively attenuated G_1/S transmission, DNA synthesis and proliferation of VSMC (Stabile et al., 2003). Additionally, stenting-associated mechanical stretch activates the PI3K-Akt-GSK3 signalling pathway and induces neointimal formation in the stented rat aorta (Zhou et al., 2003). Overall, these data emphasise the important role that the PI3K/Akt signalling plays in VSMC proliferation and vascular remodelling.

GPCR activation of the PI3K/Akt pathway is achieved via activation of $PI3K_{\gamma}$ ($p110_{\gamma}$) by the $G_{\alpha q}$ subunit (Murga et al., 1998; Xie et al., 2000) and by βγ subunits (Stoyanov et al., 1995; Stephens et al., 1997). Indeed, Liu et al. (2003) reported that $ET1/ET_B$-mediated NO production through βγ subunits recruitment and activation of PI3K with downstream phosphorylation of Akt (Liu et al., 2003). Furthermore, infection of endothelial cells with an adenovirus expressing the carboxyl terminus of GRK2 (GRK2CT), which binds to βγ subunits and prevents their downstream signalling (Koch et al., 1993), abolished ET1-induced Akt phosphorylation (Liu et al., 2003). The ability of GPCRs to activate PI3K was further confirmed by our finding, which shows that AngII, ET1 and UTP all induced PI3K/Akt signalling pathway activation. Stimulation of RASM cells with AngII and/or ET1 activates Akt producing a peak at 5 min followed by a sustained phase of pAkt between 10 and 60 min. These results are supported by the previous observations that Akt was phosphorylated after a 5 min stimulation with AngII

(Ushio-Fukai et al., 1999) or ET1 in VSMC (Daou and Srivastava, 2004). Furthermore, our findings show that AngII/AT_1 and ET1/ET_A phosphorylate PI3K/Akt downstream substrates GSK3-α and GSK3-β. In accordance with the present results, previous studies have demonstrated that PI3K/Akt signalling can be activated by AngII and ET1 in VSMC (Takahashi et al., 1999; Huang et al., 2011; Jia et al., 2012). Hence, these data suggested that the PI3K/Akt pathway is involved in vasoconstrictor-driven VSMC proliferation. This idea was further confirmed by the data from [^{3}H]-thymidine incorporation assay, which showed that LY294002, a potent PI3K inhibitor (Vlahos et al., 1994), attenuated AngII, ET1 and NA induced cell proliferation. This finding is in line with earlier reports showing that inhibition of PI3K attenuates GPCR-stimulated VSMC proliferation (Saward Peter Zahradka, 1997; Liu et al., 2010a).

Although GSK3 exists as two closely related isoforms GSK3-α and GSK3-β, each isoform displays distinct roles in many diverse cellular processes and physiological functions (Lee et al., 2007; Pardo et al., 2016). GSK3-α has been reported to regulate inflammatory responses (Silva-García et al., 2018), hepatic glucose metabolism (MacAulay et al., 2007) and glycogen metabolism in cardiac myocytes (Mora et al., 2005). While GSK3-β has been reported play roles in regulating cardiac myocyte growth and development (Haq et al., 2000; Hardt and Sadoshima, 2002; Michael et al., 2004), and participates in hippocampal neural precursor cell proliferation (Pardo et al., 2016). Moreover, knockout of GSK3-β led to embryonic lethality in GSK3-$\beta^{-/-}$ mice (Hoeflich et al., 2000). It has been revealed that Akt, a major upstream regulator of GSK3, controls cell cycle progression via GSK3-β inactivation (Liang and Slingerland, 2003). Such evidence suggests that GSK3-β isoform may participates in vasoconstrictors-stimulated cell proliferation as a down-stream modulator from PI3K/Akt pathway. This idea was further confirmed by findings that the PI3K inhibitor, LY294002, blocked AngII and ET1-induced GSK3-β phosphorylation. This is consistent with other studies which showed that LY294002 abolished Akt phosphorylation as well as downstream GSK3 phosphorylation in VSMC (Ushio-Fukai et al., 1999; Zhou et al., 2003; Rahmani et al., 2005). Collectively, these data strongly suggest that PI3K-Akt-GSK3 signalling plays a fundamental role in vasoconstrictor-stimulated VSMC proliferation.

As previously stated, GRK2 expression is upregulated in hypertension (Eckhart et al., 2002; Willets et al., 2015), a process regulated by a complex interaction of diverse signal transduction pathways (Ramos-Ruiz et al., 2000; Penela et al., 2010b).

Interestingly, PI3K/Akt activation diminishes GRK2 degradation (Salcedo et al., 2006), suggesting that enhanced PI3K/Akt activity in hypertension (Ljuca et al., 2001; Northcott Carrie et al., 2002) could contribute to the up-regulation of GRK2 expression observed in hypertension. Moreover, GRK2 expression and its activity are required for VSMC growth (see *Chapter 4*) which combined with reports that GRK2 interacts directly with PI3K (Naga Prasad et al., 2002a; Robinson and Pitcher, 2013), suggests that GRK2 could be regulating PI3K recruitment to AngII/AT_1 and ET1/ET_A induced Akt signalling in VSMC. Here, I propose that GRK2 plays an essential role in the PI3K/Akt signalling process to mediate proliferation. Furthermore, I hypothesise that increased GRK2 expression in hypertension may participate in VSMC proliferation, vascular wall thickening, and ultimately hypertensive vascular remodelling via PI3K-Akt-GSK3 activation. The effect of GRK2 on AngII, ET1 and UTP induced Akt activation have been investigated in GRK2-depleted RASM cells. Consistent with previous findings (Nash et al., 2018), anti-GRK2 siRNA knockdown reduced GRK2 expression by ~70-80% without affecting other non-targeted GRK expression (see *Section 4.3.6*). siRNA knockdown of GRK2 did not affect the peak phase (5 min) of Akt activation, but a significantly reduced in the prolonged phase response to AngII and ET1, but not UTP. These findings highlight the importance of GRK2 expression in the sustained Akt activation induced by pro-proliferative vasoconstrictors (i.e. AngII and ET1). To further validate the potential role of GRK2 expression in AngII and ET1 stimulated PI3K/Akt signalling activation, I examined the effect of GRK2 knockdown on GSK3-β as a downstream mediator from PI3K/Akt. Depletion of GRK2 expression resulted in a marked reduction in AngII and ET1 induced GSK3-β phosphorylation. These results confirm that GRK2 expression plays an important role in PI3K/Akt downstream signalling to GSK3-β. Therefore, the process of AngII/AT_1 and ET1/ET_A induced sustained PI3K/Akt signalling activation appears to be GRK2 dependent. This finding is consistent with that of Schlegel et al. (2017) who showed that AngII-promoted cardiac myocyte hypertrophy is mediated via GRK2-PI3K$_\gamma$-Akt pathway activation and subsequently inactivation of GSK3-β, also that GRK2-PI3K$_\gamma$ interaction was enhanced following GRK2 overexpression. Furthermore, they revealed that PI3K$_\gamma$-Akt pathway activation was enhanced by GRK2 overexpression and attenuated by GRK2 depletion in neonatal rat ventricular cardiac myocytes (Schlegel et al., 2017). Moreover, elevated GRK2 expression is associated with enhanced PI3K activity, particularly the PI3K$_\gamma$ isoform, in the porcine heart failure model (Perrino et al., 2005). Consistent with this notion, Nogues et al., (2016) noted that GRK2 overexpression

correlated with Akt activation in breast cancer cells (Nogues et al., 2016). These studies highlight a positive relationship between GRK2 expression and PI3K/Akt activation, and together with our results, suggest a strong relationship between GRK2 expression and PI3K-Akt-GSK3 activation.

Additionally, the findings in this chapter revealed that inhibition of GRK2 activity with COMP101 resulted in a slight (non-significant) increase on AngII, ET1 or UTP induced Akt activation. This result may be explained by the fact that activation of AT_1, ET_A and $P2Y_2$ receptors recruits GRK2 (Kim et al., 2009; Morris et al., 2010; Morris et al., 2011), consequently inhibition of GRK2 catalytic activity resulted in increased AT_1, ET_A and $P2Y_2$ receptor activity, and enhanced Akt signalling via G protein-dependent mechanism. Thus, GRK2 catalytic activity is not required for vasoconstrictor-induced prolonged Akt phosphorylation which indicates that the involvement of GRK2 in the PI3K/Akt signalling cascade is via phosphorylation-independent mechanisms. Moreover, as GRK2 interacts directly with PI3K (Naga Prasad et al., 2002a; Robinson and Pitcher, 2013), the results provide evidence for the function of GRK2 expression as a scaffold involved in AngII/AT_1 and ET1/ET_A mediated PI3K/Akt signalling. Thus, I propose a model in which AngII/AT_1 and ET1/ET_A stimulation would cause $G_{\beta\gamma}$-dependent translocation of GRK2 to the plasma membrane (Tesmer et al., 2005) and GRK2-mediated recruitment and interaction with PI3K (Naga Prasad et al., 2001). GRK2-PI3K interaction enables phosphorylation of downstream targets Akt and GSK3 resulting in activation of VSMC proliferation process. This model predicts that alterations in GRK2 protein expression would alter GRK2-dependent PI3K/Akt signalling. Therefore, increased expression of GRK2 in hypertension could be a key to enhancing PI3K/Akt signalling and thus increased VSMC proliferation. In future investigations, to further validate the idea of a positive relationship between GRK2 expression and PI3K/Akt activation in VSMC, it might be possible to examine the effect of GRK2 over-expression on AngII and ET1-stimulated PI3K/Akt signalling activation, which could be achieved by infection of RASM cells with adenoviral GRK2 constructs or using aortic smooth muscle cells isolated from SHR, in which GRK2 expression is already elevated. Additionally, in this context, further experiments are needed to identify the potential interaction between GRK2 and PI3K, and how this interaction influences Akt activation in response to pro-proliferative vasoconstrictor stimuli.

In contrast, to the data indicating a positive relationship between GRK2 expression and PI3K/Akt activation in VSMC, previous reports suggest that GRK2 plays a negative role in modulating endothelial cell function and endothelium-dependent relaxation via its effect on Akt/endothelial nitric oxide synthase (eNOS) signalling (Taguchi et al., 2011; Taguchi et al., 2012; Taguchi et al., 2014). Furthermore, GRK2 has been shown to directly interact with Akt and inhibit eNOS generation in cardiac myocytes (Brinks et al., 2010; Huang et al., 2013). It has been reported that elevated GRK2 expression and activity attenuates Akt phosphorylation and reduces eNOS activity in diabetic mouse aortae (Taguchi et al., 2011). These contradictory results may highlight alternative regulatory processes in different cell types, e.g. endothelial cells or cardiac myocytes, and/or changes in signalling patterns under different pathological conditions. Even though these studies show a negative effect of GRK2 on Akt activation, they indicate that GRK2 is a central player in PI3K/Akt signalling. Taguchi et al., (2011) reported that $G_{\beta\gamma}$ subunits mediate the membrane localization and activation of GRK2 (Taguchi et al., 2011), thereby supporting our hypothesis that GRK2 acts as scaffold for GPCR mediated PI3K/Akt signalling.

Published studies emphasise the importance of PI3K/Akt in the phosphorylation and inactivation of GSK3-α and GSK3-β by GPCRs agonists (Takahashi et al., 1999; Morisco et al., 2000). However, GSK3 may be regulated, phosphorylated and inactivated by other protein kinases (Beurel et al., 2015). For example, Moore et al., (2013) demonstrated that PKC and Akt regulate and phosphorylate GSK3-α and GSK3-β in thrombin-mediated (via the AP1 receptor) platelet activation (Moore et al., 2013). Even though the observed effects of AngII and ET1 on GSK3-α and GSK3-β are mediated through AT_1 and ET_A receptors, the involvement of PKC in GSK3-α, and GSK3-β phosphorylation may not be needed since our results show that inhibition of PI3K markedly attenuates both GSK3-α and GSK3-β isoforms phosphorylation. Additionally, similar effects were seen following GRK2 depletion, indicating that GSK3-α and GSK3-β isoform phosphorylation is mediated through the GRK2-PI3K-Akt pathway, not PKC. Furthermore, it has been reported that GRK2 regulates GSK3 via its effect on RTK downstream signalling (Usui et al., 2004). For example, GRK2 negatively regulates insulin-induced GSK3-α phosphorylation to regulate glycogen synthesis in liver cells (Shahid and Hussain, 2007). Therefore, in VSMC, GRK2 could be a novel regulator of GSK3-α as well as GSK3-β signalling stimulated through a GPCR or RTK-dependent

process. Thus, GRK2-dependent regulation of GSK3 in the response to various stimuli highlights GRK2 as a fundamental player in GSK3-mediated diverse cellular processes, physiological functions and pathological conditions.

Results from *Section 4.3.6* revealed that GRK6 depletion attenuated the ability of vasoconstrictors to induce Ki67 expression, suggesting that GRK6 may regulate VSMC proliferation. However, depletion of GRK6 had no effect upon ET1-promoted Akt activation indicating that GRK6 does not regulate ET1-stimulated PI3K/Akt signalling. Thus, the involvement of PI3K/Akt signalling in GRK6-mediated effects on Ki67 expression is unexpected and at present unexplained in VSMC. Even though several published reports show that GRK6 expression is correlated with cell proliferation in some types of cancer (Li, 2013; Xu et al., 2017; Che et al., 2018), there is no published evidence which investigates the relation between GRK6 expression and PI3K/Akt signalling. A further study with more focus on the effect of GRK6 on cell proliferation is therefore suggested.

Prior evidence suggests that arrestins can regulate AngII and ET1 stimulated Akt signalling (Cianfrocca et al., 2010; Kendall et al., 2014). Of particular interest in this regard, is that PI3K and Akt can be activated in an arrestin-dependent manner (Mehta and Griendling, 2007). Hence, arrestins might be involved in vasoconstrictor-mediated activation of GRK2-PI3K-Akt signalling in VSMC. To interrogate this hypothesis the effect of arrestins on AngII induced Akt activation were examined in arrestin2 and arrestin3 depleted VSMCs cells. Consistent with previous findings (Morris et al., 2012), anti-arrestin2 and anti-arrestin3 siRNA reduced targeted arrestin expression by ~70-80% (see *Section 5.3.5*). Even though arrestin3 has been shown to mediate AngII-stimulated Akt activation in VSMC (Ahn et al., 2009), our data show that arrestins depletion had no effect on Akt phosphorylation. However, it should be noted that in these experiments AngII-stimulated Akt phosphorylation curves were different, displaying an absence of the sustained phase of Akt signalling. Thus it is difficult to accurately determine whether depletion of arrestins played any role in Akt signalling. These differences can possible be explained by the fact that the arrestin experiments were conducted on higher passage cells, and subsequently we later discovered that younger RASM cells (passage 1-4) produced optimal results for PI3K/Akt/GSK3 studies. Therefore, in order to further verify the effect of arrestins on vasoconstrictors stimulated PI3K/Akt signalling, it would be prudent to repeat these experiments using a lower passage RASM cells.

Overall, this chapter illustrated three key aspects of PI3K/Akt signalling in VSMC. Firstly, that vasoconstrictors can activate PI3K-Akt-GSK3 signalling in VSMC, indicating that PI3K/Akt signalling participates in vasoconstrictor-induced VSMC proliferation. Secondly, these data highlight the essential requirement for GRK2 expression to enable pro-proliferative agonists such as AngII and ET1 to stimulate prolonged Akt signalling, thus identifying a novel molecular mechanism by which GRK2 expression can regulate RASM cell growth. To our knowledge, this is the first report documenting a mechanism linking GRK2 expression directly to vasoconstrictor-mediated Akt and GSK3 phosphorylation in VSMCs, thus we postulate that AngII and ET1 might stimulate VSMC proliferation via GRK2-PI3K-Akt-GSK3 signalling pathway (**Figure 6.12**). Thirdly, GRK2 catalytic activity had no effect on vasoconstrictor-stimulated Akt phosphorylation in VSMCs. Therefore, as GRK2 interacts directly with PI3K (Naga Prasad et al., 2002a; Robinson and Pitcher, 2013), GRK2 is likely acting as a scaffold for PI3K to enable AngII/AT_1 and ET1/ET_A mediated PI3K/Akt signalling. Therefore, it is probable that increased GRK2 expression observed in hypertension (Willets et al., 2015) may contribute to VSMC proliferation, vascular wall thickening, and ultimately hypertensive vascular remodelling via PI3K-Akt-GSK3 activation.

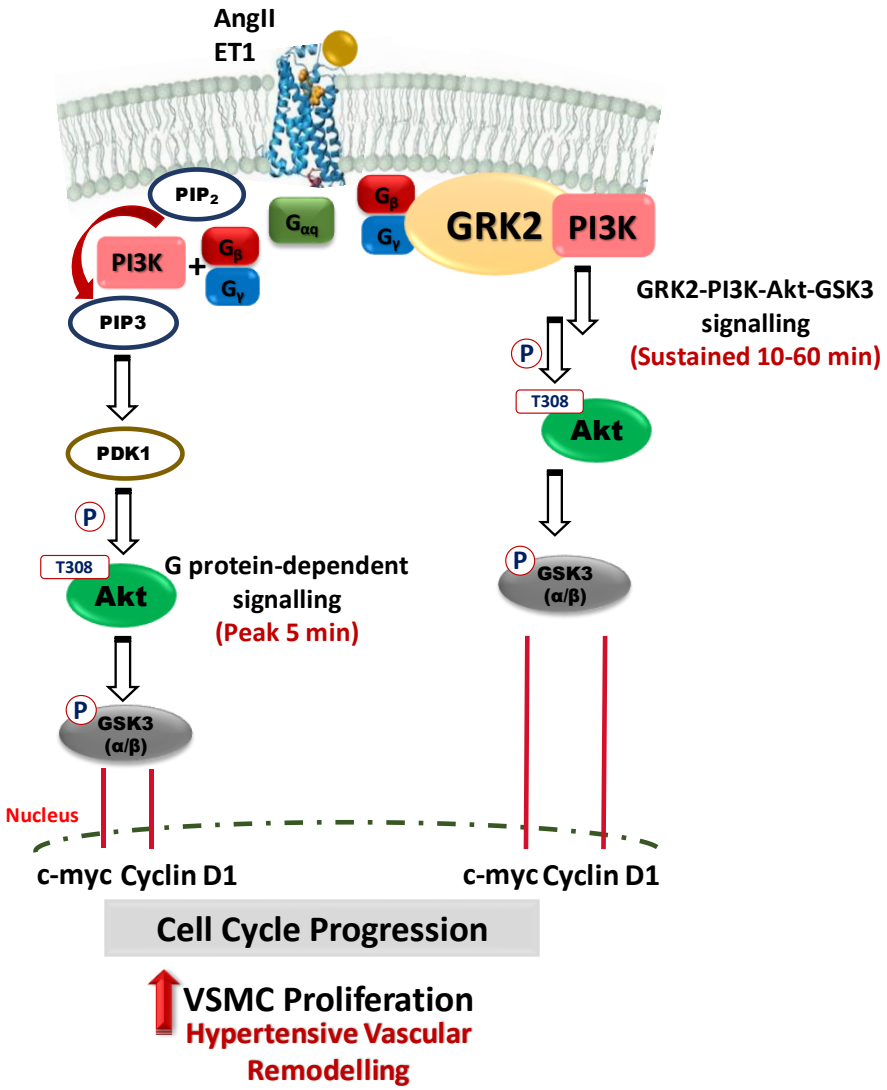


Figure 6.12 Hypothetical model for the possible role of GRK2 in AngII and ET1-mediated PI3K/Akt signalling in VSMC.

Suggested mechanisms by which AngII/AT_1 and ET1/ET_A mediate PI3K-Akt-GSK3 cascade activation. Peak (5 min) Akt activation possibly is G protein-dependent in where G protein subunits activate PI3K (Stephens et al., 1997; Murga et al., 1998), resulting in Akt activation, phosphorylation of GSK3 (inhibition) and thus the regulation of cell-cycle progression. GRK2 can directly interact with PI3K (Naga Prasad et al., 2002b), which mediates scaffolding for sustained Akt activation and GSK3 phosphorylation in a GRK2-PI3K-Akt-GSK3 dependent manner. PI3K, phosphoinositide 3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate lipid; PDK1, 3' phosphoinositide dependent-kinase 1; GSK3, glycogen synthase kinase 3.

Chapter Seven

7. Concluding discussion and future directions

7.1. Discussion

Pathological VSMC proliferation is a major contributor to arterial wall thickening and hypertensive vascular remodelling (De Mey, 1995; Nemenoff et al., 2011). The process of VSMC proliferation is regulated by a complex network of cellular signalling mechanisms (Wang et al., 2018a). Among the network of receptors and signalling molecules regulating vascular tone in hypertension, GPCRs function to modulate cell proliferation by transducing a wide range of signals that participate in VSMC growth processes and thus arterial wall remodelling (Hirata et al., 1989; Hahn et al., 1993; Qin et al., 2004; Lacolley et al., 2009). The currently available pharmacological options for prevention and treatment of hypertensive vascular remodelling include blockade of RAAS, which has been shown to provide some protection from hypertension-induced vascular remodelling and end-organ damage (Weir, 2007; Cohn and Goldman, 2008). However, pathological VSMC proliferation contributes to other cardiovascular diseases including the progression of atherosclerosis and development of restenosis after surgical intervention (Wang et al., 2018a). Therefore, prevention and treatment of pathological VSMC proliferation remains a significant clinical problem.

In VSMC GRK2 acts as a key GPCR signalling regulator, terminating G protein-dependent signalling, and initiating other G protein-independent signalling pathways (Reiter and Lefkowitz, 2006; Brinks and Eckhart, 2010). Indeed, mounting evidence highlights GRK2 as the key regulator of a number of vasoconstrictor GPCRs, including the α_{1D}-adrenoceptor, ET_A, AT_1 and $P2Y_2$ receptors (Mayor et al., 1998; Olivares-Reyes et al., 2001; Morris et al., 2010; Morris et al., 2011). Apart from the classical mechanism of modulating GPCR signalling, GRK2 has multiple functions independent of the classical GPCR desensitization process (Penela et al., 2010b). Accumulating evidence strongly suggests that GRK2 is able to interact with a diversity of cellular proteins implicated in signal transduction in a non-classical GPCR signalling manner (Penela et al., 2010b; Shenoy and Lefkowitz, 2011). In particular, these could be involved in sustained GPCR signalling. Of particular importance, enhanced GRK2 expression and altered functionality have been found in hypertension where abnormal GRK2 expression or activity appears to contribute to disease progression through various molecular

mechanisms (Cohn et al., 2008; Cohn et al., 2009; Willets et al., 2015). In this context, investigation of GRK2 function beyond GPCR desensitization could provide new insights in understanding its mechanisms in the pathophysiology of hypertensive vascular remodelling. In this chapter, I will discuss the main findings of this project and their implications as therapeutic strategies for the management of uncontrolled VSMC proliferation in patients with hypertension or complications of hypertension.

The main goal of the current research was to define GRK2 functions in VSMC growth and proliferation. In other terms, to what degree does GRK2 expression and/or its kinase activity affect with vasoconstrictor-induced VSMC proliferation? Research presented in this thesis has identified that depletion of GRK2 expression, or inhibition of its catalytic activity effectively attenuates vasoconstrictor-mediated VSMC proliferation in a GPCR-specific manner (as shown in *Chapter 4*). This indicates the potential connectivity of GRK2 with hypertension induced changes in vascular wall thickness. Furthermore, the increased GRK2 expression and activity in hypertension (Gros Robert, 1997; Eckhart et al., 2002; Cohn et al., 2009; Willets et al., 2015) may help to drive the enhanced VSMC proliferation and increased wall thickening seen in vascular remodelling.

The second aim of this study was to investigate the effects of GRK2 on two different independent proliferation signalling pathways [MAPK/ERK and PI3K/Akt] during the VSMC proliferation process, and determine how GRK2 differentially regulates vasoconstrictor GPCRs signalling pathways. Starting with the MAPK/ERK cascade, the data show that both GRK2 expression and catalytic activity are required for transient and sustained ERK activation induced by AngII and ET1 in VSMC. Furthermore, modulation of GRK2 function differentially alters vasoconstrictor-induced downstream signalling. Although activation of AT_1, ET_A and $P2Y_2$ receptors recruits GRK2 (Kim et al., 2009; Morris et al., 2010; Morris et al., 2011), GRK2 expression and activity are required for AngII and ET1 (proliferative vasoconstrictor)-induced ERK activation, but not for UTP-induced ERK signalling (as shown in *Chapter 5*). Moreover, since the data indicate a proportional relationship between GRK2 expression and ERK signal activation in SHR, increased GRK2 expression seen in hypertension could be an underlying factor for long-term MAPK/ERK signalling cascade activation and subsequently, contribute to VSMC proliferation, vascular wall thickening, and ultimately hypertensive vascular remodelling.

Another significant finding was that GRK2 could regulate the PI3K/Akt signalling pathway. Current research findings demonstrated that GRK2 expression regulates

vasoconstrictor-mediated Akt and GSK3 phosphorylation in VSMC, whereas GRK2 catalytic activity had no effect on vasoconstrictor-stimulated Akt phosphorylation in VSMC. Hence, as GRK2 interacts directly with PI3K (Naga Prasad et al., 2002a; Robinson and Pitcher, 2013), GRK2 is likely acting as a scaffold for PI3K to enable AngII/AT_1 and ET1/ET_A mediated PI3K/Akt/GSK3 signalling activation (as shown in *Chapter 6*). Thus we suggest that AngII and ET1 stimulate VSMC proliferation via GRK2-PI3K-Akt-GSK3 signalling pathway. Consequently, enhanced GRK2 expression observed in hypertension (Willets et al., 2015) likely promotes VSMC proliferation, vascular wall thickening and eventually hypertensive vascular remodelling via PI3K-Akt-GSK3 activation.

In light of these results, GRK2 represents a crucial intracellular signalling molecule which drives multiple mechanisms in the VSMC proliferation process. The fact that GRK2 plays a role in VSMC growth makes it an attractive target for prevention or treatment of pathological VSMC proliferation. Thus, targeting GRK2 expression or inhibition of its catalytic activity with siRNA or a specific inhibitor respectively could be utilized to expand the therapeutic strategies that aim to prevent or treat hypertensive vascular remodelling. One of the potential therapeutic approaches might be targeting GRK2 expression by using gene therapy. The concept of gene therapy is based on using designer vectors to transfer genes of interest into a specific cell type in the cardiovascular system (Kieserman et al., 2019). These vectors can be non-pathogenic viral such as lentiviral or adenoviral vectors, or can be non-viral such as circular plasmid DNA vectors (Kieserman et al., 2019). In this context, inhibition of GRK2 using the βARKct gene has been used in an animal model of heart failure. Adeno-associated virus serotype 6 vectors (AAV6.βARKct) were used to deliver the βARKct gene to the heart, resulting in overexpression of βARKct peptide. Overexpressed βARKct showed beneficial effects on cardiac function and left ventricular remodelling (Raake et al., 2013). It can, therefore, be assumed that the using of gene therapy to carry a particular GRK2 domain (such as βARKct) to the smooth muscle cells in the blood vessels is applicable. However, some issues could arise from inhibiting GRK2 activity in blood vessels such as enhanced receptor activation which could affect vascular tone. As GRK2 regulates a number of GPCRs and due to the heterogeneity of GRK2 structures (multi-domain) (Pitcher et al., 1998), investigation of the roles of each domain of GRK2 and their related downstream signalling may enable the development of potent drugs that inhibit some signalling

pathways without affecting others. Furthermore, in order to avoid the unwanted effects of transferred GRK2 gene into other tissue or organs, virus-mediated GRK2 gene delivery could be prepared in a tissue-specific manner by using specific promoters for VSMC. Alternatively, using this technique it may be possible to target GRK2 in a specific (limited) area such as sites of atherosclerosis or injured vessels by using advanced drug delivery systems such as a drug-eluting balloon or drug-eluting stent.

Another therapeutic approach that could be applied is chemical inhibition of GRK2 catalytic activity. Aptamers, an RNA-based molecule, which inhibit GRK2 catalytic activity with a high degree of selectively by stabilizing an inactive conformation of GRK2 have been identified, such as the C13 molecule (Mayer et al., 2008; Tesmer et al., 2012; Thal et al., 2012). However, these types of molecules have limited systemic bioavailability and stability, also their ability to cross cell membranes is limited, which could restrict their therapeutic implications (Murga et al., 2019). A more promising route might be to use small molecule inhibitors. At present a number of small molecule inhibitors of GRK2 with varying degrees of potency, have been identified. These include the natural product balanol (IC_{50}: 50 nM), CCG215022 (IC_{50}: 2.95 µM) and CCG224063 (IC_{50}: 46 nM) (Thal et al., 2011; Waldschmidt et al., 2016; Rainbow et al., 2018). The *Takeda Pharmaceutical Company* characterized several compounds that inhibit GRK2 activity including CMPD101 (IC_{50}: 35 nM) and CMPD103A (IC_{50}: 0.05 µM) (Thal et al., 2011). Furthermore, the highly selective GRK2 inhibitor, 12n [CCG-224406] has an IC_{50} of 130 nM and >700 fold selectivity over other GRK family members (Waldschmidt et al., 2016). Rainbow et al. (2018) illustrated the effectiveness of Takeda's CMPD101 and the Tesmer compounds CCG215022 and CCG224063 as GRK2 inhibitors in smooth muscle cells. These compounds attenuated AT_1 and $P2Y_2$ receptor desensitization of AngII or UTP mediated contractile responses in mesenteric arterial rings, as well as attenuating $P2Y_2$ and H_1 histamine receptor desensitization of UTP or histamine mediated PLC-β signalling respectively in mesenteric arterial smooth muscle cells and human ULTR myometrial cells (Rainbow et al., 2018). However, the concern about these molecules is that although they are functional *in vitro*, to date there are no published data on their efficacy *in vivo*. In addition, their stability, systemic bioavailability, as well as their effects on cellular signalling, cross-reactivity with other kinases and possible side effects are still unknown, which limits their therapeutic potential and clinical application. Alternatively, paroxetine is an FDA-approved SSRI for treatment of depression (Tomita

et al., 2014), which displays GRK2 inhibitory effects (Thal et al., 2011) with an IC_{50} of 1 μM (Rainbow et al., 2018). Data in this thesis demonstrate that paroxetine has anti-proliferative potential in VSMC through inhibition of GRK2 kinase activity (see *Chapter 4*). Thus, paroxetine could be used as a therapeutic approach for targeting GRK2 catalytic activity. In this study, 5 μM paroxetine inhibited GRK2 catalytic activity in VSMC. However, this concentration is greater than that of the therapeutic concentration of paroxetine. The therapeutic plasma concentration range of paroxetine is 20-60 ng/mL for patients with major depression (Tomita et al., 2014), which is much lower than the required concentration to inhibit GRK2 and would not, therefore, be expected to inhibit GRK2 catalytic activity. Even so, Rainbow et al. (2018) revealed that a lower concentration of paroxetine (500 nM) induced inhibition of GRK2 catalytic activity in isolated cells (Rainbow et al., 2018). Moreover, paroxetine exerts a prolonged inhibitory effect on GRK2 activity, i.e. paroxetine triggered extended inhibition of $P2Y_2$ receptor desensitization and sustainably inhibits the loss of arterial responsiveness to UTP-mediated mesenteric arterial contractions (Rainbow et al., 2018). Clinically, the steady-state paroxetine plasma concentration is normally achieved within 14 days of initiation therapy (Tulloch and Johnson, 1992), indicating that patients with depression should use paroxetine chronically for a long period of time (could last for ≥ one year) to obtain full effectiveness (Ballesteros-Zebadua et al., 2013). Also, its plasma protein binding is ~95% at therapeutically relevant concentrations, with only 1% remaining free in the systemic circulation (Bourin et al., 2001), indicating that paroxetine accumulates in the tissues. Additionally, paroxetine is widely distributed throughout the body, and as a lipophilic compound, is likely to accumulate in tissues (Bourin et al., 2001). These findings suggest that paroxetine is probably accumulated in tissues where the concentrations could be higher than plasma level. Therefore, long-term exposure to a low dose of paroxetine could inhibit GRK2 catalytic activity *in vivo* and potentially provide a protective role against the development of vascular remodelling. Further study is required to identify the effect of low dose paroxetine therapy for extended periods on GRK2 catalytic activity. Otherwise, in the case of atherosclerosis, an advanced drug delivery system could be used, such as drug-eluting stents (DES), which is a controlled way to release anti-proliferative drugs into a particular area in the arterial wall (Bozsak et al., 2015). Indeed this therapeutic approach using sirolimus-eluting or paclitaxel-eluting stents, has considerably decreased the rate of VSMC proliferation and restenosis (Stone et al., 2007). A DES with the required paroxetine concentration could be applied locally for the narrowed areas of

the vessel to decrease VSMC proliferation at that particular site and to avoid restenosis after surgical intervention.

A further potential therapeutic approach that could be investigated is targeting GRK2-mediated signalling. Theoretically, identification of GRK2 sites responsible for the binding of PI3K, MEK or ERK enable the search for small molecules that can bind to that specific site and selectively inhibit such interactions. For example, PI3K has been shown to interact with GRK2 at amino acid 197 in the PIK domain, resulting in GRK2-mediated recruitment of PI3K to the cell membrane (Naga Prasad et al., 2001). Targeting the GRK2-PI3K interaction could attenuate GRK2-mediated scaffolded signalling. This possibility needs to be explored.

The implications of the present findings could be expanded to other pathological conditions that are associated with increased GRK2 expression, such as breast cancer (Nogues et al., 2016). Elevated GRK2 expression in breast cancer plays a major role in tumour cell proliferation and survival (Nogues et al., 2016). Since our results show that GRK2 is fundamental for cell proliferation and plays an important role in the proliferative signalling pathways [PI3K/Akt and MAPK/ERK], which are the main pathways targeted by cancer chemotherapeutic agents (Roberts and Der, 2007; Almstedt and Schmidt, 2015), GRK2 could be also be targeted in such diseases. These implications reflect those of Cho et al. (2019) who recently found that paroxetine has anticancer potential in human breast cancer cells (Cho et al., 2019). Although they show that paroxetine mediates anticancer effects through ROS (Cho et al., 2019), the findings of the current study suggest that paroxetine could exert anti-proliferative effects via inhibition of GRK2 catalytic activity (see *Chapter 4*). Thus, targeting GRK2 could contribute to the development of future therapeutic strategies for cancer therapy.

In conclusion, the current study provides novel evidence to explain the pathological mechanisms by which vasoconstrictors can induce VSMC proliferation. Demonstrating that GRK2, which is better known as a negative regulator of vasoconstrictor GPCRs, acts as a mediator of this mechanism implicates a pathogenic role in hypertensive vascular remodelling. The current project findings indicate that GRK2 expression and activity are implicated in vasoconstrictor-induced cell proliferation. Moreover, this study provides novel insights into two major GRK2-dependent mechanisms by which vasoconstrictors induce VSMC proliferation (**Figure 1.7**). This knowledge highlights the pharmacological

basis for targeting GRK2 for controlling VSMC growth. Therefore, selective regulation of GRK2 appears promising as a therapeutic approach. Many different approaches could be applied, including small molecule inhibitors, gene therapy and the use of advanced drug delivery systems to potentially improve the treatment of pathological VSMC proliferation. Collectively, pharmacological intervention of GRK2 provides a novel concept in the treatment of uncontrolled cell growth and a possible future target for vascular disease therapies.

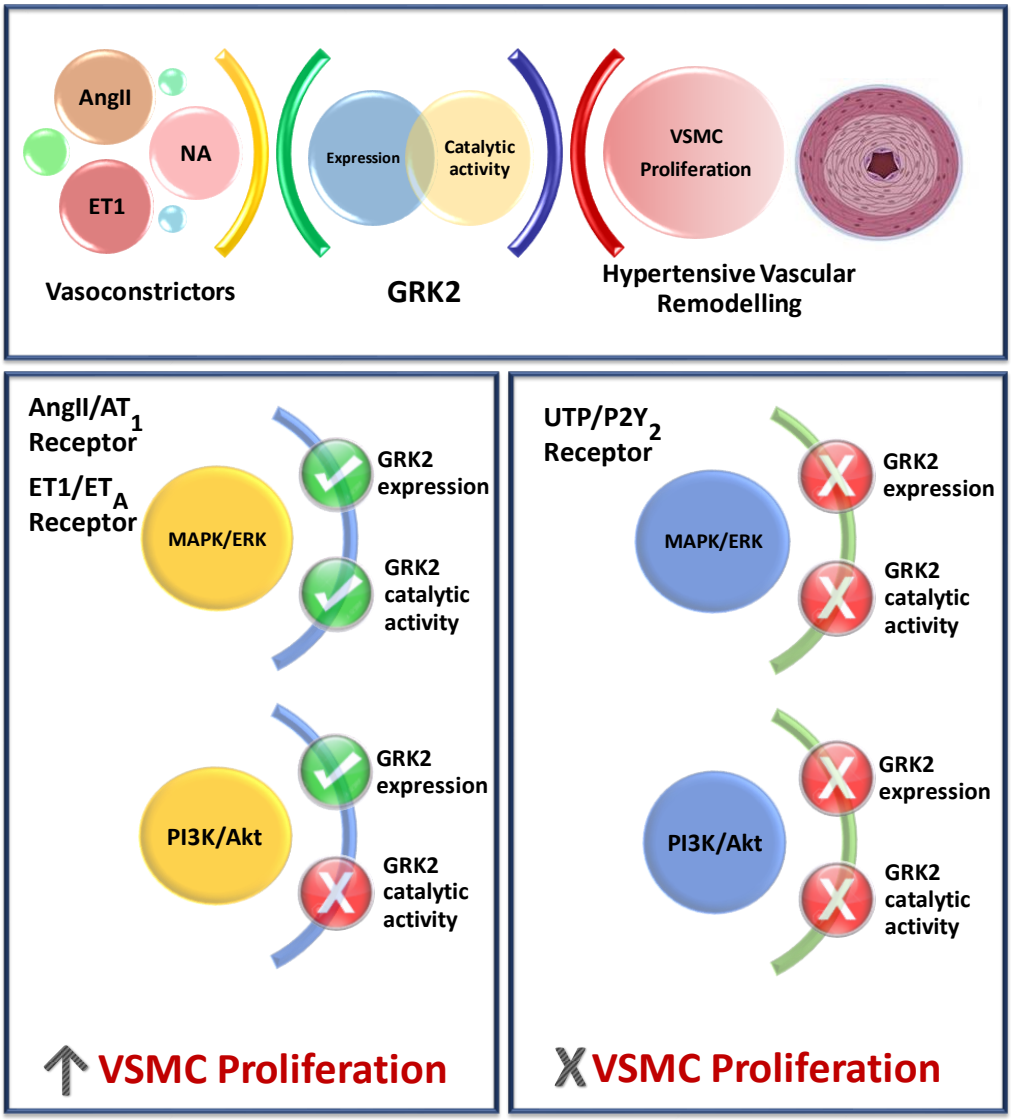


Figure 7.1 Summary showing for GRK2 effects on VSMC proliferation and vasoconstrictor GPCR signalling.

GRK2 expression and kinase activity mediate AngII, ET1 and NA stimulated VSMC proliferation. Although AT_1, ET_A and $P2Y_2$ receptor-mediated PLC signalling is negatively regulated by GRK2, modulation of GRK2 protein expression or its catalytic activity differentially change downstream signalling. GRK2 expression and its catalytic activity are essential to enable prolonged AngII and ET1-stimulated MAPK/ERK signalling. GRK2 expression, not catalytic activity, is required for AngII and ET1-stimulated PI3K/Akt signalling. However, neither GRK2 expression nor activity are required for UTP mediated MAPK/ERK or PI3K/Akt signalling.

7.2. Future investigations

Since work in this thesis has revealed that GRK2 plays a central role in mediating vasoconstrictor-stimulated VSMC proliferation and potentially, therefore, a role in hypertensive vascular remodelling, it is critical that to find a suitable way to target GRK2 *in vivo*. A further study could assess the long-term effects of low concentrations of paroxetine on GRK2 catalytic activity in VSMC and the functions consequence of this. Additionally, clinical trials could be applied to investigate the incidence of hypertension-induced cardiovascular complication in patients who already use paroxetine for extended periods of time. Confirming the ability of paroxetine to inhibit GRK2 *in vivo* could provide a new indication for paroxetine as a protective drug against the development of vascular remodelling.

Impetus Important future investigation for the development of new molecules targeting GRK2-mediated downstream signalling could arise from the study of the direct interaction mechanisms between GRK2 and ERK as well as GRK2 and PI3K interactions in VSMC. Identification of the specific GRK2 domains responsible for these interactions could help to design a molecule in order to specifically target interfaces of GRK2 with specific downstream signalling proteins relevant to VSMC proliferation. It would also be worth determining whether GRK2 overexpression enhances PI3K/Akt signalling including GSK3 isoform phosphorylation. This would further confirm the suggestion that GRK2 acts as a scaffold for PI3K-Akt-GSK3 signal activation with subsequent effects on VSMC proliferation in hypertension. It will also be important to further understand the effect of GRK2 on growth factor-promoted VSMC proliferation such as PDGF and IGF-I that mediate their effect via RTKs, as seen in atherosclerosis (Singh et al., 2002; Zhao et al., 2011). This is of particular relevance since GRK2 has been reported to regulate RTKs (Hildreth et al., 2004) and mediate the cross-talk between GPCRs and RTKs (Chen et al., 2008; Guo et al., 2009). Future studies could be directed toward understanding how GRK2 mediates the balance and cross-talk between these two distinct receptor mediated signalling pathways and how these influence VSMC growth. This knowledge could help to design a molecule targeting GRK2 in a way that modulates VSMC proliferation promoted by two different receptors classes.

Current research findings, together with published research, reveal a strong relationship between GRK2 and cell cycle progression (Penela et al., 2010a; Wei et al., 2013). Additional studies will be needed to investigate GRK2 localization in the nucleus. In addition, examining the effect of GRK2 protein expression and catalytic activity on

each phase of cell cycle progression, cell-cycle checkpoints, cell cycle regulating genes and cyclin-dependent kinases will add considerably to our understanding. A better knowledge of the GRK2 function in each stage of cell cycle progression may help to develop approaches that particularly target nuclear GRK2 without affecting up-stream signalling and receptor activation/desensitization mechanisms. Furthermore, GRK2 has been reported to be phosphorylated by CDK2 at serine 670, promoting degradation of GRK2 and allowing cells to progress from G_2 to M phase of division (Penela et al., 2010a). This potential interaction could be tested in RASM cells, by assessing GRK2 phosphorylation (S670) status and how this affects GRK2 expression during the stages of the cell cycle. Understanding of such mechanisms may help to identify small molecules that modulate such phosphorylation and thus control GRK2-mediated pathological cell proliferation. These lines of research will help to develop a fuller picture of the effects of GRK2 on cell cycle progression and would ultimately enable a better understanding of GRK2 effects on cell proliferation.

Bibliography

Abdi H (2007). The Bonferonni and Šidák Corrections for Multiple Comparisons. *Encyclopedia of measurement and statistics,* 3.

Adachi M, Fukuda M & Nishida E (1999). Two co-existing mechanisms for nuclear import of MAP kinase: passive diffusion of a monomer and active transport of a dimer. *The EMBO journal,* 18**:** 5347-5358.

Adhikari N, Shekar KC, Staggs R, Win Z, Steucke K, Lin YW, et al. Hall JL (2015). Guidelines for the isolation and characterization of murine vascular smooth muscle cells. A report from the International Society of Cardiovascular Translational Research. *J Cardiovasc Transl Res,* 8**:** 158-63.

Agapitov AV & Haynes WG (2002). Role of endothelin in cardiovascular disease. *Journal of the Renin-Angiotensin-Aldosterone System,* 3**:** 1-15.

Ahmet I, Krawczyk M, Zhu W, Woo AY, Morrell C, Poosala S, et al. Talan MI (2008). Cardioprotective and survival benefits of long-term combined therapy with β2 adrenoreceptor (AR) agonist and β1 AR blocker in dilated cardiomyopathy postmyocardial infarction. *J Pharmacol Exp Ther,* 325**:** 491-9.

Ahn S, Shenoy SK, Wei H & Lefkowitz RJ (2004). Differential kinetic and spatial patterns of β-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem,* 279**:** 35518-25.

Ahn S, Kim J, Hara MR, Ren XR & Lefkowitz RJ (2009). β-Arrestin-2 Mediates Anti-apoptotic Signaling through Regulation of BAD Phosphorylation. *J Biol Chem,* 284**:** 8855-65.

Aktas MK, Ozduran V, Pothier CE, Lang R & Lauer MS (2004). Global risk scores and exercise testing for predicting all-cause mortality in a preventive medicine program. *JAMA,* 292**:** 1462-8.

Alderman MH, Cohen H, Roque R & Madhavan S (1997). Effect of long-acting and short-acting calcium antagonists on cardiovascular outcomes in hypertensive patients. *Lancet,* 349**:** 594-8.

Alessi DR, Cuenda A, Cohen P, Dudley DT & Saltiel AR (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J Biol Chem,* 270**:** 27489-94.

Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG, et al. Bownes M (1997). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol,* 7**:** 776-89.

Alexander MR & Owens GK (2012). Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. *Annu Rev Physiol,* 74**:** 13-40.

Allen BG & Walsh MP (1994). The biochemical basis of the regulation of smooth-muscle contraction. *Trends Biochem Sci,* 19**:** 362-8.

Almstedt K & Schmidt M (2015). Targeted Therapies Overcoming Endocrine Resistance in Hormone Receptor-Positive Breast Cancer. *Breast Care (Basel),* 10**:** 168-72.

Alonso G, Galibert E, Boulay V, Guillou A, Jean A, Compan V & Guillon G (2009). Sustained elevated levels of circulating vasopressin selectively stimulate the proliferation of kidney tubular cells via the activation of V2 receptors. *Endocrinology,* 150**:** 239-50.

Anand-Srivastava MB (1992). Enhanced expression of inhibitory guanine nucleotide regulatory protein in spontaneously hypertensive rats. Relationship to adenylate cyclase inhibition. *Biochem J,* 288 (Pt 1)**:** 79-85.

Anand-Srivastava MB, Picard S & Thibault C (1991). Altered expression of inhibitory guanine nucleotide regulatory proteins (Gi α) in spontaneously hypertensive rats. *Am J Hypertens,* 4**:** 840-3.

Anderson KM, Eckhart AD, Willette RN & Koch WJ (1999). The myocardial β-adrenergic system in spontaneously hypertensive heart failure (SHHF) rats. *Hypertension,* 33**:** 402-7.

Aoyagi M, Yamamoto M, Wakimoto H, Azuma H, Hirakawa K & Yamamoto K (1995). Immunohistochemical detection of Ki-67 in replicative smooth muscle cells of rabbit carotid arteries after balloon denudation. *Stroke,* 26**:** 2328-31; discussion 2331-2.

Aplin M, Christensen GL, Schneider M, Heydorn A, Gammeltoft S, Kjolbye AL, et al. Hansen JL (2007). Differential extracellular signal-regulated kinases 1 and 2 activation by the angiotensin type 1 receptor supports distinct phenotypes of cardiac myocytes. *Basic Clin Pharmacol Toxicol,* 100**:** 296-301.

Armaly Z, Assady S & Abassi Z (2013). Corin: a new player in the regulation of salt-water balance and blood pressure. *Curr Opin Nephrol Hypertens,* 22**:** 713-22.

Asano M, Nakajima T, Iwasawa K, Asakura Y, Morita T, Nakamura F, et al. Okuda Y (1999). Eicosapentaenoic acid inhibits vasopressin-activated Ca2+ influx and cell proliferation in rat aortic smooth muscle cell lines. *European Journal of Pharmacology,* 379**:** 199-209.

Atlas SA (2007). The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. *J Manag Care Pharm,* 13**:** 9-20.

Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, et al. Lefkowitz RJ (1992). β-arrestin2, a novel member of the arrestin/β-arrestin gene family. *J Biol Chem,* 267**:** 17882-90.

Avendano MS, Lucas E, Jurado-Pueyo M, Martinez-Revelles S, Vila-Bedmar R, Mayor F, Jr., et al. Murga C (2014). Increased nitric oxide bioavailability in adult GRK2 hemizygous mice protects against angiotensin II-induced hypertension. *Hypertension,* 63**:** 369-75.

Bader M, Peters J, Baltatu O, Muller DN, Luft FC & Ganten D (2001). Tissue renin-angiotensin systems: new insights from experimental animal models in hypertension research. *J Mol Med (Berl),* 79**:** 76-102.

Baldwin JM (1993). The probable arrangement of the helices in G protein-coupled receptors. *EMBO J,* 12**:** 1693-703.

Ballesteros-Zebadua P, Manjarrez-Marmolejo J & Franco-Perez J (2013). Chronic paroxetine treatment: effects on other non-serotonergic neurotransmitter systems. *CNS Neurol Disord Drug Targets,* 12**:** 1226-32.

Barja F, Coughlin C, Belin D & Gabbiani G (1986). Actin isoform synthesis and mRNA levels in quiescent and proliferating rat aortic smooth muscle cells in vivo and in vitro. *Lab Invest,* 55**:** 226-33.

Barreras A & Gurk-Turner C (2003). Angiotensin II receptor blockers. *Proceedings (Baylor University. Medical Center),* 16**:** 123-126.

Baskar K, Sur S, Selvaraj V & Agrawal DK (2015). Functional constituents of a local serotonergic system, intrinsic to the human coronary artery smooth muscle cells. *Molecular biology reports,* 42**:** 1295-1307.

Baumbach GL & Heistad DD (1989). Remodeling of cerebral arterioles in chronic hypertension. *Hypertension,* 13**:** 968-72.

Baumbach GL & Ghoneim S (1993). Vascular remodeling in hypertension. *Scanning Microsc,* 7**:** 137-42; discussion 143.

Bazan E, Campbell AK & Rapoport RM (1992). Protein kinase C activity in blood vessels from normotensive and spontaneously hypertensive rats. *Eur J Pharmacol,* 227**:** 343-8.
Beevers G, Lip GYH & Brien E (2001). The pathophysiology of hypertension. *BMJ,* 322**:** 912.

Bellacosa A, Testa JR, Moore R & Larue L (2004). A Portrait of AKT Kinases: Human Cancer and Animal Models Depict a Family with Strong Individualities. *Cancer Biology & Therapy,* 3**:** 268-275.

Benovic JL, Onorato JJ, Arriza JL, Stone WC, Lohse M, Jenkins NA, et al. Lefkowitz RJ (1991). Cloning, expression, and chromosomal localization of β-adrenergic receptor kinase 2. A new member of the receptor kinase family. *J Biol Chem,* 266**:** 14939-46.

Bernhem K, Krishnan K, Bondar A, Brismar H, Aperia A & Scott L (2017). AT1-receptor response to non-saturating Ang-II concentrations is amplified by calcium channel blockers. *BMC Cardiovascular Disorders,* 17**:** 126.

Beurel E, Grieco SF & Jope RS (2015). Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacology & therapeutics,* 148**:** 114-131.

Bhagani S, Kapil V & Lobo MD (2018). Hypertension. *Medicine,* 46**:** 509-515.

Bhattacharya R, Kwon J, Li X, Wang E, Patra S, Bida JP, et al. Mukhopadhyay D (2009). Distinct role of PLCβ3 in VEGF-mediated directional migration and vascular sprouting. *J Cell Sci,* 122**:** 1025-34.

Biasin V, Chwalek K, Wilhelm J, Best J, Marsh LM, Ghanim B, et al. Kwapiszewska G (2014). Endothelin-1 driven proliferation of pulmonary arterial smooth muscle cells is c-fos dependent. *The International Journal of Biochemistry & Cell Biology,* 54**:** 137-148.

Blajeski AL, Phan VA, Kottke TJ & Kaufmann SH (2002). G1 and G2 cell-cycle arrest following microtubule depolymerization in human breast cancer cells. *The Journal of Clinical Investigation,* 110**:** 91-99.

Bockaert J & Philippe Pin J (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. *The EMBO Journal,* 18**:** 1723-1729.

Bokemeyer D, Schmitz U & Kramer HJ (2000). Angiotensin II-induced growth of vascular smooth muscle cells requires an Src-dependent activation of the epidermal growth factor receptor1. *Kidney International,* 58**:** 549-558.

Bolivar JJ (2013). Essential hypertension: an approach to its etiology and neurogenic pathophysiology. *Int J Hypertens,* 2013**:** 547809.

Bornfeldt KE, Campbell JS, Koyama H, Argast GM, Leslie CC, Raines EW, et al. Ross R (1997). The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. *J Clin Invest,* 100**:** 875-85.

Bourin M, Chue P & Guillon Y (2001). Paroxetine: A Review. *CNS Drug Reviews,* 7**:** 25-47.

Bozsak F, Gonzalez-Rodriguez D, Sternberger Z, Belitz P, Bewley T, Chomaz JM & Barakat AI (2015). Optimization of Drug Delivery by Drug-Eluting Stents. *PLoS One,* 10**:** e0130182.

Brater DC (2000). Pharmacology of diuretics. *Am J Med Sci,* 319**:** 38-50.

Braun-Dullaeus RC, Mann MJ, Seay U, Zhang L, Von Der Leyen HE, Morris RE & Dzau VJ (2001). Cell cycle protein expression in vascular smooth muscle cells in vitro and in vivo is regulated through phosphatidylinositol 3-kinase and mammalian target of rapamycin. *Arterioscler Thromb Vasc Biol,* 21**:** 1152-8.

Brenner BM, Ballermann BJ, Gunning ME & Zeidel ML (1990). Diverse biological actions of atrial natriuretic peptide. *Physiol Rev,* 70**:** 665-99.

Brinks H, Boucher M, Gao E, Chuprun JK, Pesant S, Raake PW, et al. Koch WJ (2010). Level of G protein-coupled receptor kinase-2 determines myocardial ischemia/reperfusion injury via pro- and anti-apoptotic mechanisms. *Circ Res,* 107**:** 1140-9.

Brinks HL & Eckhart AD (2010). Regulation of GPCR signaling in hypertension. *Biochim Biophys Acta,* 1802**:** 1268-75.

Brown NJ & Vaughan DE (1998). Angiotensin-converting enzyme inhibitors. *Circulation,* 97**:** 1411-20.

Brunet A, Roux D, Lenormand P, Dowd S, Keyse S & Pouyssegur J (1999). Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO J,* 18**:** 664-74.

Bunkenburg B, Van Amelsvoort T, Rogg H & Wood JM (1992). Receptor-mediated effects of angiotensin II on growth of vascular smooth muscle cells from spontaneously hypertensive rats. *Hypertension,* 20**:** 746-754.

Byrom FB (1974). The evolution of acute hypertensive arterial disease. *Progress in Cardiovascular Diseases,* 17**:** 31-37.

Byrom FB (1976). The third Volhard lecture. Tension and the artery: the experimental elucidation of pseudo-uraemia and malignant nephrosclerosis. *Clin Sci Mol Med Suppl,* 3**:** 35-115.

Campbell-Boswell M & Robertson AL, Jr. (1981). Effects of angiotensin II and vasopressin on human smooth muscle cells in vitro. *Exp Mol Pathol,* 35**:** 265-76.

Campese VM (1981). Minoxidil: A Review of its Pharmacological Properties and Therapeutic Use. *Drugs,* 22**:** 257-278.

Carey RM & Siragy HM (2003). Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation. *Endocr Rev,* 24**:** 261-71.

Carnevale D & Lembo G (2012). PI3Kγ in hypertension: a novel therapeutic target controlling vascular myogenic tone and target organ damage. *Cardiovasc Res,* 95**:** 403-8.

Carretero OA (2005). Vascular remodeling and the kallikrein-kinin system. *The Journal of Clinical Investigation,* 115**:** 588-591.

Carretero OA & Oparil S (2000). Essential hypertension. Part I: definition and etiology. *Circulation,* 101**:** 329-35.

Case AJ, Roessner CT, Tian J & Zimmerman MC (2016). Mitochondrial Superoxide Signaling Contributes to Norepinephrine-Mediated T-Lymphocyte Cytokine Profiles. *PloS one,* 11**:** e0164609-e0164609.

Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, et al. Mccubrey JA (2003). Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia,* 17**:** 590-603.

Chang S, Song S, Lee J, Yoon J, Park J, Choi S, et al. Choi C (2014). Phenotypic modulation of primary vascular smooth muscle cells by short-term culture on micropatterned substrate. *PLoS One,* 9**:** e88089.

Charles L, Triscott J & Dobbs B (2017). Secondary Hypertension: Discovering the Underlying Cause. *Am Fam Physician,* 96**:** 453-461.

Che X, Zhang G, Zhang X & Xue J (2018). Overexpression of G Protein-Coupled Receptor Kinase 6 (GRK6) Is Associated with Progression and Poor Prognosis of Papillary Thyroid Carcinoma. *Medical science monitor : international medical journal of experimental and clinical research,* 24**:** 3540-3548.

Chen B, Xue J, Meng X, Slutzky JL, Calvert AE & Chicoine LG (2014). Resveratrol prevents hypoxia-induced arginase II expression and proliferation of human pulmonary artery smooth muscle cells via Akt-dependent signaling. *Am J Physiol Lung Cell Mol Physiol,* 307**:** L317-25.

Chen Q-W, Edvinsson L & Xu C-B (2009). Role of ERK/MAPK in endothelin receptor signaling in human aortic smooth muscle cells. *BMC cell biology,* 10**:** 52-52.

Chen S, Qiong Y & Gardner DG (2006). A role for p38 mitogen-activated protein kinase and c-myc in endothelin-dependent rat aortic smooth muscle cell proliferation. *Hypertension,* 47**:** 252-8.

Chen X, Zhu H, Yuan M, Fu J, Zhou Y & Ma L (2010). G-protein-coupled receptor kinase 5 phosphorylates p53 and inhibits DNA damage-induced apoptosis. *J Biol Chem,* 285**:** 12823-30.

Chen Y, Long H, Wu Z, Jiang X & Ma L (2008). EGF transregulates opioid receptors through EGFR-mediated GRK2 phosphorylation and activation. *Mol Biol Cell,* 19**:** 2973-83.

Cho YW, Kim EJ, Nyiramana MM, Shin EJ, Jin H, Ryu JH, et al. Kang D (2019). Paroxetine Induces Apoptosis of Human Breast Cancer MCF-7 Cells through Ca(2+)-and p38 MAP Kinase-Dependent ROS Generation. *Cancers (Basel),* 11.

Choi D-J, Koch WJ, Hunter JJ & Rockman HA (1997). Mechanism of β-Adrenergic Receptor Desensitization in Cardiac Hypertrophy Is Increased β-Adrenergic Receptor Kinase. *Journal of Biological Chemistry,* 272**:** 17223-17229.

Chou CT, He S & Jan CR (2007). Paroxetine-induced apoptosis in human osteosarcoma cells: activation of p38 MAP kinase and caspase-3 pathways without involvement of [Ca2+]i elevation. *Toxicol Appl Pharmacol,* 218**:** 265-73.

Chuang TT, Levine H, 3rd & De Blasi A (1995). Phosphorylation and activation of β-adrenergic receptor kinase by protein kinase C. *J Biol Chem,* 270**:** 18660-5.

Cianfrocca R, Rosano L, Spinella F, Di Castro V, Natali PG & Bagnato A (2010). β-arrestin-1 mediates the endothelin-1-induced activation of Akt and integrin-linked kinase. *Can J Physiol Pharmacol,* 88**:** 796-801.

Clarke MCH, Figg N, Maguire JJ, Davenport AP, Goddard M, Littlewood TD & Bennett MR (2006). Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nature Medicine,* 12**:** 1075.

Cohn HI, Xi Y, Pesant S, Harris DM, Hyslop T, Falkner B & Eckhart AD (2009). G protein-coupled receptor kinase 2 expression and activity are associated with blood pressure in black Americans. *Hypertension,* 54**:** 71-6.

Cohn HI, Harris DM, Pesant S, Pfeiffer M, Zhou RH, Koch WJ, et al. Eckhart AD (2008). Inhibition of vascular smooth muscle G protein-coupled receptor kinase 2 enhances α1D-adrenergic receptor constriction. *Am J Physiol Heart Circ Physiol,* 295**:** H1695-704.

Cohn JN & Goldman JM (2008). Establishing a new option for target-organ protection: rationale for ARB plus ACE inhibitor combination therapy. *Am J Hypertens,* 21**:** 248-56.

Cohn JN, Mcinnes GT & Shepherd AM (2011). Direct-Acting Vasodilators. *The Journal of Clinical Hypertension,* 13**:** 690-692.

Cohuet G & Struijker-Boudier H (2006). Mechanisms of target organ damage caused by hypertension: Therapeutic potential. *Pharmacology & Therapeutics,* 111**:** 81-98.

Coll-Bonfill N, De La Cruz-Thea B, Pisano MV & Musri MM (2016). Noncoding RNAs in smooth muscle cell homeostasis: implications in phenotypic switch and vascular disorders. *Pflugers Arch,* 468**:** 1071-87.

Collaboration NRF (2017). Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19.1 million participants. *Lancet,* 389**:** 37-55.

Consigny PM (1991). Vascular smooth muscle contraction and relaxation: pathways and chemical modulation. *J Vasc Interv Radiol,* 2**:** 309-17.

Cookonyx SJ & Mccormick F (1996). Kinetic and biochemical correlation between sustained p44ERK1 (44 kDa extracellular signal-regulated kinase 1) activation and lysophosphatidic acid-stimulated DNA synthesis in Rat-1 cells. *Biochemical Journal,* 320**:** 237.

Csiszar A, Lehoux S & Ungvari Z (2009). Hemodynamic forces, vascular oxidative stress, and regulation of BMP-2/4 expression. *Antioxid Redox Signal,* 11**:** 1683-97.

Dahlöf B (2003). End-organ damage: does it really matter how we prevent it? *European Heart Journal Supplements,* 5**:** F33-F39.

Daou GB & Srivastava AK (2004). Reactive oxygen species mediate Endothelin-1-induced activation of ERK1/2, PKB, and Pyk2 signaling, as well as protein synthesis, in vascular smooth muscle cells. *Free Radic Biol Med,* 37**:** 208-15.

Datta SR, Brunet A & Greenberg ME (1999). Cellular survival: a play in three Akts. *Genes Dev,* 13**:** 2905-27.

Davis MJ (2012). Perspective: physiological role(s) of the vascular myogenic response. *Microcirculation,* 19**:** 99-114.

Dawson CW & Young LS (2001). In vitro assays to study epithelial cell growth. *Methods Mol Biol,* 174**:** 165-72.

De Mey JGR (1995). - Smooth Muscle Cell Proliferation in Hypertension: Possible Contribution to Arterial Remodeling. *In:* SCHWARTZ, S. M. & MECHAM, R. P. (eds.) *The Vascular Smooth Muscle Cell.* San Diego: Academic Press.

Deng LY & Schiffrin EL (1992). Effects of endothelin on resistance arteries of DOCA-salt hypertensive rats. *Am J Physiol,* 262**:** H1782-7.

Dickhout JG & Lee RM (1997). Structural and functional analysis of small arteries from young spontaneously hypertensive rats. *Hypertension,* 29**:** 781-9.

Downes GB & Gautam N (1999). The G protein subunit gene families. *Genomics,* 62**:** 544-52.

Druey KM, Sullivan BM, Brown D, Fischer ER, Watson N, Blumer KJ, et al. Kehrl JH (1998). Expression of GTPase-deficient Giα2 results in translocation of cytoplasmic RGS4 to the plasma membrane. *J Biol Chem,* 273**:** 18405-10.

Duca L, Blaise S, Romier B, Laffargue M, Gayral S, El Btaouri H, et al. Maurice P (2016). Matrix ageing and vascular impacts: focus on elastin fragmentation. *Cardiovasc Res,* 110**:** 298-308.

Dugourd C, Gervais M, Corvol P & Monnot C (2003). Akt Is a Major Downstream Target of PI3-Kinase Involved in Angiotensin II–Induced Proliferation. *Hypertension,* 41**:** 882-890.

Dunn OJ (1964). Multiple Comparisons Using Rank Sums. *Technometrics,* 6**:** 241-252.
Dunnett CW (1964). New Tables for Multiple Comparisons with a Control. *Biometrics,* 20**:** 482-491.

Duprez DA (2006). Role of the renin-angiotensin-aldosterone system in vascular remodeling and inflammation: a clinical review. *J Hypertens,* 24**:** 983-91.

Eckhart AD, Ozaki T, Tevaearai H, Rockman HA & Koch WJ (2002). Vascular-targeted overexpression of G protein-coupled receptor kinase-2 in transgenic mice attenuates β-adrenergic receptor signaling and increases resting blood pressure. *Mol Pharmacol,* 61**:** 749-58.

Eddahibi S, Guignabert C, Barlier-Mur AM, Dewachter L, Fadel E, Dartevelle P, et al. Adnot S (2006). Cross talk between endothelial and smooth muscle cells in pulmonary hypertension: critical role for serotonin-induced smooth muscle hyperplasia. *Circulation,* 113**:** 1857-64.

Ediger TL & Toews ML (2000). Synergistic stimulation of airway smooth muscle cell mitogenesis. *J Pharmacol Exp Ther,* 294**:** 1076-82.

Eguchi S, Dempsey PJ, Frank GD, Motley ED & Inagami T (2001). Activation of MAPKs by angiotensin II in vascular smooth muscle cells. Metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAPK but not for JNK. *J Biol Chem,* 276**:** 7957-62.

Eguchi S, Iwasaki H, Inagami T, Numaguchi K, Yamakawa T, Motley ED, et al. Hirata Y (1999). Involvement of PYK2 in angiotensin II signaling of vascular smooth muscle cells. *Hypertension,* 33**:** 201-6.

Eichmann T, Lorenz K, Hoffmann M, Brockmann J, Krasel C, Lohse MJ & Quitterer U (2003). The amino-terminal domain of G-protein-coupled receptor kinase 2 is a regulatory Gβγ binding site. *J Biol Chem,* 278**:** 8052-7.

El Mabrouk M, Touyz RM & Schiffrin EL (2001). Differential ANG II-induced growth activation pathways in mesenteric artery smooth muscle cells from SHR. *American Journal of Physiology-Heart and Circulatory Physiology,* 281**:** H30-H39.

Elliott WJ (1996). Higher incidence of discontinuation of angiotensin converting enzyme inhibitors due to cough in black subjects. *Clin Pharmacol Ther,* 60**:** 582-8.

Erlinge D & Burnstock G (2008). P2 receptors in cardiovascular regulation and disease. *Purinergic Signal,* 4**:** 1-20.

Evron T, Daigle TL & Caron MG (2012). GRK2: multiple roles beyond G protein-coupled receptor desensitization. *Trends Pharmacol Sci,* 33**:** 154-64.

Fang H, Yang S, Luo Y, Zhang C, Rao Y, Liu R, et al. Yu J (2018). Notoginsenoside R1 inhibits vascular smooth muscle cell proliferation, migration and neointimal hyperplasia through PI3K/Akt signaling. *Scientific Reports,* 8**:** 7595.

Feihl F, Liaudet L, Waeber B & Levy BI (2006). Hypertension: a disease of the microcirculation? *Hypertension,* 48**:** 1012-7.

Feldman RD (2002). Deactivation of vasodilator responses by GRK2 overexpression: a mechanism or the mechanism for hypertension? *Mol Pharmacol,* 61**:** 707-9.

Feldman RD & Gros R (1998). Impaired Vasodilator Function in Hypertension The Role of Alterations in Receptor–G Protein Coupling. *Trends Cardiovasc Med* 8**:** 297–305.

Feng W, Dell'italia LJ & Sanders PW (2017). Novel Paradigms of Salt and Hypertension. *J Am Soc Nephrol,* 28**:** 1362-1369.

Fernandez-Hernando C, Jozsef L, Jenkins D, Di Lorenzo A & Sessa WC (2009). Absence of Akt1 reduces vascular smooth muscle cell migration and survival and induces features of plaque vulnerability and cardiac dysfunction during atherosclerosis. *Arterioscler Thromb Vasc Biol,* 29**:** 2033-40.

Fernández-Hernando C, József L, Jenkins D, Di Lorenzo A & Sessa William C (2009). Absence of Akt1 Reduces Vascular Smooth Muscle Cell Migration and Survival and Induces Features of Plaque Vulnerability and Cardiac Dysfunction During Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 29**:** 2033-2040.

Ferrer-Alcon M, La Harpe R & Garcia-Sevilla JA (2004). Decreased immunodensities of micro-opioid receptors, receptor kinases GRK 2/6 and β-arrestin-2 in postmortem brains of opiate addicts. *Brain Res Mol Brain Res,* 121**:** 114-22.

Fink KB & Gothert M (2007). 5-HT receptor regulation of neurotransmitter release. *Pharmacol Rev,* 59**:** 360-417.

Frank GD & Eguchi S (2003). Activation of Tyrosine Kinases by Reactive Oxygen Species in Vascular Smooth Muscle Cells: Significance and Involvement of EGF Receptor Transactivation by Angiotensin II. *Antioxidants & Redox Signaling,* 5**:** 771-780.

Franklin SS (1999). Ageing and hypertension: the assessment of blood pressure indices in predicting coronary heart disease. *J Hypertens Suppl,* 17**:** S29-36.

Fredriksson R, Lagerström MC, Lundin L-G & Schiöth HB (2003). The G-Protein-Coupled Receptors in the Human Genome Form Five Main Families. Phylogenetic Analysis, Paralogon Groups, and Fingerprints. *Molecular Pharmacology,* 63**:** 1256-1272.

Freeman EJ & Tallant EA (1994). Vascular smooth-muscle cells contain AT1 angiotensin receptors coupled to phospholipase D activation. *The Biochemical journal,* 304 (Pt 2)**:** 543-548.

Frishman WH & Alwarshetty M (2002). β-Adrenergic Blockers in Systemic Hypertension. *Clinical Pharmacokinetics,* 41**:** 505-516.

Fukunaga K, Kume H, Oguma T, Shigemori W, Tohda Y, Ogawa E & Nakano Y (2016). Involvement of Ca(2+) Signaling in the Synergistic Effects between Muscarinic Receptor Antagonists and β_2-Adrenoceptor Agonists in Airway Smooth Muscle. *International journal of molecular sciences,* 17**:** 1590.

Gabbiani G, Kocher O, Bloom WS, Vandekerckhove J & Weber K (1984). Actin expression in smooth muscle cells of rat aortic intimal thickening, human atheromatous plaque, and cultured rat aortic media. *J Clin Invest,* 73**:** 148-52.

Gadea A, Schinelli S & Gallo V (2008). Endothelin-1 Regulates Astrocyte Proliferation and Reactive Gliosis via a JNK/c-Jun Signaling Pathway. *The Journal of Neuroscience,* 28**:** 2394.

Gao J, Li J & Ma L (2005). Regulation of EGF-induced ERK/MAPK activation and EGFR internalization by G protein-coupled receptor kinase 2. *Acta Biochim Biophys Sin (Shanghai),* 37**:** 525-31.

Gavras I & Gavras H (2002). Angiotensin II as a cardiovascular risk factor. *J Hum Hypertens,* 16 Suppl 2**:** S2-6.

Geisterfer AA, Peach MJ & Owens GK (1988). Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circulation Research,* 62**:** 749-756.

Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U & Stein H (1984). Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *The Journal of Immunology,* 133**:** 1710-1715.

Gether U (2000). Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr Rev,* 21**:** 90-113.

Ghosh D, Syed AU, Prada MP, Nystoriak MA, Santana LF, Nieves-Cintron M & Navedo MF (2017). Calcium Channels in Vascular Smooth Muscle. *Adv Pharmacol,* 78**:** 49-87.
Gibbons GH & Dzau VJ (1994). The emerging concept of vascular remodeling. *N Engl J Med,* 330**:** 1431-8.

Gibbons GH, Pratt RE & Dzau VJ (1992). Vascular smooth muscle cell hypertrophy vs. hyperplasia. Autocrine transforming growth factor-β 1 expression determines growth response to angiotensin II. *The Journal of Clinical Investigation,* 90**:** 456-461.

Giles TD, Materson BJ, Cohn JN & Kostis JB (2009). Definition and Classification of Hypertension: An Update. *The Journal of Clinical Hypertension,* 11**:** 611-614.

Giles TD, Berk BC, Black HR, Cohn JN, Kostis JB, Izzo JL, Jr. & Weber MA (2005). Expanding the definition and classification of hypertension. *J Clin Hypertens (Greenwich),* 7**:** 505-12.

Glagov S, Weisenberg E, Zarins CK, Stankunavicius R & Kolettis GJ (1987). Compensatory enlargement of human atherosclerotic coronary arteries. *N Engl J Med,* 316**:** 1371-5.

Goncharova EA, Lim P, Goncharov DA, Eszterhas A, Panettieri RA, Jr. & Krymskaya VP (2006). Assays for in vitro monitoring of proliferation of human airway smooth

muscle (ASM) and human pulmonary arterial vascular smooth muscle (VSM) cells. *Nat Protoc,* 1**:** 2905-8.

Gottlieb SS, Mccarter RJ & Vogel RA (1998). Effect of β-blockade on mortality among high-risk and low-risk patients after myocardial infarction. *N Engl J Med,* 339**:** 489-97.

Govindan S, Taylor EJA & Taylor CW (2010). Ca($^{2+}$) signalling by P2Y receptors in cultured rat aortic smooth muscle cells. *British journal of pharmacology,* 160**:** 1953-1962.

Grainger DJ, Witchell CM, Weissberg PL & Metcalfe JC (1994). Mitogens for adult rat aortic vascular smooth muscle cells in serum-free primary culture. *Cardiovasc Res,* 28**:** 1238-42.

Grassi G (2003). Involvement of neuroadrenergic factors in hypertensive vascular remodelling: myth or reality? *Journal of Hypertension,* 21**:** 2239-2241.

Grassi G (2006). Sympathetic overdrive and cardiovascular risk in the metabolic syndrome. *Hypertens Res,* 29**:** 839-47.

Greenberg A (2000). Diuretic complications. *Am J Med Sci,* 319**:** 10-24.

Greif DM (2013). Chapter 1 - Vascular Embryology and Angiogenesis. *In:* CREAGER, M. A., BECKMAN, J. A. & LOSCALZO, J. (eds.) *Vascular Medicine: A Companion to Braunwald's Heart Disease (Second Edition).* Philadelphia: W.B. Saunders.

Griendling Kathy K, Ushio-Fukai M, Lassègue B & Alexander RW (1997). Angiotensin II Signaling in Vascular Smooth Muscle. *Hypertension,* 29**:** 366-370.

Gros R, Benovic JL, Tan CM & Feldman RD (1997). G-protein-coupled receptor kinase activity is increased in hypertension. *J Clin Invest,* 99**:** 2087-93.

Gros R, Chorazyczewski J, Meek MD, Benovic JL, Ferguson SS & Feldman RD (2000). G-Protein-coupled receptor kinase activity in hypertension : increased vascular and lymphocyte G-protein receptor kinase-2 protein expression. *Hypertension,* 35**:** 38-42.

Gros Robert BJL, Tan Christopher M., Feldman Ross D. (1997). G-Protein–coupled Receptor Kinase Activity Is Increased in Hypertension. *J Clin Invest. ,* 99**:** 2087–2093.

Guillermet-Guibert J, Bjorklof K, Salpekar A, Gonella C, Ramadani F, Bilancio A, et al. Vanhaesebroeck B (2008). The p110β isoform of phosphoinositide 3-kinase signals downstream of G protein-coupled receptors and is functionally redundant with p110γ. *Proc Natl Acad Sci U S A,* 105**:** 8292-7.

Gunther S, Alexander RW, Atkinson WJ & Gimbrone MA, Jr. (1982). Functional angiotensin II receptors in cultured vascular smooth muscle cells. *J Cell Biol,* 92**:** 289-98.

Guo J, Chen H, Ho J, Mancini J, Sontag T, Laporte SA, et al. Lebrun JJ (2009). TGFβ-induced GRK2 expression attenuates AngII-regulated vascular smooth muscle cell proliferation and migration. *Cell Signal,* 21**:** 899-905.

Guo S, Carter RL, Grisanti LA, Koch WJ & Tilley DG (2017). Impact of paroxetine on proximal β-adrenergic receptor signaling. *Cell Signal,* 38**:** 127-133.

Hafizi S, Allen SP, Goodwin AT, Chester AH & Yacoub MH (1999). Endothelin-1 stimulates proliferation of human coronary smooth muscle cells via the ETA receptor and is co-mitogenic with growth factors. *Atherosclerosis,* 146**:** 351-359.

Hahn AW, Resink TJ, Kern F & Buhler FR (1993). Peptide vasoconstrictors, vessel structure, and vascular smooth-muscle proliferation. *J Cardiovasc Pharmacol,* 22 Suppl 5**:** S37-43.

Haq S, Choukroun G, Kang ZB, Ranu H, Matsui T, Rosenzweig A, et al. Force T (2000). Glycogen synthase kinase-3β is a negative regulator of cardiomyocyte hypertrophy. *Journal of Cell Biology,* 151**:** 117-129.

Hardt SE & Sadoshima J (2002). Glycogen synthase kinase-3β: a novel regulator of cardiac hypertrophy and development. *Circ Res,* 90**:** 1055-63.

Harper S, Webb TE, Charlton SJ, Ng LL & Boarder MR (1998). Evidence that P2Y4 nucleotide receptors are involved in the regulation of rat aortic smooth muscle cells by UTP and ATP. *Br J Pharmacol,* 124**:** 703-10.

Harris DM, Cohn HI, Pesant S & Eckhart AD (2008). GPCR signalling in hypertension: role of GRKs. *Clin Sci (Lond),* 115**:** 79-89.

Harris DM, Cohn HI, Pesant S, Zhou RH & Eckhart AD (2007). Vascular smooth muscle G(q) signaling is involved in high blood pressure in both induced renal and genetic vascular smooth muscle-derived models of hypertension. *Am J Physiol Heart Circ Physiol,* 293**:** H3072-9.

Harrison DG & Luther JM (2013). Chapter 6 - Vascular Pharmacology. *In:* CREAGER, M. A., BECKMAN, J. A. & LOSCALZO, J. (eds.) *Vascular Medicine: A Companion to Braunwald's Heart Disease (Second Edition).* Philadelphia: W.B. Saunders.

Harvey A, Montezano AC & Touyz RM (2015). Vascular biology of ageing-Implications in hypertension. *J Mol Cell Cardiol,* 83**:** 112-21.

Hausdorff WP, Caron MG & Lefkowitz RJ (1990). Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J,* 4**:** 2881-9.

Havelka GE & Kibbe MR (2011). The Vascular Adventitia: Its Role in the Arterial Injury Response. *Vascular and Endovascular Surgery,* 45**:** 381-390.

Hazra A & Gogtay N (2016). Biostatistics Series Module 3: Comparing Groups: Numerical Variables. *Indian journal of dermatology,* 61**:** 251-260.

Heeneman S, Haendeler J, Saito Y, Ishida M & Berk BC (2000). Angiotensin II induces transactivation of two different populations of the platelet-derived growth factor β receptor. Key role for the p66 adaptor protein Shc. *J Biol Chem,* 275**:** 15926-32.

Hein L, Meinel L, Pratt RE, Dzau VJ & Kobilka BK (1997). Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. *Mol Endocrinol,* 11**:** 1266-77.

Henry JB, Miller MC, Kelly KC & Champney D (2002). Mean arterial pressure (MAP): an alternative and preferable measurement to systolic blood pressure (SBP) in patients for hypotension detection during hemapheresis. *J Clin Apher,* 17**:** 55-64.

Herr D, Rodewald M, Fraser HM, Hack G, Konrad R, Kreienberg R & Wulff C (2008). Regulation of endothelial proliferation by the renin–angiotensin system in human umbilical vein endothelial cells. *REPRODUCTION,* 136**:** 125-130.

Hildreth KL, Wu JH, Barak LS, Exum ST, Kim LK, Peppel K & Freedman NJ (2004). Phosphorylation of the platelet-derived growth factor receptor-β by G protein-coupled receptor kinase-2 reduces receptor signaling and interaction with the Na(+)/H(+) exchanger regulatory factor. *J Biol Chem,* 279**:** 41775-82.

Hill-Eubanks DC, Werner ME, Heppner TJ & Nelson MT (2011). Calcium signaling in smooth muscle. *Cold Spring Harb Perspect Biol,* 3**:** a004549.

Hirata Y, Takagi Y, Fukuda Y & Marumo F (1989). Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Atherosclerosis,* 78**:** 225-8.

Ho J, Cocolakis E, Dumas VM, Posner BI, Laporte SA & Lebrun J-J (2005a). The G protein-coupled receptor kinase-2 is a TGFβ-inducible antagonist of TGFβ signal transduction. *The EMBO journal,* 24**:** 3247-3258.

Ho J, Cocolakis E, Dumas VM, Posner BI, Laporte SA & Lebrun JJ (2005b). The G protein-coupled receptor kinase-2 is a TGFβ-inducible antagonist of TGFbeta signal transduction. *EMBO J,* 24**:** 3247-58.

Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O & Woodgett JR (2000). Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation. *Nature,* 406**:** 86-90.

Hoeppner CZ, Cheng N & Ye RD (2012). Identification of a nuclear localization sequence in β-arrestin-1 and its functional implications. *The Journal of biological chemistry,* 287**:** 8932-8943.

Hollmann Markus wMDPD, Strumper DMDPD, Herroeder SMD & Durieux Marcel eMDPD (2005). Receptors, G Proteins, and Their Interactions. *Anesthesiology: The Journal of the American Society of Anesthesiologists,* 103**:** 1066-1078.

Homan KT & Tesmer JJG (2014). Structural insights into G protein-coupled receptor kinase function. *Current Opinion in Cell Biology,* 27**:** 25-31.

Hong H-J, Liu J-C, Huang M-T, Chan P, Juan S-H, Cheng T-H & Lin J-G (2004). Angiotensin II induces endothelin-1 gene expression via extracellular signal-regulated kinase pathway in rat aortic smooth muscle cells. *Cardiovascular Research,* 61**:** 159-168.

Hopfner M, Maaser K, Barthel B, Von Lampe B, Hanski C, Riecken EO, et al. Scherubl H (2001). Growth inhibition and apoptosis induced by P2Y2 receptors in human colorectal carcinoma cells: involvement of intracellular calcium and cyclic adenosine monophosphate. *Int J Colorectal Dis,* 16**:** 154-66.

Hsueh WA & Anderson PW (1992). Hypertension, the endothelial cell, and the vascular complications of diabetes mellitus. *Hypertension,* 20**:** 253-63.

Hua H, Munk S & Whiteside CI (2003). Endothelin-1 activates mesangial cell ERK1/2 via EGF-receptor transactivation and caveolin-1 interaction. *Am J Physiol Renal Physiol,* 284**:** F303-12.

Huang H, Zhang P, Wang Z, Tang F & Jiang Z (2011). Activation of Endothelin-1 Receptor Signaling Pathways Is Associated With Neointima Formation, Neoangiogenesis and Irreversible Pulmonary Artery Hypertension in Patients With Congenital Heart Disease. *Circulation Journal,* 75**:** 1463-1471.

Huang ZM, Gao E, Fonseca FV, Hayashi H, Shang X, Hoffman NE, et al. Koch WJ (2013). Convergence of G protein-coupled receptor and S-nitrosylation signaling determines the outcome to cardiac ischemic injury. *Sci Signal,* 6**:** ra95.

Hubbard SR & Miller WT (2007). Receptor tyrosine kinases: mechanisms of activation and signaling. *Current opinion in cell biology,* 19**:** 117-123.

Humphrey JD (2008). Mechanisms of arterial remodeling in hypertension: coupled roles of wall shear and intramural stress. *Hypertension (Dallas, Tex. : 1979),* 52**:** 195-200.

Hupfeld CJ & Olefsky JM (2007). Regulation of receptor tyrosine kinase signaling by GRKs and β-arrestins. *Annu Rev Physiol,* 69**:** 561-77.

Iaccarino G, Barbato E, Cipolletta E, De Amicis V, Margulies KB, Leosco D, et al. Koch WJ (2005). Elevated myocardial and lymphocyte GRK2 expression and activity in human heart failure. *Eur Heart J,* 26**:** 1752-8.

Ikeda S, Keneko M, Fujiwara S. (2007), inventors; Takeda Pharmaceutical Company Ltd., Ikeda S, Keneko M, Fujiwara S, assignees. , assignees. Cardiotonic agent comprising GRK inhibitor. World patent WO2007034846 2007. March 29.

Immink RV, Van Den Born BJ, Van Montfrans GA, Koopmans RP, Karemaker JM & Van Lieshout JJ (2004). Impaired cerebral autoregulation in patients with malignant hypertension. *Circulation,* 110**:** 2241-5.

Inagami T & Eguchi S (2000). Angiotensin II-mediated vascular smooth muscle cell growth signaling. *Braz J Med Biol Res,* 33**:** 619-24.

Ishizaka N, Alexander RW, Laursen JB, Kai H, Fukui T, Oppermann M, et al. Griendling KK (1997). G protein-coupled receptor kinase 5 in cultured vascular smooth muscle cells and rat aorta. Regulation by angiotensin II and hypertension. *J Biol Chem,* 272**:** 32482-8.

Islam KN, Bae JW, Gao E & Koch WJ (2013). Regulation of nuclear factor κB (NF-κB) in the nucleus of cardiomyocytes by G protein-coupled receptor kinase 5 (GRK5). *J Biol Chem,* 288**:** 35683-9.

Israili ZH, Hernandez-Hernandez R & Valasco M (2007). The future of antihypertensive treatment. *Am J Ther,* 14**:** 121-34.

Issaq HJ, Xiao Z & Veenstra TD (2007). Serum and plasma proteomics. *Chem Rev,* 107**:** 3601-20.

Iwasaki H, Eguchi S, Ueno H, Marumo F & Hirata Y (1999). Endothelin-mediated vascular growth requires p42/p44 mitogen-activated protein kinase and p70 S6 kinase cascades via transactivation of epidermal growth factor receptor. *Endocrinology,* 140**:** 4659-68.

Jaber M, Koch WJ, Rockman H, Smith B, Bond RA, Sulik KK, et al. Giros B (1996). Essential role of β-adrenergic receptor kinase 1 in cardiac development and function. *Proc Natl Acad Sci U S A,* 93**:** 12974-9.

Jackson R & Bellamy M (2015). Antihypertensive drugs. *BJA Education,* 15**:** 280-285.

Jackson WF (2000). Ion channels and vascular tone. *Hypertension,* 35**:** 173-8.

Janakidevi K, Fisher MA, Del Vecchio PJ, Tiruppathi C, Figge J & Malik AB (1992). Endothelin-1 stimulates DNA synthesis and proliferation of pulmonary artery smooth muscle cells. *American Journal of Physiology-Cell Physiology,* 263**:** C1295-C1301.

Jarajapu YP & Knot HJ (2005). Relative contribution of Rho kinase and protein kinase C to myogenic tone in rat cerebral arteries in hypertension. *Am J Physiol Heart Circ Physiol,* 289**:** H1917-22.

Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, et al. Et Al. (1992). Molecular basis of human hypertension: role of angiotensinogen. *Cell,* 71**:** 169-80.

Jia L, Wang R & Tang DD (2012). Abl regulates smooth muscle cell proliferation by modulating actin dynamics and ERK1/2 activation. *American journal of physiology. Cell physiology,* 302**:** C1026-C1034.

Jiang L-P, Fan S-Q, Xiong Q-X, Zhou Y-C, Yang Z-Z, Li G-F, et al. Chen Y-B (2018). GRK5 functions as an oncogenic factor in non-small-cell lung cancer. *Cell Death & Disease,* 9**:** 295.

Jiang X, Yang P & Ma L (2009). Kinase activity-independent regulation of cyclin pathway by GRK2 is essential for zebrafish early development. *Proc Natl Acad Sci U S A,* 106**:** 10183-8.

Jimenez-Sainz MC, Murga C, Kavelaars A, Jurado-Pueyo M, Krakstad BF, Heijnen CJ, et al. Aragay AM (2006). G protein-coupled receptor kinase 2 negatively regulates chemokine signaling at a level downstream from G protein subunits. *Mol Biol Cell,* 17**:** 25-31.

Jing L, Zhang J, Sun J, Guo F, An X, Yang K & Li PA (2011). Inhibition of extracellular signal-regulated kinases ameliorates hypertension-induced renal vascular remodeling in rat models. *Int J Mol Sci,* 12**:** 8333-46.

John SW, Krege JH, Oliver PM, Hagaman JR, Hodgin JB, Pang SC, et al. Smithies O (1995). Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *Science,* 267**:** 679-81.

Johnsen DD, Kacimi R, Anderson BE, Thomas TA, Said S & Gerdes AM (2005). Protein kinase C isozymes in hypertension and hypertrophy: insight from SHHF rat hearts. *Mol Cell Biochem,* 270**:** 63-9.

Julius S & Nesbitt S (1996). Sympathetic overactivity in hypertension. A moving target. *Am J Hypertens,* 9**:** 113S-120S.
Jurikova M, Danihel L, Polak S & Varga I (2016). Ki67, PCNA, and MCM proteins: Markers of proliferation in the diagnosis of breast cancer. *Acta Histochem,* 118**:** 544-52.

Kamal FA, Travers JG & Blaxall BC (2012). G protein-coupled receptor kinases in cardiovascular disease: why "where" matters. *Trends Cardiovasc Med,* 22**:** 213-9.

Kannel WB (2004). Hypertensive Risk Assessment:Cardiovascular Risk Factors and Hypertension. *The Journal of Clinical Hypertension,* 6**:** 393-399.

Karnik SS, Unal H, Kemp JR, Tirupula KC, Eguchi S, Vanderheyden PML & Thomas WG (2015). International Union of Basic and Clinical Pharmacology. XCIX. Angiotensin Receptors: Interpreters of Pathophysiological Angiotensinergic Stimuli. *Pharmacological Reviews,* 67**:** 754-819.

Kawahara Y, Sunako M, Tsuda T, Fukuzaki H, Fukumoto Y & Takai Y (1988). Angiotensin II induces expression of the c-fos gene through protein kinase C activation and calcium ion mobilization in cultured vascular smooth muscle cells. *Biochem Biophys Res Commun,* 150**:** 52-9.

Kawai T, Forrester SJ, O'brien S, Baggett A, Rizzo V & Eguchi S (2017). AT1 receptor signaling pathways in the cardiovascular system. *Pharmacological Research,* 125**:** 4-13.

Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK & He J (2005). Global burden of hypertension: analysis of worldwide data. *Lancet,* 365**:** 217-23.

Kelly E, Bailey CP & Henderson G (2008). Agonist-selective mechanisms of GPCR desensitization. *British journal of pharmacology,* 153 Suppl 1**:** S379-S388.

Kendall RT, Lee MH, Pleasant DL, Robinson K, Kuppuswamy D, Mcdermott PJ & Luttrell LM (2014). Arrestin-dependent angiotensin AT1 receptor signaling regulates Akt and mTor-mediated protein synthesis. *J Biol Chem,* 289**:** 26155-66.

Khalil RA (2013). Protein Kinase C Inhibitors as Modulators of Vascular Function and their Application in Vascular Disease. *Pharmaceuticals (Basel),* 6**:** 407-39.

Khalil RA, Lajoie C, Resnick MS & Morgan KG (1992). Ca(2+)-independent isoforms of protein kinase C differentially translocate in smooth muscle. *Am J Physiol,* 263**:** C714-9.

Kieserman JM, Myers VD, Dubey P, Cheung JY & Feldman AM (2019). Current Landscape of Heart Failure Gene Therapy. *J Am Heart Assoc,* 8**:** e012239.

Kim J-Y, Kim K-H, Lee W-R, An H-J, Lee S-J, Han S-M, et al. Park K-K (2015). Apamin inhibits PDGF-BB-induced vascular smooth muscle cell proliferation and migration through suppressions of activated Akt and Erk signaling pathway. *Vascular Pharmacology,* 70**:** 8-14.

Kim J, Ahn S, Rajagopal K & Lefkowitz RJ (2009). Independent β-arrestin2 and Gq/protein kinase Czeta pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. *J Biol Chem,* 284**:** 11953-62.

Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H & Lefkowitz RJ (2005a). Functional antagonism of different G protein-coupled receptor kinases for β-arrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci U S A,* 102**:** 1442-7.

Kim J, Lee YR, Lee CH, Choi WH, Lee CK, Kim J, et al. Kim B (2005b). Mitogen-activated protein kinase contributes to elevated basal tone in aortic smooth muscle from hypertensive rats. *Eur J Pharmacol,* 514**:** 209-15.

Kim S-J, Masaki T, Rowley R, Leypoldt JK, Mohammad SF & Cheung AK (2005c). Different responses by cultured aortic and venous smooth muscle cells to γ radiation. *Kidney International,* 68**:** 371-377.

Kim S, Murakami T, Izumi Y, Yano M, Miura K, Yamanaka S & Iwao H (1997). Extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activities are continuously and differentially increased in aorta of hypertensive rats. *Biochem Biophys Res Commun,* 236**:** 199-204.

Kim TK (2015). T test as a parametric statistic. *Korean journal of anesthesiology,* 68**:** 540-546.

Kindy MS & Sonenshein GE (1986). Regulation of oncogene expression in cultured aortic smooth muscle cells. Post-transcriptional control of c-myc mRNA. *Journal of Biological Chemistry,* 261**:** 12865-12868.

King DW, Steinmetz R, Wagoner HA, Hannon TS, Chen LY, Eugster EA & Pescovitz OH (2003). Differential expression of GRK isoforms in nonmalignant and malignant human granulosa cells. *Endocrine,* 22**:** 135-42.

Kirby RS (2003). Effect of Doxazosin GITS on Blood Pressure in Hypertensive and Normotensive Patients: A Review of Hypertension and BPH Studies AU - LUND-JOHANSEN, PER. *Blood Pressure,* 12**:** 5-13.

Klemke RL, Cai S, Giannini AL, Gallagher PJ, De Lanerolle P & Cheresh DA (1997). Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol,* 137**:** 481-92.

Klöppel G & La Rosa S (2018). Ki67 labeling index: assessment and prognostic role in gastroenteropancreatic neuroendocrine neoplasms. *Virchows Archiv,* 472**:** 341-349.

Koch WJ, Inglese J, Stone WC & Lefkowitz RJ (1993). The binding site for the βγ subunits of heterotrimeric G proteins on the β-adrenergic receptor kinase. *Journal of Biological Chemistry,* 268**:** 8256-8260.

Kodama D & Togari A (2013). Noradrenaline stimulates cell proliferation by suppressing potassium channels via G(i/o) -protein-coupled α(1B) -adrenoceptors in human osteoblasts. *British journal of pharmacology,* 168**:** 1230-1239.

Kohan DE & Barton M (2014). Endothelin and endothelin antagonists in chronic kidney disease. *Kidney international,* 86**:** 896-904.

Komuro I, Kurihara H, Sugiyama T, Yoshizumi M, Takaku F & Yazaki Y (1988). Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS Lett,* 238**:** 249-52.

Korsgaard N & Mulvany MJ (1988). Cellular hypertrophy in mesenteric resistance vessels from renal hypertensive rats. *Hypertension,* 12**:** 162-7.

Korsgaard N, Aalkjaer C, Heagerty AM, Izzard AS & Mulvany MJ (1993). Histology of subcutaneous small arteries from patients with essential hypertension. *Hypertension,* 22**:** 523-6.

Kosch M, Hausberg M, Barenbrock M, Posadzy-Malaczynska A, Rahn KH & Kisters K (2001). Increased membraneous calcium concentrations in primary hypertension: a causal link to pathogenesis? *J Hum Hypertens,* 15**:** 37-40.

Krum H, Viskoper RJ, Lacourciere Y, Budde M & Charlon V (1998). The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. Bosentan Hypertension Investigators. *N Engl J Med,* 338**:** 784-90.

Kruskal WH & Wallis WA (1952). Use of Ranks in One-Criterion Variance Analysis. *Journal of the American Statistical Association,* 47**:** 583-621.

Kubo A, Satoh C, Kishioka H, Teng J, Kanmatsuse K, Nakayama M & Fukuda N (2000). Angiotensin II regulates the cell cycle of vascular smooth muscle cells from SHR*. *American Journal of Hypertension,* 13**:** 1117-1124.

Kubo T, Ibusuki T, Chiba S, Kambe T & Fukumori R (2002). Altered mitogen-activated protein kinase activation in vascular smooth muscle cells from spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol,* 29**:** 537-43.

Lacolley P, Safar ME, Regnault V & Frohlich ED (2009). Angiotensin II, mechanotransduction, and pulsatile arterial hemodynamics in hypertension. *Am J Physiol Heart Circ Physiol,* 297**:** H1567-75.

Laragh J (2001). Laragh's lessons in pathophysiology and clinical pearls for treating hypertension*. *American Journal of Hypertension,* 14**:** 491-503.

Laurent S (2017). Antihypertensive drugs. *Pharmacol Res,* 124**:** 116-125.

Laurent S & Boutouyrie P (2015). The structural factor of hypertension: large and small artery alterations. *Circ Res,* 116**:** 1007-21.

Lee H-C, Tsai J-N, Liao P-Y, Tsai W-Y, Lin K-Y, Chuang C-C, et al. Tsai H-J (2007). Glycogen synthase kinase 3 α and 3 β have distinct functions during cardiogenesis of zebrafish embryo. *BMC developmental biology,* 7**:** 93-93.

Lee S, Lim HJ, Park HY, Lee KS, Park JH & Jang Y (2006). Berberine inhibits rat vascular smooth muscle cell proliferation and migration in vitro and improves neointima formation after balloon injury in vivo. Berberine improves neointima formation in a rat model. *Atherosclerosis,* 186**:** 29-37.

Lefkowitz RJ (1993). G protein-coupled receptor kinases. *Cell,* 74**:** 409-412.

Lefkowitz RJ & Shenoy SK (2005). Transduction of Receptor Signals by β-Arrestins. *Science,* 308**:** 512.

Leicht M, Briest W & Zimmer H-G (2003). Regulation of norepinephrine-induced proliferation in cardiac fibroblasts by interleukin-6 and p42/p44 mitogen activated protein kinase. *Molecular and Cellular Biochemistry,* 243**:** 65-72.

Leonard BE (1995). SSRI differentiation: Pharmacology and pharmacokinetics. *Hum Psychopharmacol,* 10 Suppl 3**:** S149-S158.

Lesh RE, Nixon GF, Fleischer S, Airey JA, Somlyo AP & Somlyo AV (1998). Localization of ryanodine receptors in smooth muscle. *Circ Res,* 82**:** 175-85.

Leslie NR & Downes CP (2004). PTEN function: how normal cells control it and tumour cells lose it. *The Biochemical journal,* 382**:** 1-11.

Li JS, Schurch W & Schiffrin EL (1996a). Renal and vascular effects of chronic endothelin receptor antagonism in malignant hypertensive rats. *Am J Hypertens,* 9**:** 803-11.

Li JS, Knafo L, Turgeon A, Garcia R & Schiffrin EL (1996b). Effect of endothelin antagonism on blood pressure and vascular structure in renovascular hypertensive rats. *Am J Physiol,* 271**:** H88-93.

Li L, Liu D, Bu D, Chen S, Wu J, Tang C, et al. Jin H (2013). Brg1-dependent epigenetic control of vascular smooth muscle cell proliferation by hydrogen sulfide. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1833**:** 1347-1355.

Li LT, Jiang G, Chen Q & Zheng JN (2015). Ki67 is a promising molecular target in the diagnosis of cancer (review). *Mol Med Rep,* 11**:** 1566-72.

Li T, Li D, Xu H, Zhang H, Tang D & Cao H (2016). Wen-Xin Decoction ameliorates vascular endothelium dysfunction via the PI3K/AKT/eNOS pathway in experimental atherosclerosis in rats. *BMC Complement Altern Med,* 16**:** 27.

Li Y, Levesque LO & Anand-Srivastava MB (2010). Epidermal growth factor receptor transactivation by endogenous vasoactive peptides contributes to hyperproliferation of vascular smooth muscle cells of SHR. *Am J Physiol Heart Circ Physiol,* 299**:** H1959-67.

Li YP (2013). GRK6 expression in patients with hepatocellular carcinoma. *Asian Pac J Trop Med,* 6**:** 220-3.

Li Z, Yu C, Han Y, Ren H, Shi W, Fu C, et al. Jose PA (2008). Inhibitory effect of D1-like and D3 dopamine receptors on norepinephrine-induced proliferation in vascular smooth muscle cells. *American journal of physiology. Heart and circulatory physiology,* 294**:** H2761-H2768.

Liang J & Slingerland JM (2003). Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell cycle (Georgetown, Tex.),* 2**:** 339-345.

Liao H, Gong J, Zhang W & Guo X (2012). Valsartan inhibits angiotensin II-induced proliferation of vascular smooth muscle cells via regulating the expression of mitofusin 2. *Journal of Huazhong University of Science and Technology [Medical Sciences],* 32**:** 31-35.

Liu C, Su T, Li F, Li L, Qin X, Pan W, et al. Chen L (2010a). PI3K/Akt signaling transduction pathway is involved in rat vascular smooth muscle cell proliferation induced by apelin-13. *Acta Biochim Biophys Sin (Shanghai),* 42**:** 396-402.

Liu G, Hitomi H, Hosomi N, Lei B, Pelisch N, Nakano D, et al. Nishiyama A (2010b). Mechanical stretch potentiates angiotensin II-induced proliferation in spontaneously hypertensive rat vascular smooth muscle cells. *Hypertens Res,* 33**:** 1250-7.

Liu K, Southall N, Titus SA, Inglese J, Eskay RL, Shinn P, et al. Zheng W (2010c). A multiplex calcium assay for identification of GPCR agonists and antagonists. *Assay and drug development technologies,* 8**:** 367-379.

Liu R, Zhang Q, Luo Q, Qiao H, Wang P, Yu J, et al. Qu L (2017). Norepinephrine stimulation of α1D-adrenoceptor promotes proliferation of pulmonary artery smooth muscle cells via ERK-1/2 signaling. *The International Journal of Biochemistry & Cell Biology,* 88**:** 100-112.

Liu S, Premont RT, Kontos CD, Huang J & Rockey DC (2003). Endothelin-1 activates endothelial cell nitric-oxide synthase via heterotrimeric G-protein βγ subunit signaling to protein jinase B/Akt. *J Biol Chem,* 278**:** 49929-35.

Liu S, Premont RT, Kontos CD, Zhu S & Rockey DC (2005). A crucial role for GRK2 in regulation of endothelial cell nitric oxide synthase function in portal hypertension. *Nature Medicine,* 11**:** 952-958.

Ljuca F & Drevensek G (2010). Endothelin-1 induced vascular smooth muscle cell proliferation is mediated by cytochrome p-450 arachidonic acid metabolites. *Bosn J Basic Med Sci,* 10**:** 223-6.

Ljuca F, Nuhbegovic S, Ljuca D, Karamehic J & Halilbasic A (2001). [PI-3 kinase/AKT/PKB signal transduction pathway in kidneys mediates angiotensin II-induced hypertension]. *Med Arh,* 55**:** 189-91.

Lodowski DT, Pitcher JA, Capel WD, Lefkowitz RJ & Tesmer JJG (2003). Keeping G Proteins at Bay: A Complex Between G Protein-Coupled Receptor Kinase 2 and Gßγ. *Science,* 300**:** 1256-1262.

Loirand G & Pacaud P (2010). The role of Rho protein signaling in hypertension. *Nat Rev Cardiol,* 7**:** 637-47.

Lombardi MS, Kavelaars A, Cobelens PM, Schmidt RE, Schedlowski M & Heijnen CJ (2001). Adjuvant arthritis induces down-regulation of G protein-coupled receptor kinases in the immune system. *J Immunol,* 166**:** 1635-40.

Lovic D, Manolis AJ, Lovic B, Stojanov V, Lovic M, Pittaras A & Jakovljevic B (2014). The pathophysiological basis of carotid baroreceptor stimulation for the treatment of resistant hypertension. *Curr Vasc Pharmacol,* 12**:** 16-22.

Lowe JD, Sanderson HS, Cooke AE, Ostovar M, Tsisanova E, Withey SL, et al. Bailey CP (2015). Role of G Protein-Coupled Receptor Kinases 2 and 3 in mu-Opioid Receptor Desensitization and Internalization. *Mol Pharmacol,* 88**:** 347-56.

Lozinskaya I, Matsuda K & Cox RH (1997). Augmented Contributions of Voltage-Gated Ca2 Channels to Contractile Responses in Spontaneously Hypertensive Rat Mesenteric Arteries*. *American Journal of Hypertension,* 10**:** 1231-1239.

Lu MH, Chao CF, Huang CG & Chang LT (2003). Coculture of Vascular Endothelial Cells and Smooth Muscle Cells from Spontaneously Hypertensive Rats. *Clinical and Experimental Hypertension,* 25**:** 413-425.

Luo Q, Wang X, Liu R, Qiao H, Wang P, Jiang C, et al. Qu L (2018). α1A-adrenoceptor is involved in norepinephrine-induced proliferation of pulmonary artery smooth muscle cells via CaMKII signaling. *Journal of Cellular Biochemistry,* 0.

Luscher TF, Sturzenegger M & Noll G (1996). [Heart, brain and hypertension]. *Schweiz Med Wochenschr,* 126**:** 1449-56.

Luttrell LM & Miller WE (2013). Arrestins as regulators of kinases and phosphatases. *Prog Mol Biol Transl Sci,* 118**:** 115-47.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL & Lefkowitz RJ (2001). Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds. *Proc Natl Acad Sci U S A,* 98**:** 2449-54.

Lymperopoulos A & Bathgate A (2013). Arrestins in the cardiovascular system. *Prog Mol Biol Transl Sci,* 118**:** 297-334.

Ma KT, Li XZ, Li L, Jiang XW, Chen XY, Liu WD, et al. Si JQ (2014). Role of gap junctions in the contractile response to agonists in the mesenteric artery of spontaneously hypertensive rats. *Hypertens Res,* 37**:** 110-5.

Ma L & Pei G (2007). β-arrestin signaling and regulation of transcription. *Journal of Cell Science,* 120**:** 213-218.

Maaser K, Hopfner M, Kap H, Sutter AP, Barthel B, Von Lampe B, et al. Scherubl H (2002). Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors. *Br J Cancer,* 86**:** 636-44.

Mabrouk ME, Touyz RM & Schiffrin EL (2001). Differential ANG II-induced growth activation pathways in mesenteric artery smooth muscle cells from SHR. *American Journal of Physiology-Heart and Circulatory Physiology,* 281**:** H30-H39.

Macaulay K, Doble BW, Patel S, Hansotia T, Sinclair EM, Drucker DJ, et al. Woodgett JR (2007). Glycogen Synthase Kinase 3α-Specific Regulation of Murine Hepatic Glycogen Metabolism. *Cell Metabolism,* 6**:** 329-337.

Magnussen CG (2017). Carotid artery intima-media thickness and hypertensive heart disease: a short review. *Clin Hypertens,* 23**:** 7.

Mahmood SS, Levy D, Vasan RS & Wang TJ (2014). The Framingham Heart Study and the epidemiology of cardiovascular disease: a historical perspective. *Lancet (London, England),* 383**:** 999-1008.

Mahmood T & Yang PC (2012). Western blot: technique, theory, and trouble shooting. *N Am J Med Sci,* 4**:** 429-34.

Mancia G & Grassi G (2014). The Autonomic Nervous System and Hypertension. *Circulation Research,* 114**:** 1804-1814.

Mancia G, Fagard R, Narkiewicz K, Redon J, Zanchetti A, Böhm M, et al. Wood DA (2013). 2013 ESH/ESC guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *European Heart Journal,* 34**:** 2159-2219.

Marieb EN (2013). *Human anatomy and physiology,* Boston, Mass aLondon, Pearson.

Markou T, Cullingford TE, Giraldo A, Weiss SC, Alsafi A, Fuller SJ, et al. Sugden PH (2008). Glycogen synthase kinases 3α and 3β in cardiac myocytes: Regulation and consequences of their inhibition. *Cellular Signalling,* 20**:** 206-218.

Martelli AM, Tabellini G, Bressanin D, Ognibene A, Goto K, Cocco L & Evangelisti C (2012). The emerging multiple roles of nuclear Akt. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1823**:** 2168-2178.

Martini JS, Raake P, Vinge LE, Degeorge BR, Jr., Chuprun JK, Harris DM, et al. Koch WJ (2008). Uncovering G protein-coupled receptor kinase-5 as a histone deacetylase kinase in the nucleus of cardiomyocytes. *Proc Natl Acad Sci U S A,* 105**:** 12457-62.

Marx SO, Totary-Jain H & Marks AR (2011). Vascular smooth muscle cell proliferation in restenosis. *Circ Cardiovasc Interv,* 4**:** 104-11.

Mayer G, Wulffen B, Huber C, Brockmann J, Flicke B, Neumann L, et al. Blind M (2008). An RNA molecule that specifically inhibits G-protein-coupled receptor kinase 2 in vitro. *RNA (New York, N.Y.),* 14**:** 524-534.

Mayet J & Hughes A (2003). Cardiac and vascular pathophysiology in hypertension. *Heart,* 89**:** 1104-9.

Mayor F, Jr., Penela P & Ruiz-Gomez A (1998). Role of G protein-coupled receptor kinase 2 and arrestins in β-adrenergic receptor internalization. *Trends Cardiovasc Med,* 8**:** 234-40.

Mebratu Y & Tesfaigzi Y (2009). How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle,* 8**:** 1168-1175.

Mehta PK & Griendling KK (2007). Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *American Journal of Physiology-Cell Physiology,* 292**:** C82-C97.

Meloche S, Seuwen K, Pages G & Pouyssegur J (1992). Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation between late phase activation and mitogenicity. *Mol Endocrinol,* 6**:** 845-54.

Meng D, Lynch MJ, Huston E, Beyermann M, Eichhorst J, Adams DR, et al. Baillie GS (2009). MEK1 binds directly to βarrestin1, influencing both its phosphorylation by ERK and the timing of its isoprenaline-stimulated internalization. *J Biol Chem,* 284**:** 11425-35.

Messerli FH, Williams B & Ritz E (2007). Essential hypertension. *The Lancet,* 370**:** 591-603.

Metaye T, Menet E, Guilhot J & Kraimps JL (2002). Expression and activity of g protein-coupled receptor kinases in differentiated thyroid carcinoma. *J Clin Endocrinol Metab,* 87**:** 3279-86.

Michael A, Haq S, Chen X, Hsich E, Cui L, Walters B, et al. Force T (2004). Glycogen synthase kinase-3β regulates growth, calcium homeostasis, and diastolic function in the heart. *J Biol Chem,* 279**:** 21383-93.

Michal AM, So CH, Beeharry N, Shankar H, Mashayekhi R, Yen TJ & Benovic JL (2012). G Protein-coupled receptor kinase 5 is localized to centrosomes and regulates cell cycle progression. *J Biol Chem,* 287**:** 6928-40.

Mitchell GF, Guo CY, Benjamin EJ, Larson MG, Keyes MJ, Vita JA, et al. Levy D (2007). Cross-sectional correlates of increased aortic stiffness in the community: the Framingham Heart Study. *Circulation,* 115**:** 2628-36.

Molloy CJ, Pawlowski JE, Taylor DS, Turner CE, Weber H & Peluso M (1996). Thrombin receptor activation elicits rapid protein tyrosine phosphorylation and stimulation of the raf-1/MAP kinase pathway preceding delayed mitogenesis in cultured rat aortic smooth muscle cells: evidence for an obligate autocrine mechanism promoting cell proliferation induced by G-protein-coupled receptor agonist. *J Clin Invest,* 97**:** 1173-83.

Moore SF, Van Den Bosch MTJ, Hunter RW, Sakamoto K, Poole AW & Hers I (2013). Dual regulation of glycogen synthase kinase 3 (GSK3)α/β by protein kinase C (PKC)α and Akt promotes thrombin-mediated integrin αIIbβ3 activation and granule secretion in platelets. *The Journal of biological chemistry,* 288**:** 3918-3928.

Mora A, Sakamoto K, Mcmanus EJ & Alessi DR (2005). Role of the PDK1-PKB-GSK3 pathway in regulating glycogen synthase and glucose uptake in the heart. *FEBS Lett,* 579**:** 3632-8.

Morisco C, Zebrowski D, Condorelli G, Tsichlis P, Vatner SF & Sadoshima J (2000). The Akt-glycogen synthase kinase 3β pathway regulates transcription of atrial natriuretic factor induced by β-adrenergic receptor stimulation in cardiac myocytes. *Journal of Biological Chemistry,* 275**:** 14466-14475.

Morris GE, Nelson CP, Standen NB, Challiss RA & Willets JM (2010). Endothelin signalling in arterial smooth muscle is tightly regulated by G protein-coupled receptor kinase 2. *Cardiovasc Res,* 85**:** 424-33.

Morris GE, Nelson CP, Brighton PJ, Standen NB, Challiss RA & Willets JM (2012). Arrestins 2 and 3 differentially regulate ETA and P2Y2 receptor-mediated cell signaling and migration in arterial smooth muscle. *Am J Physiol Cell Physiol,* 302**:** C723-34.

Morris GE, Nelson CP, Everitt D, Brighton PJ, Standen NB, Challiss RA & Willets JM (2011). G protein-coupled receptor kinase 2 and arrestin2 regulate arterial smooth muscle P2Y-purinoceptor signalling. *Cardiovasc Res,* 89**:** 193-203.

Mulvany MJ (1999). Vascular remodelling of resistance vessels: can we define this? *Cardiovascular Research,* 41**:** 9-13.

Mulvany MJ (2002). Small artery remodeling in hypertension. *Curr Hypertens Rep,* 4**:** 49-55.

Mulvany MJ (2012). Small artery remodelling in hypertension. *Basic Clin Pharmacol Toxicol,* 110**:** 49-55.

Murga C, Laguinge L, Wetzker R, Cuadrado A & Gutkind JS (1998). Activation of Akt/Protein Kinase B by G Protein-coupled Receptors: A ROLE FOR α AND βγ SUBUNITS OF HETEROTRIMERIC G PROTEINS ACTING THROUGH PHOSPHATIDYLINOSITOL-3-OH KINASEγ. *Journal of Biological Chemistry,* 273**:** 19080-19085.

Murga C, Arcones AC, Cruces-Sande M, Briones AM, Salaices M & Mayor F, Jr. (2019). G Protein-Coupled Receptor Kinase 2 (GRK2) as a Potential Therapeutic Target in Cardiovascular and Metabolic Diseases. *Frontiers in pharmacology,* 10**:** 112-112.

Murphy CJ, Campbell S, Araki-Sasaki K & Marfurt CF (1998). Effect of norepinephrine on proliferation, migration, and adhesion of SV-40 transformed human corneal epithelial cells. *Cornea,* 17**:** 529-36.

Murphy LO, Smith S, Chen RH, Fingar DC & Blenis J (2002). Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol,* 4**:** 556-64.

Murthy KS & Makhlouf GM (1998). Coexpression of ligand-gated P2X and G protein-coupled P2Y receptors in smooth muscle. Preferential activation of P2Y receptors coupled to phospholipase C (PLC)-β1 via Gαq/11 and to PLC-β3 via Gβγi3. *J Biol Chem,* 273**:** 4695-704.

Muthalif MM, Benter IF, Uddin MR & Malik KU (1996). Calcium/calmodulin-dependent protein kinase IIα mediates activation of mitogen-activated protein kinase and cytosolic phospholipase A2 in norepinephrine-induced arachidonic acid release in rabbit aortic smooth muscle cells. *J Biol Chem,* 271**:** 30149-57.

Muto A, Fitzgerald TN, Pimiento JM, Maloney SP, Teso D, Paszkowiak JJ, et al. Dardik A (2007). Smooth muscle cell signal transduction: implications of vascular biology for vascular surgeons. *J Vasc Surg,* 45 Suppl A**:** A15-24.

Naftilan AJ, Pratt RE & Dzau VJ (1989a). Induction of platelet-derived growth factor A-chain and c-myc gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. *J Clin Invest,* 83**:** 1419-24.

Naftilan AJ, Gilliland GK, Eldridge CS & Kraft AS (1990). Induction of the proto-oncogene c-jun by angiotensin II. *Mol Cell Biol,* 10**:** 5536-40.

Naftilan AJ, Pratt RE, Eldridge CS, Lin HL & Dzau VJ (1989b). Angiotensin II induces c-fos expression in smooth muscle via transcriptional control. *Hypertension,* 13**:** 706-11.

Naga Prasad SV, Barak LS, Rapacciuolo A, Caron MG & Rockman HA (2001). Agonist-dependent Recruitment of Phosphoinositide 3-Kinase to the Membrane by β-Adrenergic Receptor Kinase 1: A ROLE IN RECEPTOR SEQUESTRATION. *Journal of Biological Chemistry,* 276**:** 18953-18959.

Naga Prasad SV, Laporte SA, Chamberlain D, Caron MG, Barak L & Rockman HA (2002a). Phosphoinositide 3-kinase regulates β2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/β-arrestin complex. *J Cell Biol,* 158**:** 563-75.

Naga Prasad SV, Laporte SA, Chamberlain D, Caron MG, Barak L & Rockman HA (2002b). Phosphoinositide 3-kinase regulates β2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/β-arrestin complex. *The Journal of cell biology,* 158**:** 563-575.

Nagano K, Toba K, Akishita M, Watanabe T, Kozaki K, Eto M, et al. Ouchi Y (2000). Prostanoids regulate proliferation of vascular smooth muscle cells induced by arginine vasopressin. *Eur J Pharmacol,* 389**:** 25-33.

Nakamura T, Fujii S, Hoshino J, Saito Y, Mizuno H, Saito Y & Kurabayashi M (2005). Selective Angiotensin Receptor Antagonism with Valsartan Decreases Arterial Stiffness Independently of Blood Pressure Lowering in Hypertensive Patients. *Hypertension Research,* 28**:** 937.

Nambi V, Chambless L, He M, Folsom AR, Mosley T, Boerwinkle E & Ballantyne CM (2012). Common carotid artery intima-media thickness is as good as carotid intima-media thickness of all carotid artery segments in improving prediction of coronary heart disease risk in the Atherosclerosis Risk in Communities (ARIC) study. *Eur Heart J,* 33**:** 183-90.

National Center for Biotechnology Information. PubChem Database. Paroxetine, CID=43815, https://pubchem.ncbi.nlm.nih.gov/compound/Paroxetine.

National Center for Biotechnology Information. PubChem Database. Fluoxetine, CID=3386, https://pubchem.ncbi.nlm.nih.gov/compound/Fluoxetine.

National Center for Biotechnology Information. PubChem Database. Takeda compound 101, CID=11677079, https://pubchem.ncbi.nlm.nih.gov/compound/Takeda-compound-101.

Naryzhny SN & Lee H (2007). Characterization of proliferating cell nuclear antigen (PCNA) isoforms in normal and cancer cells: There is no cancer-associated form of PCNA. *FEBS Letters,* 581**:** 4917-4920.

Nash CA, Nelson CP, Mistry R, Moeller-Olsen C, Christofidou E, Challiss RaJ & Willets JM (2018). Differential regulation of β2-adrenoceptor and adenosine A2B receptor signalling by GRK and arrestin proteins in arterial smooth muscle. *Cell Signal,* 51**:** 86-98.

Nelson PR, Yamamura S, Mureebe L, Itoh H & Kent KC (1998). Smooth muscle cell migration and proliferation are mediated by distinct phases of activation of the intracellular messenger mitogen-activated protein kinase. *J Vasc Surg,* 27**:** 117-25.

Nemenoff RA, Horita H, Ostriker AC, Furgeson SB, Simpson PA, Vanputten V, et al. Weiser-Evans MC (2011). SDF-1α induction in mature smooth muscle cells by inactivation of PTEN is a critical mediator of exacerbated injury-induced neointima formation. *Arterioscler Thromb Vasc Biol,* 31**:** 1300-8.

Nogues L, Reglero C, Rivas V, Salcedo A, Lafarga V, Neves M, et al. Penela P (2016). G Protein-coupled Receptor Kinase 2 (GRK2) Promotes Breast Tumorigenesis Through a HDAC6-Pin1 Axis. *EBioMedicine,* 13**:** 132-145.

Northcott Carrie A, Poy Matthew N, Najjar Sonia M & Watts Stephanie W (2002). Phosphoinositide 3-Kinase Mediates Enhanced Spontaneous and Agonist-Induced Contraction in Aorta of Deoxycorticosterone Acetate-Salt Hypertensive Rats. *Circulation Research,* 91**:** 360-369.

Nygaard R, Frimurer TM, Holst B, Rosenkilde MM & Schwartz TW (2009). Ligand binding and micro-switches in 7TM receptor structures. *Trends Pharmacol Sci,* 30**:** 249-59.

O'leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL & Wolfson SK, Jr. (1999). Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. *N Engl J Med,* 340**:** 14-22.

Oakley RH, Laporte SA, Holt JA, Caron MG & Barak LS (2000). Differential Affinities of Visual Arrestin, βArrestin1, and βArrestin2 for G Protein-coupled Receptors Delineate Two Major Classes of Receptors. *Journal of Biological Chemistry,* 275**:** 17201-17210.

Obrenovich ME, Morales LA, Cobb CJ, Shenk JC, Méndez GM, Fischbach K, et al. Aliev G (2009). Insights into cerebrovascular complications and Alzheimer disease through the selective loss of GRK2 regulation. *Journal of cellular and molecular medicine,* 13**:** 853-865.

Oeckler RA, Kaminski PM & Wolin MS (2003). Stretch enhances contraction of bovine coronary arteries via an NAD(P)H oxidase-mediated activation of the extracellular signal-regulated kinase mitogen-activated protein kinase cascade. *Circ Res,* 92**:** 23-31.

Ohira K & Miyakawa T (2011). Chronic treatment with fluoxetine for more than 6 weeks decreases neurogenesis in the subventricular zone of adult mice. *Molecular brain,* 4**:** 10-10.

Ohlstein EH, Arleth A, Bryan H, Elliott JD & Cheng Po S (1992). The selective endothelin ETA receptor antagonist BQ123 antagonizes endothelin-1-mediated mitogenesis. *European Journal of Pharmacology: Molecular Pharmacology,* 225**:** 347-350.

Ohtsu H, Dempsey PJ & Eguchi S (2006). ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. *Am J Physiol Cell Physiol,* 291**:** C1-10.

Olivares-Reyes JA, Smith RD, Hunyady L, Shah BH & Catt KJ (2001). Agonist-induced signaling, desensitization, and internalization of a phosphorylation-deficient AT1A angiotensin receptor. *J Biol Chem,* 276**:** 37761-8.

Oparil S, Zaman M & Calhoun DA (2003). PAthogenesis of hypertension. *Annals of Internal Medicine,* 139**:** 761-776.
Oparil S, Acelajado MC, Bakris GL, Berlowitz DR, Cífková R, Dominiczak AF, et al. Whelton PK (2018). Hypertension. *Nature Reviews Disease Primers,* 4**:** 18014.

Oppermann M, Freedman NJ, Alexander RW & Lefkowitz RJ (1996). Phosphorylation of the type 1A angiotensin II receptor by G protein-coupled receptor kinases and protein kinase C. *J Biol Chem,* 271**:** 13266-72.

Owens GK (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev,* 75**:** 487-517.

Owens GK & Schwartz SM (1983). Vascular smooth muscle cell hypertrophy and hyperploidy in the Goldblatt hypertensive rat. *Circ Res,* 53**:** 491-501.

Owens GK & Reidy MA (1985). Hyperplastic growth response of vascular smooth muscle cells following induction of acute hypertension in rats by aortic coarctation. *Circ Res,* 57**:** 695-705.

Owens GK, Rabinovitch PS & Schwartz SM (1981). Smooth muscle cell hypertrophy versus hyperplasia in hypertension. *Proceedings of the National Academy of Sciences of the United States of America,* 78**:** 7759-7763.

Owens GK, Kumar MS & Wamhoff BR (2004). Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev,* 84**:** 767-801.

Pagès G, Lenormand P, L'allemain G, Chambard JC, Meloche S & Pouysségur J (1993). Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. *Proceedings of the National Academy of Sciences of the United States of America,* 90**:** 8319-8323.

Panza JA, Quyyumi AA, Brush JE, Jr. & Epstein SE (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med,* 323**:** 22-7.

Panza JA, Casino PR, Badar DM & Quyyumi AA (1993). Effect of increased availability of endothelium-derived nitric oxide precursor on endothelium-dependent vascular relaxation in normal subjects and in patients with essential hypertension. *Circulation,* 87**:** 1475-81.

Paquet JL, Baudouin-Legros M, Brunelle G & Meyer P (1990). Angiotensin II-induced proliferation of aortic myocytes in spontaneously hypertensive rats. *J Hypertens,* 8**:** 565-72.

Pardo M, Abrial E, Jope RS & Beurel E (2016). GSK3β isoform-selective regulation of depression, memory and hippocampal cell proliferation. *Genes, brain, and behavior,* 15**:** 348-355.

Park JB & Schiffrin EL (2001). Small artery remodeling is the most prevalent (earliest?) form of target organ damage in mild essential hypertension. *J Hypertens,* 19**:** 921-30.

Park KW, Yang HM, Youn SW, Yang HJ, Chae IH, Oh BH, et al. Walsh K (2003). Constitutively active glycogen synthase kinase-3β gene transfer sustains apoptosis, inhibits proliferation of vascular smooth muscle cells, and reduces neointima formation after balloon injury in rats. *Arterioscler Thromb Vasc Biol,* 23**:** 1364-9.

Paul M, Mehr AP & Kreutz R (2006). Physiology of Local Renin-Angiotensin Systems. *Physiological Reviews,* 86**:** 747-803.

Pawlowski JE, Taylor DS, Valentine M, Hail ME, Ferrer P, Kowala MC & Molloy CJ (1997). Stimulation of activin A expression in rat aortic smooth muscle cells by thrombin and angiotensin II correlates with neointimal formation in vivo. *J Clin Invest,* 100**:** 639-48.

Penela P, Ribas C & Mayor F (2003). Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cellular Signalling,* 15**:** 973-981.

Penela P, Rivas V, Salcedo A & Mayor F, Jr. (2010a). G protein-coupled receptor kinase 2 (GRK2) modulation and cell cycle progression. *Proc Natl Acad Sci U S A,* 107**:** 1118-23.

Penela P, Murga C, Ribas C, Lafarga V & Mayor F, Jr. (2010b). The complex G protein-coupled receptor kinase 2 (GRK2) interactome unveils new physiopathological targets. *Br J Pharmacol,* 160**:** 821-32.

Peppel K, Jacobson A, Huang X, Murray John P, Oppermann M & Freedman Neil J (2000). Overexpression of G Protein–Coupled Receptor Kinase-2 in Smooth Muscle Cells Attenuates Mitogenic Signaling via G Protein–Coupled and Platelet-Derived Growth Factor Receptors. *Circulation,* 102**:** 793-799.

Peppel K, Zhang L, Huynh TT, Huang X, Jacobson A, Brian L, et al. Freedman NJ (2002). Overexpression of G protein-coupled receptor kinase-2 in smooth muscle cells reduces neointimal hyperplasia. *J Mol Cell Cardiol,* 34**:** 1399-1409.

Perrino C, Naga Prasad SV, Schroder JN, Hata JA, Milano C & Rockman HA (2005). Restoration of β-adrenergic receptor signaling and contractile function in heart failure by disruption of the βARK1/phosphoinositide 3-kinase complex. *Circulation,* 111**:** 2579-87.

Pieper R, Gatlin CL, Makusky AJ, Russo PS, Schatz CR, Miller SS, et al. Steiner S (2003). The human serum proteome: display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. *Proteomics,* 3**:** 1345-64.

Pierce KL, Luttrell LM & Lefkowitz RJ (2001). New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene,* 20**:** 1532-9.

Pinto E (2007). Blood pressure and ageing. *Postgraduate medical journal,* 83**:** 109-114.

Pitcher JA, Freedman NJ & Lefkowitz RJ (1998). G protein-coupled receptor kinases. *Annu Rev Biochem,* 67**:** 653-92.

Prelich G, Tan CK, Kostura M, Mathews MB, So AG, Downey KM & Stillman B (1987). Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. *Nature,* 326**:** 517-20.

Premont RT & Gainetdinov RR (2007). Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu Rev Physiol,* 69**:** 511-34.

Premont RT, Macrae AD, Stoffel RH, Chung N, Pitcher JA, Ambrose C, et al. Lefkowitz RJ (1996). Characterization of the G protein-coupled receptor kinase GRK4. Identification of four splice variants. *J Biol Chem,* 271**:** 6403-10.

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C & Ullrich A (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature,* 402**:** 884-8.

Prewitt RL, Rice DC & Dobrian AD (2002). Adaptation of resistance arteries to increases in pressure. *Microcirculation,* 9**:** 295-304.

Prisant LM (2008). Nebivolol: pharmacologic profile of an ultraselective, vasodilatory β1-blocker. *J Clin Pharmacol,* 48**:** 225-39.

Psaty BM, Heckbert SR, Koepsell TD, Siscovick DS, Raghunathan TE, Weiss NS, et al. Et Al. (1995). The risk of myocardial infarction associated with antihypertensive drug therapies. *JAMA,* 274**:** 620-5.

Puar THK, Mok Y, Debajyoti R, Khoo J, How CH & Ng AKH (2016). Secondary hypertension in adults. *Singapore medical journal,* 57**:** 228-232.

Puetz S, Lubomirov LT & Pfitzer G (2009). Regulation of smooth muscle contraction by small GTPases. *Physiology (Bethesda),* 24**:** 342-56.

Pugh EN & Lamb TD (2000). Chapter 5 Phototransduction in vertebrate rods and cones: Molecular mechanisms of amplification, recovery and light adaptation. *In:* STAVENGA, D. G., DEGRIP, W. J. & PUGH, E. N. (eds.) *Handbook of Biological Physics.* North-Holland.

Qin XP, Ye F, Hu CP, Liao DF, Deng HW & Li YJ (2004). Effect of calcitonin gene-related peptide on angiotensin II-induced proliferation of rat vascular smooth muscle cells. *Eur J Pharmacol,* 488**:** 45-9.

Qiu G, Helmeste DM, Samaranayake AN, Lau WM, Lee TM, Tang SW & So KF (2007). Modulation of the suppressive effect of corticosterone on adult rat hippocampal cell proliferation by paroxetine. *Neurosci Bull,* 23**:** 131-6.

Qiu Y, Liu Y, Li W-H, Zhang H-Q, Tian X-X & Fang W-G (2018). P2Y2 receptor promotes the migration and invasion of breast cancer cells via EMT-related genes Snail and E-cadherin. *Oncology reports,* 39**:** 138-150.

Quelle DE, Ashmun RA, Shurtleff SA, Kato JY, Bar-Sagi D, Roussel MF & Sherr CJ (1993). Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev,* 7**:** 1559-71.

Raake PW, Schlegel P, Ksienzyk J, Reinkober J, Barthelmes J, Schinkel S, et al. Muller OJ (2013). AAV6.βARKct cardiac gene therapy ameliorates cardiac function and normalizes the catecholaminergic axis in a clinically relevant large animal heart failure model. *Eur Heart J,* 34**:** 1437-47.

Raasch W, Betge S, Dendorfer A, Bartels T & Dominiak P (2001). Angiotensin converting enzyme inhibition improves cardiac neuronal uptake of noradrenaline in spontaneously hypertensive rats. *J Hypertens,* 19**:** 1827-33.

Rahman A, Davis B, Lovdahl C, Hanumaiah VT, Feil R, Brakebusch C & Arner A (2014). The small GTPase Rac1 is required for smooth muscle contraction. *J Physiol,* 592**:** 915-26.

Rahmani M, Read JT, Carthy JM, Mcdonald PC, Wong BW, Esfandiarei M, et al. Mcmanus BM (2005). Regulation of the versican promoter by the β-catenin-T-cell factor complex in vascular smooth muscle cells. *J Biol Chem,* 280**:** 13019-28.

Rahuel J, Rasetti V, Maibaum J, Rüeger H, Göschke R, Cohen NC, et al. Grütter MG (2000). Structure-based drug design: the discovery of novel nonpeptide orally active inhibitors of human renin. *Chemistry & Biology*, 7**:** 493-504.

Rainbow RD, Brennan S, Jackson R, Beech AJ, Bengreed A, Waldschmidt HV, et al. Willets JM (2018). Small-Molecule G Protein-Coupled Receptor Kinase Inhibitors Attenuate G Protein-Coupled Receptor Kinase 2-Mediated Desensitization of Vasoconstrictor-Induced Arterial Contractions. *Mol Pharmacol,* 94**:** 1079-1091.

Rajagopal K, Whalen EJ, Violin JD, Stiber JA, Rosenberg PB, Premont RT, et al. Lefkowitz RJ (2006). β-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. *Proc Natl Acad Sci U S A,* 103**:** 16284-9.

Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y, Sakthisekaran D, Sethi G & Nishigaki I (2013). The vascular endothelium and human diseases. *International journal of biological sciences,* 9**:** 1057-1069.

Ram CV (2002). Antihypertensive drugs: an overview. *Am J Cardiovasc Drugs,* 2**:** 77-89.

Ramos-Ruiz R, Penela P, Penn RB & Mayor F, Jr. (2000). Analysis of the human G protein-coupled receptor kinase 2 (GRK2) gene promoter: regulation by signal transduction systems in aortic smooth muscle cells. *Circulation,* 101**:** 2083-9.

Ray JL, Leach R, Herbert JM & Benson M (2001). Isolation of vascular smooth muscle cells from a single murine aorta. *Methods Cell Sci,* 23**:** 185-8.

Reiter E & Lefkowitz RJ (2006). GRKs and β-arrestins: roles in receptor silencing, trafficking and signaling. *Trends Endocrinol Metab,* 17**:** 159-65.

Ren XR, Reiter E, Ahn S, Kim J, Chen W & Lefkowitz RJ (2005). Different G protein-coupled receptor kinases govern G protein and β-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci U S A,* 102**:** 1448-53.

Rengo G, Galasso G, Femminella GD, Parisi V, Zincarelli C, Pagano G, et al. Leosco D (2014). Reduction of lymphocyte G protein-coupled receptor kinase-2 (GRK2) after

exercise training predicts survival in patients with heart failure. *Eur J Prev Cardiol,* 21**:** 4-11.

Rensen SSM, Doevendans PaFM & Van Eys GJJM (2007). Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. *Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation,* 15**:** 100-108.

Reudelhuber TL (2005). The renin-angiotensin system: peptides and enzymes beyond angiotensin II. *Curr Opin Nephrol Hypertens,* 14**:** 155-9.

Riaz A, Huang Y & Johansson S (2016). G-Protein-Coupled Lysophosphatidic Acid Receptors and Their Regulation of AKT Signaling. *International journal of molecular sciences,* 17**:** 215-215.

Ribas C, Penela P, Murga C, Salcedo A, Garcia-Hoz C, Jurado-Pueyo M, et al. Mayor F, Jr. (2007). The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. *Biochim Biophys Acta,* 1768**:** 913-22.

Ringvold HC & Khalil RA (2017). Protein Kinase C as Regulator of Vascular Smooth Muscle Function and Potential Target in Vascular Disorders. *Adv Pharmacol,* 78**:** 203-301.

Rizzoni D, Castellano M, Porteri E, Bettoni G, Muiesan ML & Agabiti-Rosei E (1994). Vascular structural and functional alterations before and after the development of hypertension in SHR. *Am J Hypertens,* 7**:** 193-200.

Rizzoni D, Porteri E, Boari GE, De Ciuceis C, Sleiman I, Muiesan ML, et al. Agabiti-Rosei E (2003). Prognostic significance of small-artery structure in hypertension. *Circulation,* 108**:** 2230-5.

Roberts PJ & Der CJ (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene,* 26**:** 3291-310.

Roberts RE (2012). The extracellular signal-regulated kinase (ERK) pathway: a potential therapeutic target in hypertension. *Journal of experimental pharmacology,* 4**:** 77-83.

Robinson JD & Pitcher JA (2013). G protein-coupled receptor kinase 2 (GRK2) is a Rho-activated scaffold protein for the ERK MAP kinase cascade. *Cell Signal,* 25**:** 2831-9.

Roerecke M, Kaczorowski J, Tobe SW, Gmel G, Hasan OSM & Rehm J (2017). The effect of a reduction in alcohol consumption on blood pressure: a systematic review and meta-analysis. *Lancet Public Health,* 2**:** e108-e120.

Rogier Van Der Velde A, Meijers WC & De Boer RA (2015). Cardiovascular Biomarkers. 167-183.

Roman MJ, Saba PS, Pini R, Spitzer M, Pickering TG, Rosen S, et al. Devereux RB (1992). Parallel cardiac and vascular adaptation in hypertension. *Circulation,* 86**:** 1909-18.

Roovers K, Davey G, Zhu X, Bottazzi ME & Assoian RK (1999). α5β1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. *Mol Biol Cell,* 10**:** 3197-204.

Ross EM & Wilkie TM (2000). GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem,* 69**:** 795-827.

Roy J, Kazi M, Hedin U & Thyberg J (2001). Phenotypic modulation of arterial smooth muscle cells is associated with prolonged activation of ERK1/2. *Differentiation,* 67**:** 50-8.

Rubenstein DA, Yin W & Frame MD (2012). Chapter 5 - Blood Flow in Arteries and Veins. *In:* RUBENSTEIN, D. A., YIN, W. & FRAME, M. D. (eds.) *Biofluid Mechanics.* Boston: Academic Press.

Rudijanto A (2007). The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. *Acta Med Indones,* 39**:** 86-93.

Salamanca DA & Khalil RA (2005). Protein kinase C isoforms as specific targets for modulation of vascular smooth muscle function in hypertension. *Biochemical pharmacology,* 70**:** 1537-1547.

Salcedo A, Mayor F, Jr. & Penela P (2006). Mdm2 is involved in the ubiquitination and degradation of G-protein-coupled receptor kinase 2. *EMBO J,* 25**:** 4752-62.

Sari Y & Zhou FC (2003). Serotonin and its transporter on proliferation of fetal heart cells. *Int J Dev Neurosci,* 21**:** 417-24.

Savoia C, Sada L, Zezza L, Pucci L, Lauri FM, Befani A, et al. Volpe M (2011). Vascular inflammation and endothelial dysfunction in experimental hypertension. *Int J Hypertens,* 2011**:** 281240.

Saward Peter Zahradka L (1997). Angiotensin II Activates Phosphatidylinositol 3-Kinase in Vascular Smooth Muscle Cells. *Circulation Research,* 81**:** 249-257.

Scheerer P & Sommer ME (2017). Structural mechanism of arrestin activation. *Current Opinion in Structural Biology,* 45**:** 160-169.

Schiffrin EL (1995). Endothelin: potential role in hypertension and vascular hypertrophy. *Hypertension,* 25**:** 1135-43.

Schiffrin EL & Deng LY (1992). Effects of Endothelin-1 and Vasopressin on Resistance Arteries of Spontaneously Hypertensive Rats. *American Journal of Hypertension,* 5**:** 817-822.

Schiffrin EL, Deng LY & Larochelle P (1993). Morphology of resistance arteries and comparison of effects of vasoconstrictors in mild essential hypertensive patients. *Clin Invest Med,* 16**:** 177-86.

Schlegel P, Reinkober J, Meinhardt E, Tscheschner H, Gao E, Schumacher SM, et al. Raake PW (2017). G protein-coupled receptor kinase 2 promotes cardiac hypertrophy. *PloS one,* 12**:** e0182110-e0182110.

Schlyer S & Horuk R (2006). I want a new drug: G-protein-coupled receptors in drug development. *Drug Discov Today,* 11**:** 481-93.

Schmieder RE (2010). End organ damage in hypertension. *Deutsches Arzteblatt international,* 107**:** 866-873.

Schonwasser DC, Marais RM, Marshall CJ & Parker PJ (1998). Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol Cell Biol,* 18**:** 790-8.

Schreiner CE, Kumerz M, Gesslbauer J, Schachner D, Joa H, Erker T, et al. Dirsch VM (2010). Resveratrol blocks Akt activation in angiotensin II- or EGF-stimulated vascular smooth muscle cells in a redox-independent manner. *Cardiovascular Research,* 90**:** 140-147.

Schumacher SM, Gao E, Zhu W, Chen X, Chuprun JK, Feldman AM, et al. Koch WJ (2015). Paroxetine-mediated GRK2 inhibition reverses cardiac dysfunction and remodeling after myocardial infarction. *Sci Transl Med,* 7**:** 277ra31.

Schuster C, Fernbach N, Rix U, Superti-Furga G, Holy M, Freissmuth M, et al. Sexl V (2007). Selective serotonin reuptake inhibitors--a new modality for the treatment of lymphoma/leukaemia? *Biochem Pharmacol,* 74**:** 1424-35.

Schutzer WE, Reed JF & Mader SL (2005). Decline in caveolin-1 expression and scaffolding of G protein receptor kinase-2 with age in Fischer 344 aortic vascular smooth muscle. *Am J Physiol Heart Circ Physiol,* 288**:** H2457-64.

Schwartz SM, Geary RL & Adams LD (2003). Vascular failure: a hypothesis. *Curr Atheroscler Rep,* 5**:** 201-7.

Sear JW (2019). 26 - Antihypertensive Drugs and Vasodilators. *In:* HEMMINGS, H. C. & EGAN, T. D. (eds.) *Pharmacology and Physiology for Anesthesia (Second Edition).* Philadelphia: Elsevier.

Shahid G & Hussain T (2007). GRK2 Negatively Regulates Glycogen Synthesis in Mouse Liver FL83B Cells. *Journal of Biological Chemistry,* 282**:** 20612-20620.

Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. Owens GK (2015). KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. *Nat Med,* 21**:** 628-37.

Sharma D & Parameswaran N (2015). Multifaceted role of β-arrestins in inflammation and disease. *Genes Immun,* 16**:** 499-513.

Shenoy SK & Lefkowitz RJ (2011). β-arrestin-mediated receptor trafficking and signal transduction. *Trends in Pharmacological Sciences,* 32**:** 521-533.

Shiekh GA, Ayub T, Khan SN, Dar R & Andrabi KI (2011). Reduced nitrate level in individuals with hypertension and diabetes. *J Cardiovasc Dis Res,* 2**:** 172-6.

Shigematsu K, Koyama H, Olson NE, Cho A & Reidy MA (2000). Phosphatidylinositol 3-kinase signaling is important for smooth muscle cell replication after arterial injury. *Arterioscler Thromb Vasc Biol,* 20**:** 2373-8.

Shiina T, Arai K, Tanabe S, Yoshida N, Haga T, Nagao T & Kurose H (2001). Clathrin box in G protein-coupled receptor kinase 2. *J Biol Chem,* 276**:** 33019-26.

Sica DA & Grubbs R (2005). Transdermal clonidine: therapeutic considerations. *J Clin Hypertens (Greenwich),* 7**:** 558-62.

Siehler S (2007). G12/13-dependent signaling of G-protein-coupled receptors: disease context and impact on drug discovery. *Expert Opinion on Drug Discovery,* 2**:** 1591-1604.

Silva-García O, Rico-Mata R, Maldonado-Pichardo MC, Bravo-Patiño A, Valdez-Alarcón JJ, Aguirre-González J & Baizabal-Aguirre VM (2018). Glycogen Synthase Kinase 3α Is the Main Isoform That Regulates the Transcription Factors Nuclear Factor-κB and cAMP Response Element Binding in Bovine Endothelial Cells Infected with Staphylococcus aureus. *Frontiers in immunology,* 9**:** 92-92.

Silver PJ, Cumiskey WR & Harris AL (1992). Vascular protein kinase C in Wistar-Kyoto and spontaneously hypertensive rats. *Eur J Pharmacol,* 212**:** 143-9.

Simon G (2004). Pathogenesis of structural vascular changes in hypertension. *J Hypertens,* 22**:** 3-10.

Singh RB, Mengi SA, Xu Y-J, Arneja AS & Dhalla NS (2002). Pathogenesis of atherosclerosis: A multifactorial process. *Experimental and clinical cardiology,* 7**:** 40-53.

Smith PA, Graham LN, Mackintosh AF, Stoker JB & Mary DaSG (2004). Relationship between central sympathetic activity and stages of human hypertension*. *American Journal of Hypertension,* 17**:** 217-222.

Somlyo AP & Somlyo AV (2003). Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev,* 83**:** 1325-58.

Sorriento D, Santulli G, Franco A, Cipolletta E, Napolitano L, Gambardella J, et al. Ciccarelli M (2015). Integrating GRK2 and NFκB in the Pathophysiology of Cardiac Hypertrophy. *Journal of Cardiovascular Translational Research,* 8**:** 493-502.

Spieker LE, Flammer AJ & Luscher TF (2006). The vascular endothelium in hypertension. *Handb Exp Pharmacol***:** 249-83.

Stabile E, Zhou YF, Saji M, Castagna M, Shou M, Kinnaird TD, et al. Fuchs S (2003). Akt controls vascular smooth muscle cell proliferation in vitro and in vivo by delaying G1/S exit. *Circ Res,* 93**:** 1059-65.

Stadel JM, Nambi P, Shorr RG, Sawyer DF, Caron MG & Lefkowitz RJ (1983). Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase is associated with phosphorylation of the β-adrenergic receptor. *Proceedings of the National Academy of Sciences,* 80**:** 3173-3177.

Stanton A (2003). Therapeutic potential of renin inhibitors in the management of cardiovascular disorders. *Am J Cardiovasc Drugs,* 3**:** 389-94.

Stephens LR, Eguinoa A, Erdjument-Bromage H, Lui M, Cooke F, Coadwell J, et al. Hawkins PT (1997). The G βγ sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell,* 89**:** 105-14.

Stepulak A, Rzeski W, Sifringer M, Brocke K, Gratopp A, Kupisz K, et al. Ikonomidou C (2008). Fluoxetine inhibits the extracellular signal regulated kinase pathway and suppresses growth of cancer cells. *Cancer Biology & Therapy,* 7**:** 1685-1693.

Stevens VJ, Obarzanek E, Cook NR, Lee IM, Appel LJ, Smith West D, et al. Cohen J (2001). Long-term weight loss and changes in blood pressure: results of the Trials of Hypertension Prevention, phase II. *Ann Intern Med,* 134**:** 1-11.

Stone GW, Moses JW, Ellis SG, Schofer J, Dawkins KD, Morice MC, et al. Leon MB (2007). Safety and efficacy of sirolimus- and paclitaxel-eluting coronary stents. *N Engl J Med,* 356**:** 998-1008.

Stouffer GA & Owens GK (1992). Angiotensin II-induced mitogenesis of spontaneously hypertensive rat-derived cultured smooth muscle cells is dependent on autocrine production of transforming growth factor-β. *Circulation Research,* 70**:** 820-828.

Stoyanov B, Volinia S, Hanck T, Rubio I, Loubtchenkov M, Malek D, et al. Et Al. (1995). Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science,* 269**:** 690-3.

Sung CP, Arleth AJ, Storer BL & Ohlstein EH (1994). Angiotensin type 1 receptors mediate smooth muscle proliferation and endothelin biosynthesis in rat vascular smooth muscle. *Journal of Pharmacology and Experimental Therapeutics,* 271**:** 429.

Suzuki N, Hajicek N & Kozasa T (2009). Regulation and physiological functions of G12/13-mediated signaling pathways. *Neuro-Signals,* 17**:** 55-70.

Taguchi K, Matsumoto T, Kamata K & Kobayashi T (2012). G protein-coupled receptor kinase 2, with β-arrestin 2, impairs insulin-induced Akt/endothelial nitric oxide synthase signaling in ob/ob mouse aorta. *Diabetes,* 61**:** 1978-85.

Taguchi K, Kobayashi T, Takenouchi Y, Matsumoto T & Kamata K (2011). Angiotensin II causes endothelial dysfunction via the GRK2/Akt/eNOS pathway in aortas from a murine type 2 diabetic model. *Pharmacol Res,* 64**:** 535-46.

Taguchi K, Sakata K, Ohashi W, Imaizumi T, Imura J & Hattori Y (2014). Tonic inhibition by G protein-coupled receptor kinase 2 of Akt/endothelial nitric-oxide synthase signaling in human vascular endothelial cells under conditions of hyperglycemia with high insulin levels. *J Pharmacol Exp Ther,* 349**:** 199-208.

Tajsic T & Morrell NW (2011). Smooth muscle cell hypertrophy, proliferation, migration and apoptosis in pulmonary hypertension. *Compr Physiol,* 1**:** 295-317.

Takahashi T, Taniguchi T, Konishi H, Kikkawa U, Ishikawa Y & Yokoyama M (1999). Activation of Akt/protein kinase B after stimulation with angiotensin II in vascular smooth muscle cells. *American Journal of Physiology-Heart and Circulatory Physiology,* 276**:** H1927-H1934.

Tanner FC, Greutert H, Barandier C, Frischknecht K & Luscher TF (2003). Different cell cycle regulation of vascular smooth muscle in genetic hypertension. *Hypertension,* 42**:** 184-8.

Tantini B, Manes A, Fiumana E, Pignatti C, Guarnieri C, Zannoli R, et al. Galiè N (2005). Antiproliferative effect of sildenafil on humanpulmonary artery smooth muscle cells. *Basic Research in Cardiology,* 100**:** 131-138.

Taylor DS, Cheng X, Pawlowski JE, Wallace AR, Ferrer P & Molloy CJ (1999). Epiregulin is a potent vascular smooth muscle cell-derived mitogen induced by angiotensin II, endothelin-1, and thrombin. *Proceedings of the National Academy of Sciences of the United States of America,* 96**:** 1633-1638.

Tennant M & Mcgeachie JK (1990). Blood vessel structure and function: a brief update on recent advances. *Aust N Z J Surg,* 60**:** 747-53.

Tesmer VM, Lennarz S, Mayer G & Tesmer JJ (2012). Molecular mechanism for inhibition of g protein-coupled receptor kinase 2 by a selective RNA aptamer. *Structure,* 20**:** 1300-9.

Tesmer VM, Kawano T, Shankaranarayanan A, Kozasa T & Tesmer JJG (2005). Snapshot of Activated G Proteins at the Membrane: The Gα_q-GRK2-Gßγ Complex. *Science,* 310**:** 1686-1690.

Thal DM, Yeow RY, Schoenau C, Huber J & Tesmer JJ (2011). Molecular mechanism of selectivity among G protein-coupled receptor kinase 2 inhibitors. *Mol Pharmacol,* 80**:** 294-303.

Thal DM, Homan KT, Chen J, Wu EK, Hinkle PM, Huang ZM, et al. Tesmer JJ (2012). Paroxetine is a direct inhibitor of g protein-coupled receptor kinase 2 and increases myocardial contractility. *ACS Chem Biol,* 7**:** 1830-9.

Theilade J, Lerche Hansen J, Haunso S & Sheikh SP (2002). Extracellular signal-regulated kinases control expression of G protein-coupled receptor kinase 2 (GRK2). *FEBS Lett,* 518**:** 195-9.

Theilade J, Hansen JL, Haunso S & Sheikh SP (2005). MAP kinase protects G protein-coupled receptor kinase 2 from proteasomal degradation. *Biochem Biophys Res Commun,* 330**:** 685-9.

Tomita T, Yasui-Furukori N, Nakagami T, Tsuchimine S, Ishioka M, Kaneda A, et al. Kaneko S (2014). Therapeutic reference range for plasma concentrations of paroxetine in patients with major depressive disorders. *Ther Drug Monit,* 36**:** 480-5.

Touyz RM & Montezano AC (2015). Hypertensive Vasculopathy. *In:* LANZER, P. (ed.) *PanVascular Medicine.* Berlin, Heidelberg: Springer Berlin Heidelberg.

Touyz RM, Deng LY & Schiffrin EL (1995). Ca^{2+} and contractile responses of resistance vessels of WKY rats and SHR to endothelin-1. *J Cardiovasc Pharmacol,* 26 Suppl 3**:** S193-6.

Touyz RM, Deng LY, He G, Wu XH & Schiffrin EL (1999). Angiotensin II stimulates DNA and protein synthesis in vascular smooth muscle cells from human arteries: role of extracellular signal-regulated kinases. *J Hypertens,* 17**:** 907-16.

Touyz RM, He G, El Mabrouk M, Diep Q, Mardigyan V & Schiffrin EL (2001). Differential activation of extracellular signal-regulated protein kinase 1/2 and p38 mitogen activated-protein kinase by AT1 receptors in vascular smooth muscle cells from Wistar-Kyoto rats and spontaneously hypertensive rats. *J Hypertens,* 19**:** 553-9.

Touyz RM, Alves-Lopes R, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A & Montezano AC (2018). Vascular smooth muscle contraction in hypertension. *Cardiovasc Res,* 114**:** 529-539.

Traynham CJ, Cannavo A, Zhou Y, Vouga AG, Woodall BP, Hullmann J, et al. Koch WJ (2015). Differential Role of G Protein-Coupled Receptor Kinase 5 in Physiological Versus Pathological Cardiac Hypertrophy. *Circulation research,* 117**:** 1001-1012.

Tulloch IF & Johnson AM (1992). The pharmacologic profile of paroxetine, a new selective serotonin reuptake inhibitor. *J Clin Psychiatry,* 53 Suppl**:** 7-12.

Tuna BG, Bakker EN & Vanbavel E (2012). Smooth muscle biomechanics and plasticity: relevance for vascular calibre and remodelling. *Basic Clin Pharmacol Toxicol,* 110**:** 35-41.

Turner AJ & Hooper NM (2002). The angiotensin–converting enzyme gene family: genomics and pharmacology. *Trends in Pharmacological Sciences,* 23**:** 177-183.

Uehara Y, Numabe A, Kawabata Y, Takada S, Hirawa N, Nagata T, et al. Omata M (1993). Inhibition of protein synthesis and antiproliferative effect of the angiotensin converting enzyme inhibitor trandolaprilat in rat vascular smooth muscle cells. *J Hypertens,* 11**:** 1073-81.

Ungerer M, Bohm M, Elce JS, Erdmann E & Lohse MJ (1993). Altered expression of β-adrenergic receptor kinase and β1-adrenergic receptors in the failing human heart. *Circulation,* 87**:** 454-63.

Urschel K, Cicha I, Daniel WG & Garlichs CD (2012). Shear stress patterns affect the secreted chemokine profile in endothelial cells. *Clin Hemorheol Microcirc,* 50**:** 143-52.

Ushio-Fukai M, Griendling KK, Becker PL, Hilenski L, Halleran S & Alexander RW (2001). Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol,* 21**:** 489-95.

Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K & Griendling KK (1999). Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J Biol Chem,* 274**:** 22699-704.

Usui I, Imamura T, Satoh H, Huang J, Babendure JL, Hupfeld CJ & Olefsky JM (2004). GRK2 is an endogenous protein inhibitor of the insulin signaling pathway for glucose transport stimulation. *The EMBO journal,* 23**:** 2821-2829.

Valente AJ, Yoshida T, Murthy SN, Sakamuri SSVP, Katsuyama M, Clark RA, et al. Chandrasekar B (2012). Angiotensin II enhances AT1-Nox1 binding and stimulates arterial smooth muscle cell migration and proliferation through AT1, Nox1, and interleukin-18. *American journal of physiology. Heart and circulatory physiology,* 303**:** H282-H296.

Van Haaften EE, Bouten CVC & Kurniawan NA (2017). Vascular Mechanobiology: Towards Control of In Situ Regeneration. *Cells,* 6.

Van Varik BJ, Rennenberg RJMW, Reutelingsperger CP, Kroon AA, De Leeuw PW & Schurgers LJ (2012). Mechanisms of arterial remodeling: lessons from genetic diseases. *Frontiers in genetics,* 3**:** 290-290.

Vanhaesebroeck B, Leevers SJ, Panayotou G & Waterfield MD (1997). Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci,* 22**:** 267-72.

Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, et al. Waterfield MD (2001). Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem,* 70**:** 535-602.

Vecchione C, Patrucco E, Marino G, Barberis L, Poulet R, Aretini A, et al. Lembo G (2005). Protection from angiotensin II-mediated vasculotoxic and hypertensive response in mice lacking PI3Kγ. *J Exp Med,* 201**:** 1217-28.

Vierhapper H, Wagner O, Nowotny P & Waldhausl W (1990). Effect of endothelin-1 in man. *Circulation,* 81**:** 1415-8.

Violin JD, Dewire SM, Barnes WG & Lefkowitz RJ (2006). G protein-coupled receptor kinase and β-arrestin-mediated desensitization of the angiotensin II type 1A receptor elucidated by diacylglycerol dynamics. *J Biol Chem,* 281**:** 36411-9.

Vlahos CJ, Matter WF, Hui KY & Brown RF (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem,* 269**:** 5241-8.

Vroon A, Kavelaars A, Limmroth V, Lombardi MS, Goebel MU, Van Dam AM, et al. Heijnen CJ (2005). G protein-coupled receptor kinase 2 in multiple sclerosis and experimental autoimmune encephalomyelitis. *J Immunol,* 174**:** 4400-6.

Walcher D, Babiak C, Poletek P, Rosenkranz S, Bach H, Betz S, et al. Marx N (2006). C-Peptide Induces Vascular Smooth Muscle Cell Proliferation. *Circulation Research,* 99**:** 1181-1187.

Waldschmidt HV, Homan KT, Cruz-Rodríguez O, Cato MC, Waninger-Saroni J, Larimore KM, et al. Larsen SD (2016). Structure-Based Design, Synthesis, and Biological Evaluation of Highly Selective and Potent G Protein-Coupled Receptor Kinase 2 Inhibitors. *Journal of Medicinal Chemistry,* 59**:** 3793-3807.

Walsh MP (2011). Vascular smooth muscle myosin light chain diphosphorylation: mechanism, function, and pathological implications. *IUBMB Life,* 63**:** 987-1000.

Walsh MP, Horowitz A, Clement-Chomienne O, Andrea JE, Allen BG & Morgan KG (1996). Protein kinase C mediation of Ca^{2+}-independent contractions of vascular smooth muscle. *Biochem Cell Biol,* 74**:** 485-502.

Wang D, Uhrin P, Mocan A, Waltenberger B, Breuss JM, Tewari D, et al. Atanasov AG (2018a). Vascular smooth muscle cell proliferation as a therapeutic target. Part 1: molecular targets and pathways. *Biotechnol Adv,* 36**:** 1586-1607.

Wang L, Karlsson L, Moses S, Hultgardh-Nilsson A, Andersson M, Borna C, et al. Erlinge D (2002). P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol,* 40**:** 841-53.

Wang P, Wu Y, Ge X, Ma L & Pei G (2003). Subcellular localization of β-arrestins is determined by their intact N domain and the nuclear export signal at the C terminus. *J Biol Chem,* 278**:** 11648-53.

Wang P, Xu T-Y, Guan Y-F, Su D-F, Fan G-R & Miao C-Y (2009). Perivascular adipose tissue-derived visfatin is a vascular smooth muscle cell growth factor: role of nicotinamide mononucleotide. *Cardiovascular Research,* 81**:** 370-380.

Wang Z, Li R & Zhong R (2018b). Extracellular matrix promotes proliferation, migration and adhesion of airway smooth muscle cells in a rat model of chronic obstructive pulmonary disease via upregulation of the PI3K/AKT signaling pathway. *Molecular medicine reports,* 18**:** 3143-3152.

Watanabe T, Pakala R, Katagiri T & Benedict CR (2001). Angiotensin II and serotonin potentiate endothelin-1-induced vascular smooth muscle cell proliferation. *J Hypertens,* 19**:** 731-9.

Watts SW (1998). Activation of the mitogen-activated protein kinase pathway via the 5-HT2A receptor. *Ann N Y Acad Sci,* 861**:** 162-8.

Watts SW, Yang P, Banes AK & Baez M (2001). Activation of Erk mitogen-activated protein kinase proteins by vascular serotonin receptors. *J Cardiovasc Pharmacol,* 38**:** 539-51.

Webb RC (2003). Smooth muscle contraction and relaxation. *Adv Physiol Educ,* 27**:** 201-6.

Weber H, Taylor DS & Molloy CJ (1994). Angiotensin II induces delayed mitogenesis and cellular proliferation in rat aortic smooth muscle cells. Correlation with the expression of specific endogenous growth factors and reversal by suramin. *J Clin Invest,* 93**:** 788-98.

Weber JD, Raben DM, Phillips PJ & Baldassare JJ (1997). Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. *Biochem J,* 326 (Pt 1)**:** 61-8.

Weber MA, Neutel JM & Cheung DG (1989). Hypertension in the aged: a pathophysiologic basis for treatment. *Am J Cardiol,* 63**:** 25H-32H.

Wedgwood S, Dettman RW & Black SM (2001). ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 281**:** L1058-L1067.

Wehrwein EA & Joyner MJ (2013). Chapter 8 - Regulation of blood pressure by the arterial baroreflex and autonomic nervous system. *In:* BUIJS, R. M. & SWAAB, D. F. (eds.) *Handbook of Clinical Neurology.* Elsevier.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM & Lefkowitz RJ (2003). Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America,* 100**:** 10782-10787.

Wei Z, Hurtt R, Gu T, Bodzin AS, Koch WJ & Doria C (2013). GRK2 negatively regulates IGF-1R signaling pathway and cyclins' expression in HepG2 cells. *J Cell Physiol,* 228**:** 1897-901.

Weir MR (2007). Effects of renin-angiotensin system inhibition end-organ protection: Can we do better? *Clinical Therapeutics,* 29**:** 1803-1824.

Wen Y, Nadler JL, Gonzales N, Scott S, Clauser E & Natarajan R (1996). Mechanisms of ANG II-induced mitogenic responses: role of 12-lipoxygenase and biphasic MAP kinase. *Am J Physiol,* 271**:** C1212-20.

Whelton PK, He J, Cutler JA, Brancati FL, Appel LJ, Follmann D & Klag MJ (1997). Effects of oral potassium on blood pressure. Meta-analysis of randomized controlled clinical trials. *JAMA,* 277**:** 1624-32.

Whelton SP, Chin A, Xin X & He J (2002). Effect of aerobic exercise on blood pressure: a meta-analysis of randomized, controlled trials. *Ann Intern Med,* 136**:** 493-503.

White DG, Cannon TR, Garratt H, Mundin JW, Sumner MJ & Watts IS (1993). Endothelin ETA and ETB receptors mediate vascular smooth-muscle contraction. *J Cardiovasc Pharmacol,* 22 Suppl 8**:** S144-8.

White PJ, Kumari R, Porter KE, London NJ, Ng LL & Boarder MR (2000). Antiproliferative effect of UTP on human arterial and venous smooth muscle cells. *Am J Physiol Heart Circ Physiol,* 279**:** H2735-42.

Whitehurst AW, Wilsbacher JL, You Y, Luby-Phelps K, Moore MS & Cobb MH (2002). ERK2 enters the nucleus by a carrier-independent mechanism. *Proc Natl Acad Sci U S A,* 99**:** 7496-501.

Who (2013). A global brief on hypertension. *Available at: http://apps.who.int/iris/bitstream/10665/79059/1/WHO_DCO_WHD_2013.2_eng.pdf. Accessed Jan 15, 2019.*

Widmann C, Gibson S, Jarpe MB & Johnson GL (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev,* 79**:** 143-80.

Willets JM, Brighton PJ, Mistry R, Morris GE, Konje JC & Challiss RA (2009). Regulation of oxytocin receptor responsiveness by G protein-coupled receptor kinase 6 in human myometrial smooth muscle. *Mol Endocrinol,* 23: 1272-80.

Willets JM, Challiss RA & Nahorski SR (2003). Non-visual GRKs: are we seeing the whole picture? *Trends Pharmacol Sci,* 24**:** 626-33.

Willets JM, Nash MS, Challiss RA & Nahorski SR (2004). Imaging of muscarinic acetylcholine receptor signaling in hippocampal neurons: evidence for phosphorylation-

dependent and -independent regulation by G-protein-coupled receptor kinases. *J Neurosci,* 24**:** 4157-62.

Willets JM, Nash CA, Rainbow RD, Nelson CP & Challiss RA (2015). Defining the roles of arrestin2 and arrestin3 in vasoconstrictor receptor desensitization in hypertension. *Am J Physiol Cell Physiol,* 309**:** C179-89.

Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, et al. Group ESCSD (2018). 2018 ESC/ESH Guidelines for the management of arterial hypertension. *European Heart Journal,* 39**:** 3021-3104.

Willis AI, Pierre-Paul D, Sumpio BE & Gahtan V (2004). Vascular smooth muscle cell migration: current research and clinical implications. *Vasc Endovascular Surg,* 38**:** 11-23.

Woodard GE & Rosado JA (2008). Natriuretic peptides in vascular physiology and pathology. *Int Rev Cell Mol Biol,* 268**:** 59-93.

Wort SJ, Woods M, Warner TD, Evans TW & Mitchell JA (2001). Endogenously Released Endothelin-1 from Human Pulmonary Artery Smooth Muscle Promotes Cellular Proliferation. *American Journal of Respiratory Cell and Molecular Biology,* 25**:** 104-110.

Wu JH, Goswami R, Kim LK, Miller WE, Peppel K & Freedman NJ (2005). The platelet-derived growth factor receptor-β phosphorylates and activates G protein-coupled receptor kinase-2. A mechanism for feedback inhibition. *J Biol Chem,* 280**:** 31027-35.

Wu JH, Goswami R, Cai X, Exum ST, Huang X, Zhang L, et al. Freedman NJ (2006). Regulation of the platelet-derived growth factor receptor-β by G protein-coupled receptor kinase-5 in vascular smooth muscle cells involves the phosphatase Shp2. *J Biol Chem,* 281**:** 37758-72.

Wu JH, Zhang L, Fanaroff AC, Cai X, Sharma KC, Brian L, et al. Freedman NJ (2012). G protein-coupled receptor kinase-5 attenuates atherosclerosis by regulating receptor tyrosine kinases and 7-transmembrane receptors. *Arterioscler Thromb Vasc Biol,* 32**:** 308-16.

Wu X & Lu Q (2013). Expression and significance of α-SMA and PCNA in the vascular adventitia of balloon-injured rat aorta. *Experimental and therapeutic medicine,* 5**:** 1671-1676.

Wynne BM, Chiao C-W & Webb RC (2009). Vascular Smooth Muscle Cell Signaling Mechanisms for Contraction to Angiotensin II and Endothelin-1. *Journal of the American Society of Hypertension : JASH,* 3**:** 84-95.

Xie P, Browning DD, Hay N, Mackman N & Ye RD (2000). Activation of NF-κB by bradykinin through a Gα(q)- and Gβγ-dependent pathway that involves phosphoinositide 3-kinase and Akt. *J Biol Chem,* 275**:** 24907-14.

Xu B-E, Robinson F, Pearson G, Berman K, Karandikar M, Beers Gibson T & Cobb MH (2001). Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions*. *Endocrine Reviews,* 22**:** 153-183.

Xu L-Q, Tan S-B, Huang S, Ding H-Y, Li W-G, Zhang Y, et al. Wang T (2017). G protein-coupled receptor kinase 6 is overexpressed in glioma and promotes glioma cell proliferation. *Oncotarget,* 8**:** 54227-54235.

Xu S, Fu J, Chen J, Xiao P, Lan T, Le K, et al. Liu P (2009). Development of an optimized protocol for primary culture of smooth muscle cells from rat thoracic aortas. *Cytotechnology,* 61**:** 65-72.

Yaghini FA, Song CY, Lavrentyev EN, Ghafoor HUB, Fang XR, Estes AM, et al. Malik KU (2010). Angiotensin II-induced vascular smooth muscle cell migration and growth are mediated by cytochrome P450 1B1-dependent superoxide generation. *Hypertension (Dallas, Tex. : 1979),* 55**:** 1461-1467.

Yamamoto T, Ebisuya M, Ashida F, Okamoto K, Yonehara S & Nishida E (2006). Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression. *Curr Biol,* 16**:** 1171-82.

Yamanaka Y, Hayashi KI, Komurasaki T, Morimoto S, Ogihara T & Sobue K (2001). EGF Family Ligand-Dependent Phenotypic Modulation of Smooth Muscle Cells through EGF Receptor. *Biochemical and Biophysical Research Communications,* 281**:** 373-377.

Yoshizumi M, Tsuchiya K, Kirima K, Kyaw M, Suzaki Y & Tamaki T (2001). Quercetin Inhibits Shc- and Phosphatidylinositol 3-Kinase-Mediated c-Jun N-Terminal Kinase Activation by Angiotensin II in Cultured Rat Aortic Smooth Muscle Cells. *Molecular Pharmacology,* 60**:** 656.

Yu S, Chen Y, Chen S, Ye N, Li Y & Sun Y (2018). Regulation of angiotensin II-induced B-cell lymphoma-2-associated athanogene 3 expression in vascular smooth muscle cells. *Mol Med Rep,* 17**:** 6156-6162.

Zacchetti A, Van Garderen E, Teske E, Nederbragt H, Dierendonck JH & Rutteman GR (2003). Validation of the use of proliferation markers in canine neoplastic and non-neoplastic tissues: comparison of KI-67 and proliferating cell nuclear antigen (PCNA) expression versus in vivo bromodeoxyuridine labelling by immunohistochemistry. *APMIS,* 111**:** 430-8.

Zhang D, Zhao Q & Wu B (2015). Structural Studies of G Protein-Coupled Receptors. *Molecules and cells,* 38**:** 836-842.

Zhang F, Hu Y, Xu Q & Ye S (2010). Different effects of angiotensin II and angiotensin-(1-7) on vascular smooth muscle cell proliferation and migration. *PLoS One,* 5**:** e12323.

Zhang F, Ren X, Zhao M, Zhou B & Han Y (2016). Angiotensin-(1–7) abrogates angiotensin II-induced proliferation, migration and inflammation in VSMCs through inactivation of ROS-mediated PI3K/Akt and MAPK/ERK signaling pathways. *Scientific Reports,* 6**:** 34621.

Zhang H, Chalothorn D, Jackson Leslie F, Lee David C & Faber James E (2004). Transactivation of Epidermal Growth Factor Receptor Mediates Catecholamine-Induced Growth of Vascular Smooth Muscle. *Circulation Research,* 95**:** 989-997.

Zhang R & Xie X (2012). Tools for GPCR drug discovery. *Acta pharmacologica Sinica,* 33**:** 372-384.

Zhang W & Liu HT (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res,* 12**:** 9-18.

Zhang ZD & Cox G (1996). MTT assay overestimates human airway smooth muscle cell number in culture. *Biochem Mol Biol Int,* 38**:** 431-6.

Zhao D, Letterman J & Schreiber BM (2001). β-Migrating very low density lipoprotein (β VLDL) activates smooth muscle cell mitogen-activated protein (MAP) kinase via G protein-coupled receptor-mediated transactivation of the epidermal growth factor (EGF) receptor: effect of MAP kinase activation on β VLDL plus EGF-induced cell proliferation. *J Biol Chem,* 276**:** 30579-88.

Zhao Y, Liu J, Li L, Liu L & Wu L (2005). Role of Ras/PKCzeta/MEK/ERK1/2 signaling pathway in angiotensin II-induced vascular smooth muscle cell proliferation. *Regul Pept,* 128**:** 43-50.

Zhao Y, Biswas SK, Mcnulty PH, Kozak M, Jun JY & Segar L (2011). PDGF-induced vascular smooth muscle cell proliferation is associated with dysregulation of insulin receptor substrates. *Am J Physiol Cell Physiol,* 300**:** C1375-85.

Zheng H, Worrall C, Shen H, Issad T, Seregard S, Girnita A & Girnita L (2012). Selective recruitment of G protein-coupled receptor kinases (GRKs) controls signaling of the insulin-like growth factor 1 receptor. *Proc Natl Acad Sci U S A,* 109**:** 7055-60.

Zheng X, Baker H, Hancock WS, Fawaz F, Mccaman M & Pungor Jr E (2006). Proteomic Analysis for the Assessment of Different Lots of Fetal Bovine Serum as a Raw Material

for Cell Culture. Part IV. Application of Proteomics to the Manufacture of Biological Drugs. *Biotechnology Progress,* 22**:** 1294-1300.

Zhou RH, Lee TS, Tsou TC, Rannou F, Li YS, Chien S & Shyy JY (2003). Stent implantation activates Akt in the vessel wall: role of mechanical stretch in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol,* 23**:** 2015-20.

Zhu L-A, Fang N-Y, Gao P-J, Jin X, Wang H-Y & Liu Z (2015). Differential ERK1/2 Signaling and Hypertrophic Response to Endothelin-1 in Cardiomyocytes from SHR and Wistar-Kyoto Rats: A Potential Target for Combination Therapy of Hypertension. *Current vascular pharmacology,* 13**:** 467-474.