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Effect of Hypoxia on the Cardiovascular Sphingolipid System

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Submitted in fulfilment of the requirements of the degree of
Doctor of Philosophy in the Institute of Cardiovascular and
Medical Sciences, University of Glasgow

Institute of Cardiovascular and Medical Sciences,
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H. Alganga 2017

Author's Declaration

I hereby declare that this thesis has been written solely by me with the research entirely generated by myself with the exception of the western blot which was in collaboration with Ms. Ruth Muir. The work presented has not been previously submitted for any other degree. All experiments were conducted in the Institute of Cardiovascular and Medical Sciences at the University of Glasgow, under the supervision of Dr. Simon Kennedy.

Husam S. F. Alganga

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Tables of Contents

Effect of Hypoxia on the Cardiovascular Sphingolipid System	i
Author's Declaration	ii
Acknowledgements.....	iii
Tables of Contents	iv
List of Figures.....	viii
List of Tables.....	x
Publications	xi
List of abbreviations	xii
Abstract.....	xv
Chapter one	1
1 General introduction	1
1.1 Cardiovascular system	2
1.1.1 Structural organisation	2
1.1.2 Histology of the arterial wall	2
1.1.3 Vascular endothelium function	2
1.2 Cardiovascular disease.....	9
1.2.1 Coronary heart disease (CHD)	9
1.2.2 Pathophysiology of coronary heart disease	9
1.3 Endothelium and myocardial ischaemia.....	10
1.3.1 Mechanism of reperfusion-induced vascular endothelium dysfunction 11	
1.3.2 Mechanism of myocardial ischaemia/reperfusion injury.....	13
1.3.3 The relationship between endothelial cells and myocardial injury .	16
1.3.4 Cardioprotection against ischaemia/reperfusion insult.....	16
1.4 Sphingosine-1-Phosphate (S1P)	18
1.4.1 S1P overview	18
1.4.2 Sphingosine-1-phosphate synthesis and degradation	19
1.4.3 S1P in circulation	19
1.4.4 S1P receptors, Agonists and Antagonists	20
1.4.5 Physiological Role of S1P in the Cardiovascular System	23
1.5 Sphingosine kinase (s).....	32
1.5.1 Role and regulation of SK1	33
1.5.2 Regulation of SK2	35
1.5.3 Degradation of SKs	36
1.5.4 Sphingosine kinase inhibitors	37
1.6 Role of sphingosine kinase/S1P in cardioprotection.....	39

1.6.1	Studies of S1P in cardiomyocyte cell culture.....	39
1.6.2	Role of sphingosine kinase in cardioprotection	40
1.6.3	S1P Lyase	42
1.6.4	Cardioprotective effects of High Density Lipoprotein (HDL)	43
1.6.5	FTY720	44
	Hypothesis and aims.....	46
	Chapter two	47
2	Materials and methods	47
2.1	Animal source	48
2.2	Chemicals and reagents	48
2.3	Confocal microscopy	48
2.3.1	The confocal microscope	49
2.3.2	Principle of fluorescence	49
2.3.3	Tissue preparation for immunofluorescence studies	50
2.3.4	Label protocol.....	50
2.3.5	Hypoxia protocol.....	51
2.3.6	Slide mounting	52
2.3.7	Imaging.....	52
2.3.8	Image analysis.....	53
2.4	Functional studies (wire myography)	56
2.4.1	Vessel preparation and mounting	56
2.4.2	Experimental protocols	57
2.4.3	Statistical analysis.....	60
2.5	Cell culture and tissue preparation	60
2.5.1	Human Umbilical Vein Endothelial Cells (HUVEC) culture	60
2.5.2	Treatment of Human Umbilical Vein Endothelial Cells	60
2.5.3	Preparation of heart tissue.....	61
2.5.4	Quantification of protein concentration	61
2.5.5	Western blotting.....	62
	Chapter three	65
3	Effect of hypoxia on Sphingosine Kinase 1 expression in rat coronary artery	65
3.1	Introduction	66
3.1.1	Aims	67
3.2	Methods	68
3.2.1	Tissue preparation.....	68
3.2.2	Hypoxia protocol.....	68
3.2.3	Experimental protocol	68
3.2.4	Image analysis.....	69

3.3	Results	70
3.3.1	Expression of SK1 enzyme, S1P ₁ and S1P ₃ receptor subtypes in rat coronary artery endothelium.....	70
3.3.2	Effect of hypoxia on SK1 enzyme expression in vascular endothelium. 75	
3.3.3	Effect of cycloheximide on hypoxia-induced increase in SK1 expression.....	75
3.3.4	Effect of sphingosine kinase inhibitors on hypoxia-induced increase in SK1 expression.....	75
3.3.5	Expression of SK1 in presence of degradation inhibitors in vascular endothelium.	81
3.3.6	Effect of SKi on degradation of SK1.....	82
3.4	Discussion	88
3.5	Conclusion	92
	Chapter four.....	93
4	The effect of hypoxia-induced increase in SK1 on vascular function.....	93
4.1	Introduction	94
4.1.1	Aims	96
4.2	Methods	97
4.2.1	Tissue preparation for immunofluorescent studies.....	97
4.2.2	Experimental protocols	97
4.2.3	Image analysis.....	97
4.2.4	Tissue preparation for myography	98
4.2.5	Experimental protocols for myography.....	98
4.3	Results	101
4.3.1	Effect of hypoxia on contractile responses to U46619 in rat aorta.	101
4.3.2	S1P-induces vascular relaxation in an endothelium dependent manner.	105
4.3.3	TRPV1 channels are involved in S1P-stimulated relaxation.	110
4.3.4	Effect of hypoxia on SK1 enzyme expression in rat aortic endothelium.	111
4.3.5	Effect of cycloheximide on hypoxia-induced increase in SK1 expression.....	111
4.3.6	Effect of hypoxia on S1P-stimulated vascular relaxation.....	111
4.3.7	Effect of sphingosine kinase inhibitors on hypoxia-induced increase in SK1 expression.....	119
4.3.8	Effect of inhibitors and antagonists on S1P-stimulated relaxation.	119
4.3.9	S1P-stimulated vascular relaxation in rat aorta from spontaneously hypertensive rats.....	128
4.4	Discussion	131
4.5	Conclusion	136

Chapter five	139
5 Influence of hypoxia on SK1 expression in cultured endothelial cells and cardiac tissue	139
5.1 Introduction	140
5.1.1 Aims	141
5.2 Methods	142
5.2.1 Cell culture	142
5.2.2 Experimental protocols	142
5.2.3 Preparation of heart tissue.....	143
5.2.4 Statistical analysis.....	143
5.3 Results	144
5.3.1 Expression of SK1 in HUVECs in presence and absence of different inhibitory agents under normoxia and hypoxia	144
5.3.2 Expression of SK1 in cardiac tissue following treatment with specific inhibitory agents under normoxia and hypoxia	144
5.4 Discussion	153
5.5 Conclusion	154
Chapter six	156
6 General discussion	156
6.1 General Discussion	157
6.2 Limitations and future work	160
6.3 Clinical Relevance	162
6.4 Conclusion	163
Chapter seven.....	164
7 References	164

List of Figures

Figure 1-1 Schematic representation of the coagulation cascade.	4
Figure 1-2 Flow chart shows the main mechanisms of ischaemia/reperfusion injury.	15
Figure 1-3 Summary of the steps involved in sphingosine-1-phosphate synthesis and breakdown.	22
Figure 1-4 Diverse physiological processes are regulated by S1P.	31
Figure 1-5 Flow chart shows the mechanism of S1P-induced cardioprotection in cardiomyocytes.	45
Figure 2-1 Fluorescence generation	49
Figure 2-2 Indirect immunofluorescence	51
Figure 2-3 Slide preparation	52
Figure 2-4 Representative picture of a rat aortic ring mounted in the small artery wire myograph.	56
Figure 2-5 Representative experimental recordings showing isometric tension (g) plotted against time in rat thoracic aorta.	59
Figure 3-1 Representative images showing the difference between endothelial and smooth muscle cell nuclei of rat coronary artery.	71
Figure 3-2 Expression of SK1 in endothelial cells of rat coronary artery.	71
Figure 3-3 Anti-SK1 antibody specificity.	72
Figure 3-4 S1P ₁ and S1P ₃ receptor expression in rat coronary artery endothelium.	73
Figure 3-5 Expression of SK1 enzyme and S1P ₃ receptors in rat CA endothelium.	73
Figure 3-6 Anti- α smooth muscle actin antibody.	74
Figure 3-7 Effect of hypoxia on SK1 expression levels.	76
Figure 3-8 Effect of hypoxia on pSK1 levels.	77
Figure 3-9 Effect of cycloheximide on hypoxia-induced increase in SK1 expression.	78
Figure 3-10 Effect of Sphingosine Kinase inhibitors on SK1 expression in rat coronary artery endothelium under normoxia.	79
Figure 3-11 Effect of Sphingosine Kinase inhibitors on SK1 expression in rat coronary artery endothelium under hypoxia.	80
Figure 3-12 Effect of MG132, Lactacystin, CA-074ME and a combination of MG132 and CA-074ME on SK1 in rat coronary artery endothelium under normoxia.	84
Figure 3-13 Effect of MG132, Lactacystin, CA-074ME and a combination of MG132 and CA-074ME on SK1 in rat coronary artery endothelium under hypoxia.	85
Figure 3-14 Effect of degradation inhibitors on SKi-induced downregulation of SK1 in rat CA endothelium under normoxia.	86
Figure 3-15 Effect of degradation inhibitors on SKi-induced downregulation of SK1 in rat CA endothelium under hypoxia.	87
Figure 4-1 Diagram showing the experimental protocols used for the myography experiments.	100
Figure 4-2 Contractile responses to U46619 in rat aorta under control and hypoxic conditions.	102
Figure 4-3 Contractile responses to U46619 in rat aorta in presence and absence of SKi under control conditions.	103
Figure 4-4 Contractile responses to U46619 in rat aorta in presence and absence of SKi and under hypoxic conditions.	104
Figure 4-5 S1P stimulated aortic relaxation in an endothelium dependent manner.	106
Figure 4-6 S1P ₁ receptor expression in rat aortic endothelium.	107
Figure 4-7 S1P ₃ receptor expression in rat aortic endothelium.	108
Figure 4-8 S1P and CYM 5541 induce aortic relaxation in an endothelium-dependent manner.	109
Figure 4-9 Role of TRPV1 channels in S1P-mediated relaxation.	110

Figure 4-10 Expression of SK1 in endothelial cells of rat aorta.	112
Figure 4-11 Effect of hypoxia on SK1 expression levels.	113
Figure 4-12 Effect of hypoxia on pSK1 levels.....	114
Figure 4-13 Effect of cycloheximide on hypoxia-induced increase in SK1 expression. ...	115
Figure 4-14 Representative trace showing the vasorelaxation response to S1P following exposure to hypoxia.	117
Figure 4-15 Effect of hypoxia on S1P-induced relaxation in rat aorta.	118
Figure 4-16 Effect of SKi on hypoxia-induced increase in SK1 expression.	121
Figure 4-17 Effect of SKi on S1P-induced relaxation in rat aorta.	122
Figure 4-18 Effect of PF543 on hypoxia-induced increase in SK1 expression.	123
Figure 4-19 S1P-induced relaxation in rat aorta in presence and absence of PF543.	124
Figure 4-20 S1P-induced relaxation in rat aorta in presence and absence of ROME.	125
Figure 4-21 S1P-induced relaxation in rat aorta in presence and absence of L-NNA.	126
Figure 4-22 S1P-induced relaxation in rat aorta in presence and absence of CAY10444.	127
Figure 4-23 S1P-stimulated relaxation in rat aorta from normal and hypertensive rats. ...	129
Figure 4-24 S1P-induced relaxation in rat aorta from hypertensive rats.	130
Figure 4-25 A schematic diagram showing the mechanism of S1P-induced relaxation....	138
Figure 5-1 Expression of SK1b in HUVECs under normoxia and hypoxia.	146
Figure 5-2 Effect of SKi, cycloheximide, MG132 and CA-074ME on SK1 in HUVECs under normoxia.	147
Figure 5-3 Effect of SKi, cycloheximide, MG132 and CA-074ME on SK1 in HUVECs under hypoxia.	148
Figure 5-4 Expression of SK1a in rat heart tissue under normoxia and hypoxia.	149
Figure 5-5 Effect of SKi, PF543 and cycloheximide on SK1 in rat cardiac tissue under normoxia.	150
Figure 5-6 Effect of SKi, PF543 and cycloheximide on SK1 in rat cardiac tissue under hypoxia.	151
Figure 5-7 Effect of hypoxia on pSK1 in rat cardiac tissue.	152

List of Tables

Table 2-1 List of drugs and concentrations used	54
Table 2-2 Primary antibodies used for immunofluorescence	55
Table 2-3 Secondary detection agents for immunofluorescence	55
Table 2-4 Primary antibodies used for western blotting	64
Table 2-5 Secondary detection agent for western blotting.....	64
Table 3-1 Integrated density for SK1 in presence of degradation inhibitors under normoxia	81
Table 3-2 Integrated density for SK1 in presence of degradation inhibitors under hypoxia	81
Table 3-3 Effect of SKi on degradation of SK1 under normoxic conditions.....	83
Table 3-4 Effect of SKi on degradation of SK1 under hypoxic conditions	83
Table 4-1 Effect of enzyme inhibitors and antagonists (all used at 10 μ M concentration except PF543 which was used at 100 nM) on U46619-induced contraction (3×10^{-8} M) in rat aortae under control conditions.....	116
Table 4-2 Effect of enzyme inhibitors and antagonists (all used at 10 μ M concentration except PF543 which was used at 100 nM) on U46619-induced contraction (5×10^{-9} M) in rat aortae under hypoxic conditions.	116

Publications

M. A. Ewart, T.A.M. Almagrouk, A.B. Uguşman, O. Katwan, **H. Alganga**, S. Currie and S. Kennedy (2017) Changes in IP3 receptor expression and function in aortic smooth muscle of atherosclerotic mice. Accepted for publication in Journal of Vascular Research.

Abstracts:

H. Alganga, S. Pyne, N.J. Pyne and S. Kennedy (2014) Distribution of cannabinoid, S1P receptors and sphingosine kinase 1 in the rat coronary artery under normoxia and hypoxia. Scottish Cardiovascular Forum (Poster).

H. Alganga, S. Pyne, N.J. Pyne and S. Kennedy (2015) Effect of hypoxia on sphingosine kinase 1 expression in the rat coronary artery. Scottish Cardiovascular Forum, Edinburgh (Poster).

H. Alganga, S. Pyne, N.J. Pyne and S. Kennedy (2015) Effect of hypoxia on the vascular sphingolipid system. British Pharmacology Society Meeting, London, (Pharmacology 2015). pA2 online, 13(3),301P.

Azizah B. Uguşman, **H. Alganga**, Tarek A.M. Almagrouk, Veronika Zhelyazkova and Simon Kennedy (2016) Modulation of aortic perivascular adipose tissue function by peroxynitrite. British Pharmacology Society Meeting, London, (Pharmacology 2016).

List of abbreviations

13-HODE	13-hydroxyoctadecadienoic acid
ABC	ATP-binding cassette transporters
ADMA	Asymmetrical dimethylarginine
ADP	Adenosine di-phosphate
ATP	Adenosine tri-phosphate
BACE1	beta-site amyloid precursor protein cleaving enzyme 1
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanine monophosphate
CGRP	Calcitonin gene-related peptide
CHD	Coronary heart disease
CIB1	Calcium and integrin-binding protein 1
COX	Cyclooxygenase
CVD	Cardiovascular disease
CVS	Cardiovascular system
DDAH	Dimethylarginine dimethylaminohydrolase
EC	Endothelial cell
eNOS	Endothelial nitric oxide synthase
ET	Endothelin
FHL-2	Four and a half LIM domains protein 2
GM-1	Monoganglioside-1
GP	Glycoprotein
GSK-3 β	Glycogen synthase kinase-3 β
HDL	High-density lipoprotein
HIF	Hypoxia-inducible factor
hTERT	Human telomerase reverse transcriptase
HUVEC	Human umbilical vein endothelial cell
ICa,L	L-type calcium channels
ICAM-1	Intracellular adhesion molecule
iNOS	Inducible nitric oxide synthase
IPC	Ischaemic preconditioning
IPost	Ischaemic postconditioning
IR	Ischaemia/reperfusion
JAM	Junctional adhesion molecule
MCP-1	Monocyte chemoattractant protein-1
MEFs	Mouse embryonic fibroblasts
MI	Myocardial infarction

mPTP	mitochondrial permeability transition pore
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PCI	Primary percutaneous coronary intervention
PDEs	Phosphodiesterases
PDGF	Platelet-derived growth factor
PECAM-1	Platelet-endothelial cell adhesion molecule
PGH ₂	Prostaglandin H ₂
PGI ₂	Prostacyclin
PHB2	Prohibitin 2
PKC	Protein kinase C
PMT	Photomultiplier tube
PP2A	Phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
PTKs	Protein tyrosine kinases
PTX	Pertussis toxin
ROS	Reactive oxygen species
S1P	Sphingosine-1-Phosphate
sGC	Soluble guanylyl cyclase
SK	Sphingosine kinase
SKIP	SK1 interacting protein
SPL	S1P lyase
Spns2	Spinster homolog 2
TBS	Tris-buffered saline solution
TBST	Tris-buffered saline solution with Tween-20
TF	Tissue factor
THI	Tetrahydroxybutylimidazole
TNF- α	Tumour necrosis factor alpha
TP	Thromboxane-prostanoid
t-PA	Tissue-plasminogen activator
TRAF2	TNF receptor-associated factor 2
TRPV	Transient receptor potential channels, of vanilloid subtype
TXA ₂	Thromboxane A ₂
u-PA	Urokinase-plasminogen activator

VECAM-1	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VGCC	Voltage gated calcium channel
VSMC	Vascular smooth muscle cell
vWF	Von Willebrand factor

Abstract

Sphingosine kinase 1 (SK1) catalyses the synthesis of the important bioactive sphingolipid sphingosine-1-phosphate (S1P), that has an important role in vascular tone regulation and cardioprotection against ischaemia/reperfusion injury. The work presented in this thesis describes the influence of short periods of hypoxia on expression of SK1 in vascular endothelium and how this may regulate vascular function. The aims were achieved by using wire myography to study vascular function and confocal microscopy for the studies of expression and distribution of SK1 under normoxic and hypoxic conditions.

In the first study, it was found that exposure of isolated rat coronary artery to a short period of hypoxia increases SK1 expression and ser225 phosphorylation. It was also demonstrated that the hypoxia-induced increase in SK1 expression was reduced by pre-treatment with cycloheximide, a protein synthesis inhibitor, SKi, a non-selective SK inhibitor and PF543, a selective SK1 inhibitor. However, pre-treatment with proteasomal and/or lysosomal inhibitors did not increase SK1 expression under normoxia or hypoxia. Similarly, SK1 expression was also increased in aortic endothelium following exposure to short-term hypoxia and this effect was also inhibited by cycloheximide, SKi and PF543. Collectively, these data suggest that hypoxia increases SK1 synthesis in coronary and aortic endothelium. Moreover, the SKi-induced reduction in SK1 expression in coronary endothelium was reversed by proteasomal and/or lysosomal inhibitors, indicating that SKi stimulates both proteasomal and lysosomal degradation of SK1 under normoxia and hypoxia. In chapter two, it was demonstrated that S1P and CYM5541, an S1P₃ agonist, induced dose-dependent relaxation in endothelium-intact aortic rings, whereas the S1P₁ agonist SEW2871 was without effect. The S1P stimulated relaxation was significantly enhanced in endothelium-intact aortic rings subjected to short-term hypoxia and this effect was entirely endothelium-dependent. Interestingly, the vasorelaxation response to S1P was inhibited by pre-treatment with SKi and PF543 but not ROME, a selective SK2 inhibitor under both normoxia and hypoxia. A nitric oxide synthase inhibitor also inhibited the S1P-induced relaxation in aortic rings. Moreover, the enhanced relaxation response to S1P due to hypoxia was maintained in aortae obtained from spontaneously hypertensive Wistar Kyoto rats. These findings suggest that the vasorelaxation response to S1P under normoxia and the enhanced response under hypoxia are mediated by SK1 and NO.

In chapter five, it was found that hypoxia did not change the SK1b expression in HUVECs and pre-treatment with SKi or cycloheximide exerted no effect under both normoxia and

hypoxia. However, proteasomal and/or lysosomal inhibitors increased SK1 expression under hypoxic conditions. In heart tissue, no significant difference was seen in expression of SK1 following exposure to hypoxia. However, SK1 expression was reduced by pre-treatment with SK inhibitors and cycloheximide under normoxia but not hypoxia. SK1a was identified in heart tissue, which is more sensitive to the degradation-induced by SK inhibitors than SK1b.

In summary, the results of this study imply that short-term hypoxia induces an increase in SK1 expression in coronary and aortic vascular endothelium. The increased SK1 induced by hypoxia appears to mediate the enhanced vasorelaxation response to S1P in endothelium-intact aortae. In HUVECs and heart tissue, it is likely that hypoxia induces resistance of SK1 to SK inhibitor-induced downregulation through a compensatory increase in SK1 expression.

Chapter one

1 General introduction

1.1 Cardiovascular system

1.1.1 Structural organisation

The cardiovascular system (CVS) is responsible for transporting essential substances including gases, nutrients, and hormones to various cells and tissues in the body. It is also responsible for removing the waste products of metabolism and plays a significant role in the regulation of body temperature by transport of heat as well as monitoring the water content of the cells. This system is composed of the heart, blood and an intricate network of blood vessels including arteries, veins and capillaries. The heart is the key organ in the cardiovascular system. Its main function is to maintain adequate circulation of oxygenated blood around the vascular network of the body. The oxygen-rich blood is ejected from the left ventricle through the aorta at high pressure to be distributed to the peripheral tissues. The deoxygenated blood returns to right atrium via the inferior and superior vena cava and is then pumped into the lung from the right ventricle via the pulmonary artery where the gas exchange process takes place.

1.1.2 Histology of the arterial wall

The arterial wall consists of three concentric layers: the tunica intima, tunica media and tunica adventitia. The innermost layer, tunica intima, is a thin single sheet of endothelial cells attached to a thin layer of connective tissue; the basal lamina. Endothelial cells line all blood vessels from the heart to the capillaries and regulate the exchanges between the bloodstream and surrounding tissue. The tunica media is composed mainly of vascular smooth muscle cells (VSMCs) arranged roughly in spiral layers and located between the internal and external elastic laminae. The tunica media is responsible for regulating the total peripheral resistance (vascular tone) and the distribution of blood flow throughout the body. The outermost layer, the tunica adventitia, is made chiefly of longitudinally arranged collagen fibres and surrounds the tunica media. The adventitia serves to anchor the blood vessels to nearby organs, giving it stability.

1.1.3 Vascular endothelium function

The endothelium is a thin monolayer that lines the inner surface of the entire vascular system, separating the circulating blood from the tissues. This continuous layer constitutes approximately 1% of body mass (1 kg) (Galley and Webster, 2004). The shape of endothelial cells varies across the vascular tree, but generally they are thin and slightly

elongated, approximately 50-70 μm long, 10-30 μm wide, and 0.1-10 μm thick. In the blood vessel wall, endothelial cells are orientated along the axis of the vascular wall and this helps to minimize the shear stress forces induced by the blood flow. Up to the early 1970s, the endothelium was thought of as a mere diffusion barrier preventing the access of the blood cells to the vascular matrix, but it has now become clear that this monolayer plays a predominant role in the control of blood fluidity, platelet activation, cellular adhesion, vascular tone and angiogenesis.

1.1.3.1 Blood fluidity, thrombosis and thrombolysis

In the heart, arteries and veins and under physiological conditions, the endothelium plays an important role in maintaining the proper haemostatic balance. Under normal conditions, endothelial cells help to maintain blood fluidity by means of different antiplatelet and anticoagulant mechanisms. These cells prevent adhesion, aggregation and activation of platelets and enhance platelet de-aggregation via expression of 13-hydroxyoctadecadienoic acid (13-HODE) on their surface cells, releasing nitric oxide (NO) and prostacyclin, hydrolysing ATP and ADP by membrane ectonucleotidases and inhibiting the action of thrombin, a potent aggregating enzyme (Michiels, 2003, Verhamme and Hoylaerts, 2006). They also inhibit the action of thrombin by expressing the thrombin receptor thrombomodulin on their surface, which promotes conversion of thrombin from a procoagulant to an anticoagulant enzyme. The thrombomodulin-bound thrombin activates protein C/protein S pathway leading to inactivation of coagulant factors Va and VIIIa and ultimately blocks the coagulation cascade (Rajendran et al., 2013).

Moreover, endothelial cells play a pivotal role in all major haemostatic pathways upon vascular injury and restrict clot formation to the areas where haemostasis is required to restore vascular integrity. Disruption of endothelial continuity results in the exposure of blood to the subendothelial matrix and collagen fibres and this triggers platelet adhesion to these structures. Von Willebrand factor (vWF), a multimeric protein, is mainly generated by endothelial cells and plays a crucial role in initial platelet aggregation and clot formation. It acts as a bridge between the tissue and the platelet through GPIb/IX/V glycoprotein receptor, a platelet receptor for vWF and other molecules including thrombin, P-selectin, factor XI and factor XII. The rapid formation of a platelet plug provides an initial defence mechanism against vessel wall injury and bleeding. Furthermore, the activated platelets secrete different platelet agonists such as thromboxane A₂, ADP and serotonin that mediate vascular smooth muscle cell contraction and therefore induce

vasoconstriction. Platelet-mediated primary haemostasis goes hand in hand with the activation of the coagulation system. Clot formation also occurs in response to exposure of blood to subendothelial tissue upon endothelial damage. Endothelial cells are involved in regulation of the haemostatic response, both in the initiation and amplification of thrombin formation and in the anticoagulant pathways that control the haemostatic response. The initiation of the coagulation cascade is primarily mediated by tissue factor (TF). In healthy blood vessels, TF is mainly located in the adventitial layer and the subcutaneous tissue. Direct contact of the blood with TF and the subsequent binding to factor VIIa initiates the coagulation cascade. The TF-VIIa complex converts circulating factor X and factor XI into active enzymes, namely factors Xa and XIa. Factor XIa promotes the activation of factor X. Factor X catalyses the generation of thrombin. Thrombin plays a pivotal role in amplification of the coagulation cascade via cleavage of fibrinogen to fibrin, activation of factors V, VIII, XI, XIII, and platelet activation (Figure 1-1).

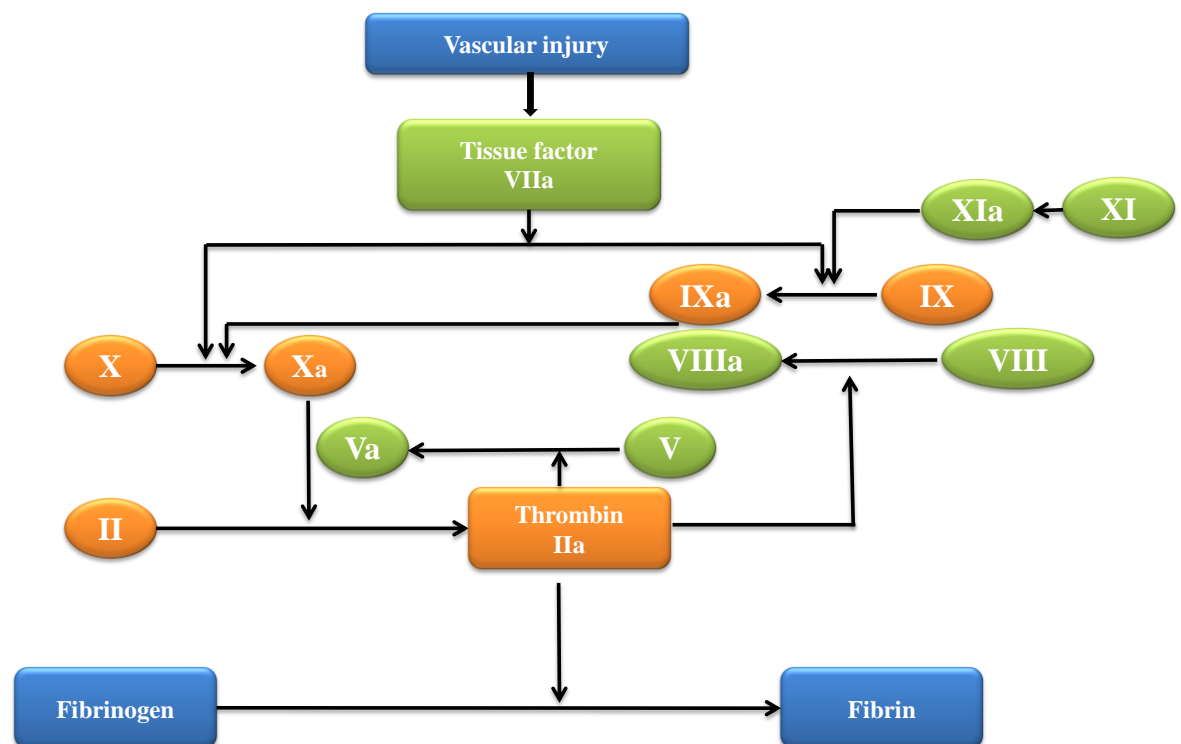


Figure 1-1 Schematic representation of the coagulation cascade.

In injured blood vessels, the tissue factor binds factor VIIa to activate factors X and IX: activated factor IX enhances the activation of factor X and the later catalyses the generation of thrombin. The thrombin that is generated in the initiation phase promotes amplification of the coagulation cascade via cleavage of fibrinogen to fibrin, activation of factors V, VIII.

When the blood clot is no longer needed for haemostasis, the fibrinolytic system degrades the clot and restores the patency of the blood vessel. The fibrinolytic system dissolves the

clot via action of plasmin which degrades the fibrin component of the blood clot into soluble degradation products. The plasmin is present as an inactive proenzyme, plasminogen, which is converted to active plasmin via two different activators, tissue-plasminogen activator (t-PA) and urokinase-plasminogen activator (u-PA). Both t-PA and u-PA are synthesized and released by endothelial cells.

1.1.3.2 Leukocyte trafficking

The interaction between leukocytes and vascular endothelium contributes to several physiological and pathophysiological processes including the immune response, wound repair and thrombosis as well as to acute and chronic inflammation. Transmigration of leukocytes from the circulation to the surrounding tissue is a multi-step process involving the capture, rolling and arrest of the leukocytes, followed by their extravasation to the surrounding tissue (or diapedesis) (Ley and Reutershan, 2006). The recruitment of leukocytes at sites of vascular injury is a very rapid response and mostly depends on the expression of the selectin family of adhesion molecules, in both endothelial cells and leukocytes. The capture and rolling of leukocytes is mainly regulated by E-selectin and P-selectin. Following rolling, endothelial chemokines stimulate conformational changes in integrins; adhesion receptors expressed by leukocytes. Integrins bind to their ligands on the endothelial cells such as vascular cell adhesion molecule (VECAM-1), platelet-endothelial cell adhesion molecule (PECAM-1), intracellular adhesion molecule (ICAM-1) or junctional adhesion molecule (JAM). Then, leukocytes migrate through the endothelium preferentially via inter-endothelial junctions and this process is regulated mainly by PECAM-1, ICAM-1 and VECAM-1 (Ley and Reutershan, 2006).

1.1.3.3 Regulation of vascular tone

The endothelium acts not only as a physical barrier between the circulation and the tissue, but it plays an important role in regulation of vascular tone by secreting a variety of regulatory substances. Endothelial cells control the vascular tone by releasing vasodilators such as nitric oxide (NO) and prostacyclin (PGI₂), as well as vasoconstrictors including endothelin, thromboxane A₂, angiotensin II, superoxide, and platelet-activating factor. These different compounds are not stored in intracellular granules. Rather, their major physiological effects are controlled by specific receptors on vascular cells, via their rapid metabolism, or at the level of gene transcription. Endothelial cells generate NO through oxidation of the amino acid L-arginine to NO and this process is catalysed by nitric oxide synthase (NOS) (Palmer et al., 1988). Three isoforms of NOS are identified: neuronal

isoform (nNOS) which forms NO to work as a neuronal messenger that modulates synaptic neuronal transmitter release (Prast and Philippu, 2001), Macrophage or Inducible isoform (iNOS) which is only induced in cells which have been exposed to particular inflammatory mediators that activate the macrophages (Michel and Feron, 1997), and endothelial NOS (eNOS) which synthesizes nitric oxide in vasculature (Lamas et al., 1992). These isoforms are not only expressed in the cells they were originally found in but they also exist in other cells. For example, all three NOS can be expressed in the vascular wall. nNOS is present not only in perivascular nerves but can also be expressed in endothelial and vascular smooth muscle (Gonzalez et al., 1997, Boulanger et al., 1998). iNOS expression has been reported in all nucleated cells in the cardiovascular system (Papapetropoulos et al., 1999) and eNOS can be detected in cardiomyocytes and platelets (Balligand et al., 1993, Sase and Michel, 1995). It is known that the dilation of the blood vessels is largely dependent on the activity of eNOS; therefore this introduction will now focus on this isoform.

Inactive eNOS is attached to the protein caveolin and is located in caveolae; small invaginations in the cell membrane (Bucci et al., 2000). eNOS is detached from caveolin and activated when the intracellular level of Ca^{2+} is increased (Bucci et al., 2000). Several receptor-dependent agonists such as bradykinin, acetylcholine, thrombin, adenosine diphosphate (ADP), adenosine tri-phosphate (ATP) and substance P can stimulate the detachment of eNOS from caveolin by releasing Ca^{2+} from the endoplasmic reticulum (Bae et al., 2003, Sandoo et al., 2010). Depletion of intracellular Ca^{2+} stores leads to opening of Ca^{2+} channels allowing Ca^{2+} influx from the extracellular space (Schilling et al., 1992, Schilling and Elliott, 1992). Ca^{2+} binds to the protein calmodulin in the cytoplasm of the cells and this leads to structural changes which enables it to attach to eNOS (Fleming and Busse, 1999). Activated eNOS then converts L-arginine to NO and so this pathway of NO generation depends upon the intracellular levels of Ca^{2+} in the endoplasmic reticulum as well as Ca^{2+} influx into the cells from extracellular stores. A decrease in Ca^{2+} results in dissociation of the calcium-calmodulin complex from eNOS which in turn attaches to caveolin and becomes inactivated (Fleming and Busse, 1999). However, there are additional mechanisms which can activate eNOS including phosphorylation via protein kinases such as protein kinase A (Bae et al., 2003) and cyclic guanosine-3', 5'-monophosphate (cGMP) protein kinase dependent II (Butt et al., 2000). Shear stress can also activate eNOS via the action of protein kinase A and Akt (Boo et al., 2002). Shear stress is caused by increased blood flow in the vessel and can elevate NO generation by eNOS phosphorylation or an increase in intracellular Ca^{2+} levels through stimulation of Ca^{2+} -activated K^+ channels on the endothelial cell surface; allowing K^+ efflux and Ca^{2+}

influx into the cells (Tran et al., 2000). Once generated, NO diffuses to the underlying smooth muscle where it activates the enzyme soluble guanylyl cyclase (sGC) (Ignarro et al., 1986). This enzyme catalyses the conversion of guanosine triphosphate (GTP) to cGMP, which decreases Ca^{2+} release from the sarcoplasmic reticulum in the smooth muscle cells and also helps to restore Ca^{2+} to the sarcoplasmic reticulum (Cornwell et al., 1991). Both actions induce relaxation of smooth muscle cells. Apart from its vasodilator effect, endothelium-derived NO also prevents leukocyte adhesion to endothelium and decreases inflammatory mediators in response to vascular injury (Peng et al., 1998).

Furthermore, prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) are also involved in regulation of vascular function (Bunting et al., 1983). Their synthesis is catalysed by cyclooxygenase enzymes (COX), of which there are two isoforms: COX-1 and COX-2. COX-1 is generated continuously in endothelial cells; in contrast COX-2 is only produced when the endothelium is injured and exposed to inflammatory cytokines (FitzGerald, 1991, Needleman and Isakson, 1997). COX enzymes catalyse the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) and the latter is converted to PGI_2 by prostacyclin synthase (McAdam et al., 1999). Then PGI_2 binds to the prostacyclin receptors (IP) on smooth muscle cells to activate adenylate cyclase which produces cyclic adenosine monophosphate (cAMP). The latter then activates protein kinase A, which allows relaxation of the smooth muscle by the same mechanism as for NO (Stitham et al., 2004). Prostacyclin receptors are also expressed on platelets and their activation by PGI_2 leads to inhibition of platelet aggregation (Higgs et al., 1978). In contrast to PGI_2 , TxA_2 induces vasoconstriction and platelet aggregation (Thomas et al., 1998). It is produced by converting arachidonic acid to PGH_2 and then TxA_2 is generated by thromboxane synthase (Bunting et al., 1983). TxA_2 induces its effects through thromboxane-prostanoid (TP) receptors which are expressed on platelets and whose stimulation enhances platelet aggregation (FitzGerald, 1991). The TP receptors are also located on smooth muscle cells and their activation by TxA_2 results in an increase in intracellular Ca^{2+} levels, leading to vasoconstriction (Cogolludo et al., 2003). The balance in the activity between PGI_2 and TxA_2 is important in maintaining homeostasis in the healthy vessel.

Endothelin-1 (ET-1) is another vasoconstrictor which is released by endothelial cells. ET-1 is synthesized by converting big ET-1 to ET-1 via endothelin converting enzyme (Yanagisawa et al., 1988). ET-1 synthesis and release is stimulated by inflammatory mediators such as interleukins and $\text{TNF-}\alpha$ and reduced by NO and PGI_2 (Alonso and Radomski, 2003). Shear stress causes a reduction in ET-1 expression, after initially

enhancing it. ET-1 receptors (ET_A and ET_{B2}) have been identified on smooth muscle cells and (ET_{B1}) receptors on endothelial cells (Davenport et al., 1995). Activation of ET_A and ET_{B2} by ET-1 results in Ca²⁺ influx into smooth muscle cells, leading to vasoconstriction in a similar mechanism to TxA₂. In contrast, stimulation of ET_{B1} receptors on the endothelium causes vasodilatation by releasing NO and PGI₂ (de Nucci et al., 1988).

1.1.3.4 Angiogenesis

Recovery of cardiac function and prevention of heart failure following myocardial infarction remains a challenge and there is no satisfactory strategy to overcome such complications. In the last two decades, proangiogenic therapy to enhance reperfusion and function of ischaemic hearts appeared a promising therapeutic intervention in preclinical studies. However, in clinical trials, this strategy has shown disappointing results, increasing the need for better understanding of angiogenic mechanisms in ischaemic hearts. The endothelium plays an important role in blood vessel formation. When a primitive network has been formed via vasculogenesis, it expands and remodels into a functional vascular network. This process involves angiogenesis; the growing of microvessels from pre-existing vessels. In the adult, angiogenesis is involved in regulation of reproduction, in wound repair and in response to stimuli such as inflammation and hypoxia. Several diseases are caused by abnormal angiogenesis or conversely by insufficient angiogenesis or vessel regression (Fischer et al., 2006). Although endothelial cells are normally quiescent, during angiogenesis they can proliferate rapidly with a turnover time less than 5 days (Folkman and D'Amore, 1996). Angiogenesis is a sophisticated phenomenon remarkably regulated in a spatial and temporal manner. In response to angiogenic factors produced by surrounding hypoxic tissues, endothelial cells degrade the extracellular matrix, proliferate, migrate towards these angiogenic signals and create tubular structures, the basis of the new blood vessels (Egeblad and Werb, 2002). Vascular endothelial growth factor (VEGF) plays a pivotal role in this phenomenon with additional contribution of other factors including angiopoietin-1 and integrins. VEGF and angiopoietin might also be involved in tube formation whereas ephrins, Eph receptor ligands, guide the forming vessel toward its target. Moreover, VEGF also promotes survival of the endothelial cell-constituted new blood vessel. However, the angiogenic process can differ markedly depending upon the origin of the neovascularization (physiological or pathological) and the vascular bed involved (Fischer et al., 2006).

1.2 Cardiovascular disease

Cardiovascular disease (CVD) is a general term that describes all diseases involving the heart and blood vessels including coronary heart disease (CHD), stroke, peripheral arterial disease and aortic disease. CVD is one of the leading causes of death in the United Kingdom. In 2014, CVD was the second most common cause of death in the UK, with a total of around 155,000 deaths (British Heart Foundation, 2015). Among them 41,000 were premature deaths (people below the age of 75). CVD is a multifactorial disease with interaction of both genetic and environmental risk factors such as smoking, diet and lifestyle. The most common causes of CVD deaths are CHD and stroke. In 2014, 44% of CVD deaths were caused by CHD and 25% were from stroke in the UK (British Heart Foundation, 2015).

1.2.1 Coronary heart disease (CHD)

Coronary artery disease (CAD) constitutes a major public health problem and is one of the leading causes of morbidity and mortality among adults worldwide. Each year more than 29 million people suffer a myocardial infarction, leading to 17 million deaths around the world. Moreover, coronary artery disease is the most common aetiology of sudden cardiac death which is responsible for 300 000 to 400 000 deaths every year in the United States alone (Santini et al., 2007). In 2014, CHD was responsible for 69,000 deaths in UK and Scotland had the highest rate of premature CHD deaths for both men (83/100,000) and women (28/100,000)(British Heart Foundation, 2015). There are several risk factors that have been confirmed to increase the risk of coronary heart disease including: hypertension, high cholesterol levels, cigarette smoking, and diabetes (Gordon et al., 1977, Kannel and McGee, 1979, Gordon and Kannel, 1982, Khot et al., 2003). Other factors such as obesity, left ventricular hypertrophy, previous family history of CHD, and oral contraception have also been considered in defining CHD risk (Kannel, 1991, Wilson et al., 1998, Logue et al., 2011).

1.2.2 Pathophysiology of coronary heart disease

Coronary atherosclerosis is by far the most common cause of ischaemic heart disease, and plaque disruption with superimposed thrombosis is the leading cause of acute coronary syndromes including unstable angina, myocardial infarction and sudden death (Arbab-Zadeh et al., 2012). Coronary events can also be caused by coronary dissection, coronary spasm, arteritis and thromboembolism without obvious coronary artery disease (Davies,

2000). Coronary thrombosis is most commonly triggered by plaque disruption or endothelial erosion. In white males more than 85% of major coronary thrombi are associated with vulnerable plaque rupture. In contrast, endothelial erosion is responsible for $\geq 50\%$ of coronary thrombi in women (Burke et al., 1997, Arbustini et al., 1998). Several studies have demonstrated that the atherosclerotic plaque type at the greatest risk of rupture is thin-cap fibroatheroma, characterized by a large necrotic core surrounded by a thin fibrous layer of cap (Virmani et al., 2006, Calvert et al., 2011, Stone et al., 2011). During the atherosclerotic process, the necrotic core increases in volume as a result of macrophage infiltration, the demise of macrophages and intraplaque bleeding. This leads to separation of the necrotic core from the flowing blood by an ultra-thin layer of fibrous tissue that is depleted of smooth muscle cells and is infiltrated by inflammatory cells. Thus, plaque growth, the inflammatory process within the fibrous cap and external shear stress may disrupt the thinnest part of the fibroatheroma, causing tearing and exposure of the highly thrombogenic material to the blood (Virmani et al., 2006). This ultimately results in thrombus formation and, in severe cases, occlusion of the coronary artery.

1.3 Endothelium and myocardial ischaemia

Vascular endothelial cells play an essential role in regulation of vascular tone, angiogenesis, as well as of platelet and neutrophil function. Several experimental and clinical studies suggest that these important physiological functions of the endothelium deteriorate in numerous pathophysiological conditions, such as hypertension, hyperlipidaemia or diabetes. Such dysfunction can be manifested by a decrease in endothelial release of nitric oxide (NO) and an increased generation of oxygen-derived free radicals (Laude et al., 2001). A similar impairment in the endothelial production of NO has also been observed at the level of the coronary circulation after myocardial ischaemia and reperfusion (Yang et al., 2013). This may lead to marked deleterious consequences for the coronary arterial wall.

Ischaemia is a process in which the demand for oxygen is greater than the available supply, most commonly caused by inadequate blood flow. In myocardial infarction, partial or total occlusion of a large epicardial coronary artery results in disruption of blood flow to the myocardium. In order to prevent irreversible myocardial tissue damage, it is essential to restore blood supply after severe ischaemia. However, reperfusion itself results in further tissue damage which might be more severe than the damage produced by ischaemia alone. Evidence that myocardial ischaemia causes coronary endothelial dysfunction was first

observed by Ku who showed that exposure to a defined period of ischaemia (90 min) followed by reperfusion for 1 to 2 hr led to a decrease in endothelium-dependent relaxation to thrombin in canine coronary arteries (Ku, 1982). Similar findings were also reported with other endothelium-dependent vasodilators such as acetylcholine (VanBenthuyzen et al., 1987) whereas the response to nitroprusside, an endothelium-independent vasodilator, was intact. This impairment of endothelium-dependent relaxation to acetylcholine appeared to be a manifestation of reperfusion injury as ischaemia alone did not alter the relaxant response to acetylcholine (Tsao et al., 1990). It is likely that such prolonged endothelial impairment was a consequence of dysfunctional regenerated endothelium whereas the acute changes were a result of structural injury to endothelial cells (Pearson et al., 1990, Kaeffer et al., 1996).

1.3.1 Mechanism of reperfusion-induced vascular endothelium dysfunction

Although endothelial cells (EC) are less sensitive to ischaemia alone, they are quite sensitive to an IR insult in the heart (Brutsaert, 2003). It has been demonstrated that reperfusion causes a variety of cellular abnormalities that occur quickly such as increased reactive oxygen species (ROS) production, decreased NO bioavailability, induction of inflammatory processes and impaired Ca^{2+} homeostasis and these abnormalities enhance apoptosis of the endothelial cells. ROS are negatively charged short lived oxygen species that induce nonspecific and irreparable oxidative damage. It has been demonstrated that exposure of human umbilical vein ECs (HUVECs) to 2 hr hypoxia with glucose depletion followed by 1hr normoxia with glucose increased ROS production (Therade-Matharan et al., 2004, Therade-Matharan et al., 2005). The major source of endothelial ROS during an IR insult is the mitochondrial electron transport chain (Coudray et al., 1992, Quintero et al., 2006). ROS generation occurs rapidly during reperfusion and is a critical mediator for many downstream events. In cultured ECs, ROS production appears within minutes of reoxygenation based on electron paramagnetic resonance spectroscopy (Zweier et al., 1994). Increased ROS production is associated with a decrease in vasoreactivity (Szocs, 2004). In this regard, ROS release may overwhelm the endogenous antioxidant environment, reduce NO bioavailability, and consequently impair endothelium-dependent vasorelaxation (Szocs, 2004). Moreover, increased ROS release during IR insult might lead to expression of surface adhesion molecules (Patel et al., 1991, Gaboury et al., 1994) and complement activation (Lucchesi, 1994), which collectively enhance neutrophil adhesion to the EC surface. At the onset of ischaemia, NO release increases within 5-10 seconds and

this induces vasodilation but this effect decreases within 5-10 min (Vinten-Johansen et al., 1999, Schulz et al., 2004, Davidson and Duchon, 2007). During reperfusion NO release again increases rapidly but reduces within the same time frame as for ischaemia (Wang and Zweier, 1996). Furthermore, several studies have demonstrated that delivery of an NO donor (e.g. sodium nitroprusside, nitroglycerin) or arginine, a substrate required for NO synthesis, protects the heart against IR injury via at least 2 mechanisms (Vinten-Johansen et al., 1999). First, NO activates the soluble guanylyl cyclase and this leads to activation of a host of cGMP kinases including protein kinase G, ion channels (e.g. Mito K_{ATP} channel) and phosphodiesterases (PDEs) (Grover and Garlid, 2000, Friebe and Koesling, 2003). Consequently this results in vasodilation and reduced platelet adhesion. NO has also direct effects on neutrophil adhesion independent of its influence on cGMP activity (Schulz et al., 2004). There is also evidence that endogenous NO synthase inhibitors may have an important role in ischaemia/reperfusion injury. Impaired NO production may result from increased levels of asymmetrical dimethylarginine (ADMA), an endogenous competitive inhibitor of NOS (Cooke, 2000). It has been observed that ADMA is elevated in newly diagnosed patients with acute myocardial infarction compared to healthy subjects (Bae et al., 2005). Stühlinger et al observed that increased ADMA levels during early phase of reperfusion is due to inhibition of its metabolism by dimethylarginine dimethylaminohydrolase (DDAH) (Stühlinger et al., 2007). The end result is reduction of NO bioavailability and endothelial dysfunction and this effect can be reversed by administration of exogenous L-arginine or partial elimination of ADMA in a genetic mouse model (Stühlinger et al., 2007). Solid evidence exists that eNOS may contribute to protection against myocardial injury. It was observed that IR injury increased in eNOS knock out mice compared to wild type mice (Huang et al., 1995).

Moreover, Ca^{2+} homeostasis may play a pivotal role in endothelial cell IR injury. Under normal conditions, Ca^{2+} levels in both cytosol and mitochondria are maintained in a narrow range (Davidson and Duchon, 2007, Peters and Piper, 2007). In contrast, during ischaemia intracellular Ca^{2+} increases slowly and increases further during reperfusion in both endothelial cells and cardiomyocytes (Symons and Schaefer, 2001, Peters and Piper, 2007). Several studies assessed the contribution of Ca^{2+} overload to IR-induced myocardial injury and coronary vascular dysfunction in pigs and rats using cariporide, a Na^+-H^+ pump inhibitor (Symons et al., 1998, Symons and Schaefer, 2001, Scheule et al., 2003). Specifically, coronary relaxation in response to acetylcholine but not sodium nitroprusside was impaired after IR injury, suggesting an IR-induced endothelial dysfunction (Symons and Schaefer, 2001). Pre-treatment with cariporide preserved endothelial function in

coronary arteries after exposure to an IR insult and their relaxation in response to acetylcholine was depressed to a lesser degree (Symons and Schaefer, 2001). This suggests that pharmacological attenuation of calcium overload during ischaemia-reperfusion may be a promising strategy for cardioprotection.

1.3.2 Mechanism of myocardial ischaemia/reperfusion injury

Restoration of blood supply after the onset of acute myocardial ischaemia is crucial to preserve viable myocardium, limit myocardial infarct size, salvage left ventricular systolic function and prevent the onset of heart failure. However, the reperfusion itself can independently cause cardiomyocyte death (Braunwald and Kloner, 1985, Piper et al., 1998, Hausenloy and Yellon, 2007). IR induces four forms of myocardial injury: arrhythmia, myocardial stunning, microvascular obstruction and ultimately death of cardiomyocytes. First, sudden reperfusion of ischaemic myocardium might be associated with ventricular arrhythmia, which is usually reversible without treatment (Hearse and Tosaki, 1987). Myocardial stunning, reversible myocardial contractile dysfunction, occurs on reperfusion of acute ischaemic myocardium as a result of oxidative stress and intracellular Ca^{2+} overload in myocardium (Kloner et al., 1998). Reopening of the occluded artery for reperfusion of ischaemic myocardium does not guarantee that the blood flow will return to pre-occlusion values. In severe cases, even in the presence of a completely patent artery, no-reflow phenomenon may occur secondary to disrupted coronary microcirculation (Menger et al., 1992, Reffelmann and Kloner, 2004, Ito, 2006). The major contributing factors to disrupted coronary microcirculation include capillary damage with impaired vasodilation, evoked mechanical capillary compression by endothelial cells and cardiomyocyte swelling, neutrophil trapping and /or platelet aggregation and micro-embolization by friable material released from the atherosclerotic plaque. Ultimately, reperfusion causes death of cardiomyocytes (Piper et al., 1998). This could potentially result from oxidative stress, calcium overload, mitochondrial permeability transition pore (mPTP) opening and hypercontracture (Yellon and Hausenloy 2007). Reperfusion-induced cardiomyocyte death has been observed in both experimental myocardial infarction models and in patients with STEMI, ST-segment elevation myocardial infarction, by the observation that therapeutic intervention applied only at the onset of reperfusion decreased MI size by 40%-50% (Yellon and Hausenloy 2007).

Therefore, it is important to understand the mechanism of IR injury at the level of the cell. During acute myocardial ischaemia, cessation of oxygen delivery switches cell metabolism

to anaerobic respiration and this leads to an increase in lactate production and a decrease in intracellular pH to less than 7.0. In contrast, normal pH is rapidly restored by the washout of lactate and stimulation of $\text{Na}^+ - \text{H}^+$ exchanger at reperfusion (Lemasters et al., 1996). This contributes to reperfusion-induced cardiomyocyte death by permitting mPTP opening and cardiomyocyte rigor hypercontracture. Moreover, the electron transport chain is reactivated during reperfusion, generating ROS (Downey, 1990, Li and Jackson, 2002, Zweier and Talukder, 2006). But ROS can also be generated in endothelial cells (xanthine oxidase) and neutrophils (NADPH oxidase). ROS contribute to myocardial reperfusion injury via opening of mPTP, damage to the cell membrane by lipid peroxidation, enhancing neutrophil recruitment, and mediating dysfunction of the sarcoplasmic reticulum (SR)(Li and Jackson, 2002, Hausenloy and Yellon, 2013). As described above, Ca^{2+} overload in cardiomyocytes is one of the major contributing factors to IR injury. This begins during myocardial ischaemia and is exacerbated at reperfusion due to disruption of the plasma membrane, dysfunction of sarcoplasmic reticulum and mitochondrial re-energization (Miyamae et al., 1996). The mPTP opening is one of the most important contributors to IR-induced myocardial and endothelial cell injury. It has been demonstrated that they remain closed during ischaemia and only open at the time of reperfusion in response to rapid restoration of physiological pH, mitochondrial Ca^{2+} overload, oxidative stress and relative ATP depletion (Griffiths and Halestrap, 1995, Halestrap et al., 2004, Ruiz-Meana et al., 2007). The opening of mPTP is associated with cell death, either necrotic or apoptotic (Mattson and Kroemer, 2003, Crow et al., 2004). The severity of damage induced by mPTP opening depends upon the duration of their opening and the number of mitochondria involved. Long lasting mPTP opening causes loss of mitochondrial membrane potential, ATP depletion and release of apoptotic factors such as cytochrome c, caspase 9 and caspase 3. Consequently, this leads to cell death and loss of oxidative phosphorylation capacity (Crompton, 1999, Mattson and Kroemer, 2003, Crow et al., 2004, Kroemer et al., 2007) (Figure 1-2).

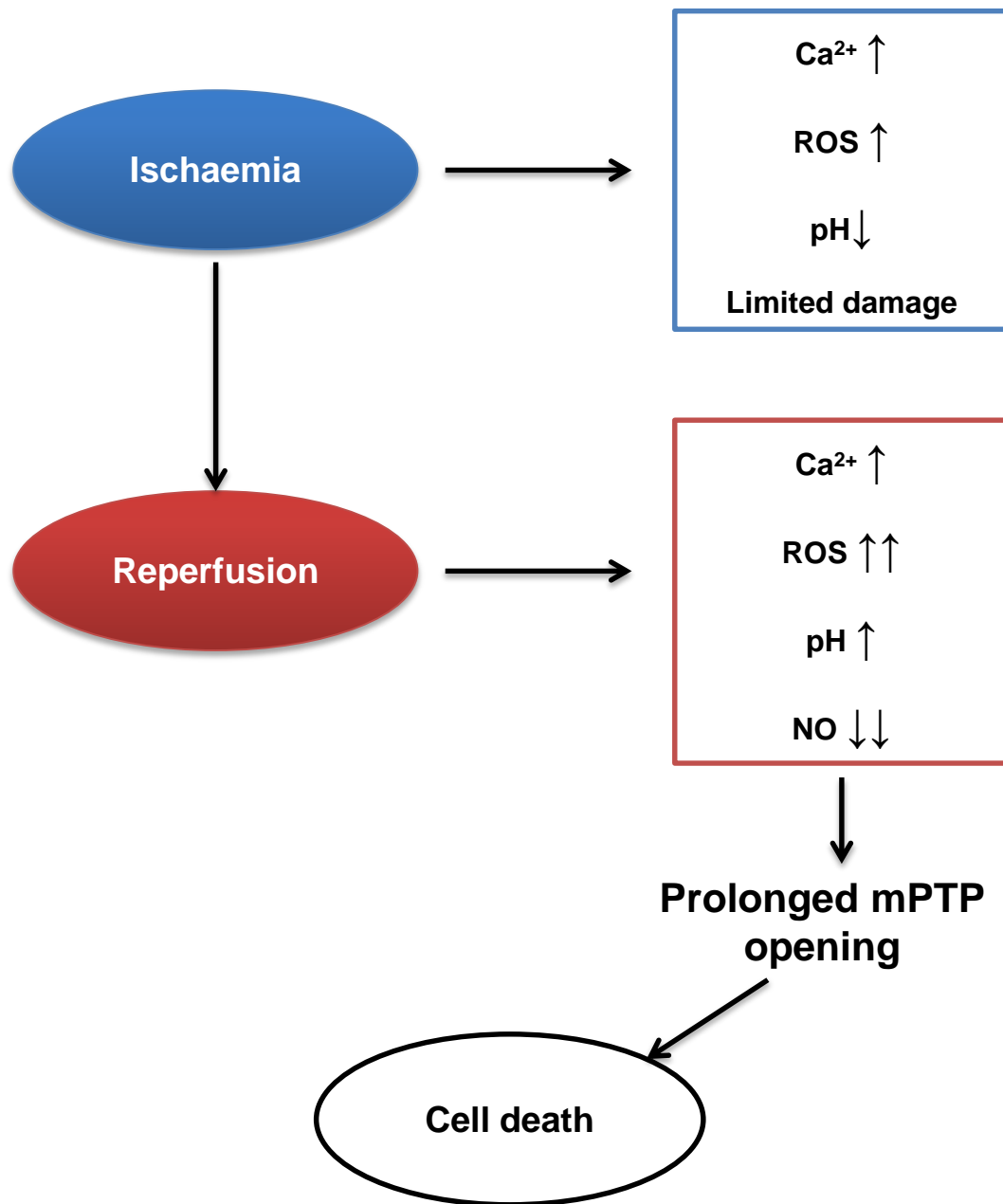


Figure 1-2 Flow chart shows the main mechanisms of ischaemia/reperfusion injury.

Ischaemia causes a slight increase in ROS and Ca²⁺ overload and the opening of mPTP during ischaemia is limited due to low pH. During reperfusion, pH returns to its baseline levels and ROS generation increases, leading to prolonged mPTP opening which mediates irreversible cell damage during reperfusion.

1.3.3 The relationship between endothelial cells and myocardial injury

ECs modulate cardiomyocyte growth, metabolism, contractility, apoptosis and arrhythmogenicity by generating and releasing many signalling molecules (Brutsaert, 2003). In addition to its vasodilatory effects, NO acts as a signalling molecule and because of the close proximity between ECs and cardiac cells, NO is able to diffuse to cardiomyocytes and plays a role in regulation of contractility, electrical activity and oxygen consumption of cardiomyocytes (Brutsaert, 2003). Other EC signalling molecules can also contribute to the interaction between ECs and cardiomyocytes and these include factors responsible for vasoregulation (e.g. prostacyclin, endothelin-1, and angiotensin II), inflammation (e.g. IL-1, TNF- α), and growth (PDGF, VEGF). Several studies have shown that a strong correlation exists between vascular and cardiac function in an isolated heart model immediately following an IR injury (Di Napoli et al., 1998, Fedalen et al., 2003, Mohara et al., 2005). In isolated rat hearts pre-treated with verapamil before subjecting the hearts to 30 min global ischaemia, preservation of coronary flow was significantly correlated with functional recovery (Mohara et al., 2005). It is still unclear how preserved endothelial function contributes to cardioprotection against IR injury, however, several mechanisms have been proposed including enhanced microcirculation and decreased capillary permeability and interstitial oedema (Di Napoli et al., 1998).

1.3.4 Cardioprotection against ischaemia/reperfusion insult

The term cardioprotection refers to protection against IR-induced myocardial injury. In this regard, several interventions have been studied to condition the heart in order to reduce the severity of myocardial injury and improve the prognosis of patients exposed to acute myocardial infarction. Indeed the most important determinant of myocardial damage during any ischaemia and reperfusion event is the myocardial infarct size (Geltman, 1984). It is therefore the most significant goal in management of acute myocardial infarction to reduce the infarct size as much as possible and avoid subsequent serious complications of AMI. Early reperfusion can help to prevent myocardial injury and reduce the infarct size (Maroko et al., 1972) and this can usually be achieved by the use of primary percutaneous coronary intervention (PCI) and thrombolytic therapy. However, as noted above, reperfusion itself paradoxically contributes to the myocardial injury and attenuates the benefits of restoration of the blood flow to the myocardium (Braunwald and Kloner, 1985, Garcia-Dorado and Piper, 2006). Therefore several studies have been carried out to develop new treatment protocols and/or surgical interventions to protect the heart against

ischaemia/reperfusion insult. In this regard, it has been shown that brief episodes of nonlethal ischaemia and reperfusion prior to sustained ischaemia can reduce myocardial infarct size and this has become known as ischaemic preconditioning (Murry et al., 1986). Moreover, brief episodes of nonlethal IR just at the onset of reperfusion have also reduced myocardial infarct size and this is termed ischaemic postconditioning (Zhao et al., 2003).

1.3.4.1 Ischaemic preconditioning (IPC) and postconditioning (IPost)

The phenomenon of myocardial ischaemic preconditioning has been confirmed in rats (Hu and Nattel, 1995), pigs (Schott et al., 1990, Vahlhaus et al., 1998), and rabbits (Liu et al., 1991, Miura et al., 1992) as well as human isolated cardiomyocytes (Ikonomidis et al., 1994). Furthermore, strong evidence exists that preconditioning may occur in vivo in human hearts (Deutsch et al., 1990, Cribier et al., 1992, Yellon et al., 1993). Ischaemic preconditioning confers several beneficial effects including infarct size limitation, reduction of IR-induced arrhythmia and contractile dysfunction and protects coronary endothelium (Minamino et al., 1998, Eisen et al., 2004, Garcia-Dorado et al., 2011). At the cellular level, the significant effects of ischaemic preconditioning are assumed to prevent cell death due to reperfusion injury. Many factors such as autacoids (eg, adenosine, bradykinin, and opioids), their receptors, kinase signalling pathways and mitochondria modulation are involved in the cardioprotective effects of ischaemic preconditioning. Short periods of ischaemia lead to production of endogenous autacoids and activation of their respective receptors. This results in activation of cardioprotective signalling pathways such as ERK1/2, PI3K/Akt, protein C and protein G, leading to inactivation of mitochondrial glycogen synthase kinase-3 β (GSK-3 β). This ultimately inhibits the opening of mPTP, which plays a pivotal role in cardiac cell apoptosis and necrosis (Miura and Tanno, 2012). Although the above studies show that IPC is effective in whole heart preparations, it has also been reported that preconditioning provides a protection at the level of the coronary endothelial cells and prevents the impaired endothelium-dependent relaxant responses to acetylcholine induced by prolonged ischaemia-reperfusion in rat models (Richard et al., 1994, Laude et al., 2002) and in humans (Luca et al., 2013).

In 2003, Zhao et al reported that short periods of coronary occlusion and reperfusion at the onset of reperfusion following 60 min of ischaemia reduced myocardial infarction by 40% in canine hearts (Zhao et al., 2003). The cardioprotective effects of ischaemic postconditioning have been confirmed in many species including humans (Staat et al., 2005, Skyschally et al., 2009). IPost protects the heart against IR through prevention of the

dramatic increase in intracellular pH during the early stages of reperfusion. Furthermore, during the early stages of reperfusion, there is robust generation of ROS in the vascular endothelium, cardiomyocytes, and mitochondria and this can be reduced by postconditioning (Sun et al., 2005, Serviddio et al., 2005). IPost also activates intracellular signal transduction in a way that is similar to preconditioning.

1.3.4.2 Pharmacological treatment

Several pharmacological agents are cardioprotective when administered before ischaemia or at the time of reperfusion. These drugs include adenosine (Whittaker et al., 1996), bradykinin (Brew et al., 1995), insulin (Sack and Yellon, 2003), Glucagon like peptide 1 (Bose et al., 2005), cariporide (Zhang et al., 2006), atorvastatin (Birnbaum et al., 2003), sphingosine-1-phosphate (Knapp, 2011, Somers et al., 2012), cyclosporine (Piot et al., 2008) and sangliferin A (Clarke et al., 2002). All these drugs likely work upstream from mPTP except cyclosporine and sangliferin A which directly regulate mPTP. One of the important strategies for cardioprotection against IR insult is to prevent microvascular dysfunction as this will improve blood flow during reperfusion. Several studies demonstrated that preservation of vascular integrity during ischaemia/reperfusion may also protect the heart through limitation of no-flow phenomenon, decrease in post-infarct inflammatory response and limit the expansion of infarct size. In this regard, a number of drugs such as angiopoietin-like 4 (Galaup et al., 2012), sevoflurane (Anneck et al., 2010) and nitroglycerin (Lisi et al., 2012) protect vascular endothelium against an ischaemia reperfusion insult and confer secondary cardioprotection during acute myocardial infarction.

1.4 Sphingosine-1-Phosphate (S1P)

1.4.1 S1P overview

The eukaryotic cell membrane consists of three main lipid types including cholesterol, glycerophospholipid, and sphingolipids. Sphingomyelin metabolites are biologically active lipids synthesized from membrane phospholipids upon cell activation. Sphingosine-1-phosphate (S1P) is one of the important phospholipid cellular metabolites that acts as an extracellular mediator and intracellular messenger. It is generated and released from many different cells by multistep enzymatic pathways in order to regulate diverse biological functions involving cell proliferation, migration, growth and survival. Although S1P has been extensively studied, much still remains to be investigated about this lysophospholipid.

1.4.2 Sphingosine-1-phosphate synthesis and degradation

S1P biosynthesis starts with generation of ceramide from sphingomyelin via the action of the enzyme sphingomyelinase (Hannun et al., 2001) or de novo. This enzyme is stimulated by growth factors, arachidonic acid and oxidative stress to catalyse the generation of ceramide (Levade and Jaffrezou, 1999). The next step is deacylation of ceramide to produce sphingosine and non-esterified fatty acid and this step is catalysed by the enzyme ceramidase. Then sphingosine kinases (SK) catalyse the phosphorylation of sphingosine to produce S1P which in turn is dephosphorylated through the action of S1P phosphatase or degraded by S1P lyase (Figure 1-3). Interestingly the effect of ceramide and S1P on cell survival is completely different as ceramide promotes cell death (apoptosis) whereas S1P enhances cell survival. The intracellular levels of S1P are regulated by the balance between SK-mediated synthesis of S1P and its breakdown by S1P lyase or phosphatase. It has been reported that S1P can act as an intracellular messenger (Olivera and Spiegel, 1993, Spiegel, 1999) or be released from cells in response to agonist stimulation to allow actions at S1P₁₋₅ receptors. De novo ceramide synthesis occurs in endoplasmic reticulum and is initiated by production of 3-Oxosphinganine from the serine palmitoyltransferase catalysed condensation of serine and palmitoyl-CoA. 3-Oxosphinganine is then reduced to dihydrosphingosine by an NAD(P)H-dependent reductase. Dihydroceramide is produced from N-acylation of dihydrosphingosine via dihydroceramide synthase. Dihydroceramide is later desaturated by dihydroceramide desaturase to produce ceramide (Pyne and Pyne, 2000).

1.4.3 S1P in circulation

S1P is present in plasma at elevated concentrations ~ 0.1 to 1.1 μM (Murata et al., 2000) relative to the interstitial fluid of tissues. This S1P gradient is important in regulation of many physiological functions provided by extracellular S1P (Olivera et al., 2013). The high level of circulating S1P is maintained mainly by erythrocytes (Pappu et al., 2007, Xiong et al., 2014). The vascular endothelium is another important contributor to plasma S1P in adults (Venkataraman et al., 2008) and the lymphatic endothelium is the main source of lymphatic S1P (Pham et al., 2010). Furthermore, other blood cells such as activated platelets, neutrophils and monocytes may also contribute by releasing a small amount of S1P to the plasma (Yatomi et al., 1995). Within the plasma, S1P is bound to protein carriers such as HDL (50-60%) (Blaho et al., 2015), albumin (30–40%), LDL (~8%) and VLDL (2-3%)(Okajima, 2002, Karuna et al., 2011). S1P is associated to HDL

specifically via apolipoprotein ApoM, which functions as an S1P chaperone that regulates the levels of S1P in blood (Christoffersen et al., 2011). Moreover, chaperones such as ApoM may also protect S1P from degradation and facilitate its delivery to S1P receptors.

1.4.4 S1P receptors, Agonists and Antagonists

The S1P receptors were originally known as endothelium differentiation gene (Edg) receptors because of the high expression of the gene encoding for this receptor in differentiating human umbilical vein endothelial cells. They are a G-protein coupled receptor family and each consists of approximately 400 amino acids (Hla and Maciag, 1990, An et al., 1997, Okamoto et al., 1999, Im et al., 2000). The common features for S1P receptors include the seven transmembrane α -helices that extend through the lipid bilayer creating a polar internal tunnel, the C terminus and three intracellular loops exposed to the interior, and the N terminus and three extracellular loops exposed to the exterior. The S1P binds to these receptors with high affinity and therefore they are now named S1P receptors instead of Edg receptors. A set of five S1P receptors has been discovered, S1P₁₋₅ but only S1P₁₋₃ receptors are expressed in the cardiovascular system (Okazaki et al., 1993, MacLennan et al., 1994, Graler et al., 1998, Glickman et al., 1999, Yamazaki et al., 2000). The S1P receptors have 50% amino acid sequence homology and display overlapping as well as different expression patterns in tissue. S1P₁ couples to particular pathways via G_{i/o} (Okamoto et al., 1998, Zondag et al., 1998). Stimulation of S1P₁ receptor evokes certain responses including adenylyl cyclase inhibition, phospholipase C activation, Ca²⁺ mobilisation (Okamoto et al., 1998) and activation of Ras-ERK and phosphatidylinositol 3-kinase (PI3K)(O'Sullivan and Dev, 2013). S1P₂ and S1P₃ receptors couple via G_{i/o}, G_q and G_{12/13} and their stimulation leads to activation of phospholipase C (Kon et al., 1999), Ca²⁺ mobilisation (Okamoto et al., 1998) and activation of Ras and extracellular signal related kinases (ERK)(An et al., 2000). S1P₄ and S1P₅ couple via G_{i/o} and G_{12/13} to activate Rho (O'Sullivan and Dev, 2013).

S1P receptors appear to be specific as they have the ability to bind only S1P and dihydro-S1P. In the past it was suggested that S1P receptors may be capable of binding lysophospholipids due to their structural similarity to S1P, however it is now widely believed that this is not the case (Contos et al., 2000). Sphingosine receptor agonists such as FTY720 have been used to study the activity of S1P receptors. The FTY720 is phosphorylated by sphingosine kinase 2 to generate its active form, phospho-FTY720, which is then able to bind to S1P receptors, with the exception of S1P₂ (Paugh et al.,

2003). FTY720 acts non-selectively and activates both S1P₁/S1P₃ receptors whereas SEW2871, AUY954 and AKP-11 can act as selective S1P₁ agonists (Pan et al., 2006, Tsukada et al., 2007, Samuvel et al., 2015) and CYM5541 can selectively bind S1P₃ receptor (Jo et al., 2012). In terms of antagonists, the S1P₃ receptor subtype can be blocked selectively by suramin (Ancellin and Hla, 1999) and BML-241 (also named CAY10444) but the receptor selectivity of the latter is still subject to debate (Salomone and Waeber, 2011, Pyne and Pyne, 2011). VPC23019 is a dual S1P₁/S1P₃ antagonist, however its use has been limited because of its poor stability and in vivo efficacy (Davis et al., 2005, Awad et al., 2006, Salomone and Waeber, 2011). Conversely, W146 is widely utilized as a selective S1P₁ receptor antagonist (Sanna et al., 2006). Although JTE-013 is capable of blocking S1P₂ receptor activation, several studies showed that it also has antagonistic activity at S1P₄ as well as non-S1PR-mediated effects (Salomone et al., 2008, Long et al., 2010, Pyne and Pyne, 2011). Furthermore S1P has also been suggested to act as an intracellular messenger regulating intracellular Ca²⁺ mobilisation, cell proliferation and survival (Ghosh et al., 1990, Zhang et al., 1991). S1P has been demonstrated to bind and activate TRAF2, an E3 ligase that plays an important role in Lys 63-linked polyubiquitination of RIP1 and this process enhances cell survival by inhibiting switching of RIP1 from a prosurvival to proapoptotic adaptor protein (Alvarez et al., 2010). Prohibitin 2 (PHB2), a highly conserved protein that plays an important role in regulation of mitochondrial assembly and function, is another intracellular target for S1P. It was shown that interaction of S1P produced by SK2 in mitochondria with PHB2 is essential for cytochrome-c oxidase assembly and mitochondrial respiration (Strub et al., 2011). Moreover, Takasugi et al showed that S1P can bind to BACE1, the rate-limiting enzyme for amyloid- β peptide (A β) production, in vitro and enhances its proteolytic activity (Takasugi et al., 2011). A recent study found that S1P generated by SK2 in fibroblast nuclei interacts with hTERT, human telomerase reverse transcriptase, promoting its stability and consequently cell proliferation (Panneer Selvam et al., 2015). SK2 inhibition or deletion or mutating the S1P binding site reduced the stability of hTERT in cultured cells and enhanced senescence and loss of telomere integrity. It has also been reported that S1P acts as a ligand for PPAR γ , Peroxisome proliferator-activated receptor γ that regulates neoangiogenesis (Parham et al., 2015). Therefore, targeting S1P signalling pathway may help to understand the regulation of multiple systems including the cardiovascular system.

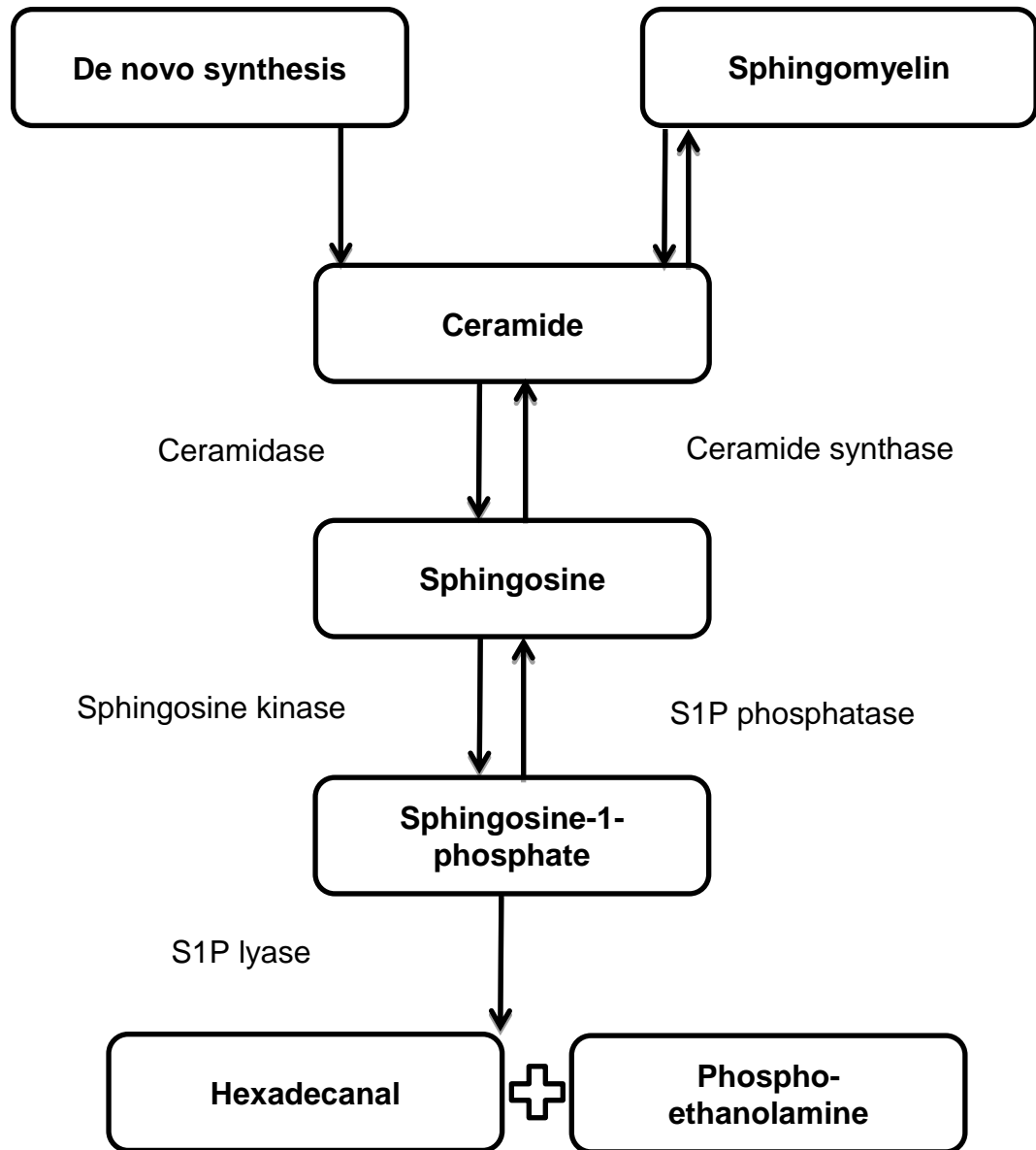


Figure 1-3 Summary of the steps involved in sphingosine-1-phosphate synthesis and breakdown.

S1P synthesis is started by generation of ceramide from sphingomyelin via the action of the enzyme sphingomyelinase. Then sphingosine is formed from ceramide through the action of ceramidase enzyme. Thereafter, sphingosine kinases catalyse the phosphorylation of sphingosine to produce S1P which in turn is dephosphorylated through the action of S1P phosphatase or degraded by S1P lyase.

1.4.5 Physiological Role of S1P in the Cardiovascular System

S1P and other sphingomyelin metabolites regulate a number of important functions in cardiac and vascular homeostasis. They may also play pivotal roles in the pathogenesis of several cardiovascular diseases including coronary artery disease, atherosclerosis, myocardial infarction, and heart failure. Therefore S1P has been extensively studied in recent years in order to elucidate its role in cardiovascular function.

1.4.5.1 Effects of S1P on vascular development and tone

Only S1P₁, S1P₂ and S1P₃ are expressed in the cardiovascular system. Endothelial cells express mainly S1P₁ receptors, less S1P₃ and only very few S1P₂ receptors under normal conditions (Ozaki et al., 2003, Saba and Hla, 2004, Alewijnse and Peters, 2008). On the other hand S1P₂ receptors are predominately expressed in vascular smooth muscle cells (VSMC) where expression of S1P₁ and S1P₃ is much lower (Alewijnse and Peters, 2008). Moreover, there are relative differences in expression of S1P receptors in endothelial cells of different vascular beds (aortic, cerebral, coronary, renal, and mesenteric vessels) (Levkau, 2008, Igarashi and Michel, 2009). It has been reported that S1P may play an important role in angiogenesis as S1P₁ knockout mice die during embryogenesis as a result of vascular haemorrhage due to a lack of support from vascular smooth muscle cell that fail to surround and support the developing vasculature (Liu et al., 2000b). This demonstrates that S1P₁ receptors in endothelial cells but not VSMC play a decisive role in recruitment of VSMC and this is confirmed by the appearance of the same phenotype in endothelial-specific S1P₁ knockout mice (Allende and Proia, 2002). SK1 and SK2 knockout mice that completely lack S1P have identical defects (Mizugishi et al., 2005). These studies provide considerable evidence that S1P has an important role in vascular morphogenesis.

S1P has been implicated in regulation of vascular tone and the first study to examine its effects on vascular tone used rat isolated mesenteric artery and intra-renal vessels. In both arteries, S1P induced vasoconstriction and this effect was mediated by a G-protein coupled receptor and associated with an increase in intracellular calcium (Bischoff et al., 2000). Since this initial demonstration of S1P's role in regulation of vascular tone, further studies have been conducted in a variety of vessels which have shown that the degree and type of vascular response to S1P varies between species and vascular beds. For instance, exogenous application of S1P to isolated rat mesenteric and cerebral arteries has been reported to promote vasoconstriction in these vessels (Hemmings et al., 2004, Salomone et

al., 2008). Furthermore, Salomone et al (2008) demonstrated that the vasoconstrictor response of rat cerebral arteries to S1P was mediated through S1P₃ receptor. It has also been reported that S1P may constrict rat coronary artery via a mechanism requiring S1P₃ receptor (Murakami et al., 2010). S1P-induced vasoconstriction has also been observed in porcine pulmonary (Hsiao et al., 2005), human placental (Hemmings et al., 2006), intrarenal (Czyborra et al., 2006) and hamster gracilis muscle resistance arteries (Keller et al., 2006). Rho-associated kinase activation (Hemmings et al., 2006) and increased reactive oxygen species production (Keller et al., 2006) are suggested mechanisms mediating contractions in these arteries (Figure 1-4). On the other hand, S1P can also induce vasorelaxation in particular vascular beds. It has been found that S1P induced relaxation of rat mesenteric artery and this response can be abolished by removal of endothelium or by addition of nitric oxide synthase inhibitor (Dantas et al., 2003), suggesting involvement of endothelial isoforms of nitric oxide synthase (eNOS) in the relaxant response (Figure 1-4). The relaxant effect of S1P has also been demonstrated in rat aorta and this response was S1P₃ receptor dependent and involved activation of the eNOS pathway (Nofer et al., 2004, Roviezzo et al., 2006). A reason behind the different effects of S1P on the same vessel preparation may be due to the differences in the receptor subtypes expressed within the vessels and the distribution of these receptors (Coussin et al., 2002). S1P is able to induce vasorelaxation via activation of eNOS and this effect is mediated by S1P₁ receptor subtype, which is highly expressed in vascular endothelium (Igarashi and Michel, 2000, Igarashi et al., 2001). In contrast, S1P₂, which is mainly expressed in vascular smooth muscle cells, can mediate vasoconstriction via increased intracellular calcium levels and activation of Rho kinase in VSMC (Hemmings et al., 2006, Peters and Alewijnse, 2007). Thus, the receptor subtype expression in the vascular bed and the balance between the vasodilator and vasoconstrictor signals stimulated by S1P play an important role in determination of the overall effect on vascular tone. Another important factor is the concentration range of agonist used in the studies. For instance, S1P-induced vasoconstriction of rat mesenteric artery only developed at high agonist concentration ($>3 \times 10^{-5}$ M) (Hedemann et al., 2004) whereas S1P-induced vasodilation developed at much lower concentration range (1×10^{-10} M - 1×10^{-7} M) (Dantas et al., 2003). Moreover, S1P had no effect on tone of rat aorta at high concentrations ($>3 \times 10^{-5}$ M) (Coussin et al., 2002) but a lower concentration range (1×10^{-8} M - 3×10^{-5} M) induced relaxation (Roviezzo et al., 2006). Therefore, S1P may have a relaxant effect at low physiological levels and cause constriction at higher, non-physiological levels. In the coronary artery, S1P may potentially have constrictor and dilator effects. S1P has been observed to induce contraction of cultured coronary smooth

muscle cells via S1P₂ (Ohmori et al., 2003). It has also been demonstrated that S1P stimulates cAMP production in these cells, which would be expected to have a relaxant effect via cAMP-induced uncoupling of excitation-contraction. cAMP production in these cells was inhibited by indomethacin and the S1P₂ receptor blocker, JTE-013 (Damirin et al., 2005). In mice hearts, S1P may reduce myocardial perfusion via its coronary vasoconstrictor effect (Levkau et al., 2004). In contrast, there is growing evidence that S1P can induce coronary artery vasorelaxation. It has been observed that sphingomyelinase, the enzyme responsible for synthesis of S1P precursors, induces dilatation of the bovine coronary artery via production of nitric oxide (Mogami et al., 2005). Furthermore, Mair et al demonstrated that S1P can induce vasorelaxation in rat coronary artery via a mechanism requiring CB₂ and S1P₃ receptors. Indeed this study has shown that there is an interaction between anandamide and S1P in mediating the relaxant effect on rat coronary artery (Mair et al., 2010). Moreover, transient receptor potential channels, of vanilloid subtype (TRPV), function as sensory mediators, being stimulated by endogenous ligands, mechanical, osmotic and heat stress (Baylie and Brayden, 2011). Within blood vessels, TRPV channels are expressed in endothelial cells, smooth muscle cells, as well as peri-vascular nerves. Activation of TRPV1 channels in endothelial cells can contribute to vasorelaxation via nitric oxide- and prostacyclin-dependent pathways (Baylie and Brayden, 2011). Several studies showed that S1P and its precursor sphingosine activate these channels and this may play a role in regulation of vascular tone (Numata et al., 2011, Langeslag et al., 2014).

1.4.5.2 Role of S1P in regulation of blood pressure

Several studies have demonstrated that S1P signalling may contribute to regulation of blood pressure. Administration of S1P and its analogue, FTY720 has been shown to induce a transient decrease in blood pressure initially and a mild increase in blood pressure following long-term use (Camm et al., 2014). It has been suggested that the initial hypotensive effect is likely due to S1P₁/S1P₃-dependent activation of eNOS, whereas downregulation of S1P₁ in endothelial cells might be the cause of long-term hypertensive response. Cantalupo et al has recently shown that S1P/S1P₁/eNOS pathway is critical for vascular function and blood pressure homeostasis (Cantalupo et al., 2015). In the same study, it has been found that deletion of Nogo B, a membrane protein of the endoplasmic reticulum that inhibits serine palmitoyltransferase, systemically or specifically in endothelial cells led to hypotension and resistance to angiotensin II-induced hypertension. Increase in eNOS activity and NO production were also reported in Nogo-B-null mice (Cantalupo et al., 2015). Moreover, inhibition of S1P₁ but not S1P₂ or S1P₃ receptors

reversed the enhanced vasorelaxation in Nogo-B-null mice. In contrast, another study has reported that knocking out or inhibition of SK1 attenuates Angiotensin II-induced intracellular Ca^{2+} rise in vascular smooth muscle cells and consequently inhibits both the rapid increase in blood pressure in response to Angiotensin II and the chronic hypertensive response to continuous administration of S1P in mice, suggesting that S1P generated by SK1 may contribute to development of hypertension (Wilson et al., 2015). Obviously, more studies are required to interrogate the role of S1P signalling in control of blood pressure and whether vascular response to S1P can differ under certain circumstances.

1.4.5.3 Influence of S1P endothelial integrity

The intimal-located ECs in the blood vessels are essential to maintain barrier integrity and thus regulate extravasation of blood cells and other molecules. The permeability of this barrier is regulated by extracellular cell-cell and cell-matrix forces as well as intracellular cytoskeleton configuration by actin-myosin interactions. S1P stimulates synthesis of actin lamellipodia and membrane ruffles and increases actin assembly leading to an enhanced barrier function (Garcia et al., 2001). Several studies have demonstrated that S1P promotes EC barrier integrity primarily through activation of S1P_1 receptor (Garcia et al., 2001, Feistritzer and Riewald, 2005, Singleton et al., 2005), which strengthens EC junctions. Moreover, the role of S1P_1 in maintaining EC barrier was confirmed by studies using S1P_1 knockout mice (Liu et al., 2000b, Allende and Proia, 2002) (Figure 1-4). In vivo studies have also shown that S1P_1 antagonism causes loss of capillary integrity in mouse skin and lung (Sanna et al., 2006). Christoffersen et al showed that HDL-associated S1P is carried by ApoM which delivers S1P to S1P_1 receptors in endothelial cells (Christoffersen et al., 2011). S1P carried by ApoM in the HDL fraction was shown to play an important role in maintaining endothelial barrier integrity via promoting endothelial cell migration and formation of endothelial adherens junctions (Christoffersen et al., 2011). Recently it has been demonstrated that the increased pulmonary vascular permeability of ApoM-deficient mice was related to reduced plasma S1P levels and rapidly reversed by addition of ApoM/S1P-enriched plasma or SEW2871, an S1P_1 agonist (Christensen et al., 2016). This study has also reported that deletion of endothelial S1P_1 receptor was associated with increased susceptibility to inflammation-induced vascular leakage. Conversely, the effect of S1P_2 and S1P_3 activation on EC barrier is still an area of controversy. Several studies ruled out a protective function for these receptors on EC barrier (Garcia et al., 2001, Lucke and Levkau, 2010). However, other studies observed that these receptors are negative

regulators of barrier integrity (Gon et al., 2005, Singleton et al., 2006, Sanchez et al., 2007).

1.4.5.4 Influence of S1P on cell adhesion

Several studies demonstrated that S1P has an inhibitory effect on leukocyte adhesion; an initial step for cell extravasation and inflammatory responses. For instance, Nofer et al showed that HDL-associated lysophospholipids decrease TNF α -induced expression of E-selectin in a partially S1P₃-dependent manner (Nofer et al., 2003). Other studies showed that the S1P₁ signalling pathway is also involved in the prevention of leukocyte adhesion via a decrease in expression of adhesion molecules such as VE-cadherin and E-selectin (Krump-Konvalinkova et al., 2005) (Figure 1-4). In addition to the inhibitory effect on the expression of adhesion molecules, S1P also reduced the production of proinflammatory cytokines. Tolle et al provided evidence that S1P is able to reduce production of monocyte chemoattractant protein-1 (MCP-1), via an action at S1P₃ receptors (Tolle et al., 2008) (Figure 1-4) and Bolick et al showed that S1P inhibits TNF α -mediated leukocyte adhesion by reduction of proinflammatory cytokine production in an S1P₁-mediated manner (Bolick et al., 2005). Moreover, cross activation of TGF- β , a potent anti-inflammatory cytokine, via S1P₃ decreases the expression of proinflammatory genes such as inducible NO synthase, secretory phospholipase A2 and matrix metalloproteinase-9 in mesangial cells (Xin et al., 2004). However, others have demonstrated that S1P may enhance adhesion molecule expression in ECs (Kimura et al., 2006) and stimulate the release of MCP-1 and interleukin-8 (IL-8) (Lin et al., 2006). It has also been reported that S1P increases E-selectin expression and leukocyte adhesion via intracellular signalling pathways in vascular endothelial cells (Weis et al., 2010) and this is in agreement with others (Lee et al., 2004b, Lin et al., 2007).

1.4.5.5 Effects of S1P on inflammation and atherosclerotic plaque instability

It has been demonstrated that treatment with FTY720, an S1P analogue, significantly decreases atherosclerotic plaques in mouse models (Keul et al., 2007b, Nofer et al., 2007). A recent study showed that deletion of S1P₁ receptors in endothelial cells enhanced the expression of proinflammatory adhesion molecules such as ICAM-1, whereas overexpression of S1P₁ exhibited protective effects against vascular inflammation in mouse aortic endothelium (Galvani et al., 2015). This study also found that HDL-S1P enhanced the formation of a cell surface S1P₁- β -arrestin 2 complex in HUVECs and reduced NF- κ B activation by proinflammatory cytokine TNF α . Micaud et al provided evidence that S1P₂

has an inhibitory effect on macrophage migration *in vitro* (Michaud et al., 2010). Moreover, S1P₂ and apoE knockout result in inhibition of macrophage pro-inflammatory activities and atherosclerosis (Wang et al., 2010, Skoura et al., 2011). Selective attenuation of toll like receptor 2 may underlie the atheroprotective effects of S1P (Duenas et al., 2008). Furthermore, the S1P receptors might be involved in rebalance alterations in the coagulation pathway (Ruf et al., 2009) and thus reduce the risk of thrombosis.

1.4.5.6 Influence of S1P on proliferation and migration of ECS and VSMCS

S1P plays an important role in regulation of proliferation and migration of many cells including ECs and VSMCs (Kimura et al., 2000, Kluk and Hla, 2001). Cell migration and proliferation is an important process not only for embryogenesis but also for inflammation, angiogenesis, wound healing and tumour growth. Kimura and co-workers demonstrated that S1P_{1/3} receptors are essential in EC proliferation (Kimura et al., 2000) (Figure 1-4). Overexpression of S1P₁ in VSMCs results in activation of p70S6 kinase and cyclin D expression and therefore promotes VMSCs proliferation (Kluk and Hla, 2001, Kluk and Hla, 2002) (Figure 1-4). In addition to positive proliferation effects, anti-proliferative responses could be shown as well. Deletion of S1P₂ in mouse embryonic fibroblasts (MEFs) enhances their proliferation compared with wild type MEFs (Goparaju et al., 2005). Therefore, it seems to be that S1P has both stimulatory and inhibitory effects on proliferation and this may depend upon the cell and receptor subtype involved. Several studies identified an important role for S1P_{1/3} receptor subtypes in regulation of EC migration (Kimura et al., 2000, Panetti et al., 2000). It could also be shown that S1P migratory effect is essential for the formation of blood vessels (Argraves et al., 2004). Moreover, Anelli et al identified SK1 as an important mediator of S1P-induced migration and tube formation in ECs *in vitro* (Anelli et al., 2010). In contrast, other studies demonstrated an inhibitory potential of S1P on the migration of ECs and VSMCs. S1P reduces the PDGF-induced chemotaxis of human VSMCs (Bornfeldt et al., 1995). Moreover, Tamama et al showed that HDL-associated S1P inhibits VSMC migration (Tamama et al., 2005). It has also been demonstrated that S1P-induced inhibitory actions were decreased by S1P₂-specific siRNA in human coronary artery smooth muscle cells (Damirin et al., 2007) and JTE-013, an S1P₂ antagonist, could promote EC and VSMC migration (Osada et al., 2002). *In vivo* studies were consistent with these findings. Deletion of S1P₂ induced large neointimal lesions after ligation of the carotid artery (Shimizu et al., 2007). Moreover, S1P enhanced VSMCs migration rate compared with

VSMCs in wild type mice (Shimizu et al., 2007). Others confirmed that inhibiting S1P₂ increases migration rate (Inoue et al., 2007).

1.4.5.7 Apoptosis

Pathological conditions affecting the blood vessels often induce endothelial dysfunction that results in an increased cell turnover rate and hence ECs undergo apoptosis. S1P is a potent factor protecting ECs against apoptosis by inhibition of caspases, cytochrome c release and DNA fragmentation (Cuvillier et al., 1996). Moreover, HDL-associated S1P can directly protect mouse cardiomyocytes and isolated hearts subjected to oxidative injury and this prosurvival signal stimulation is dependent on both S1P₁ and S1P₃ receptors (Tao et al., 2010). The protective role of S1P is not only mediated by its receptor activation and downstream G-protein-mediated signalling pathways (Radeff-Huang et al., 2004), but also through its function as an internal signalling molecule. NO is a known inhibitory factor of endothelial apoptosis and mediates protection via nitrosylation of cysteine residues in the catalytic centre of caspases and thus results in enzyme inactivation (Rössig et al., 1999, Sun et al., 2006a). Kwon provided evidence that S1P protective effects are mediated through S1P_{1/3} activation and this effect is reversed by inhibition of NO synthase (Kwon et al., 2001). Other studies showed cytoprotective effects of S1P through S1P₁₋₃ (Donati et al., 2007, Hofmann et al., 2009, Nieuwenhuis et al., 2009). In many cell types, apoptosis is enhanced by tumour necrosis factor α (TNF- α) which activates TNF-receptor 1. It has been reported that activation of SK by tumour necrosis factor α protects human umbilical vein endothelial cells from apoptosis (Xia et al., 1999). Other studies showed that an overexpression of SK inhibits apoptosis in a pertussis toxin (PTX)-independent way (Olivera et al., 1999). Another important factor inducing apoptosis in vascular diseases is the increased generation of ROS in the vasculature. It was demonstrated that S1P prevents EC apoptosis via a decrease in ROS production. Indeed, H₂O₂-induced apoptosis in ECs can be reversed by S1P pre-treatment (Moriue et al., 2008).

1.4.5.8 Role of S1P in cardiac development, function and heart rate

S1P₁, S1P₂ and S1P₃ are expressed in adult rodent heart with S1P₁ being more highly expressed than the other two (Alewijns et al., 2004) whereas in human hearts, S1P₁ and S1P₃ are expressed in similar amounts. S1P₁ is mainly expressed in ventricular, atrial and septal cardiomyocytes and in endothelial cells of coronary vessels (Mazurais et al., 2002). Moreover, the enzymes responsible for generation and breakdown of S1P also exist and are active in the heart. SK1 and SK2 are expressed in cardiac tissue as early as the linear heart

tube stage (E8.5) (Wendler and Rivkees, 2006). S1P phosphatase 1, the enzyme that catalyses S1P degradation is also expressed in the adult heart (Mandala et al., 2000, Johnson et al., 2003) and throughout the early stages of cardiac development (Wendler and Rivkees, 2006). Cardiomyocytes have been found to express more S1P lyase than cardiac fibroblasts (Bandhuvula et al., 2011). This indicates that S1P may play an important role in early cardiac morphogenesis where it regulates migration, differentiation, and survival of embryonic cardiac cells (Wendler and Rivkees, 2006).

There was no significant change in blood pressure and cardiac function in S1P₂-deficient (Lorenz et al., 2007) and S1P₃-deficient mice (Levkau et al., 2004). Systemic administration of S1P was reported to decrease heart rate in rats (Benediktsdottir et al., 2002) and mice (Sanna et al., 2004). This effect is thought to be mediated by S1P₃ receptor as in S1P₃ knockout mice, S1P had no effect on heart rate. In cardiomyocytes in vitro, S1P promotes protein synthesis and cellular hypertrophy mainly via S1P₁ receptors (Robert et al., 2001). Moreover, it has been reported that S1P activates STAT3 in ventricular cardiomyocytes (Frias et al., 2009), which is involved in the physiological hypertrophic response (Kunisada et al., 1998). Involvement of S1P in cardiac remodelling was also observed in in vivo studies. A significant association between increased S1P in the plasma and left ventricular hypertrophy was reported in patients with Fabry disease, suggesting that S1P may play a pivotal role in cardiac remodelling in these patients (Brakch et al., 2010). Zhang et al has recently shown that the SK1/S1P/S1P₁ signalling pathway is promoted within 4 weeks post MI and this markedly contributes to cardiac inflammatory response, remodelling and dysfunction (Zhang et al., 2016a). In contrast, S1P levels have been found unchanged in the peripheral circulation of patients with chronic systolic heart failure (Knapp et al., 2012). Furthermore, S1P exerts many effects on ion currents in cardiomyocytes in vitro. It activates the inward rectifier potassium current (I(K.Ach)) leading to a reduction of spontaneous pacing rate (Guo et al., 1999) and prevents the isoproterenol-induced increase in L-type calcium channel (I_{Ca,L}) currents. It also attenuates the hyperpolarization-activated inward current (I_f), thereby limiting the positive chronotropic effects of β -adrenergic stimuli in sinoatrial node cells and ventricular cardiomyocytes (Guo et al., 1999, Landeen et al., 2008).

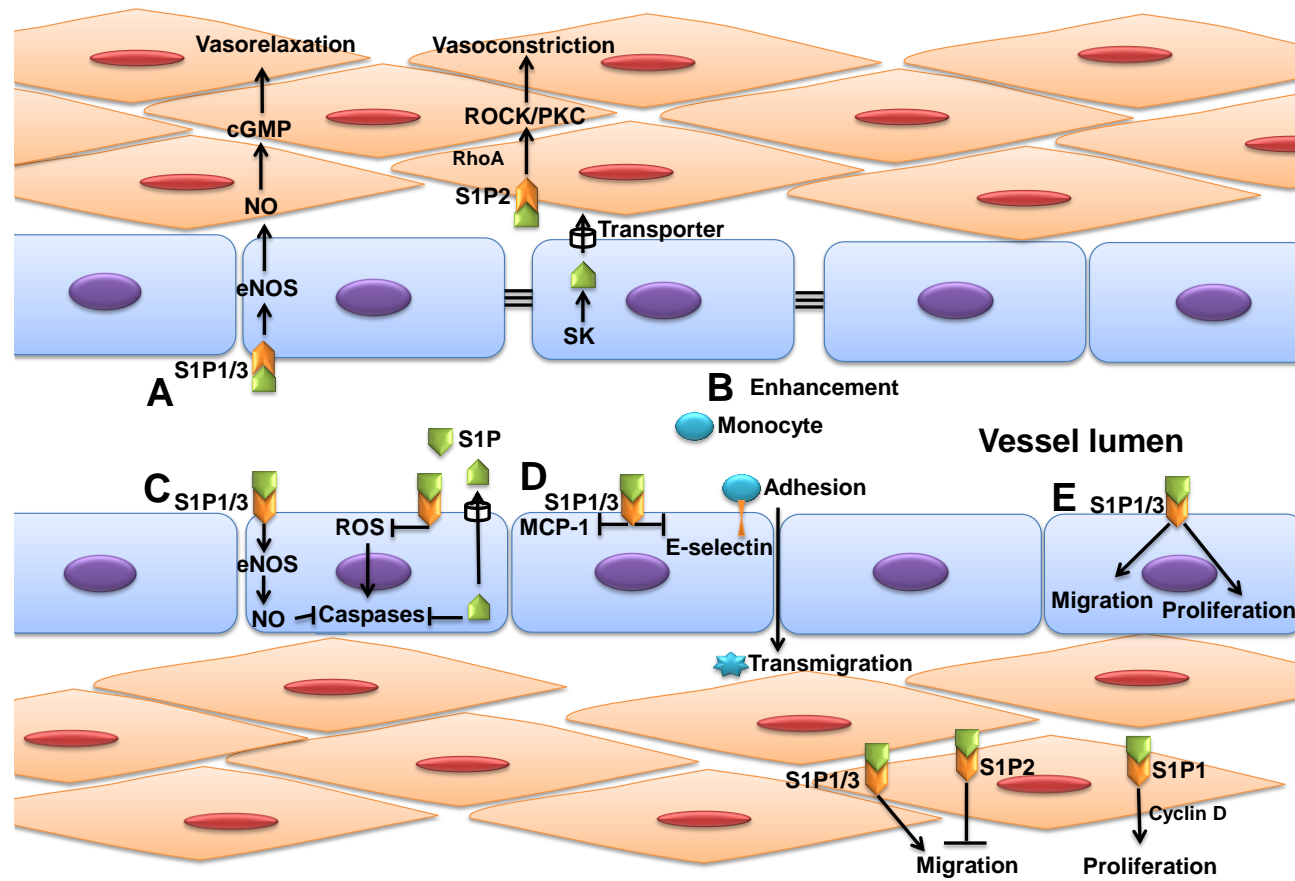


Figure 1-4 Diverse physiological processes are regulated by S1P.

(A) vascular tone, (B) endothelial integrity, (C) apoptosis, (D) cellular adhesion, (E) proliferation and migration.

1.5 Sphingosine kinase (s)

Two SK isoforms exist in mammalian cells, identified as SK1 and SK2. In humans, they are generated from two distinct genes, SPHK1 and SPHK2, which are located on chromosomes 17 and 19, respectively. Human SK1 and SK2 vary markedly in size (384 and 618 amino acid for SK1a and SK2a, respectively) (Liu et al., 2000a), but they share 80% similarity and 45% overall sequence identity. The two isoforms exhibit partial redundancy since SK1 knockout or SK2 knockout mice are phenotypically normal whereas deletion of both genes causes embryonic lethality due to vascular and neurological defects (Mizugishi et al., 2005). SK1 and SK2 have five conserved domains (C1-C5), with the catalytic domain located within C1-C3 and the ATP binding domain formed in the C2 region (Pitson et al., 2002). The activity of both SK isoforms is regulated in a spatial and temporal manner by post-translational modification and interaction with various proteins and lipids. Furthermore, both SK isoforms are ubiquitously expressed in all human tissues with highest expression of SK1 in lung, heart, spleen and leukocytes and SK2 in the liver and kidney (Melendez et al., 2000, Liu et al., 2000a). For SK1, three isoform variants (known as SK1a, SK1b, and SK1c) exist that differ only at their N termini. SK1a has a molecular mass of 42.5 kDa, while SK1b has an 86 amino acid N-terminal extension and is a 51 kDa protein (Pitson et al., 2000, Ota et al., 2004). Moreover subcellular localisation of SK1 varies between cell types. For instance, it exhibits cytoplasmic, plasma membrane, and vesicular distribution in HUVECs and it is also localised to the endoplasmic reticulum and Golgi apparatus and associated with the cytoskeleton in other cell lines such as the cultured smooth muscle cell line DDT1MF-2 (Taha et al., 2006b, Wattenberg et al., 2006). Two variants of SK2 have been described and characterized (Liu et al., 2000a, Okada et al., 2005), the shorter isoform is known as SK2a and the larger one is named SK2b which has an additional 36 amino acids at the N-terminus. Subcellular localisation of SK2 also depends on the cell type. In HeLa cells, for example, SK2 predominantly localises to the nucleus, whereas it is a cytoplasmic protein in HEK293 cells (Igarashi et al., 2003). Localisation of SK2 in endoplasmic reticulum has also been reported under stress conditions (Hait et al., 2005).

1.5.1 Role and regulation of SK1

Of the two SKs, SK1 is by far the most well studied. Several studies have demonstrated that overexpression of SK1 increases cell survival and proliferation and induces neoplastic transformation (Xia et al., 2000). SK1 is a cytoplasmic protein and its phosphorylation by Extracellular signal regulated kinase1 and 2 (ERK1/2) enhances its catalytic activity and also facilitates translocation of SK1 from cytosol towards the plasma membrane through binding to calcium and integrin-binding protein 1 (CIB1) (Pitson et al., 2003, Jarman et al., 2010). It has been demonstrated that CIB1 mediates agonist-dependent relocalisation of SK1 to the plasma membrane and a subsequent increase in S1P production in HeLa cells (Jarman et al., 2010). Moreover, knock down of CIB1 in HeLa cells led to a decrease in S1P generation, indicating that CIB1 is required for activation and translocation of SK1 to the plasma membrane.

ERK1/2 directly phosphorylates SK1 at ser225 which leads to a 14-fold rise in SK1 catalytic activity while not changing its affinity for sphingosine or ATP (Pitson et al., 2003). In most cases, the enhanced SK1 activity caused by phosphorylation is transient due to dephosphorylation by an enzyme named phosphatase 2A (PP2A) (Barr et al., 2008). At this location, SK1 catalyses the generation of S1P from plasma membrane associated sphingosine and this facilitates the extracellular transport of S1P to act on cell surface S1P receptors and the interaction with intracellular targets to mediate downstream signalling and enhance cell survival, proliferation, migration, angiogenesis and inflammation (Maceyka et al., 2009, Neubauer and Pitson, 2013, Pyne et al., 2016). Interestingly, the activity of SK1 can also be regulated via phosphorylation-independent mechanisms (ter Braak et al., 2009, Gault et al., 2012). Ser225 phosphorylation is not required for all membrane translocation of SK1. For example, ter Braak et al (2009) demonstrated that translocation of SK1 in response to muscarinic receptor activation is mediated by the G_q G-protein in HEK-293 cells. It has also been reported that translocation of SK1 in response to K-RasG12V in mouse embryonic fibroblasts does not require ser225 phosphorylation (Gault et al., 2012).

1.5.1.1 Interacting proteins that activate and inhibit SK1

Several studies have revealed that a variety of proteins can directly interact with and activate SK1. This interaction between SK1 and these proteins only leads to a modest rise in SK1 activity, but it is sufficient to initiate a number of distinct cellular responses. In most cells, two Src family protein tyrosine kinases (PTKs), Lyn and Fyn have been

demonstrated to interact with and directly promote the catalytic activity of SK1 (Urtz et al., 2004, Olivera et al., 2006). Other studies showed that δ -catenin and eukaryotic elongation factor 1A (eEF1A) could also enhance SK1 activity in vitro and in cells (Fujita et al., 2004, Leclercq et al., 2008). Moreover, adaptor protein associated with TNF- α receptor 1 (TRAF2) may also associate with and activate SK1 when overexpressed (Xia et al., 2002, Alvarez et al., 2010). This SK1-TRAF2 interaction is essential for TNF- α -induced stimulation of pro-survival, pro-inflammatory transcription factor NF- κ B. On the other hand, a number of studies have reported that some proteins can interact and inhibit the catalytic activity of SK1. For instance, FHL-2 (Sun et al., 2006b, Hayashi et al., 2009) and PECAM-1 (Fukuda et al., 2004) have been demonstrated to interact with SK1 and inhibit its activity in cardiomyocytes and vascular endothelial cells, respectively. However, SK1 dissociates from these inhibitory complexes upon stimulation with extracellular signals and becomes more active. SK1 interacting protein (SKIP) (Lacana et al., 2002) and aminoacylase 1 (Maceyka et al., 2004) are other proteins that could interact and inhibit SK1, although the physiological roles of these interactions are not known.

1.5.1.2 Interacting proteins that alter subcellular localisation of SK1

Subcellular localisation plays an essential role in the regulation and signalling functions of many signalling proteins. A growing body of evidence has shown that the cellular localisation of sphingolipids and sphingolipid-metabolising enzymes is crucial for their functions (Wattenberg et al., 2006). Given that SK1 is one of the important regulators of the sphingolipid system and that the localisation of this enzyme seems to be essential for its function, a number of studies have investigated the control of the subcellular localisation of SK1. Under basal condition, SK1 appears to reside in the cytoplasm (Pitson et al., 2003). However, phosphorylation of SK1 at Ser225 by ERK1/2 results in translocation of SK1 from cytosol to the plasma membrane. Numerous studies have suggested that this process is mediated by calmodulin since either inhibition of calmodulin or mutation of the calmodulin-binding site of SK1 prevented the plasma membrane localisation of SK1 (Young et al., 2003, Sutherland et al., 2006). Recently, it has been demonstrated that SK1 translocation to the plasma membrane is mediated by the CaM-related, calcium- and integrin-binding protein 1 (CIB1) (Jarman et al., 2010). Like other calcium-myristoyl switch proteins, translocation of CIB1 to the plasma membrane requires both its myristoylation and calcium binding, providing a mechanism for active relocalisation of SK1 to the plasma membrane following an increase in calcium levels that are known to be associated with SK1 activation (Spiegel and Milstien, 2003). The process

of SK1 translocation to the plasma membrane appears to be integral not only for cell survival and proliferation but also for cell motility. For instance, the plasma membrane translocation of SK1 to the migrating front of the cell has been revealed to be fundamental in the generation of lamellipodia and polarised cell movement in a process that is likely to be mediated by filamin A, an actin binding protein (Maceyka et al., 2008). Notably, filamin A acts as a scaffolding protein that attaches to both SK1 and p21-activated kinase and binds the complex to S1P₁ at lamellipodia to regulate cell migration (Maceyka et al., 2008).

1.5.1.3 Regulation of SK1 by phospholipids

While it is now known that relocalisation of SK1 to the plasma membrane following phosphorylation by ERK1/2 is mediated by CIB1 (Jarman et al., 2010), prevention of SK1 being redistributed to the cytosol is thought to be achieved by its interaction with phosphatidic acid (PA) and phosphatidylserine (PS) that are normally found in the inner leaflet on the plasma membrane (Delon et al., 2004, Stahelin et al., 2005). Moreover, phosphorylation of SK1 at Ser225 appears necessary not only for translocation of this enzyme to the plasma membrane but it is also required for SK1 to interact with PS (Pitson et al., 2003, Stahelin et al., 2005). It was suggested that Ser225 phosphorylation of SK1 may regulate the membrane binding through a conformational change in SK1 that leads to exposure of Thr54 and Asn89 on the surface of the enzyme and this allows these residues to interact with PS at the plasma membrane and endoplasmic reticulum (ER)/Golgi apparatus (Stahelin et al., 2005). Furthermore, the interaction of SK1 with these phospholipids also leads to enhanced SK1 activity (Olivera et al., 1996, Pitson et al., 2000). Although the functional consequence of these interactions is not yet well understood, it is possible that the interactions with these phospholipids may help to localise SK1 in close proximity to its substrate, sphingosine, in the plasma membrane.

1.5.2 Regulation of SK2

Like SK1, the catalytic activity of SK2 can be increased rapidly following exposure to a variety of agonists such as TNF- α (Mastrandrea et al., 2005), EGF (Hait et al., 2005) and IL-1 β (Mastrandrea et al., 2005). Since SK2 is closely related structurally to SK1, SK2 activation can occur via phosphorylation by ERK1/2 (Hait et al., 2007). Notably, while SK1 phosphorylation occurs at Ser225, SK2 was shown to be phosphorylated by ERK1/2 at two sites: Ser351 and Thr578 (Hait et al., 2007). Under normal conditions, SK2 resides predominantly in the nucleus and cytoplasm. Indeed, SK2 localisation appears to depend

upon cell type and cell density. For instance, SK2 mainly localises in the nucleus in HeLa cells whereas in HEK293 cells it is predominately cytoplasmic (Igarashi et al., 2003). Furthermore, it has been reported that the proportion of SK2 localised in the nucleus increased in COS-7 fibroblasts when they became more confluent in culture (Igarashi et al., 2003), suggesting that SK2 may play a role in contact inhibition responses which reduce cell proliferation. It has also been shown that SK2 localises to the endoplasmic reticulum (ER) under stress conditions (Maceyka et al., 2005). S1P generation at the ER can fuel the sphingosine salvage pathway driven by ER-localised S1P phosphatases and ceramide synthase, to ultimately produce pro-apoptotic ceramide (Maceyka et al., 2005). Moreover, mitochondrial localisation of SK2 has been demonstrated to enhance apoptosis via S1P and BAK-dependent membrane permeabilization and cytochrome c release (Strub et al., 2011, Chipuk et al., 2012). In contrast, several studies have shown that SK2 can have a physiological role in mediating survival and proliferation. Downregulation of SK2 by siRNA was associated with enhancement of apoptosis and reduction in chemotherapeutic resistance in several cancer cell lines (Sankala et al., 2007, Schnitzer et al., 2009, Dai et al., 2014). Moreover, a recent study has demonstrated that hypoxic preconditioning protected cardiomyocytes against hypoxia/reoxygenation injury through upregulation of SK2 expression and downstream activation of FAK/Akt signalling pathway (Zhang et al., 2016b).

1.5.3 Degradation of SKs

Treatment of cells with DNA damaging agents such as etoposide, actinomycin D, doxorubicin and UV irradiation has been demonstrated to decrease SK1 protein levels while having no effects on levels of SK1 mRNA. Moreover, at least for UV irradiation, this appears to occur in a p53-dependant manner (Taha et al., 2004, Heffernan-Stroud et al., 2012). This suggests that a further level of SK1 regulation occurs through its breakdown. In this regard, recent studies have shown, using a number of cell lines, that SK1 might be degraded via ubiquitin-proteasomal pathway in response to different SK1 inhibitors including 2-(p-hydroxyanilino)-4-(p-chloropenyl)thiazole (SKi), N,N-dimethylsphingosine (DMS), FTY720 and PF543 (Loveridge et al., 2010, Tonelli et al., 2010, McNaughton et al., 2016). Moreover, ABC294640, a selective SK2 inhibitor, was also demonstrated to induce proteasomal degradation of SK1a in prostate cancer cells (McNaughton et al., 2016). Furthermore, in most cells this effect was abolished when cells were treated with MG-132, a proteasomal inhibitor (Loveridge et al., 2010, Tonelli et al., 2010, McNaughton et al., 2016). However, SK1b was reported to resist degradation

induced by SKi in particular cell lines such as androgen-independent prostate cancer cells and this might be due to a compensatory upregulation of SK1b and/or modification of SK1b that decreases its sensitivity to SKi-induced proteasomal degradation (Loveridge et al., 2010). The mechanism by which SKi induces proteasomal degradation has been suggested to be due to inhibition of catalytic SK1 activity by SKi that results in an increase in ceramide levels and this leads to proteasomal degradation of SK1 (Loveridge et al., 2010). Recently, Yu et al showed that SK1 could be acetylated on Lys27 and Lys29 which increases the stability of the enzyme by preventing ubiquitin-mediated protein degradation (Yu et al., 2012). Mutation of two lysine residues inhibited SK1 ubiquitination and proteasomal degradation, suggesting that the process of acetylation and ubiquitination may compete for the same lysine residues and play a role SK1 regulation. Interestingly, however, a number of studies have demonstrated that SK1 can also be degraded through the lysosomal pathway (Taha et al., 2006a, Ren et al., 2010). This notion is supported by the experimental evidence that SKi-induced SK1 degradation can be prevented by treatment with chloroquine, a general lysosomal inhibitor, and CA-074ME, a specific cathepsin B inhibitor (Ren et al., 2010). The SK1 degradation by the lysosomal pathway was further supported by the colocalisation of SK1 with the lysosome and cathepsin B (one of the most abundant lysosomal proteases) in MCF-7 cells (Taha et al., 2005). Moreover, it has been shown that SK1 can be cleaved by cathepsin B (Taha et al., 2006a), suggesting that SK1, under some circumstances, may be degraded via the lysosomal pathway. The SK2 degradation mechanism is still unknown, although Lim et al demonstrated that SK2 is degraded by a non-proteasomal and non-lysosomal route in response to ROME, a selective SK2 inhibitor in HEK 293 cells (Lim et al., 2011). It has also been reported that SK2 can be cleaved by caspase-1 which appears to facilitate its release from apoptotic cells (Weigert et al., 2010).

1.5.4 Sphingosine kinase inhibitors

A number of SK inhibitors have been discovered which have potential for development as a treatment for several pathological conditions including cancer, inflammatory and cardiovascular diseases (Neubauer and Pitson, 2013). The majority of these inhibitors selectively inhibit SK1 or both SK isoforms. Recently, however, several SK2 inhibitors have been generated that show promising therapeutic properties. SKi is the most widely used SK inhibitor (French et al., 2003) and inhibits both SK1 and SK2. Among several findings, it has been shown that SKi reduces SIP production and induces apoptosis in tumour cells in vitro (French et al., 2003, French et al., 2006), abolishes tumour growth in

a mammary adenocarcinoma xenograft mouse model (French et al., 2006) and inhibits the S1P-induced relaxation in rat coronary artery (Mair et al., 2010). Indeed, this inhibitor has been commonly used as a direct SK1/2 inhibitor (Lee et al., 2004a, Maines et al., 2006, Nishiuma et al., 2008, Ricci et al., 2009), although many studies suggest that it works mainly to target SK1 via promoting degradation of this enzyme (Ren et al., 2010, Loveridge et al., 2010, McNaughton et al., 2016). While widely reported as an SK1-specific inhibitor, SKi actually inhibits SK2 with slightly higher affinity than SK1 (Gao et al., 2012), however, it does not enhance degradation of SK2 (Watson et al., 2013). DMS has been demonstrated to inhibit SK activity in a variety of cell lines (Yatomi et al., 1996, Edsall et al., 1998). However, it has also been shown to inhibit PKC (Kim et al., 2005), basic fibroblast growth factor (Xu et al., 2002), and platelet-derived growth factor (Katsuma et al., 2002). Safingol, L-threo-dihydrosphingosine, FTY720 and (S)-FTY720 vinylphosphonate have been found to inhibit SK1 activity in several cell types (Olivera et al., 1998, Tonelli et al., 2010). They can also inhibit protein kinase c and other kinases (Schwartz et al., 1993, Sensken and Graler, 2010) and therefore, they are not considered specific SK inhibitors. Recent studies have generated highly selective non lipid SK1 inhibitors with nanomolar potency including PF543 and VPC96091 (Kennedy et al., 2011, Schnute et al., 2012). PF543 has also been reported to induce a decrease in SK1 expression in pulmonary artery smooth muscle cells (PASMC), which was reversed by pre-treatment with MG132 (Byun et al., 2013). VPC96091 was found to selectively inhibit SK1 in nanomolar concentrations, however both PF543 and VPC96091 were not effective in reducing DNA synthesis in PASMC (Byun et al., 2013). Furthermore, recent studies showed that PF543 decreased right ventricular hypertrophy but not vascular remodelling in mice subjected to chronic hypoxia for a period of 3 weeks and this was associated with protection against cardiomyocyte apoptosis (MacRitchie et al., 2016). (2S,3R)-1-Deoxysphinganine (55-21) is another selective SK1 inhibitor that induces proteasomal degradation of SK1 and inhibits DNA synthesis, although it is a moderate potency inhibitor of SK1 (Byun et al., 2013). A number of SK2 selective inhibitors have been generated such as ABC294640, (R)-FTY720-OMe, SG-12 and trans-12a. ABC294640 is an orally bioavailable agent specifically inhibiting SK2 in a sphingosine competitive manner. No effect was reported with ABC294640 on SK1 at concentrations up to 100 μ M (French et al., 2010, Gao et al., 2012). Different studies have examined the effect of this inhibitor on a variety of disease models. It has been demonstrated that ABC294640 significantly inhibits tumour growth in vivo in different tumour models in mice (French et al., 2010, Antoon et al., 2011, Beljanski et al., 2011, Antoon et al., 2012). Moreover, ABC294640 also appears

to have therapeutic potential for other diseases. For example, it attenuates disease progression in rodent models of osteoarthritis (Fitzpatrick et al., 2011b), Crohn's disease (Maines et al., 2010), ulcerative colitis (Maines et al., 2008), rheumatoid arthritis (Fitzpatrick et al., 2011a), and diabetic retinopathy (Maines et al., 2006). Biochemically, ABC294640 has been shown to reduce S1P and increase ceramide levels in cells (French et al., 2010, Gao et al., 2012), reduce plasma S1P level in mice (Beljanski et al., 2011) and inhibit the activation of STAT3, Akt, and ERK2 (Gao et al., 2012). The mechanism by which ABC294640 induces cell death is still a conflicting subject, with a number of studies showing that apoptotic pathways are activated (French et al., 2010, Antoon et al., 2012) and others demonstrating the presence of autophagy markers (Beljanski et al., 2010, Gao et al., 2012). Moreover, McNaughton et al demonstrated that ABC294640 can indirectly induce proteasomal degradation of SK1a in prostate cancer cells (McNaughton et al., 2016). (R)-FTY720-OMe (ROME) is another selective SK2 inhibitor. It was found to competitively inhibit SK2 and show no effect on SK1 activity at 50 μ M (Lim et al., 2011). It was also found to reduce expression of SK2 and stimulate autophagy, but not apoptosis in LNCaP prostate cancer cells (Watson et al., 2013). Unlike ABC294640, (R)-FTY720-OMe failed to reduce the expression of SK1a in prostate cancer cells (McNaughton et al., 2016). SG-12 and trans-12a were also reported to inhibit SK2 in a number of cell types (Kim et al., 2005, Raje et al., 2012). SG-12 has been shown to induce cell death in CHO-KI cells, which might be a result of SK inhibition, however, it also can inhibit PKC (Kim et al., 2005).

1.6 Role of sphingosine kinase/S1P in cardioprotection

In the last decade, it has become abundantly clear that SK and S1P play an essential role in cardioprotection against acute or chronic ischaemia or ischaemia/reperfusion injury. Therefore, these molecules and their synthetic analogues have been studied extensively to interrogate their therapeutic potential as modulators of cardiac responses to both acute and chronic myocardial injury. Much remains to be studied however, for example if the S1P in HDL is responsible for the beneficial and cardioprotective properties of HDL and also the role of S1P in the remodelling process after myocardial infarction.

1.6.1 Studies of S1P in cardiomyocyte cell culture

Several studies have demonstrated that S1P has protective effects against oxidative stress-induced conditions such as acute or chronic ischaemia/hypoxia or ischaemia/reperfusion

injury. For example, exogenous S1P has been found to enhance neonatal rat cardiomyocyte survival during hypoxia (Karlner et al., 2001). Zhang et al provided evidence that S1P-enhanced survival in adult mouse cardiomyocytes exposed to prolonged in vitro hypoxia is mediated by S1P₁ receptor and G_i-dependent activation of Akt (Zhang et al., 2007). SEW2871, a selective S1P₁ receptor agonist, and the sphingosine analogue FTY720 were also as effective as S1P in protecting cardiomyocyte viability during hypoxia (Zhang et al., 2007). Indeed, another study demonstrated that deletion of both S1P₂ and S1P₃ receptors increased infarct size in mice exposed to ischaemia/reperfusion injury (Means et al., 2007). In these hearts, activation of Akt was significantly decreased compared with wild type mice, but deletion of either receptor alone had no effect on either infarct size or Akt activation after ischaemia/reperfusion injury (Means et al., 2007). S1P also enhanced Akt activity in murine cardiomyocytes, but was not effective in double knockout myocytes. Therefore, these findings suggest that S1P receptor subtypes (S1P₂ and S1P₃) might also be essential for cell survival during ischaemia/reperfusion injury (Figure 1-5).

1.6.2 Role of sphingosine kinase in cardioprotection

Deletion of SK1 gene in ventricular cardiomyocytes subjected to hypoxia resulted in increased cell death and cytochrome c release compared with wild type controls (Tao et al., 2007). Moreover, exogenous S1P was found to prevent hypoxic cell death in SK1 null cardiomyocytes. It has also been shown that monoganglioside GM-1, which increases intracellular S1P by activating SK1 was cytoprotective in wild type cardiomyocytes but not in SK1 null myocytes (Tao et al., 2007). This suggests that GM-1 activates only SK1. Furthermore, the protective effects of GM-1 on wild type cardiomyocytes were decreased by preincubation with either S1P₁ receptor antagonist or pertussis toxin, indicating that endogenous S1P was exported to the extracellular space for stimulation of its cognate G-protein coupled receptors (Tao et al., 2007). GM-1 induced increases in S1P can also be inhibited by the SK inhibitor, DMS (Cavallini et al., 1999). In isolated adult mouse hearts, exogenous S1P and GM-1 separately decreased ischaemia/reperfusion myocardial injury in wild type mouse hearts as determined by haemodynamic and infarct size measurements (Jin et al., 2002, Lecour et al., 2002). Further experiments studied the hypothesis that SK may play a role in ischaemic preconditioning (ICP) in isolated mouse hearts. It was shown that IPC sufficient to decrease infarct size in wild type hearts enhanced SK activity and translocation from cytosol to membranes (Jin et al., 2004). Moreover, pre-treatment with 10µM DMS prevented ICP-induced cardioprotection in wild type hearts (Jin et al., 2004). In contrast, low dose DMS (0.3 to 1µM) was reported to enhance cytosolic SK activity and

protect murine hearts against ischaemia/reperfusion (Jin and Karliner, 2006). DMS appears to stimulate SK in the cytosol via a PKC ϵ -dependent mechanism (Jin and Karliner, 2006). Sphingosine, the immediate precursor of S1P, has also a biphasic effect on cardioprotection depending on the concentration used. At higher concentration (5 μ M), sphingosine was cardiotoxic, but at a physiological concentration (0.4 μ M) sphingosine as well as S1P was cardioprotective against ischaemia/reperfusion when both were added before ischaemia or at the time of reperfusion (Vessey et al., 2008b).

In vivo experiments demonstrated that permanent left anterior descending coronary artery occlusion in mice led to a steady decrease in total SK activity over weeks (Yeh et al., 2009). In these experiments, SEW2871, the selective S1P₁ agonist, was given orally to mice and reduced apoptosis and improved echocardiographic ejection fraction over the first two weeks after infarction. These results suggest that oral S1P receptor agonist may have potential therapeutic benefits in the post-myocardial infarction heart. Furthermore, SK1 knockout mice were used for a series of studies to strengthen the evidence that the SK1 isoform can protect against ischaemia/reperfusion injury. Expression of SK2 increases in hearts after SK1 gene deletion, leading to total SK activity half that of wild type mice (Jin et al., 2007). SK1 knockout hearts exhibited normal hemodynamic performance under baseline conditions. However infarction and contractile dysfunction were more severe after ischaemia/reperfusion compared to that of wild type hearts. As predicted, knocking out SK1 prevented IPC-induced cardioprotection (Jin et al., 2007) but, exogenous S1P was able to induce cardioprotection in these SK1 knockout hearts. Although SK2 expression increased in the SK1 knockout hearts, treatment with DMS did not affect the infarct size, suggesting that the disruption of SK1 rather than increased expression of SK2 was crucial to the loss of cardioprotection in SK1 null hearts (Jin et al., 2007). Another study has shown that prior adenoviral gene transfer of SK1 prevented haemodynamic deterioration and decreased creatine kinase release and arrhythmias during acute ischaemia/reperfusion in isolated hearts (Duan et al., 2007). In addition, gene transfer at the time of acute left anterior descending coronary artery ligation resulted in improved left ventricular function in the treated mice, decreased infarct size, more neovascularisation, and reduced collagen content (Duan et al., 2007). Ischaemic postconditioning as well as ischaemic preconditioning is cardioprotective (Zhao et al., 2003). A number of studies examined whether the SK/S1P pathway is a determinant for successful postconditioning (Jin et al., 2008). In this study, wild type hearts and SK1 knockout hearts were subjected to ischaemia/reperfusion and selected hearts were subjected at the time of reperfusion to three brief cycles of postconditioning (5 sec of ischaemia followed by 5 sec of reperfusion). In

treated hearts, haemodynamics were improved and infarct size was decreased compared to controls (Jin et al., 2008). Phosphorylated Akt and phosphorylated ERK levels were also increased. In post-conditioned hearts SK activity at the end of reperfusion was higher than control hearts. In SK1 null hearts, none of these findings were observed. Thus SK1 may play an important role in mediating successful postconditioning (Jin et al., 2008). It has also been reported that combined low dose sphingosine, S1P and ischaemic postconditioning can protect isolated hearts from as much as 90 min of ischaemia (Vessey et al., 2008a).

Although, the significant role of S1P in cardioprotection against ischaemia/reperfusion insult has been demonstrated in many studies, S1P has also been implicated in cardiac remodelling. It was reported that S1P stimulated cellular hypertrophy in rat neonatal cardiomyocytes and this effect was inhibited by an S1P₁ antagonist (Brinkmann et al., 2002). Overexpression of SK1 in heart tissue was shown to contribute significantly to progressive myocardial degeneration and fibrosis in mice (Takuwa et al., 2010). Another study has also shown that TGF- β -stimulated increase in SK1 expression and/or activity was associated with an increase in collagen production in cardiac fibroblasts (Gellings Lowe et al., 2009). Recently, Zhang et al (2016) has demonstrated that increased S1P production and SK1 expression following myocardial infarction was associated with enhancement of cardiac remodelling and dysfunction in mice. Moreover, in the same study, inhibition of SK1 by PF543, a selective SK1 inhibitor, was protective against post-MI remodelling.

1.6.3 S1P Lyase

S1P lyase (SPL) is responsible for the degradation of S1P. By decreasing available S1P pools, SPL enhances apoptosis under stress conditions, whereas downregulation of SPL enhances cell survival. It has been shown that SPL was stimulated by ischaemia in murine hearts and hearts of heterozygous SPL null mice exhibited decreased SPL activity, increased S1P levels, smaller infarct size, and improved functional recovery after ischaemia/reperfusion injury compared with littermate hearts (Bandhuvula et al., 2011). It has also been found that giving tetrahydroxybutylimidazole (THI), an SPL inhibitor, overnight in the drinking water resulted in a 30-40% increase in serum S1P and decreased SPL activity, decreased infarct size and promoted haemodynamic recovery after ischaemia/reperfusion in ex vivo hearts (Bandhuvula et al., 2011). Thus, increased S1P induced by inhibition of SPL is sufficient to induce cardioprotection and represents a new

target in the metabolism of S1P that can be used to protect the heart against an ischaemia/reperfusion insult. In contrast Zhang et al has demonstrated that SPL inhibition may exacerbate cardiac systolic dysfunction and aggravate post MI remodelling which is likely due to an increase in expression levels of remodelling genes (Zhang et al., 2016a). Moreover, it has been shown that SPL heterozygous mice are more sensitive to hypoxia-mediated pulmonary hypertension (Chen et al., 2014) and so caution has to be exercised when considering SPL as a viable therapeutic target.

1.6.4 Cardioprotective effects of High Density Lipoprotein (HDL)

HDL is the major carrier of S1P in serum as described above (Murata et al., 2000, Okajima, 2002). It is well known that HDL plays an important role in prevention of atherogenesis. This protective effect might be due to the ability of S1P carried by HDL to preserve endothelium and reduce pro-inflammatory responses in endothelial and vascular smooth muscle (Nofer et al., 2001, Kimura et al., 2003, Tolle et al., 2007). Once FTY720, a synthetic sphingosine analogue, is phosphorylated by SK2, it acts as an agonist at S1P₁ and S1P₃ receptor subtypes and has been demonstrated to decrease atherosclerosis in both low density lipoprotein receptor deficient mice (Nofer et al., 2007) and in apolipoprotein E-deficient mice (Keul et al., 2007b). A recent study has shown that knocking out S1P₁ receptors increased immunoexpression of proinflammatory adhesion proteins such as ICAM-1 and VCAM-1 in mouse aortic endothelium and overexpression of S1P₁ receptors reduced the abundance of these proinflammatory molecules, suggesting that it has anti-inflammatory effects (Galvani et al., 2015). In the same study, it was demonstrated that HDL-S1P reduced the TNF α -induced NF- κ B activation and adhesion protein expression in HUVECs and this anti-inflammatory properties of HDL-S1P was mediated by S1P₁ receptors. Moreover, HDL-S1P stimulated the formation of an anti-inflammatory complex between S1P and β arrestin and the protective effects of HDL-S1P were antagonised by pre-treatment with P-FTY720, which enhances S1P₁ degradation (Galvani et al., 2015). In acute myocardial ischaemia/reperfusion injury, it has been shown that HDL is a direct cardioprotective agent and that this cardioprotective effect occurs independently of its atheroprotective effect (Keul et al., 2007a). The biologically active HDL- S1P appears to be responsible for the protective effects of HDL on myocardium via inhibition of adhesion molecule expression and recruitment of leukocytes to the infarcted area (Argraves and Argraves, 2007). Toa et al demonstrated that HDL, via its cargo of S1P, can directly protect adult murine cardiomyocytes against oxidative injury induced by hypoxia/reoxygenation (Tao et al., 2010). This cytoprotective effect was inhibited by S1P₁

and S1P₃ receptor antagonist VPC23019, CAY10444, an S1P₃ receptor antagonist, PD-98059, a MEK inhibitor and by the PI-3 kinase inhibitor wortmannin (Tao et al., 2010). Both Akt and ERK1/2 pathways were activated via S1P-associated HDL and both S1P₁ and S1P₃ receptors are involved in prosurvival signal activation. Moreover, HDL was found to protect neonatal rat ventricular cardiomyocytes against doxorubicin-induced apoptosis and this effect was mediated by the S1P₂ receptor, ERK1/2 and STAT3 (Frias et al., 2010). Furthermore, both genetic disruption of S1P receptors and responses to HDL have provided additional evidence for the involvement of SK/S1P pathways in cardioprotection. For example, Nofer et al demonstrated that HDL stimulated NO release in human endothelial cells and induced NO-dependent vasorelaxation through S1P₃ receptor (Nofer et al., 2004). These effects could be inhibited in S1P₃ knockout mice. Based on these results, HDL may improve myocardial perfusion and protect the heart against ischaemia/reperfusion insult in vivo through S1P₃.

1.6.5 FTY720

FTY720, a structural homologue of sphingosine, is derived from myriocin. Once FTY720 becomes phosphorylated by SK2, it acts as a potent agonist of S1P₁ receptor and also binds (though less avidly) to S1P₃ receptors (Mandala et al., 2002, Hla and Brinkmann, 2011). It is an FDA-approved drug as an oral treatment for patients with multiple sclerosis (Pelletier and Hafler 2012). Several studies have examined whether FTY720 is cardioprotective against ischaemia/reperfusion injury. For example, Zhang et al showed that FTY720 promoted survival in isolated adult murine cardiomyocytes subjected to severe long periods of hypoxia (Zhang et al., 2007). It has also been demonstrated that FTY720 prevented cardiac arrhythmia in an ex vivo rat heart model subjected to ischaemia/reperfusion (Egom et al., 2010). A subsequent study showed that FTY720 is able to protect neonatal rat ventricular cardiomyocytes against CoCl₂-induced hypoxic injury and stimulated NO release via a pertussis toxin-sensitive PI3K/Akt/eNOS pathway (Egom et al., 2011). Another study reported that treatment with FTY720 at the time of reperfusion improved recovery of left ventricular developed pressure and decreased left ventricular end-diastolic pressure in isolated rat hearts subjected to ischaemia/reperfusion (Hofmann et al., 2009). A further study reported that long term treatment with FTY720 improved survival in a mouse model of occlusive coronary atherosclerosis, MI and heart failure through enhancing cardiomyocyte resistance to ischaemic injury, oxidative stress and apoptosis and reducing inflammation in the heart (Wang et al., 2014). Recently a study showed that FTY720 exhibited cardioprotective effects against post MI cardiac

dysfunction and remodelling through interfering with SK1/S1P/S1P₁ signalling pathway (Zhang et al., 2016a). In contrast to these beneficial effects in animals, it has been identified that oral administration of FTY720 causes an initial bradycardia. Therefore the FDA advises not to prescribe this drug for patients with heart problems or stroke within the previous 6 months or patients on anti-arrhythmic treatment.

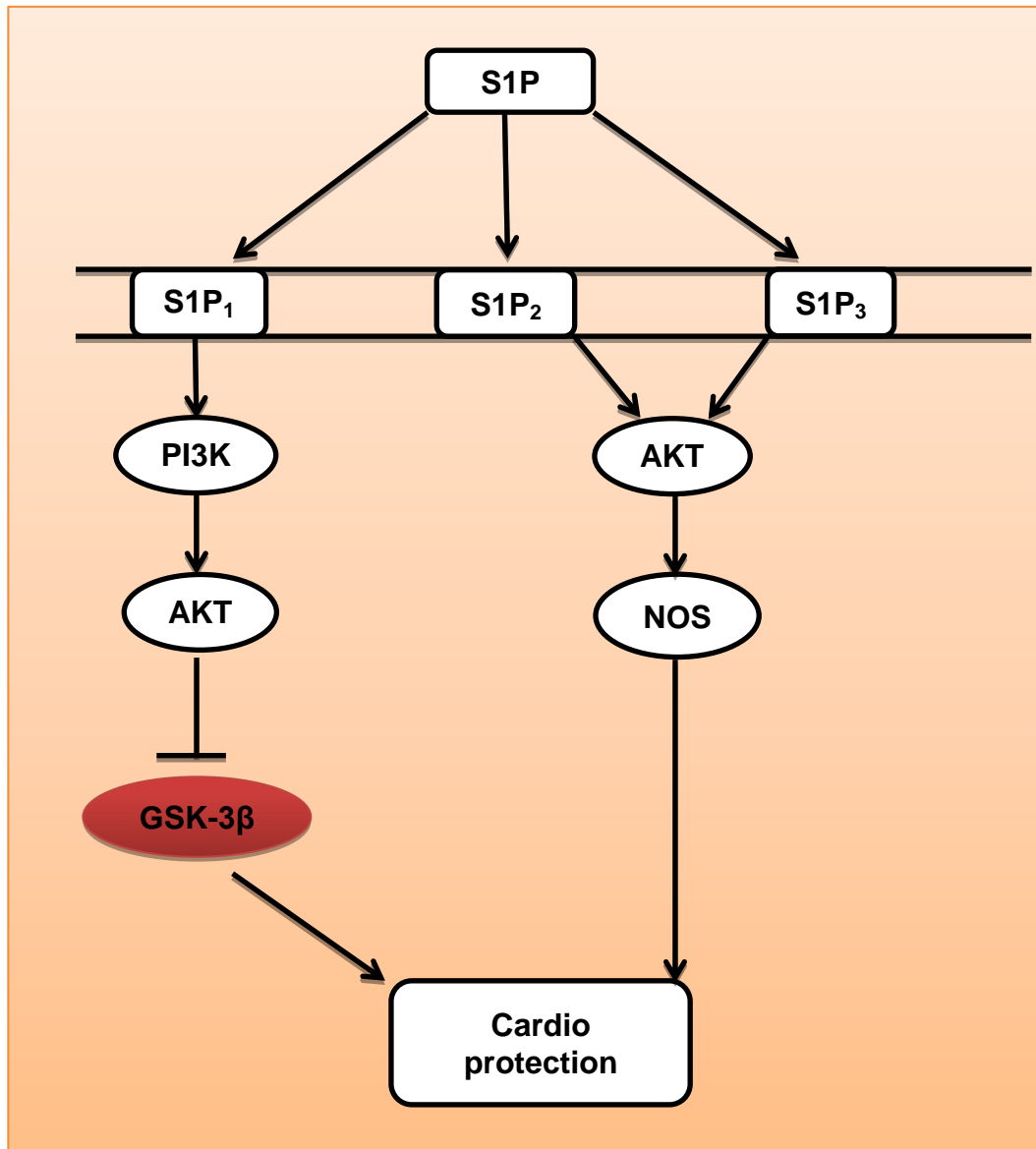


Figure 1-5 Flow chart shows the mechanism of S1P-induced cardioprotection in cardiomyocytes.

S1P₁ receptor activation enhances cardiomyocytes protection against ischaemia/reperfusion via signalling pathway involving activation of PI3K, Akt and inactivation of GSK-3 β . S1P₂ and S1P₃ receptors also enhances cardioprotection via activation of Akt which increases endothelial NO synthase.

Hypothesis and aims

Myocardial hypoxia/ischaemia increases SK1 expression and generation of endogenous S1P in coronary endothelial cells and cardiomyocytes, which may have an effect on regulation of coronary reactivity and exert a cardioprotective effect against ischaemia/reperfusion injury. Thus the principal research aim of this thesis was to investigate the influence of hypoxia on SK1 expression in rat coronary artery and how this may regulate vascular function through generation of S1P. This was achieved through the following experimental study aims:

- Examine the expression of S1P receptors within normoxic rat coronary artery.
- Examine the expression of SK1enzyme within normoxic and hypoxic rat coronary artery in the presence and absence of SK1 inhibitors.
- Compare the SK1 expression in coronary artery endothelium in the presence and absence of proteasomal and/or lysosomal inhibitors under normoxia and hypoxia.
- Investigate the effect of hypoxia on SK1 expression in rat aorta and how this may regulate vascular reactivity.
- Evaluate the influence of hypoxia on SK1 expression in HUVECS and rat cardiac tissue in the presence and absence of SK1 inhibitors.

Chapter two

2 Materials and methods

2.1 Animal source

All animal experiments were performed in accordance with the United Kingdom Home Office Legislation under the Animals (Scientific Procedure) Act 1986 (project licence 70/8572). Sprague-Dawley rats were purchased from ENVIGO (UK) and spontaneously hypertensive Wistar Kyoto rats were a generous gift from Dr Lorraine Work (ICAMS, Glasgow University). Rats were housed in the Central Research Facility at University of Glasgow and maintained on 12 hour cycles of light and dark and the room temperature was held at 21°C. Rats had free access to both food and water.

2.2 Chemicals and reagents

S1P, CYM5541, SEW2871 and AMG9810 were purchased from Tocris Bioscience (Bristol, UK). Cycloheximide, CA-074 Me, SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole) and PF543 were from Merck Biosciences Ltd (Nottingham, UK). MG132 was from Stratech Scientific Ltd (Suffolk, UK). Lactacystin was from Enzo Life Sciences (Exeter, UK) and CAY10444 was from Cayman Chemical (Cambridge, UK). The following chemicals were purchased from Sigma-Aldrich (Poole, UK): U46619, acetylcholine, and Nitric Oxide Synthase inhibitor (L-NNA, N ω -Nitro-L-arginine). ROME, (R)-FTY720 methyl ether, was a kind gift from Prof Susan Pyne (Strathclyde University) (Table 2-1, page 54). Culture supplies were obtained from PromoCell Bioscience (Heidelberg, Germany) unless otherwise stated. All Western blot materials were obtained from Life Technologies (Paisley, UK) unless otherwise stated.

2.3 Confocal microscopy

The confocal microscope was invented by Minsky in 1955 from the conventional light microscope. The aim was to produce sharp images of thin cross sections, which had not been possible with conventional microscopy. The basic confocal microscope system has been improved upon over the last decade and confocal laser scanning microscopy (CLSM) is now a commonly used technique. Many confocal studies have been carried out on cells but recently this technique has also been used on whole blood vessels (Arribas et al., 2007, Daly et al., 2010).

2.3.1 The confocal microscope

The confocal microscope is composed of a photomultiplier tube (PMT), a detector pinhole, a source pinhole, a dichroic mirror and an objective lens. It is based upon the principle that an excitation light from a laser is focused into the source pinhole and reflected by the dichroic mirror in order to pass it through the objective to the sample. The excitation light fills a converging cone as it is focused through the specimen to the object plane, and all the excitation light passes out through a similar diverging cone. The fluorophore in the specimen emits the fluorescent light in all directions. Light from the specimen returns through the objective and dichroic mirror respectively and is focused into the detector pinhole, then measured by the PMT. The detector pinhole is designed to ensure that only light from the in-focus planes reach the PMT, and prevents the light scattered from out-of-focus planes reaching the PMT. An image is produced from individual pixels using a computer with imaging software to visualise a complete image.

2.3.2 Principle of fluorescence

When molecules with luminescence properties absorb light energy of a particular wavelength, they give off light of a different wavelength. This occurs because of their atomic structure. Electrons are arranged around the atom's nucleus in different energy levels. When an electron absorbs light energy, it reaches a higher energy or excited state. The higher energy state is unstable and does not last long. The electron loses some of the absorbed energy as heat and the remaining extra energy is emitted as a photon of light (Figure 2-1). This emitted light is always of a lower energy than the excited light, so the wavelength of the emitted fluorescence is longer than that of the absorbed light.

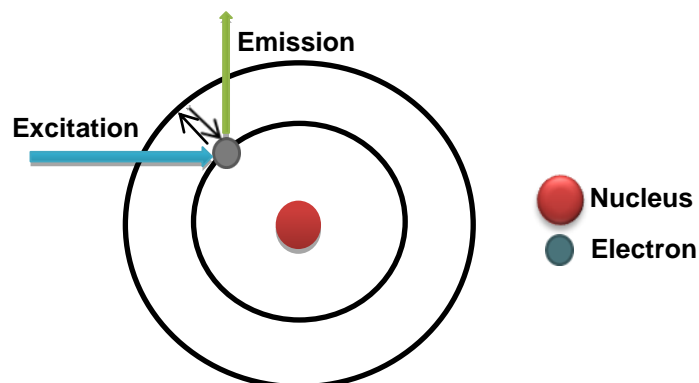


Figure 2-1 Fluorescence generation

2.3.3 Tissue preparation for immunofluorescence studies

Male Sprague-Dawley rats (200-250 g body weight) were killed by cervical dislocation. The chest cavity was opened and the heart and thoracic aorta were rapidly removed and placed into cold Krebs-Henseleit solution (NaCl 118 mM, NaHCO₃ 25 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1 mM, glucose 11 mM, and CaCl₂ 2.5 mM) previously gassed with 95% O₂, 5% CO₂ at room temperature. Under a binocular microscope, the right and left coronary arteries were carefully dissected free of connecting heart tissue with fine dissecting scissors and the thoracic aorta was also cleaned of adherent connective tissue. 1-2 mm segments of freshly isolated coronary and 2-3 mm segments of thoracic artery were carefully opened longitudinally, treated with specific inhibitory agents for half an hour (Table 2-1, page 54), then subjected to normoxia/hypoxia (see hypoxia protocol in section 2.3.5) for 30 min and fixed by 3% paraformaldehyde overnight at 4 °C. To determine the most appropriate method of preparing the coronary and aortic arteries for imaging, a series of experiments was carried out using whole vessels (without opening them longitudinally). In the whole vessels, it was not possible to focus into the endothelium and the smooth muscle layers because of the thick elastic wall of the arteries. Therefore, both coronary arteries and aorta were turned ‘inside out’ by cutting the vessel ring longitudinally, exposing the endothelium. By using this preparation, it was possible to image the vessels from the endothelium through to the media. Thus, the open preparation was applied in all experiments as it enabled imaging of the endothelium and smooth muscle cells with minimum damage.

2.3.4 Label protocol

2.3.4.1 Single label protocol

After fixation with paraformaldehyde, the samples were washed 3 times with phosphate buffered saline (PBS) and incubated in blocking solution (10% normal goat serum) for 2 hours. Thereafter, samples were incubated in a primary antibody against the protein of interest overnight at 4°C. Primary antibodies were diluted in PBS supplemented with 1% BSA (1:50 & 1:100 dilution used) (Table 2-2, page 55). Samples were then washed 3 times with PBS before incubation with an AlexaFlour 594 conjugated goat anti-rabbit secondary antibody for 1 hour at room temperature (Figure 2-2). Secondary antibody was also diluted in PBS supplemented with 1% BSA (1:250 dilution) (Table 2-3, page 55). Nuclear stain Syto 61 (1µM) was also added to the secondary antibody solution to stain the endothelial

and smooth muscle cell nuclei. Nuclear shape and position were used to distinguish endothelial from smooth muscle cells.

2.3.4.2 Double label protocol for SK1 enzyme and S1P₃ receptor

Following fixation and washing as previously described, samples were incubated in blocking solution (5% normal donkey serum) for 2 hours and then incubated with anti-S1P₃ antibody (1:50) overnight at 4 °C. Samples were then washed 3 times with PBS before incubation with AffiniPure fab fragment goat anti-rabbit antibody (1:300) for 1-2 hrs. Thereafter, samples were again washed by PBS (3 times) before incubation with AlexaFluor 488 conjugated donkey anti-goat secondary antibody (green) (1:250) for 1 hr. Samples were then washed 3 times with PBS and incubated with rabbit polyclonal anti-SK1 primary antibody (1:50) overnight at 4 °C. Following washing with PBS, samples were incubated for 1 hr at room temperature in AlexaFluor 594 conjugated donkey anti-rabbit secondary antibody (red) (1:250). Nuclear stain Syto 61 (1 μM) was also added to the secondary antibody solution to stain the endothelial and smooth muscle cell nuclei. Each staining step was performed sequentially in the dark. Primary and secondary antibodies were diluted in PBS supplemented with 1% BSA. A list of primary and secondary antibodies is presented in table 2-2 (Page 55) and table 2-3 (Page 55) respectively.

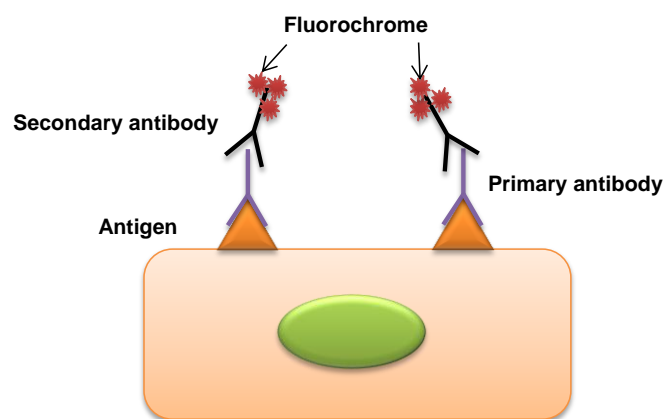


Figure 2-2 Indirect immunofluorescence

2.3.5 Hypoxia protocol

Normoxia (~ 20% O₂, pO₂ ~160 mmHg) was induced by placing the artery in 1 ml Krebs' solution which had been previously gassed with 95% O₂, 5% CO₂ (Solution was not gassed

during procedures) to keep oxygen tension at ~ 20% O₂ saturation). Hypoxia was induced by placing the artery in 1 ml Krebs' solution which had been constantly bubbled with 95% N₂/5% CO₂ for approximately 30 min to reduce oxygen tension to 1-1.5% O₂ saturation (pO₂ ~ 8-10 mmHg). An Optical Oxygen Meter-Fire Sting O₂ (Pyro Science, Germany) was used to measure oxygen tensions before and after the experiment. Since the oxygen meter repeatedly yielded the same oxygen saturation values on several occasions, it was deemed unnecessary to measure oxygen tension in all experiments. Hypoxia was maintained for 30 min and temperature was held at 37°C. Arteries were then fixed in 3% paraformaldehyde and immunostained as outlined above.

2.3.6 Slide mounting

Once the incubation period was finished, each artery segment was laid flat on a microscope slide with the endothelial side uppermost. A small well was created on the microscope slide using grease and filled with the Krebs solution (NaCl 118 mM, NaHCO₃ 25 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1 mM, glucose 11 mM, and CaCl₂ 2.5 mM) that the vessel was incubated in (Miquel et al., 2005) (Figure 2-3). A coverslip (thickness 1.5 µm) was placed on the top creating a chamber.

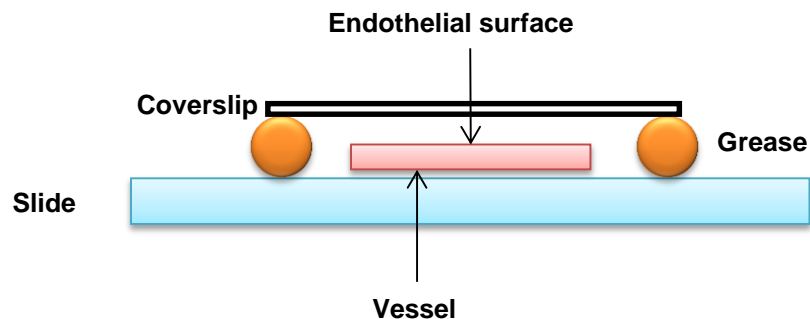


Figure 2-3 Slide preparation

2.3.7 Imaging

Initially, the objective was cleaned in order to remove any dust particles and a glass rod was used to apply oil onto the objective to prevent air bubbles forming in the immersion oil. Then, the specimen was visualised through the microscope eyepiece and the microscope (Nikon Eclipse TE300) applied conventionally to focus on the specimen. When focussed, the visualisation was changed from the microscope eyepiece to the computer. Prior to starting an exploratory scan, the scan setting for the confocal microscope was selected. A series of preliminary experiments was carried out to establish

the best scan setting for the confocal microscope that allow to visualise the protein of interest with less noise in the image background. All vessels were visualised by the Bio-Rad 2100 Confocal Laser Scanning microscope equipped with argon, HeNe and red diode lasers. x40 oil immersion and x20 oil/water immersion lenses were used for all experiments. For the rat coronary artery, the images were recorded mainly at zoom 3 and 6, HeNe laser intensity 100; a gain 30; offset 0.0 and pinhole setting of 3.0 were chosen. In the case of thoracic aorta, the gain was 40 with other settings remaining the same. The standard scan speed of 500 lines per second was used in all experiments. An image size of 512 x 512 pixels produced a field size of 289 μm x 289 μm .

Each vessel was imaged from endothelium to the media. At least three images were collected from random areas of the vessels. Each experiment was repeated at least three times in separate rats. Vessels were scanned using lambda strobing to minimize bleed-through on individual channels. Kalman frame averaging varied between 3 and 5 was used to improve the quality of the image. Each fluorescent probe was imaged as close as possible to its excitation and emission maxima: AlexaFlour 594 secondary antibody (ex. 591 nm, em. 614 nm); Syto 61 (ex. 637 nm, em. 660 nm).

2.3.8 Image analysis

2D images were produced (8 bit) and displayed spatially as pixels, where each pixel represents an intensity value between 0 (black) and 255 (white) from the Gray scale. Following image capture, Image J software (1.47 version) was used to analyse 2D images collected at zoom 3 and 6. Integrated density (the total amount of fluorescence) was measured from 3 images (zoom 3) taken from 3 different areas in each artery by Image J software (the three areas combined to give a single average value for each animal) and the mean data plotted in bar charts. Statistical comparisons between integrated densities generated in the presence and absence of hypoxia and specific inhibitory agents (Table 2-1) were determined using an unpaired t test and one way analysis of variance (ANOVA) with Dunnett's post-test or Bonferroni multiple comparison post-test. P-values were considered statistically significant when $p < 0.05$

Table 2-1 List of drugs and concentrations used

Drugs	Description	Concentration	Diluent
Cathepsin B inhibitor (CA-074ME)	Lysosomal inhibitor	10 μ M	DMSO
CAY10444	Selective S1P ₃ antagonist	10 μ M	DMSO
Cycloheximide	Protein synthesis inhibitor	10 μ M	Ethanol
CYM5541	Selective S1P ₃ agonist	1 nM–30 μ M	DMSO
Lactacystin	Proteasomal inhibitor	10 μ M	Distilled water
MG132	Proteasomal inhibitor	10 μ M	DMSO
N ω -Nitro-L-arginine (L-NNA)	Nitric Oxide Synthase Inhibitor	100 μ M	DMSO
PF543	Selective SK1 inhibitor	100 nM	DMSO
(R)-FTY720 methyl ether (ROME)	Selective SK2 inhibitor	10 μ M	DMSO
S1P	Sphingosine-1-Phosphate	1 nM–30 μ M	PBS/BSA
SEW2871	Selective S1P ₁ receptor agonist	1 nM–30 μ M	Ethanol
Sphingosine kinase inhibitor (SKi)	Non-selective SK inhibitor	10 μ M	DMSO

Table 2-2 Primary antibodies used for immunofluorescence

Epitope	Clonality	Host species	Blocking serum	Dilution	Source
Phospho SK1	Polyclonal	Rabbit	10% NGS	1:50	ECM biosciences SP1641
SK1	Polyclonal	Rabbit	10% NGS	1:50	Abcam ab71700
S1P ₁	Polyclonal	Rabbit	10% NGS	1:50	Abcam ab23695
S1P ₃	Polyclonal	Rabbit	10% NGS	1:50	Santa Cruz Biotechnology sc-30024

NGS: normal goat serum. Primary antibodies were diluted in PBS supplemented with 1% BSA and incubated at 4 °C overnight. Phospho SK1 antibodies were raised against phospho-SK1 (Ser-225) synthetic peptide corresponding to amino acids surrounding serine 225 in human SK1. SK1 antibodies were raised against a synthetic peptide corresponding to human SPHK1 aa 286-315. S1P₁ and S1P₃ antibodies were raised against a synthetic peptide corresponding to human S1P₁ aa 241-253 and human S1P₃ aa 309-378 respectively.

Table 2-3 Secondary detection agents for immunofluorescence

Linked molecule	Epitope	Clonality	Host species	Dilution	Source
Alexa Fluor® 594 (Red)	Rabbit IgG	Polyclonal	Goat	1:250	Stratech Scientific
Alexa Fluor® 488 (Green)	Goat IgG	Polyclonal	Donkey	1:250	Stratech Scientific
Alexa Fluor® 594 (Red)	Rabbit IgG	Polyclonal	Donkey	1:250	Stratech Scientific
AffiniPure fab fragment	Rabbit IgG	Polyclonal	Goat	1:300	Stratech Scientific

Secondary antibodies were diluted in PBS supplemented with 1% BSA and incubated at room temperature for 1 hour.

2.4 Functional studies (wire myography)

2.4.1 Vessel preparation and mounting

Male Sprague-Dawley rats (200-250 g body weight) and spontaneously hypertensive Wistar Kyoto rats (350-400 g body weight) were killed by cervical dislocation. The chest cavity was opened and the thoracic aorta was rapidly removed and placed into cold Krebs-Henseleit solution (NaCl 118 mM, NaHCO₃ 25 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1 mM, glucose 11 mM, and CaCl₂ 2.5 mM) previously gassed with 95% O₂, 5% CO₂ at room temperature. Then the thoracic aorta was carefully dissected free from adherent connective tissue and cut into 2-3 mm rings. Vessel rings were mounted on two stainless steel pins in a four-channel small vessel wire myograph (Danish Myo Technology, Aarhus, Denmark). One of the pins was connected to a micrometer, to adjust the tension on the ring, and the other one was attached to a force transducer, which measures the force generated by the vessel ring in milliNewtons (Figure 2-4). Vessels were incubated at 37 °C in Krebs-Henseleit buffer with constant supply of 95% O₂, 5% CO₂ gas. The vessels were then allowed to equilibrate for 15-30 min at resting tension. Thereafter, vessels were stretched until an optimum resting tension of 1 gm (9.8 mN) was reached and allowed to equilibrate for 30 min. Chart™ 5 Pro software (ADInstruments, Chalgrove, U.K.) was used to record and measure vessel responses to different reagents.



Figure 2-4 Representative picture of a rat aortic ring mounted in the small artery wire myograph.

A 3 mm segment of artery was mounted on two pins with one attached to a force transducer and a computer to measure the changes in the tone of the vessel ring. The other pin was connected to a micrometer to adjust the tension on the ring.

2.4.2 Experimental protocols

2.4.2.1 Wake up procedure

In order to assess the viability of the vessels after dissection and mounting, the vessels were exposed to two additions of 40 mM KCl (Mair et al., 2010). Once the response had reached a plateau the vessels were rinsed two to three times with Krebs-Henseleit solution and rested for 10 min between additions. Contraction to KCl of > 0.5 gram was indicative of vessel viability.

2.4.2.2 U46619 protocol

To investigate the effect of hypoxia on contraction to the thromboxane A₂ mimetic U46619, (9,11-dideoxy-11 α ,9 α -epoxymethanoprostaglandin F₂ α), aortic rings were subjected to 30 min hypoxia, then re-oxygenated and maintained under normoxic conditions. Then concentration response curves were generated by cumulative addition of U46619 (1×10^{-9} - 1×10^{-6} M) with 10 min between each addition. In experiments where the effects of SKi, a non-selective SK inhibitor (10 μ M), on U46619-induced contraction were to be investigated under hypoxia and normoxia, the SKi was added 15 min before exposure to hypoxia and was present during generation of the concentration-response curve. Hypoxia was induced by altering the bubbling of the myograph chamber from 95% O₂-5% CO₂ to 95% N₂-5% CO₂ (< 5% O₂ saturation) for 30 min and thereafter the gas mixture was returned to control conditions.

2.4.2.3 Agonist comparison

After 30 min equilibration and challenge with KCl, aortic rings were submaximally contracted with U46619 (3×10^{-8} M) (Mair et al., 2010). Once a plateau was achieved, concentration-response curves were performed by cumulative addition of agonists. S1P, CYM5541, a selective S1P₃ receptor agonist, and SEW2871, a selective S1P₁ receptor agonist, (1×10^{-9} - 3×10^{-6} M) were added in half-log molar concentration in a cumulative manner with 5 min between each addition. Maximal response was measured five minutes after addition of each concentration of drug.

2.4.2.4 Antagonists and inhibitors protocol

In experiments where the effects of antagonists or inhibitors on agonist-induced relaxation were to be assessed, the antagonist of interest was added 30 min prior to contraction with

U46619, and was present during generation of the concentration-response curve. A list of antagonists and inhibitors and their final bath concentrations used are provided in table 2-1 (Page 54).

2.4.2.5 Hypoxia protocol

In order to investigate the effect of hypoxia on the S1P-induced relaxation in thoracic aorta, thoracic aortic rings were subjected to hypoxia for 30 min just after testing the vessel viability with KCl, then reoxygenated and submaximally contracted with U46619 (5×10^{-9} M). Once a plateau was achieved, concentration response curves were performed by cumulative addition of S1P as described above. In experiments where the effects of antagonists or inhibitors on agonist-induced relaxation under hypoxic conditions were to be assessed, the antagonist or inhibitor of interest was added 30 min before exposure to hypoxia and was present during generation of the concentration-response curve. Hypoxia was induced as described above.

2.4.2.6 Endothelial denudation protocol

Vascular endothelial function was assessed at the end of the experiment by adding 1 μ M ACh. Relaxation to ACh of $> 50\%$ was indicative of a vessel with an intact endothelium. Where indicated, endothelium was removed by gentle rubbing of the intimal surface with forceps. Relaxation to ACh $< 10\%$ denoted an endothelium denuded vessel (Figure 2-5).

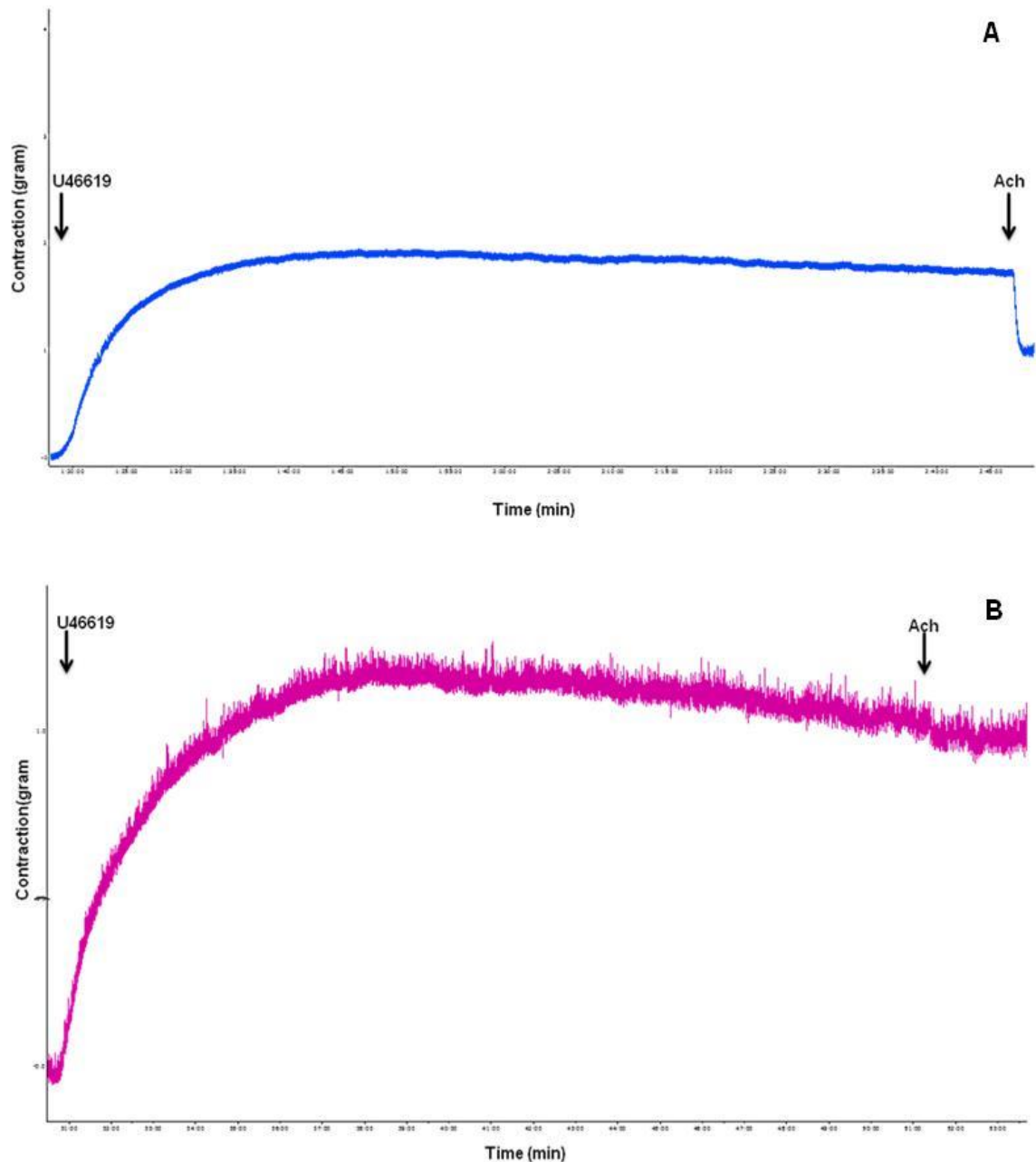


Figure 2-5 Representative experimental recordings showing isometric tension (g) plotted against time in rat thoracic aorta.

(A) Represents a recording generated by an endothelium-intact aortic ring with more than 50% relaxation to acetylcholine. (B) Represents a recording from an endothelium-denuded vessel with less than 10% relaxation to acetylcholine.

2.4.3 Statistical analysis

Graph Pad Prism (version 5) software was used to analyse all the data. Contractile responses to U46619 were expressed as mean \pm standard error of the mean (S.E.M) in grams. All relaxation responses were reported as a percentage loss of the tone induced by U46619. Results were expressed as mean \pm SEM of n, where n is the number of arterial segments from different animals. Differences between concentration-response curves were tested by two ways analysis of variance (ANOVA) with a Bonferroni's post-test. To compare the effect of enzyme inhibitors and antagonists on U44619-induced contraction in rat aorta, one-way ANOVA with Dunnett's post-test was used. In all cases, statistical significance was accepted when $p < 0.05$.

2.5 Cell culture and tissue preparation

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and immunoblotting were carried out using a Novex® NuPAGE® gel electrophoresis system (Life Technologies, Paisley, U.K.), in order to work out the relative expression of the proteins of interest.

2.5.1 Human Umbilical Vein Endothelial Cells (HUVEC) culture

HUVECs were a kind gift from Dr. Ian Salt (Institute of Cardiovascular & Medical Sciences, University of Glasgow). HUVECs were grown in 75 cm³ culture flasks and maintained in Endothelial Cell Growth Media MV / MV2 (PromoCell, Germany). The cells were kept incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The media was replaced every 2 days. Cells were routinely sub-cultured when approximately 80-90% confluency was reached and used for experiments at passage 4 to 6. For HUVECs sub-culture, the cells were washed with sterile Dulbecco's PBS (Sigma-Aldrich, Poole, U.K.) prior to incubation with 3 ml of trypsin-ethylenediamine tetra-acetic acid (trypsin-EDTA) at 37 °C for at least 1 min. The complete medium was then added to stop the action of the trypsin-EDTA solution and allow sub-culturing of the cell suspension.

2.5.2 Treatment of Human Umbilical Vein Endothelial Cells

HUVECs were seeded in 6 well plates and grown to approximately 90% confluence. The cells were then incubated for 24 hr with serum free medium. Then the cells were treated

with specific inhibitory agents and subjected to normoxia/hypoxia (as outlined in the relevant results chapter) for a particular period of time. Thereafter, the 6 well plate was placed on ice and the media was removed by aspiration. Subsequently, ice-cold lysis buffer (Cell extraction buffer (Invitrogen, CA), supplemented with 1mM of protease inhibitor cocktail (Sigma Aldrich, UK), 1mM Phenylmethylsulfonyl fluoride (PMSF) and 1mM Dithiothreitol (DTT) (Sigma Aldrich, UK) was added to each well for 5 minutes. The cell extract was collected by scraping and transferred into ice-cold centrifuge tubes. The cell lysates were then centrifuged (13300 x g, 10 min) on a bench top centrifuge. The supernatants were stored at -80 °C for Western blotting.

2.5.3 Preparation of heart tissue

Heart tissue was removed and dissected immediately after death from Male Sprague-Dawley rats (200-250 g body weight). The right ventricular wall was cut into 8 pieces and each piece of tissue was pre-incubated with a specific inhibitory compound before exposure to hypoxia or normoxia for a particular length of time (as described in results chapter). The samples were immediately snap frozen and stored at -80 °C until use. Heart tissue was pulverised in liquid nitrogen into a fine powder by using a mortar and pestle and resuspended in ice-cold lysis buffer. The extract was transferred into ice-cold centrifuge tubes and centrifuged (8000 x g, 10 min) on a bench top centrifuge. The supernatants were stored at -80 °C for Western blotting.

2.5.4 Quantification of protein concentration

Standard dilutions of bovine serum albumin (BSA) ranging from 0.1 mg/ml to 1mg/ml were used to generate a standard protein curve with distilled water as a blank. Lysates, analysed in triplicate, were diluted in distilled water at a ratio of 5:1. 10 µl of each sample and standards were added in triplicate to a 96 well plate followed by 100 µl DC™ Protein Assay Reagent (Bio-Rad Laboratories Ltd, Herts, UK). A FLUOstar OPTIMA microplate reader (BMG Labtech, Germany) was used to read absorbance at 595 nm. The mean absorbance from each sample was generated in triplicate and the protein concentration was determined by comparison with the BSA standard curve.

2.5.5 Western blotting

2.5.5.1 SDS PAGE

The lysates were mixed with 7.5 μ l of DTT and 12.5 μ l of NuPAGE® LDS sample buffer as a load dye, to a total volume of 50 μ l and heated at 70°C for 20 mins prior to separation by SDS-PAGE. Samples were then loaded on NuPAGE® Novex® 4-12 % Bis-Tris mini gels (1.0 mm thick, 15 wells) at 10 μ g of protein per well. 10 μ l Novex® sharp pre-stained protein markers were used as a standard. Gels were run at a constant voltage of 200 V for approximately 45-60 minutes in NuPAGE® MOPS SDS running buffer until the tracking dye had reached the bottom of the gel. To transfer proteins from the gel to the nitrocellulose membrane, the gel was removed from the plate and an equal-sized sheet of nitrocellulose (0.45 μ m pore size) was placed on it. They were then placed between two layers of 3 mm filter paper, prewashed with transfer buffer. After that the sandwich was inserted between the plates of the gel holder cassette and transfer was carried out at 30 V for 90 min in NuPAGE® transfer buffer containing 10 % (v/v) methanol. After removal of the nitrocellulose membrane from the transfer cassette, the efficiency of transfer was checked by the presence and intensity of pre-stained molecular weight standards using Ponceau S stain.

2.5.5.2 Immunoblotting

Non-specific sites on nitrocellulose membranes were blocked by incubation with 5 % (w/v) milk powder (Marvel) prepared in Tris-buffered saline (20mM Tris; 137mM NaCl PH 7.4) containing 0.1% (v/v) Tween-20 (TBST) at room temperature with continuous shaking for 60 minutes. After rinsing (3 x 5 min) the membranes in TBST, the membranes were incubated overnight at 4 °C in primary antibody diluted in 50% (v/v) TBST, 50% (v/v), OBB, Odyssey® blocking buffer (LI-COR, USA). The membranes were then rinsed (3 x 5 min) with TBST before incubation, with shaking, for 60 min at room temperature with IRDye® 800CW Donkey anti-Rabbit IgG antibodies (Li-COR, USA) diluted in the same way as the primary antibody. Following this, the membranes were washed with TBST (3 x 5 min) and TBS (1 x 5 min) before visualisation of immunolabelled bands using an Odyssey Sa Infrared Imaging System (LI-COR, USA) linked with Odyssey Sa Infrared Imaging System software (LI-COR, USA). Primary and secondary antibodies used in Western blotting are presented in table 2-5.

2.5.5.3 Stripping of nitrocellulose membranes

Nitrocellulose membranes were incubated in Restore Plus Western Blot Stripping Buffer (Thermoscientific, UK) for 15 min at room temperature. Membranes were then rinsed with TSBT (3 x 5 min) and TBS (1 x 5 min).

2.5.5.4 Quantification of protein bands

The intensity of the immune-detected protein bands was quantified using ImageJ software (version 1.47). The housekeeping protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as a protein loading control. Thus, data were expressed as a ratio of protein of interest to the loading control.

Table 2-4 Primary antibodies used for western blotting

Epitope	Clonality	Host species	Dilution	Diluent	Source
GAPDH	Polyclonal	Rabbit	1:2000	50% TBST 50% OBB	Thermoscientific PA1-988
Phospho SK1	Polyclonal	Rabbit	1:1000	50% TBST 50% OBB	ECM biosciences SP1641
SK1	Polyclonal	Rabbit	1:1000	50% TBST 50% OBB	Abcam ab71700

OBB: Odyssey® blocking buffer. All blots were incubated 4 °C overnight.

Table 2-5 Secondary detection agent for western blotting

Linked molecule	Epitope	Host species	Dilution	Diluent	Source
IRDye® 800CW	Rabbit IgG	Donkey	1:5000	50% TBST 50% OBB	LI-COR Biosciences 926-32213

All blots were incubated with secondary antibody for 1 h at room temperature.

Chapter three

3 Effect of hypoxia on Sphingosine Kinase 1 expression in rat coronary artery

3.1 Introduction

Sphingosine-1-phosphate (S1P) is a potent signalling lipid and exhibits a wide range of cellular activities including cell proliferation, migration and survival. Once S1P is generated by phosphorylation of sphingosine it can either be exported from cells (via transporter proteins e.g. Spns2 and particular ABC transporters) or retained within the cell. Extracellular S1P is an agonist of five GPCRs named S1P₁₋₅ (Blaho and Hla, 2014), but it can also function intracellularly as a second messenger. The level of S1P in plasma and tissue is regulated by the balance between its production and degradation (Thuy et al., 2014). S1P is degraded via reversible dephosphorylation by S1P phosphatases or irreversible hydrolysis by S1P lyase (Pyne and Pyne, 2010).

Synthesis of S1P is catalysed by two distinct isoforms of sphingosine kinase (SK1 and SK2), which are encoded by different genes and differ in their subcellular localisations; with SK1 being a mostly cytosolic protein and SK2 predominately nuclear (Siow and Wattenberg, 2011). Several stimuli can either activate or increase SK1 expression including agonists of GPCRs, tyrosine kinase receptors and immunoglobulin receptors, proinflammatory cytokines, calcium and protein kinase activators (Taha et al., 2006b, Alemany et al., 2007, Pyne et al., 2009). Hypoxia has also been demonstrated to increase SK1 expression in human pulmonary smooth muscle cells and the human endothelial cell line EA.hy926 (Ahmad et al., 2006, Schwalm et al., 2008). SK1 is activated by phosphorylation at ser225 and then the phosphorylated SK1 is translocated from the cytosol to the plasma membrane where it interacts with its substrate, sphingosine (Jarman et al., 2010). SK1 is deactivated by dephosphorylation at ser225 via the action of phosphatase 2A (Barr et al., 2008) and it can also be degraded via the ubiquitin proteasome mediated pathway and/or a cathepsin B mediated process (Taha et al., 2006a, Loveridge et al., 2010).

There is a growing body of evidence suggesting that SK and S1P may play an important role in cardioprotection against acute or chronic ischaemia or ischaemia/reperfusion injury (Karliner, 2013). Therefore, the role of these molecules or their synthetic analogues in cardioprotection against ischaemia/reperfusion has been extensively studied over the last decades. For example, exposure of SK1 knockout mouse ventricular cardiomyocytes to ischaemia/reperfusion resulted in increased cell death and cytochrome c release compared with wild type controls (Tao et al., 2007). Moreover, pretreatment of SK1 null cardiomyocytes with exogenous S1P prevented hypoxia-induced cell death while

exogenous S1P decreased infarct size in isolated adult mouse hearts subjected to ischaemia/reperfusion (Jin et al., 2002, Lecour et al., 2002). Also, it has been shown that ischaemic preconditioning (ICP) sufficient to decrease infarct size in wild type hearts promoted SK activity and relocalisation from the cytosol to the membrane (Jin et al., 2004).

In addition to damaging cardiomyocytes, ischaemia/reperfusion may cause early and severe injury within the vasculature that could prevent a return to normal coronary perfusion, leading to inadequate myocardial perfusion (the “no-reflow” phenomenon). This phenomenon was observed in 30% of patients with myocardial infarction and was associated with a higher incidence of death (Ito, 2006, Niccoli et al., 2009). Therefore, prevention of coronary microvascular dysfunction and enhancing coronary perfusion represents a promising strategy to improve ongoing therapies that may reduce infarct size and prolong survival.

Since control of vascular diameter is crucial in maintaining myocardial perfusion during hypoxia, there is a great deal of interest in the effect of endogenous vasoactive substances such as S1P and whether their activity is changed under hypoxic conditions. Several studies have reported that hypoxia induces vasodilation in human (Lynch et al., 2006) and rat coronary arteries (Kerkhof et al., 2002). It has been demonstrated that S1P induced vascular relaxation in intact rat coronary artery via a mechanism requiring S1P₃ and CB₂ receptors under normoxic conditions and this effect was inhibited by pre-treatment with SKi (Mair et al., 2010). Another study showed that SK1 is upregulated in human endothelial cell lines (Schwalm et al., 2008) and human pulmonary artery smooth muscle cells subjected to acute and chronic hypoxia (Ahmad et al., 2006). Although the effect of hypoxia/ischaemia on SK activity in cardiomyocytes has been extensively studied, little is known about the effect of hypoxia/ischaemia on SK levels in coronary arteries.

3.1.1 Aims

- Study the distribution of sphingosine kinase 1 and S1P receptors in rat coronary artery endothelium.
- Investigate the effect of hypoxia on SK1 expression in the presence and absence of different SK inhibitors.

- Examine the effect of hypoxia on SK1 expression in the presence and absence of proteasomal and lysosomal degradation inhibitors.

3.2 Methods

3.2.1 Tissue preparation

Rat coronary artery sections were prepared, fixed and immunostained as detailed in section 2.3.3. Briefly, vessels from male Sprague-Dawley rats were dissected immediately after death and placed into 1ml Krebs-Henseleit solution previously gassed with 95% O₂, 5% CO₂ at 37 °C. The segments were then treated with specific inhibitory agents for half an hour (detailed below), then subjected to normoxia/hypoxia for 30 min and fixed by 3% paraformaldehyde overnight at 4 °C. The vessels were then labelled with specific primary and secondary antibodies, placed on a glass slide and the protein of interest visualized by confocal microscopy.

3.2.2 Hypoxia protocol

Hypoxia was induced by placing the artery in 1 ml Krebs' solution which had been constantly bubbled with 95% N₂/5% CO₂ for approximately 30 min to reduce oxygen tension to 1-1.5% O₂ saturation. An optical oxygen meter (Fire StingO₂ Pyro Science, Germany) was used to measure oxygen tension before and after the experiment. Since the oxygen meter repeatedly yielded the same oxygen saturation values in several experiments, it was deemed unnecessary to measure oxygen tension on all occasions. Hypoxia was maintained for 30 min and temperature was held at 37°C. Arteries were then fixed in 3% paraformaldehyde and immunostained as outlined in section 2.3.4.

3.2.3 Experimental protocol

3.2.3.1 SK inhibitors

A series of experiments was carried out where the vessels were preincubated with 10 µM SKi, a non-selective SK inhibitor (Loveridge et al., 2010), 100 nM PF543, a selective SK1 inhibitor (Schnute et al., 2012), or 10 µM ROME, a selective SK2 inhibitor (McNaughton et al., 2016) followed by exposure to normoxia/hypoxia for 30 min.

3.2.3.2 Protein synthesis inhibitor

A number of experiments were performed where the vessel was pre-treated with 10 μ M cycloheximide, a protein synthesis inhibitor, for 30 min prior to exposure to normoxia/hypoxia for 30 min.

3.2.3.3 Proteasomal and lysosomal inhibitors

To study the effect of proteasomal and lysosomal degradation on SK1 expression under normoxic and hypoxic conditions, a series of experiments was carried out where the vessels were preincubated with 10 μ M MG132, a proteasomal inhibitor (Loveridge et al., 2010), 10 μ M lactacystin, a proteasomal inhibitor (Loveridge et al., 2010), 10 μ M CA-074ME, a cathepsin B inhibitor (Ren et al., 2010) or a combination of MG132 and CA-074ME for 30 min before exposure to normoxia/hypoxia for 30 min. Other experiments were performed where the arterial segments were pre-treated with MG132, lactacystin, CA-074ME or a combination of MG132 and CA-074ME for 30 min followed by treatment with 10 μ M SKi for 30 min and then the segments were subjected to normoxia/hypoxia for 30 min.

3.2.4 Image analysis

A Bio-Rad Radiance 2100 confocal microscope was used to visualise the protein of interest as described in detail in section 2.3.7. Following the image capture, Image J software (1.47 version) was used to analyse 2D images collected at zoom 3 and 6. Integrated density was measured from 3 images (zoom 3) taken from 3 different areas in each artery by Image J software. Statistical comparisons between integrated densities (the arithmetic sum of all the pixel values in an area) generated in the presence and absence of hypoxia and specific inhibitory agents were determined using an unpaired Student's t-test and one way analysis of variance (ANOVA) with Bonferroni multiple comparison post-test. P-values were considered statistically significant when $p < 0.05$.

3.3 Results

3.3.1 Expression of SK1 enzyme, S1P₁ and S1P₃ receptor subtypes in rat coronary artery endothelium.

Using syto 61 stain (nuclear stain) allowed discrimination of endothelial cells from vascular smooth muscle cells. Endothelial cells were oval in shape and ran on the surface of the internal elastic lamina or in the grooves of the internal elastic lamina whereas vascular smooth muscle cells were spindle shaped cells and ran underneath the internal elastic lamina and in the opposite direction (Figure 3-1). The SK1 enzyme expression was examined in rat coronary artery endothelium obtained from three different animals. SK1 enzyme was found to be expressed strongly in the endothelial cells of rat coronary artery. In figure 3-2, immunofluorescent staining of SK1 (red) appears as punctate staining in the cytosol of the cells. Specificity of SK1-labelling was examined by comparison of SK1-labelled sections which had been incubated with SK1 antibody pre-absorbed with SK1 peptide antigen (Abcam, ab187382). In those vessels pre-incubated with SK1 peptide antigen, SK1 immunostaining (red) was greatly diminished compared with controls (Figure 3-3). Moreover, S1P₁ and S1P₃ receptors are prominently expressed in endothelial cells of rat coronary artery with S1P₁ apparently located in the cytoplasm and S1P₃ on the plasma membrane (Figure 3-4). Segments of rat CA were also used to examine the co-localisation of SK1 enzyme and S1P₃ receptor subtypes in the endothelial cells. The confocal scan shows that both SK1 enzyme and S1P₃ receptor subtype are abundantly expressed in the same endothelial cells but they were not co-localised (Figure 3-5).

The SK1 immunostaining in rat coronary artery smooth muscle cells was very weak and this might be a result of poor penetration of the antibodies deep to the smooth muscle cells. To test this, I used anti- α smooth muscle actin antibody to test the antibody penetration ability. The signal with the α -actin antibody was very weak, meaning that it is likely the antibodies cannot penetrate the internal and external lamina to immunostain the medial layer (Figure 3-6).

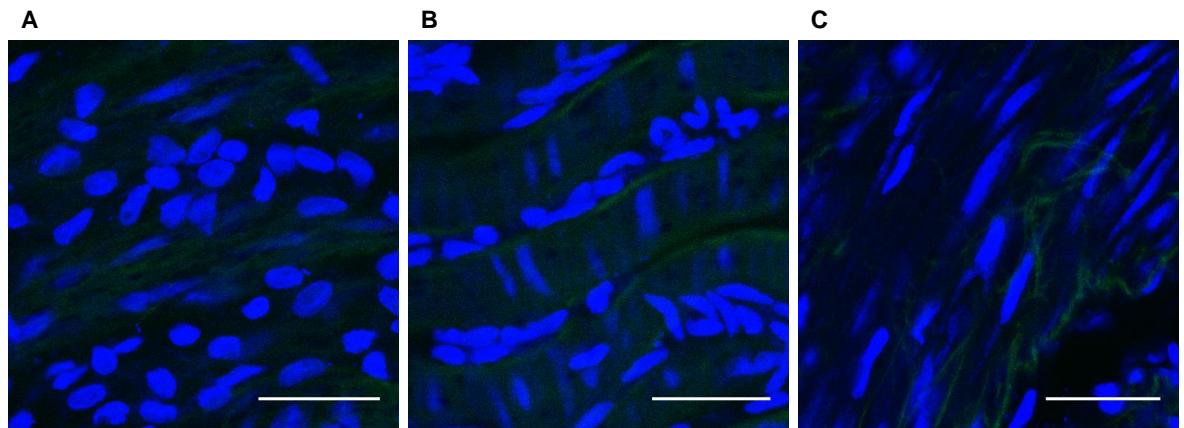


Figure 3-1 Representative images showing the difference between endothelial and smooth muscle cell nuclei of rat coronary artery.

(A) shows oval shaped endothelial cell nuclei (blue) running on the surface of internal elastic lamina (green) and between the grooves of the internal elastic lamina (B). Smooth muscle cells with spindle shaped nuclei run underneath the internal elastic lamina (C). Scale bar is 30 μm in all images. Images are representative of at least 3 experiments.

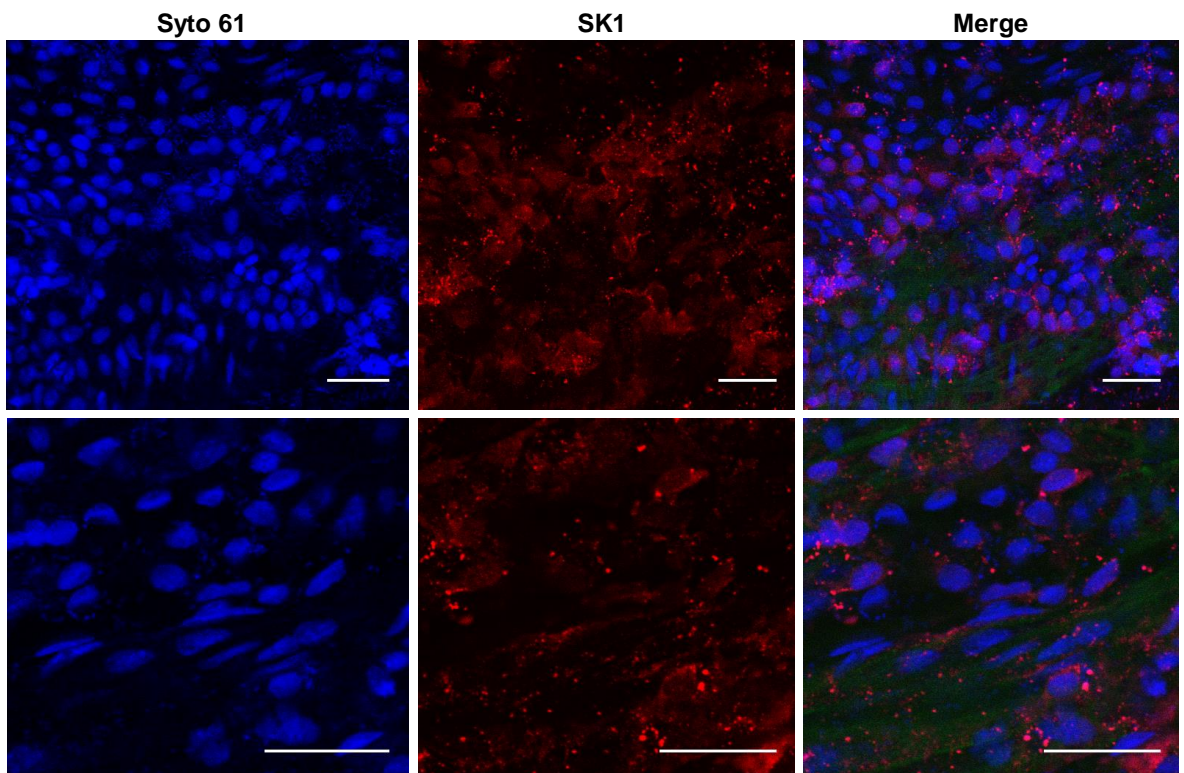


Figure 3-2 Expression of SK1 in endothelial cells of rat coronary artery.

Rat coronary artery sections were immunostained for SK1 (red) with anti-SK1 antibody. Syto 61 nuclear stain was used to stain the endothelial and smooth muscle cell nuclei (blue). The green colour in the merged image represents the autofluorescence of internal elastic lamina. Scale bar is 30 μm in all images. Images are representative of at least 3 separate experiments.

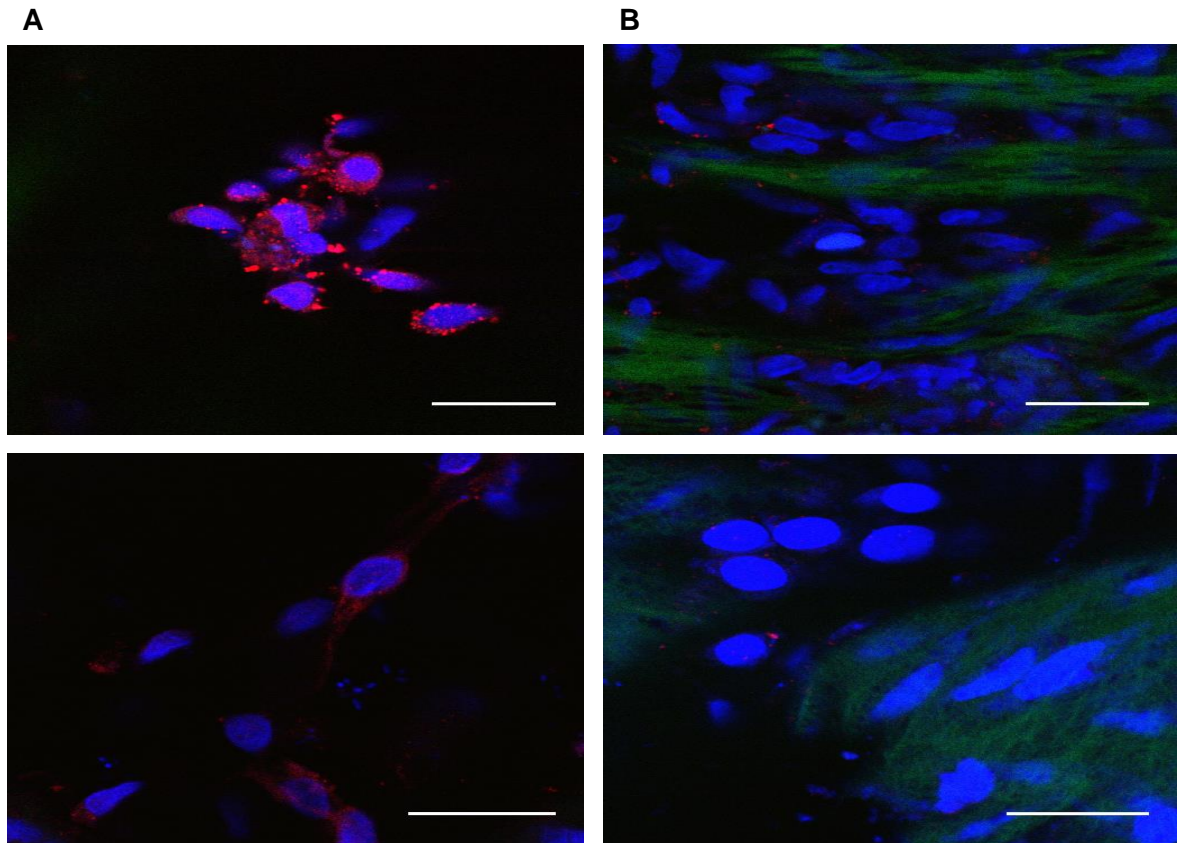


Figure 3-3 Anti-SK1 antibody specificity.

Representative immunofluorescent images for SK1 (red) expression (panel A) in control sections. Panel B demonstrates that addition of blocking peptide greatly decreases the detection of SK1 by immunofluorescence. Results are representative of 3 separate experiments. Scale bar is 20 μm for all images.

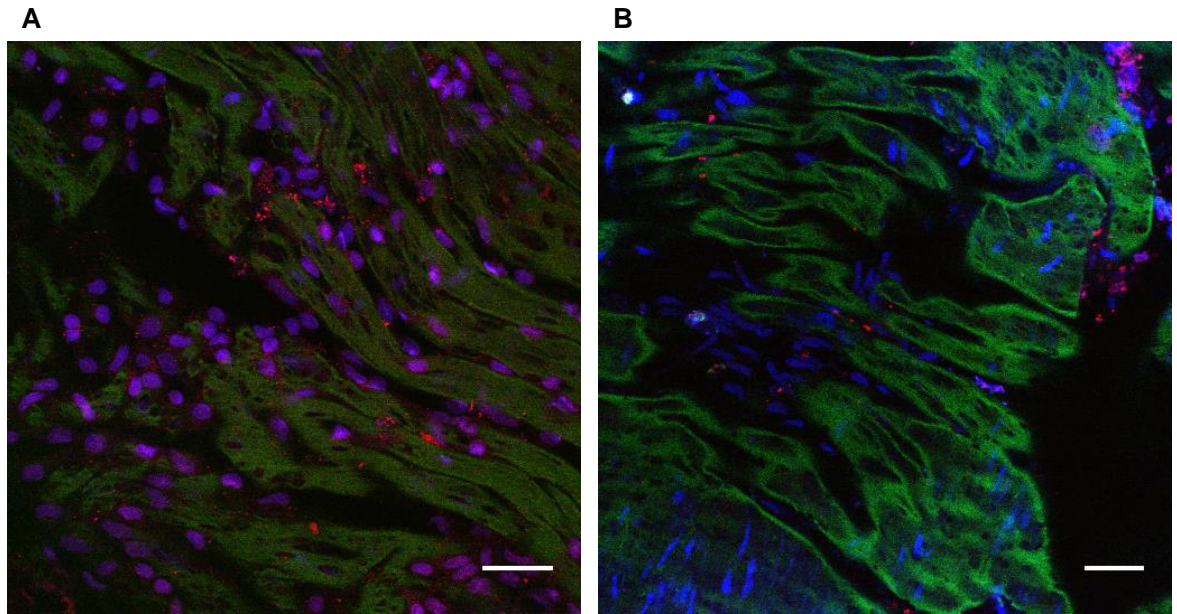


Figure 3-4 S1P₁ and S1P₃ receptor expression in rat coronary artery endothelium.

Representative immunofluorescent images (of 2 experiments) for S1P₁ receptors (red) (A) and S1P₃ receptors (red) (B). Scale bar is 30 μm for all images.

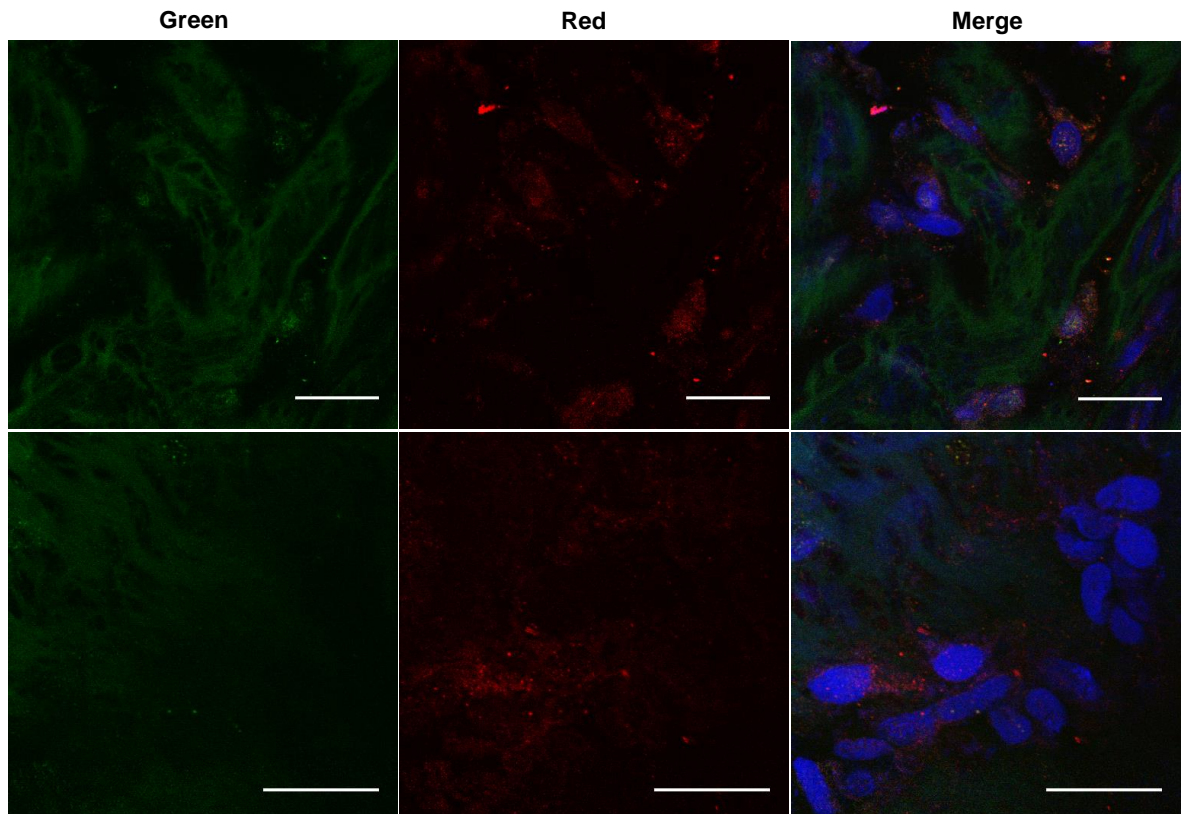


Figure 3-5 Expression of SK1 enzyme and S1P₃ receptors in rat CA endothelium.

Representative immunofluorescent images demonstrate the expression of SK1 (red) and S1P₃ receptor subtype (green) in rat CA endothelial cells. S1P₃ receptors (green) are differentiated from internal elastic lamina autofluorescence (green) by their punctate appearance. Results are representative of 3 separate experiments. Scale bar is 30 μm for all images.

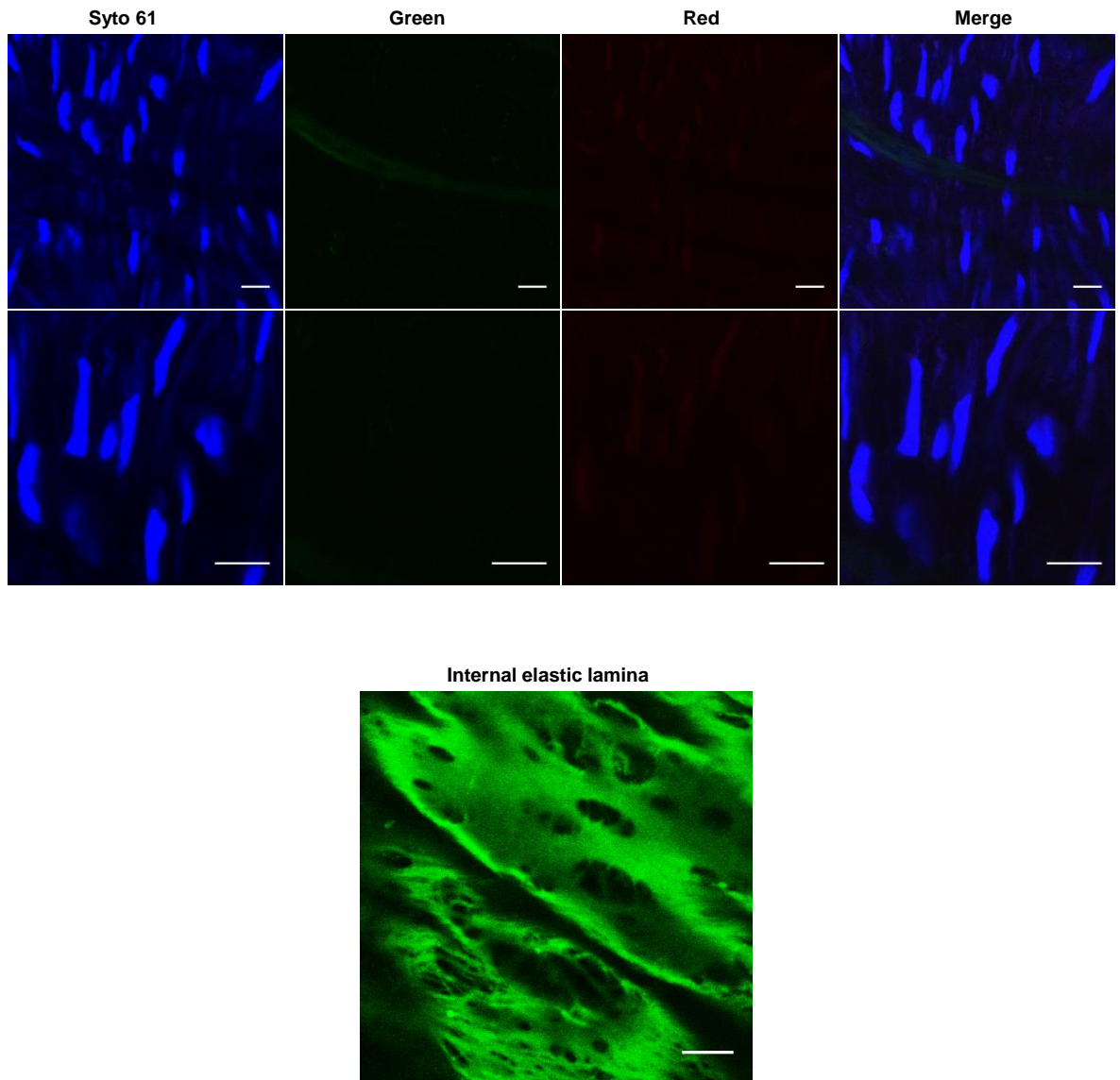


Figure 3-6 Anti- α smooth muscle actin antibody.

Immunofluorescent images showing a very weak immunostaining of α smooth muscle actin in rat CA smooth muscle cells (red). Internal (green) and external elastic lamina may reduce the antibody penetration to the smooth muscle cells. Green colour is the autofluorescence of internal elastic lamina collagen. Scale bar is 10 μ m for all images.

3.3.2 Effect of hypoxia on SK1 enzyme expression in vascular endothelium.

Exposure of rat coronary arteries to hypoxia for 30 min resulted in a significant increase (41%) in integrated density (total amount of fluorescence) $1.7 \times 10^6 \pm 2.4 \times 10^5$ AU, n=5 compared with the corresponding levels in arteries maintained under normoxic conditions ($1.0 \times 10^6 \pm 5.2 \times 10^5$ AU, n=4; Figure 3-7). There was also a significant increase in phosphorylated-SK1 (pSK1) integrated density in arteries subjected to hypoxia ($2.8 \times 10^6 \pm 2.7 \times 10^5$ AU, n=3) compared with the corresponding levels in controls ($1.5 \times 10^6 \pm 1.9 \times 10^5$ AU, n=3; Figure 3-8).

3.3.3 Effect of cycloheximide on hypoxia-induced increase in SK1 expression.

Pre-treatment of coronary arteries with cycloheximide for 30 min under normoxic conditions led to a small reduction in SK1 integrated density ($0.79 \times 10^6 \pm 2.1 \times 10^5$ AU, n=3) compared with controls ($1.0 \times 10^6 \pm 5.2 \times 10^5$ AU, n=4; Figure 3-9). In those arteries pre-treated with cycloheximide prior to exposure to hypoxia, there was a significant reduction (60%) in integrated density of SK1 ($0.68 \times 10^6 \pm 2.8 \times 10^5$ AU, n=3) compared with controls ($1.7 \times 10^6 \pm 2.4 \times 10^5$ AU, n=5; Figure 3-9).

3.3.4 Effect of sphingosine kinase inhibitors on hypoxia-induced increase in SK1 expression.

Under normoxic conditions, pre-treatment with SKi significantly decreased the integrated density of SK1 (67%) in rat CA endothelium ($0.33 \times 10^6 \pm 0.21 \times 10^5$ AU, n=3) compared with controls ($1.0 \times 10^6 \pm 5.2 \times 10^5$ AU, n=4). Treatment with the selective nM potent SK1 inhibitor PF543 visually appeared to reduce SK1 immunostaining (red punctate staining in the images) but image J software yielded no significant differences in the integrated density of SK1 and this is likely because image J is also quantifying the background noise in these images ($0.93 \times 10^6 \pm 1.7 \times 10^5$ AU, n=4). The selective SK2 inhibitor ROME had no effect on SK1 expression under normoxic conditions ($1.2 \times 10^6 \pm 2.6 \times 10^5$ AU, n=4; Figure 3-10). However, both SKi and PF543 markedly reduced the increase in SK1 expression caused by hypoxia when added 30 min prior to exposure to hypoxia ($0.41 \times 10^6 \pm 1.9 \times 10^5$ AU, n=3 (76%)) and ($0.45 \times 10^6 \pm 1.4 \times 10^5$ AU, n=4 (73%)) respectively compared with controls ($1.7 \times 10^6 \pm 2.4 \times 10^5$ AU, n=5). ROME had no effect on the hypoxia-induced increase in SK1 expression ($1.7 \times 10^6 \pm 1.9 \times 10^5$ AU, n=5; Figure 3-11).

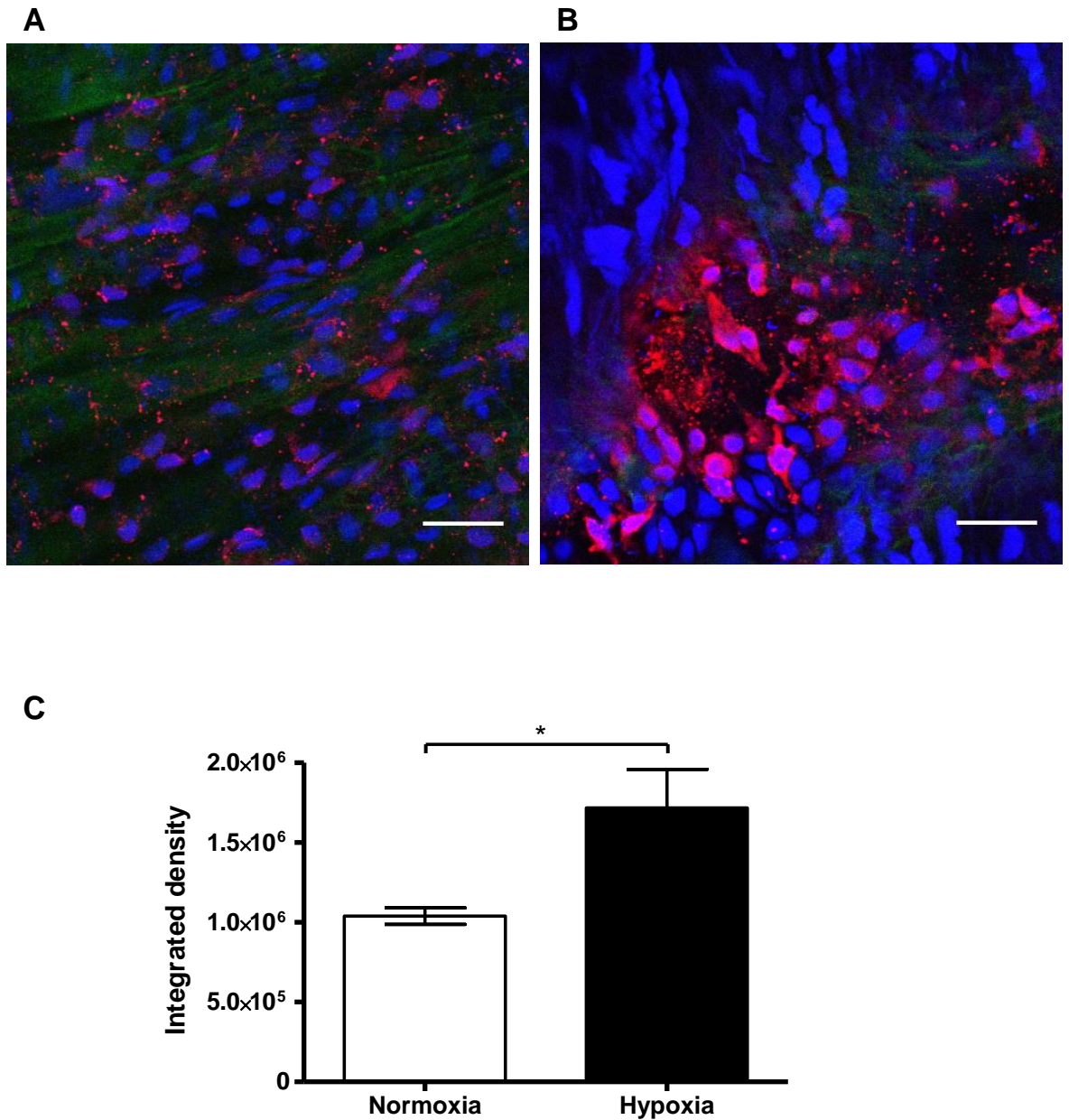


Figure 3-7 Effect of hypoxia on SK1 expression levels.

Immunofluorescent images show expression of SK1 (red) in rat coronary artery endothelium under normoxic conditions (A) and hypoxic conditions (B). (C) Quantitative fluorescence measurement of SK1 under normoxia and hypoxia. Scale bar is 30 μm for both images. * $p < 0.05$ vs normoxia as determined by unpaired t test. Results are representative of 4-5 separate experiments.

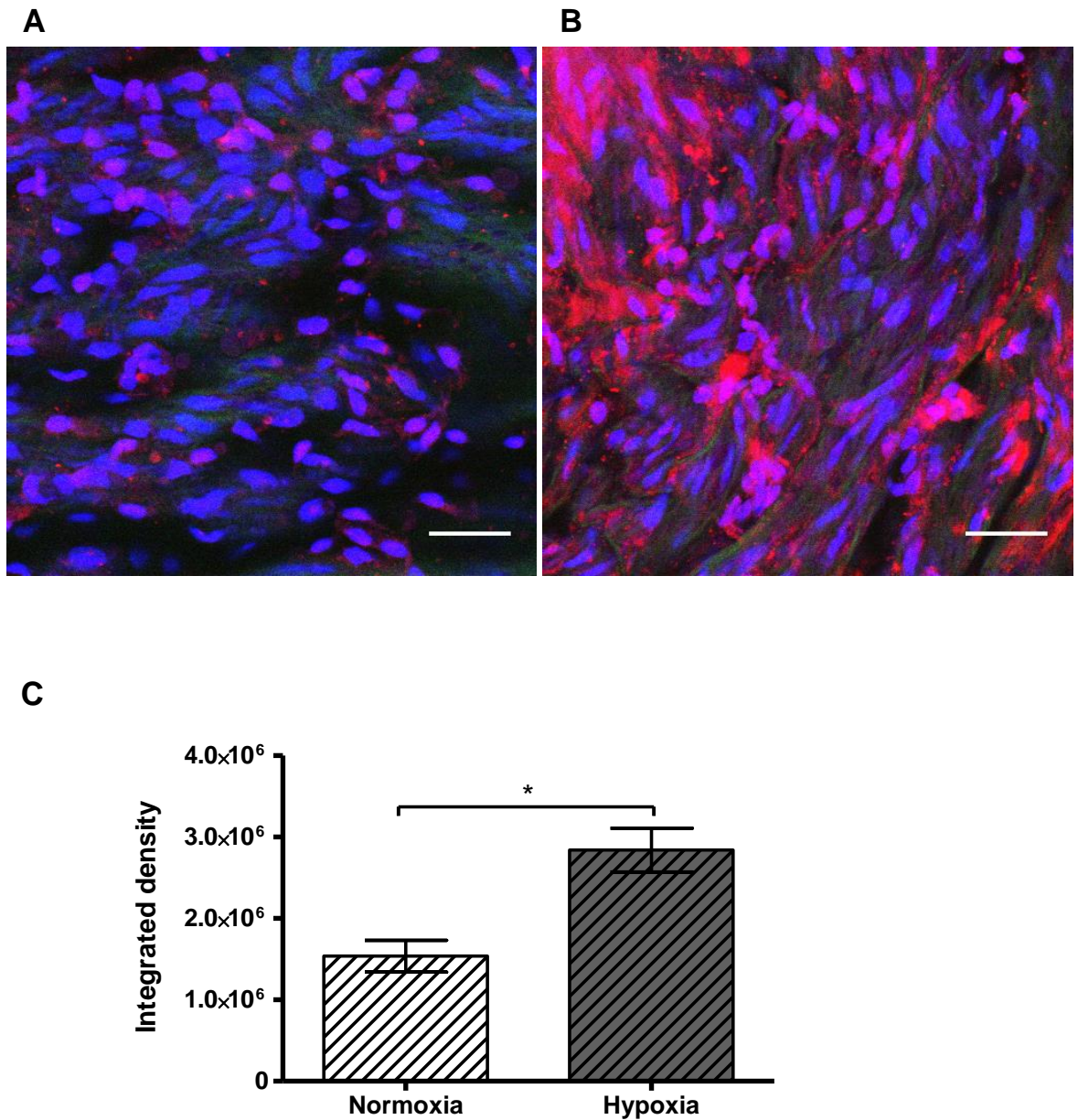


Figure 3-8 Effect of hypoxia on pSK1 levels.

Immunofluorescent images showing effect of hypoxia on pSK1 (red) level in rat coronary artery endothelium (B) compared with control (A). (C) Quantitative fluorescence measurement of pSK1 under normoxia and hypoxia. Scale bar is 30 μm for both images. * $p < 0.05$ vs normoxia as determined by unpaired t test. Results are representative of 3 separate experiments.

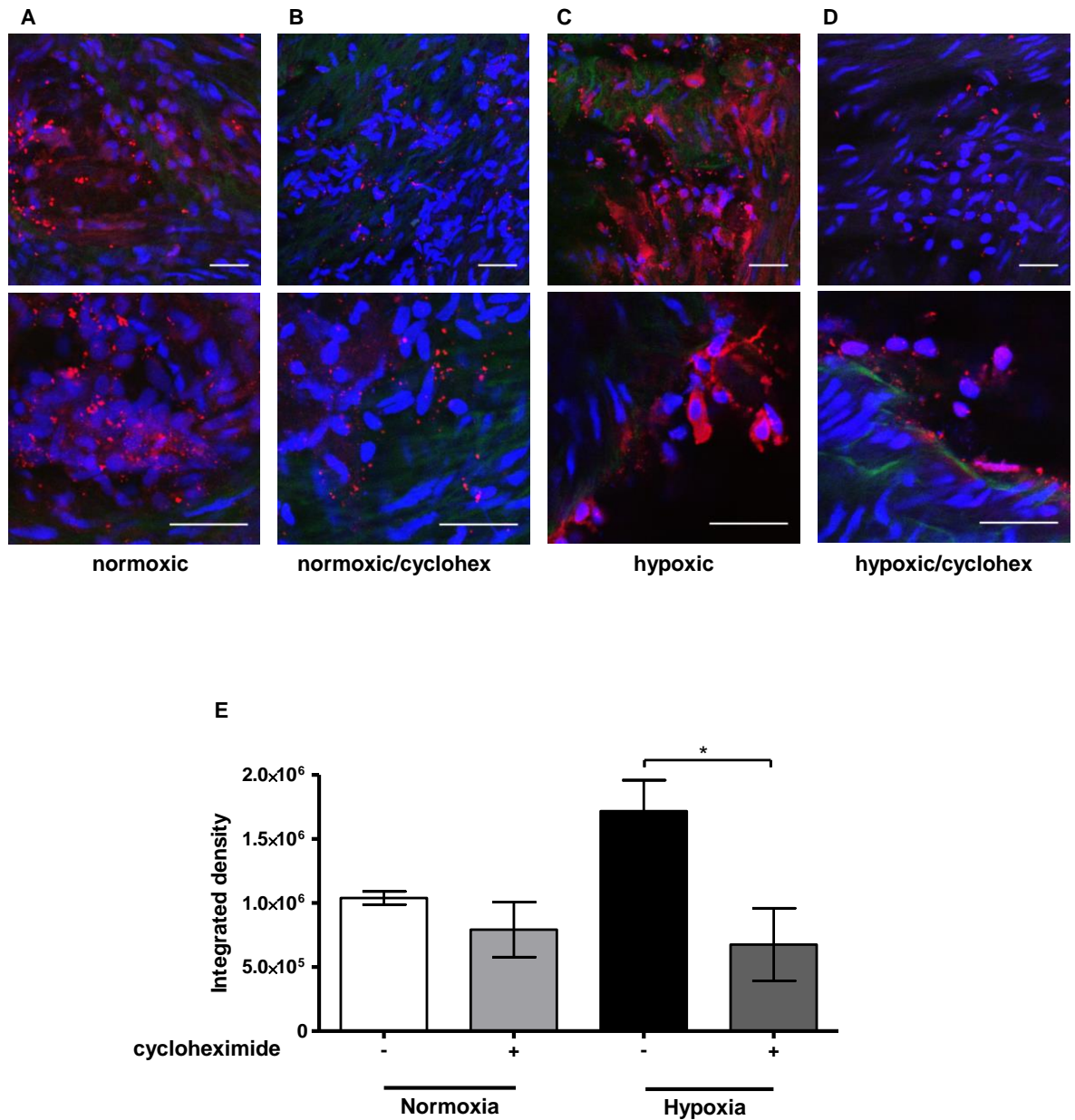


Figure 3-9 Effect of cycloheximide on hypoxia-induced increase in SK1 expression.

Immunofluorescent images show expression of SK1 (red) in rat coronary artery endothelium (A) and following pre-treatment with cycloheximide (10 μ M, 30 min) under normoxic conditions (B). Expression of SK1 in rat coronary artery subjected to 30 min hypoxia (C). Preincubation of rat coronary artery with cycloheximide reduced the hypoxia-induced increase in SK1 expression (D). (E) Quantitative fluorescence measurement of SK1 in the presence and absence of cycloheximide. * $p < 0.05$ vs hypoxia alone as determined by one way ANOVA with Bonferroni multiple comparison post-tests. Results are representative of 3-5 separate experiments. Scale bar is 30 μ m for all images.

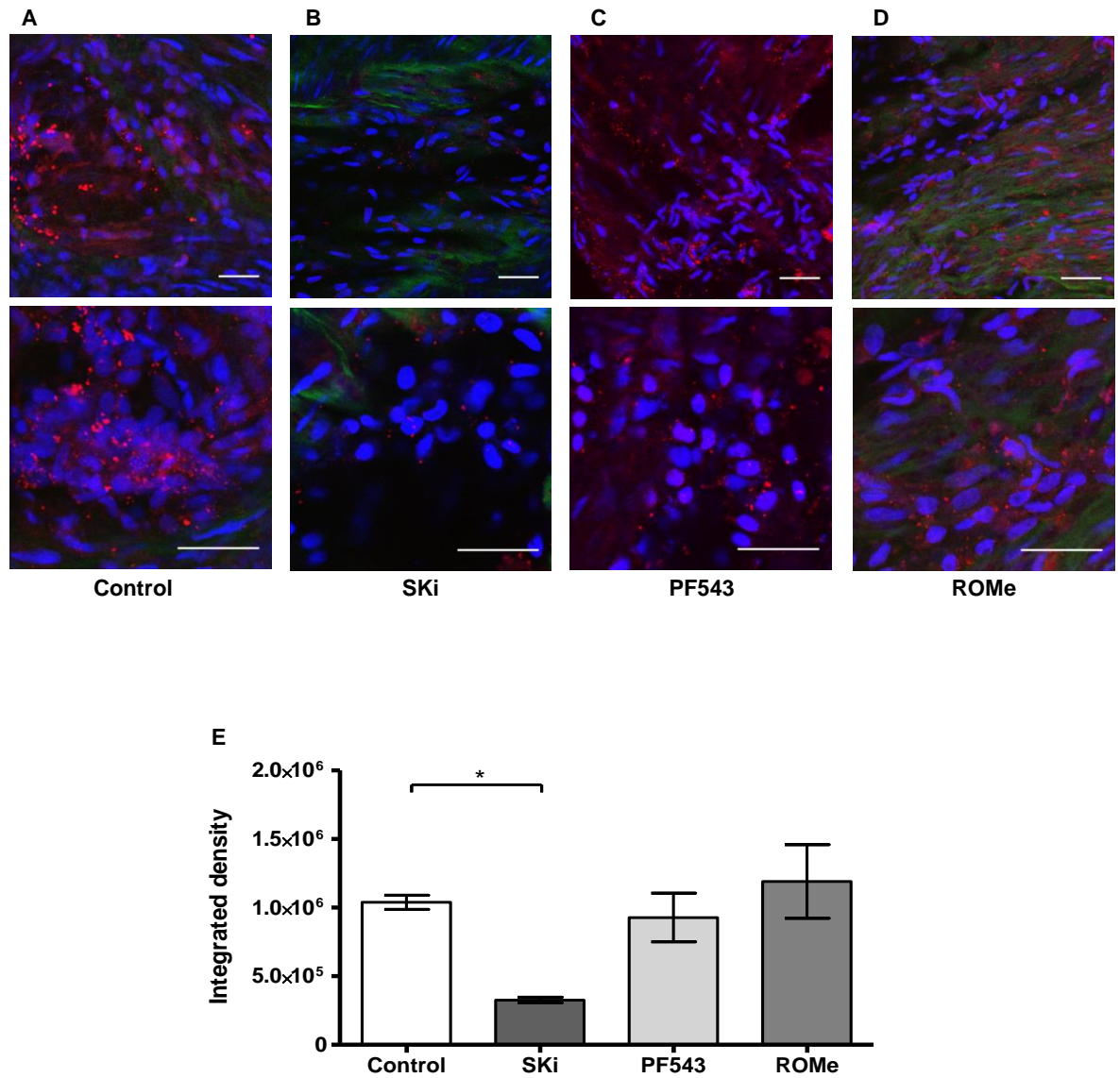


Figure 3-10 Effect of Spingosine Kinase inhibitors on SK1 expression in rat coronary artery endothelium under normoxia.

Immunofluorescent images showing expression of SK1 (red) in rat coronary artery endothelium (A) and following pre-treatment with SKi (10 μM, 30 min) (B), PF543 (100 nM, 30 min) (C) and ROME (10 μM, 30 min) (D). (E) Quantitative fluorescence measurement of SK1 in the presence and absence of Spingosine Kinase inhibitors. *p<0.05 vs control as determined by one way ANOVA with Bonferroni multiple comparison post-tests. Results are representative of 3-4 separate experiments. Scale bar is 30 μm for all images.

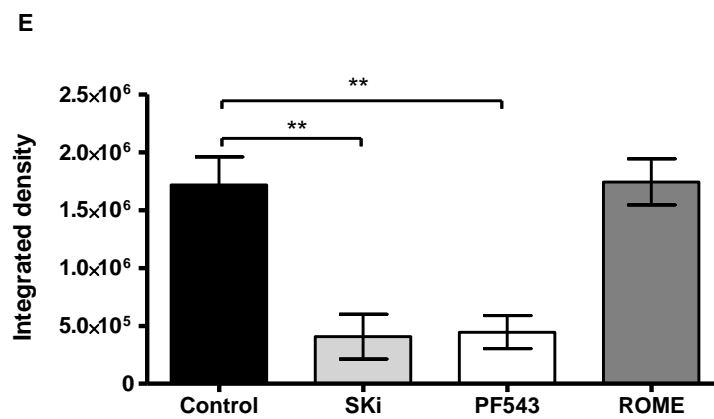
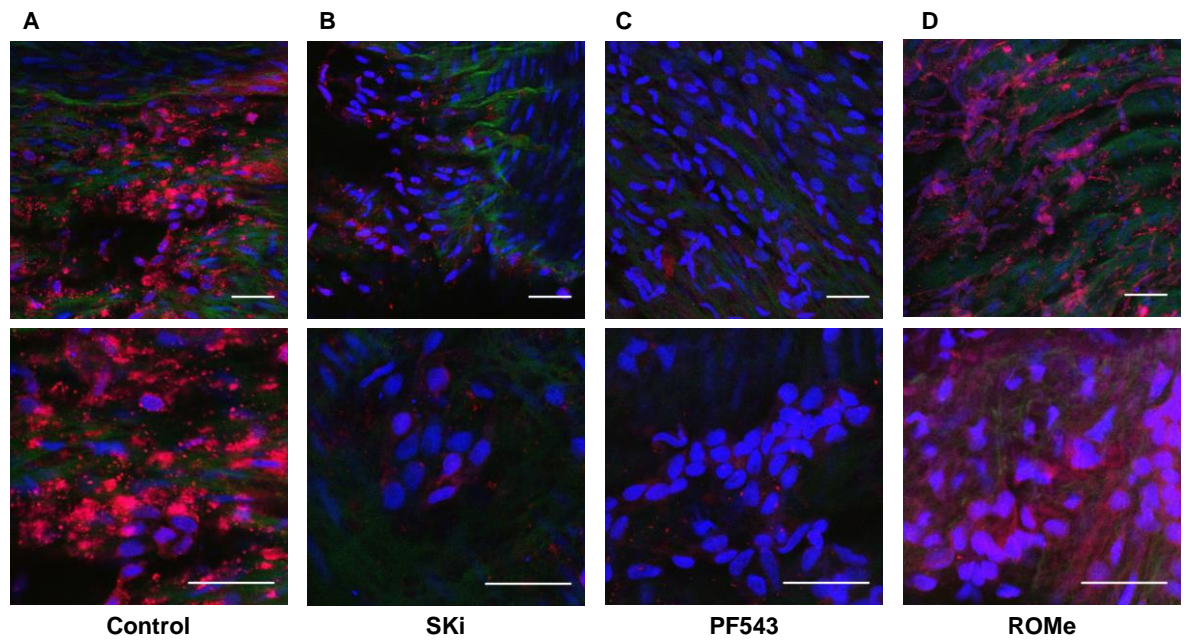


Figure 3-11 Effect of Sphingosine Kinase inhibitors on SK1 expression in rat coronary artery endothelium under hypoxia.

Immunofluorescent images showing an increase in SK1 (red) expression caused by hypoxia compared to normoxia (not shown) (A) and following pre-treatment with SKi (10 μ M, 30 min) (B), PF543 (100 nM, 30 min) (C) and ROME (10 μ M, 30 min) (D). (E) Quantitative fluorescence measurement of SK1 in the presence and absence of Sphingosine Kinase inhibitors. ** $p < 0.01$ vs control as determined by one way ANOVA with Bonferroni multiple comparison post-tests. Results are representative of 3-5 separate experiments. Scale bar is 30 μ m for all images.

3.3.5 Expression of SK1 in presence of degradation inhibitors in vascular endothelium.

Preincubation of rat coronary arteries with MG132, lactacystin, CA-074ME, and a combination of MG132 and CA-074ME had no effect on integrated density of SK1 compared with controls under normoxia (Figure 3-12 and Table 3-1) whereas under hypoxic conditions, only MG132 significantly inhibited the hypoxic-induced increase in SK1 integrated density (Figure 3-13 and Table 3-2).

Table 3-1 Integrated density for SK1 in presence of degradation inhibitors under normoxia

Agent (All agents used at 10 μ M concentration)	Integrated density (\pm S.E.) in AU	Number of separate experiments (n)
Control	$1.0 \times 10^6 \pm 0.52 \times 10^5$	4
MG132	$1.2 \times 10^6 \pm 3.6 \times 10^5$	4
Lactacystin	$1.2 \times 10^6 \pm 1.5 \times 10^5$	4
CA-074ME	$0.79 \times 10^6 \pm 0.65 \times 10^5$	3
MG132+CA-074ME	$1.5 \times 10^6 \pm 0.69 \times 10^5$	3

Table 3-2 Integrated density for SK1 in presence of degradation inhibitors under hypoxia

Agent (All agents used at 10 μ M concentration)	Integrated density (\pm S.E.) in AU	Number of separate experiments (n)
Control	$1.7 \times 10^6 \pm 2.4 \times 10^5$	5
MG132	$1.05 \times 10^6 \pm 1.2 \times 10^5$ *	6
Lactacystin	$2.0 \times 10^6 \pm 4.1 \times 10^5$	4
CA-074ME	$1.4 \times 10^6 \pm 1.4 \times 10^5$	3
MG132+CA-074ME	$1.4 \times 10^6 \pm 2.7 \times 10^5$	3

* $p < 0.05$ vs control (one way ANOVA with Bonferroni's post test).

3.3.6 Effect of SKi on degradation of SK1.

So far, it was shown that SK1 expression increased during hypoxia, an effect which involves protein synthesis and can be blocked by SK1 inhibitors but not SK2 inhibitors. A number of studies have demonstrated that SKi induces proteasomal degradation of SK1 in several cell lines such as human pulmonary artery smooth muscle cells, androgen-sensitive LNCaP prostate cancer cells, MCF-7 and MCF-7 HER2 breast cancer cells (Loveridge et al., 2010) and lysosomal degradation in human endothelial cell line EA.hy 926, glomerular podocytes and mesangial cells (Ren et al., 2010). Therefore I investigated the mechanism by which SKi decreases SK1 in rat CA endothelium. In this regard, pre-treatment of rat coronary arteries with MG132, CA-074ME or a combination of MG132 and CA-074ME significantly reversed the SKi-induced reduction in integrated density of SK1 under normoxic conditions, but pre-treatment with lactacystin had no effect (Figure 3-14)(Table 3-3). Under hypoxia, the SKi-induced reduction in SK1 integrated density was also reversed when the arteries were pre-treated with MG132, CA-074ME or a combination of both MG132 and CA-074ME, but preincubation with lactacystin did not inhibit the SKi-induced reduction in SK1 integrated density (Figure 3-15)(Table 3-4).

Table 3-3 Effect of SKi on degradation of SK1 under normoxic conditions

Agent (All agents used in 10 μ M concentration)	Integrated density (\pm S.E.) in AU	Number of separate experiments (n)
Control	$1.0 \times 10^6 \pm 0.52 \times 10^5$ *	4
SKi	$0.33 \times 10^6 \pm 0.21 \times 10^5$	3
MG132+SKi	$1.4 \times 10^6 \pm 2.9 \times 10^5$ **	3
Lactacystin+SKi	$0.65 \times 10^6 \pm 1.0 \times 10^5$	4
CA-074ME+SKi	$0.96 \times 10^6 \pm 1.9 \times 10^5$ *	5
MG132+CA-074ME+SKi	$1.7 \times 10^6 \pm 3.0 \times 10^5$ ***	4

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs SKi (one way ANOVA with Bonferroni's post test).

Table 3-4 Effect of SKi on degradation of SK1 under hypoxic conditions

Agent (All agents used in 10 μ M concentration)	Integrated density (\pm S.E.) in AU	Number of separate experiments (n)
Control	$1.7 \times 10^6 \pm 2.4 \times 10^5$ **	5
SKi	$0.41 \times 10^6 \pm 1.9 \times 10^5$	3
MG132+SKi	$1.3 \times 10^6 \pm 0.32 \times 10^5$ *	5
Lactacystin+SKi	$0.98 \times 10^6 \pm 1.4 \times 10^5$	5
CA-074ME+SKi	$1.9 \times 10^6 \pm 4.8 \times 10^5$ **	3
MG132+CA-074ME+SKi	$2.3 \times 10^6 \pm 2.9 \times 10^5$ ***	4

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs SKi (one way ANOVA with Bonferroni's post test)

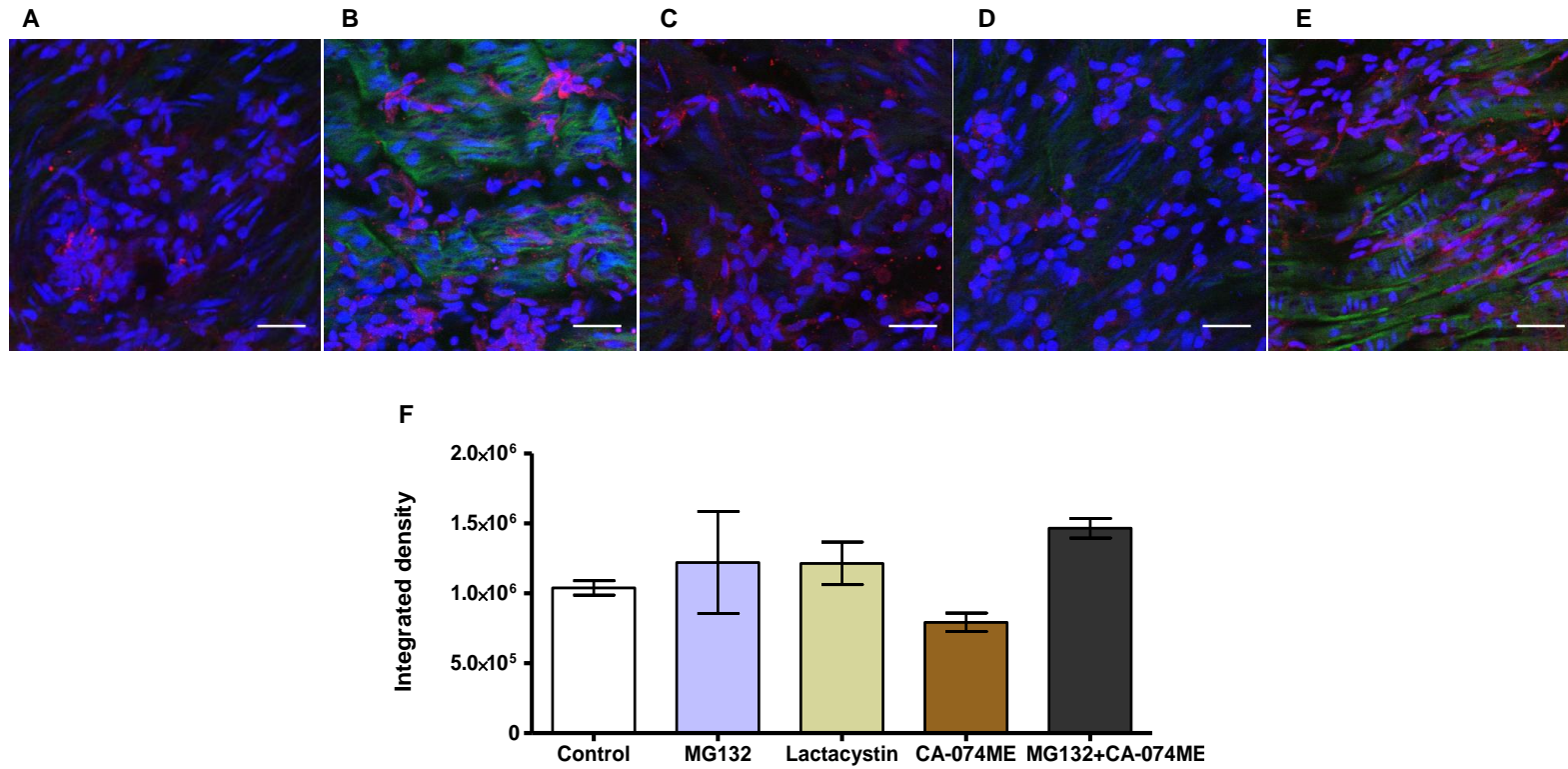


Figure 3-12 Effect of MG132, Lactacystin, CA-074ME and a combination of MG132 and CA-074ME on SK1 in rat coronary artery endothelium under normoxia.

Immunofluorescent images showing expression of SK1 in rat CA endothelium (A) and the effect of pre-treatment with (B) MG132 (10 μM, 30 min), (C) Lactacystin (10 μM, 30 min), (D) CA-074ME (10 μM, 30 min) and a combination of MG132 and CA-074ME (10 μM, 30 min)(E) on SK1 in rat CA endothelial cells under normoxic conditions. (F) Quantitative fluorescence measurement of SK1 in the presence and absence of degradation inhibitors. Results are representative of 3-4 separate experiments. Scale bar is 30 μm for all images.

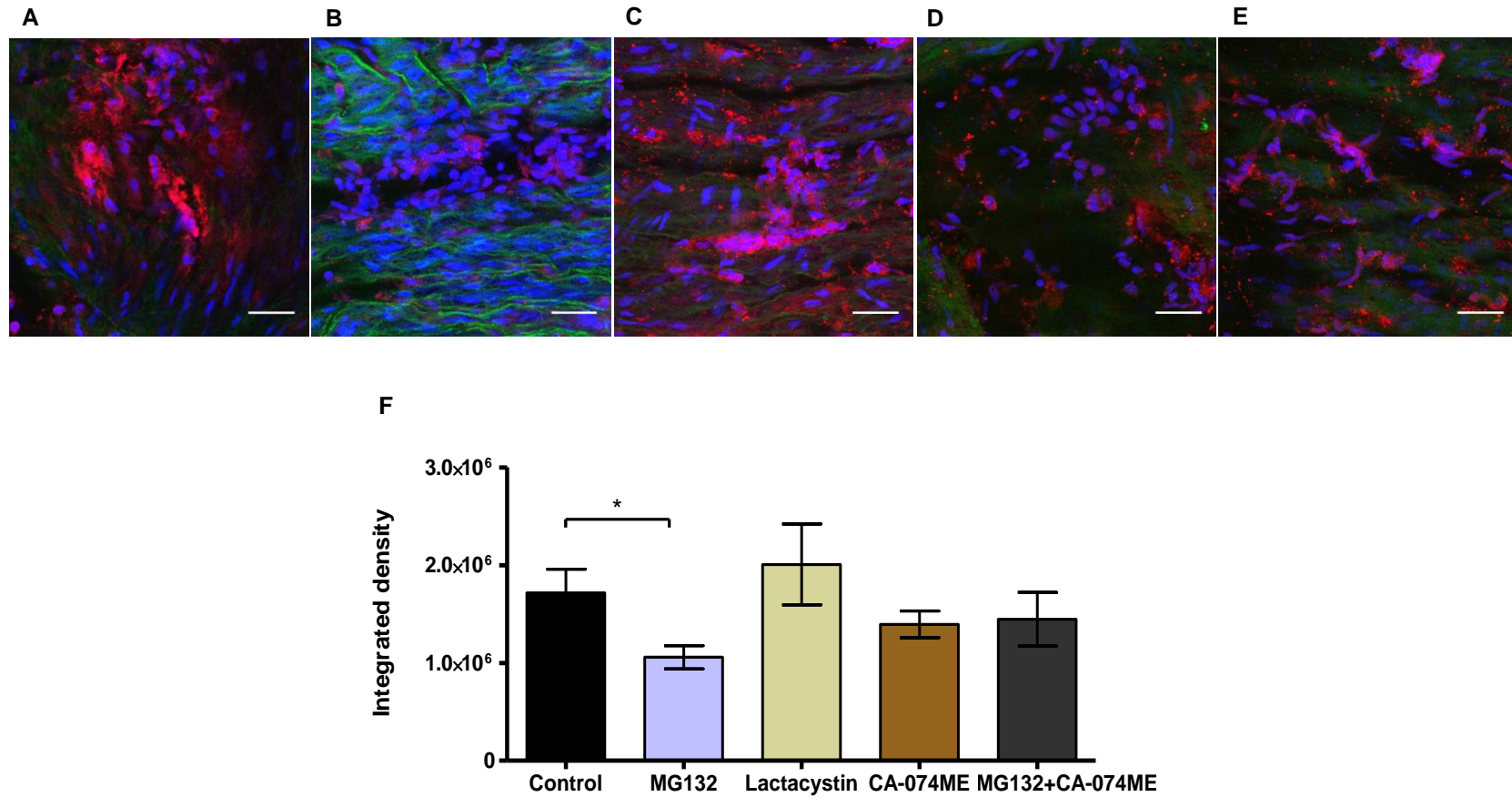


Figure 3-13 Effect of MG132, Lactacystin, CA-074ME and a combination of MG132 and CA-074ME on SK1 in rat coronary artery endothelium under hypoxia.

Immunofluorescent images showing expression of SK1 in rat CA endothelium (A) and the effect of pre-treatment with (B) MG132 (10 μ M, 30 min), (C) Lactacystin (10 μ M, 30 min), (D) CA-074ME (10 μ M, 30 min) and a combination of MG132 and CA-074ME (10 μ M, 30 min)(E) on SK1 in rat CA endothelial cells under hypoxic conditions. (F) Quantitative fluorescence measurement of SK1 in the presence and absence of degradation inhibitors. * $p < 0.05$ vs control as determined by one way ANOVA with Bonferroni's post test. Results are representative of 3-6 separate experiments. Scale bar is 30 μ m for all images.

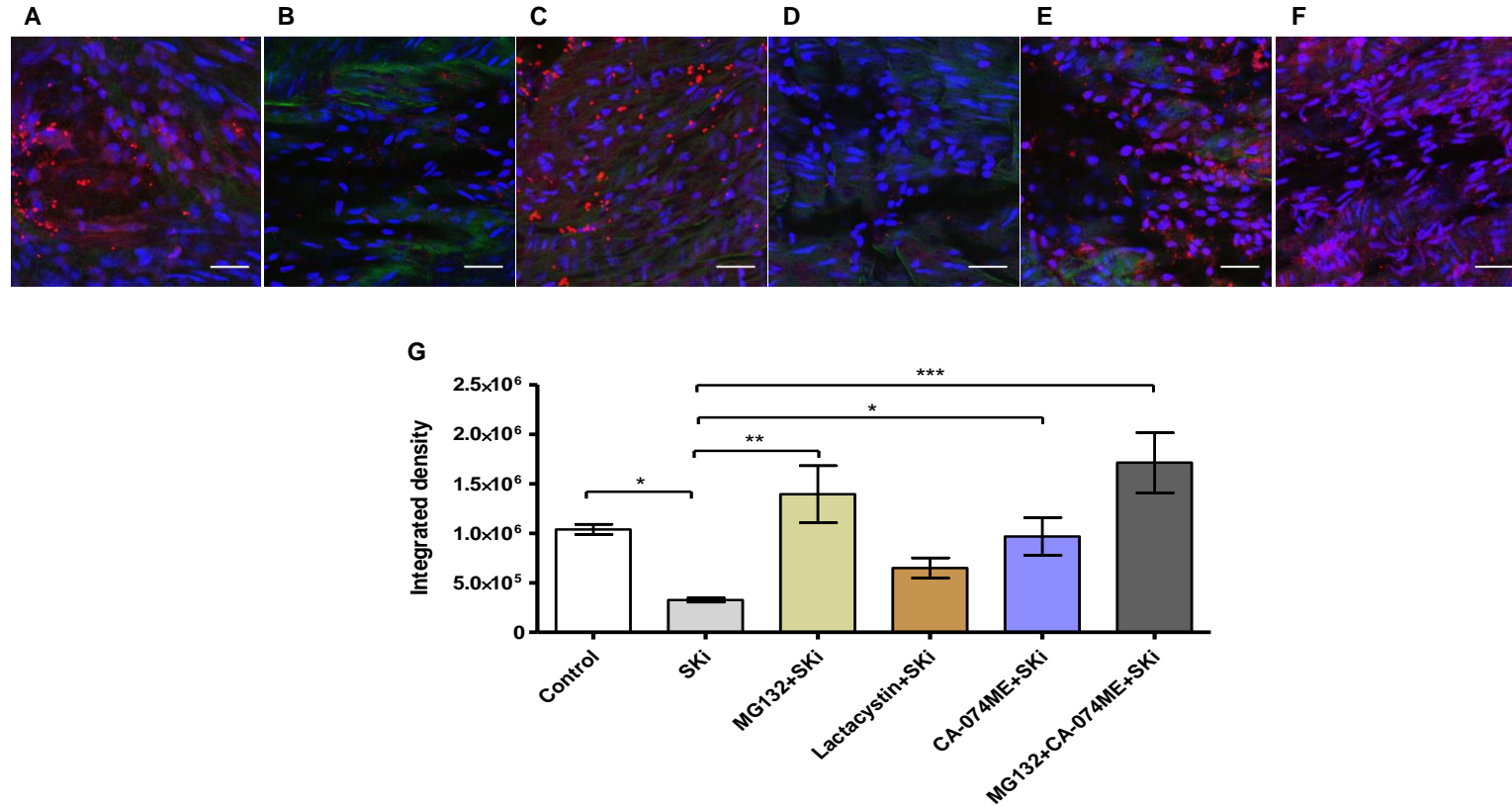


Figure 3-14 Effect of degradation inhibitors on SKi-induced downregulation of SK1 in rat CA endothelium under normoxia.

Immunofluorescent images showing expression of SK1 (red) in rat CA endothelium (A) and effect of SKi (10 μM, 30 min) on SK1 expression in rat CA endothelium (B); Significant reversal effect of pre-treatment with (C) MG132 (10 μM, 30 min); (D) Lack of reversal effect with Lactacystin pre-treatment (10 μM, 30 min); (E) Reversal by CA-074ME pre-treatment (10 μM, 30 min) of SKi (10 μM, 30 min)-induced reduction of SK1 in rat CA endothelium; (F) reversal by a combination of MG132 and CA-074ME (both 10 μM, 30 min pre-treatment) of SKi (10 μM, 30 min)-induced downregulation of SK1 in rat CA endothelium. (G) Quantitative fluorescence measurement of SK1 in the presence and absence of degradation inhibitors and SKi. * $p < 0.05$ vs SKi as determined by one way ANOVA with Bonferroni's post test. Results are representative of at least 3-5 separate experiments. Scale bar is 30 μm for all images.

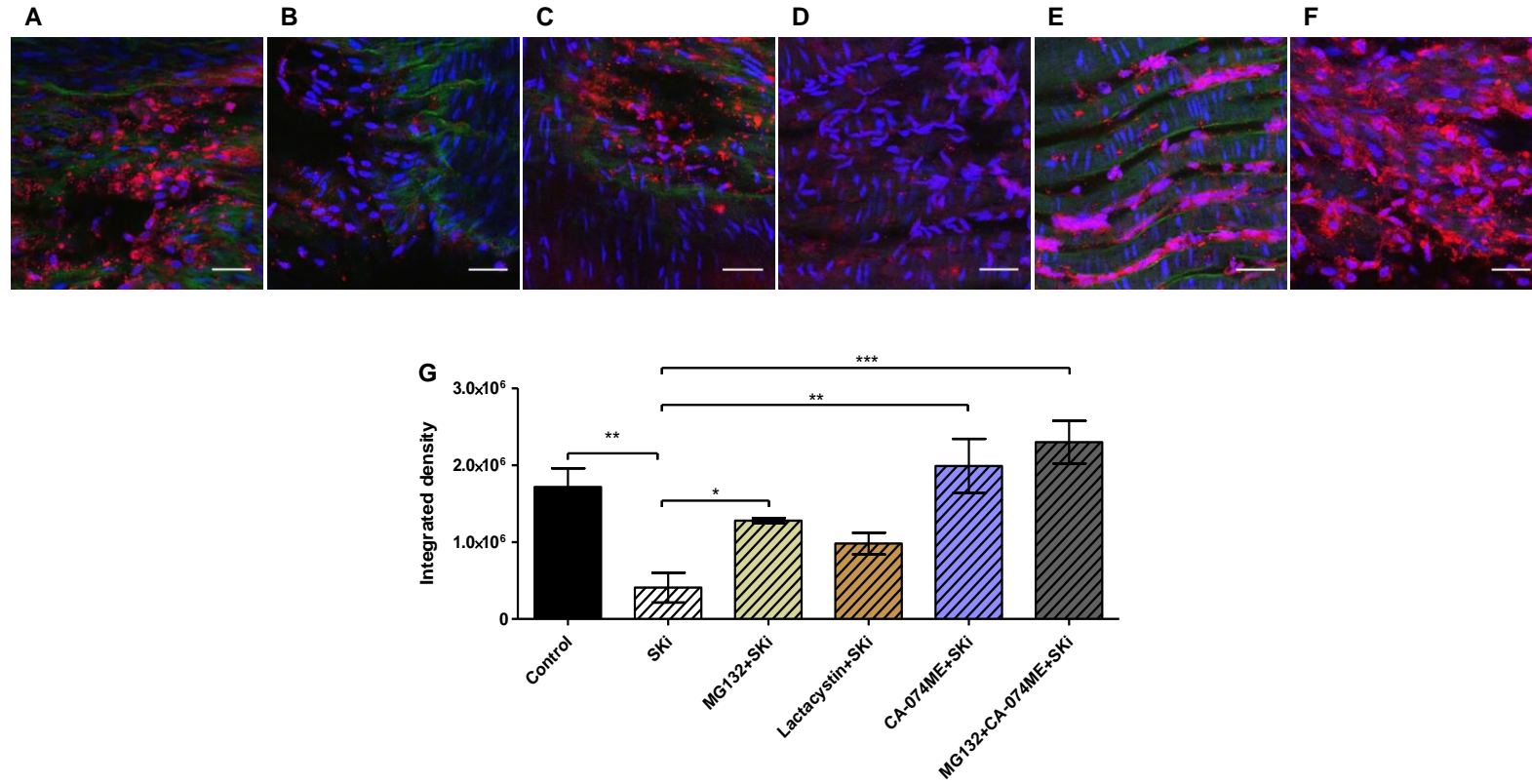


Figure 3-15 Effect of degradation inhibitors on SKI-induced downregulation of SK1 in rat CA endothelium under hypoxia.

Immunofluorescent images showing expression of SK1 (red) in rat CA endothelium (A) and effect of SKI (10 μM, 30 min) on SK1 expression in rat CA endothelium (B); Significant reversal effect of pre-treatment with (C) MG132 (10 μM, 30 min); (D) Lack of reversal by Lactacystin pre-treatment (10 μM, 30 min); (E) reversal by CA-074ME (10 μM, 30 min pre-treatment) on SKI (10 μM, 30 min)-induced reduction of SK1 in rat CA endothelium; (F) reversal by a combination of MG132 and CA-074ME (all 10 μM, 30 min pre-treatment) of SKI (10 μM, 30 min)-induced downregulation of SK1 in rat CA endothelium. (G) Quantitative fluorescence measurement of SK1 in the presence and absence of degradation inhibitors and SKI under hypoxic conditions. **p<0.01, ***p<0.001 vs SKI as determined by one way ANOVA with Bonferroni's post test. Results are representative of 3-5 separate experiments. Scale bar is 30 μm for all images.

3.4 Discussion

At the onset of this study, a series of preliminary experiments was conducted to establish the most appropriate vessel preparation and incubation conditions for the experimental protocol. It was important to use open vessel preparations to overcome the problem associated with poor penetration of lasers through the arterial wall to visualize the endothelial and smooth muscle cells. However even with open vessel preparations, it was not possible to visualize the proteins of interest present in smooth muscle cells due to poor antibody penetration to this depth. Several antibody dilutions were tested (1:250, 1:100, 1:50) to determine the most appropriate dilution to immunostain the protein of interest. Syto 61 nuclear stain was used to stain the endothelial and smooth muscle cell nuclei and allow differentiation between them by virtue of their shape and positions.

The lipid mediator S1P is produced by phosphorylation of membrane associated sphingosine by sphingosine kinase. Once generated, S1P is either transported out of the cell to exert its function via a family of five GPCRs named S1P₁₋₅ or if retained within the cell, it functions as an intracellular second messenger. In blood vessels, S1P plays an important role in enhancing endothelial cell spreading, vascular maturation/stabilization, barrier function and regulation of vascular reactivity. The role of the SK/S1P pathway in protection against ischaemic injury has been studied extensively in cardiomyocytes, however little is known about the effect of hypoxia/ischaemia on SK levels in coronary arteries. One of the aims of this part of the study was to examine the distribution of SK1 enzyme and S1P receptors in rat CA endothelial cells. Previous immunohistochemical studies using a rabbit polyclonal antibody directed against the C terminus of mouse SK1 and human SK1, found a strong positive immunostaining in different tissues such as the cerebrum, cerebellum, kidney, platelets, endothelial cells of blood vessels and lung tissue (Murate et al., 2001, Johnson et al., 2005). In the present study, a polyclonal rabbit antibody to SK1 was used to examine the immunofluorescence localisation of SK1 in rat CA endothelial cells. The specificity of the detection of SK1 by the polyclonal rabbit antibody was tested using a blocking peptide. In those arteries probed with the antibody that was immunoabsorbed with the blocking peptide, there was a great reduction in detection of SK1 (Figure 3-3). Prominent SK1 immunostaining was detected primarily in the cytosol of the endothelial cells and it had a punctate appearance in the control vessels (Figure 2-3 & Figure 3-3). Several studies have shown that S1P promotes endothelial cell survival and migration via S1P₁ and S1P₃ receptors (Kimura et al., 2001, Kimura et al.,

2003) and induces vasorelaxation of rat aorta and coronary arteries via S1P₃ receptors (Nofer et al., 2004, Mair et al., 2010). Since SK is likely to generate S1P, it was important to study if there is co-localisation of S1P receptors with the SK1 enzyme. Polyclonal antibodies to S1P₁ and S1P₃ receptors were used to examine immunoexpression of S1P₁ and S1P₃ receptor subtypes in rat CA endothelial cells. Previously, these antibodies were demonstrated to be quite immunospecific for detection of S1P₁ receptors by Western blot analysis of total cell lysate from human umbilical vein endothelial cells (Igarashi et al., 2014) and S1P₃ receptors by immunofluorescent staining method in bone marrow-derived cells (Li et al., 2009). We also found that rat CA endothelial cells exhibit an intense immunostaining for S1P₁ and S1P₃. S1P₁ receptors appear to be localised in the cytoplasm, suggesting that they have been already activated at the cell membrane and internalised, whereas S1P₃ are on the plasma membrane (Figure 3-4). However, only two independent experiments were performed and more experiments are required to examine the expression and distribution of S1P₁ and S1P₃ receptors in rat coronary endothelium. Using a double label protocol, this allowed detection of SK1 and S1P₃ receptors in the same rat CA endothelial cells but no colocalisation was observed (Figure 3-5).

Several studies have shown that hypoxia may upregulate SK1 in a variety of cell lines. For instance, exposure of human endothelial cells to hypoxia led to increased SK1 expression and enhanced activity (Schwalm et al., 2008). The same study showed that a hypoxic-induced increase in endothelial cell migration was an SK1-dependent effect. Moreover, Ahmed et al demonstrated that short-term hypoxia resulted in an expressional upregulation of SK1 and SK2 whereas chronic exposure to hypoxia increased SK1 mRNA transcript levels but not SK2 in human pulmonary smooth muscle cells (Ahmad et al., 2006). Rapid increase in SK1 immunofluorescence was also shown in rat amoeboid microglial cells after exposure to short period of hypoxia and the SK1 increase commenced 15 min after exposure to hypoxia and reached the maximum level after 1 hr (Lin et al., 2011). SK1 mRNA but not SK2 was also found to increase rapidly after hypoxia exposure from 1 hr and reached the peak level after 4 hr hypoxia in human glioma cells (Anelli et al., 2008). In the current study, it was found that exposure of rat coronary artery to short periods (30 min) of hypoxia led to a marked increase in SK1 immunostaining in rat CA endothelial cells compared with controls (Figure 3-7). Using human anti-phospho-specific sphingosine kinase 1 (Ser-225) antibody also revealed a significant increase in pSK1 immunostaining under hypoxic conditions (Figure 3-8). Moreover, pre-treatment with cycloheximide 30 min before exposure to hypoxia resulted in a significant reduction in the hypoxia-induced increase in SK1 immunostaining, suggesting that hypoxia may increase SK1 synthesis in

rat CA endothelium and this may consequently enhance S1P production (Figure 3-9). Under normoxia, a small reduction was also seen in SK1 immunostaining following treatment with cycloheximide. SKi pre-treatment led to a significant reduction in SK1 immunostaining under normoxic conditions (Figure 3-10). In four vessels obtained from four different animals which were pre-treated with PF543, it was visually very clear that PF543 had decreased SK1 immunostaining (approximately 30-40%) in two vessels but in the other vessels the background noise was high and image J software yielded no significant differences overall in the integrated density of SK1. This requires further investigation to confirm the effect of PF543 on SK1 expression under normoxic conditions. ROME did not decrease expression of SK1 in normoxic arteries. Under hypoxic conditions, preincubation with SKi and PF543 prior to exposure to hypoxia resulted in a reversal of the hypoxia-induced increase in SK1 immunostaining whereas additions of ROME did not reduce SK1 immunostaining, suggesting that hypoxia increases SK1 expression in rat CA endothelium (Figure 3-11).

A number of studies have demonstrated that SKi can not only inhibit the catalytic activity of SK1 but also it induces lysosomal and/or proteasomal degradation of SK1 depending on the cell type. For example, it was reported that SKi induced lysosomal degradation in human endothelial cells EA.hy 926, human podocytes and mesangial cells (Ren et al., 2010). Ren et al showed that the SKi triggered SK1 degradation was reversed by pre-treatment with chloroquine, a general lysosomal inhibitor, and CA-074ME, a specific inhibitor of the lysosomal protease cathepsin B, whereas pre-treatment with lactacystin, a specific proteasomal degradation inhibitor, failed to reverse the SKi-induced SK1 downregulation. Other studies, in contrast, have shown that SKi induces proteasomal degradation of SK1 in human pulmonary artery smooth muscle cells, prostate and breast cancer cells (Loveridge et al., 2010, Tonelli et al., 2013). It was shown that pre-treatment with proteasomal degradation inhibitors MG132 and lactacystin were able to reverse the effect of SKi whereas CA-074ME had no effect (Loveridge et al., 2010, Pitman et al., 2015). This effect might be due to an increase in the amount of ceramide (which may activate proteasomal degradation) as a consequence of catalytic inhibition of SK1 by SKi. In the same study and in line with this possibility, the ceramide synthase inhibitor, fumonisin B1 was able to partially reverse the effect of SKi. Other SK1 inhibitors such as FTY720, (S)-FTY720 vinylphosphonate and PF543 have also been shown to induce proteasomal degradation of SK1 in human pulmonary smooth muscle cells, prostate cancer and breast cancer cells and this effect was reversed by pre-treatment with MG132 (Tonelli et al., 2010, Byun et al., 2013, McNaughton et al., 2016). In the present study, pre-

treatment of rat coronary artery with MG132, lactacystin, CA-074ME or a combination of MG132 and CA-074ME had no effect on SK1 immunostaining compared with controls under normoxic conditions (Figure 3-12). Pre-treatment with MG132 but not lactacystin or CA-074ME prior to exposure to a short period of hypoxia significantly inhibited the hypoxia-induced increase in SK1 immunostaining whereas addition of MG132 and CA-074ME as a combination had no effect on the SK1 increase caused by hypoxia, suggesting that MG132 may direct some SK1 for lysosomal degradation (Figure 3-13). It was also found that pre-treatment with MG132, CA-074ME or a combination of MG132 and CA-074ME before addition of SKi significantly reversed the SKi triggered downregulation of SK1 under normoxic conditions, whereas lactacystin failed to reverse the SKi-induced reduction in SK1 (Figure 3-14). Moreover, the SKi-induced reduction of the hypoxia-induced increase of SK1 was significantly reversed by pre-treatment with MG132, CA-074ME or a combination of MG132 and CA-074ME but lactacystin pre-treatment did not reverse the SKi-induced reduction in SK1 (Figure 3-15). These findings suggest that SKi may induce both lysosomal and proteasomal degradation of SK1 in rat CA endothelial cells under normoxic and hypoxic conditions.

To improve the short and long term outcomes of patients with acute myocardial infarction (AMI), it is important to restore adequate myocardial perfusion as quickly as possible. Rapid restoration of blood supply in ischaemic myocardium provides oxygen and nutrients to the starved cardiac tissue, thus reducing the extent of damage. However, reperfusion also causes microvascular dysfunction, inflammation, and oxidative damage. One of the important contributors to myocardial damage after ischaemic events is increased vascular permeability (Murakami and Simons, 2009). Therefore prevention of vascular leak and oedema may help in improving microcirculation flow during reperfusion post-AMI and prevents no flow phenomenon. In this regard, several studies showed that S1P may play an important role in maintenance of vascular barrier integrity (Lee et al., 1999, Garcia et al., 2001, Sanchez et al., 2003, Jung et al., 2012). Cardiopulmonary bypass is a commonly used technique in thoracic surgery; however it may cause a number of side effects including ischaemia/reperfusion injury and systemic inflammatory response syndrome leading to vascular dysfunction. A recent study showed that pre-treatment with FTY720 or SEW2871, an S1P₁ agonist, relieved vascular dysfunction associated with cardiopulmonary bypass in the coronary and mesenteric arteries (Samarska et al., 2014). Moreover, it has been demonstrated that anandamide-stimulated relaxation was inhibited by SKi pre-treatment under normoxic conditions in rat coronary artery, suggesting that SK or SK-derived S1P may mediate the relaxation response to particular vasoactive agents

(Mair et al., 2010). Thus, increased SK1 levels and activities during myocardial ischaemia could play an important part in coronary protection through generation of more S1P which enhances vascular endothelial integrity and dilates coronary arteries to increase myocardial blood supply. Hypoxia-induced increase in SK1 may also be exploited in hypoxic preconditioning and postconditioning strategies to protect vascular endothelial cells from hypoxia/reoxygenation injury. Furthermore, vascular endothelium has been reported to contribute to circulating S1P concentration in mice (Hisano et al., 2012) and humans (Baranowski et al., 2015). Thus, increases in plasma S1P levels during an ischaemic attack may help induce cardioprotection in part by stimulating coronary vasodilatation and decreasing heart rate.

3.5 Conclusion

In summary, we found very distinct immunostaining for SK1, S1P₁ receptors and S1P₃ receptors in rat CA endothelial cells. Hypoxia increases the SK1 expression in rat coronary artery endothelial cells. This effect can be reduced by cycloheximide, suggesting that hypoxia may enhance SK1 protein synthesis. The increased SK1 level induced by hypoxia may enhance generation of the cardioprotective lipid mediator S1P, which can play a role in protection against microvascular dysfunction, inflammation, and oxidative damage caused by ischaemia/reperfusion injury. Hypoxia-stimulated increase in SK1 was also inhibited by pre-treatment with SKi and PF543 but not ROME. Furthermore, the data obtained using SKi, CA-074ME, and MG132 suggests that SKi might reduce SK1 expression by promoting the proteasomal and lysosomal degradation of SK1.

Chapter four

4 The effect of hypoxia-induced increase in SK1 on vascular function

4.1 Introduction

S1P plays an important role in regulating vascular tone through binding to and activating S1P receptors in endothelial and vascular smooth muscle cells. ECs express mainly S1P₁ and S1P₃ receptors, whereas S1P₂ receptors are predominately expressed in VSMCs (Ozaki et al., 2003, Saba and Hla, 2004, Alewijnse and Peters, 2008). Generally, S1P is capable of stimulating both vasodilation and vasoconstriction but the net effect of S1P on vascular tone is dependent on the specific vascular bed, species, S1P concentration and the S1P receptors expressed and stimulated. For instance, exogenous application of S1P to isolated rat mesenteric, cerebral and coronary arteries has been reported to promote vasoconstriction via a mechanism requiring S1P₃ receptors (Hemmings et al., 2004, Salomone et al., 2008, Murakami et al., 2010). On the other hand, it has been demonstrated that S1P induces a NO-mediated vasorelaxation in rat aorta and mesenteric arteries (Dantas et al., 2003, Nofer et al., 2004). Moreover, Mair et al showed that S1P stimulates vasorelaxation in rat coronary artery through a mechanism requiring CB₂ and S1P₃ receptors (Mair et al., 2010). Furthermore, there is evidence that TRPV channel activation within the blood vessels may contribute to vasorelaxation via nitric oxide, prostacyclin, and intermediate/small conductance potassium channel-dependent pathways (Baylie and Brayden, 2011). A number of studies have demonstrated that S1P and its precursor sphingosine activate these channels and this may play a role in the regulation of vascular tone (Numata et al., 2011, Langeslag et al., 2014).

In chapter 3, I showed that exposure of rat coronary arteries to short-term hypoxia induced an increase in SK1 expression in endothelial cells of rat coronary artery. This increase in SK1 expression was significantly reduced by pre-treatment with the protein synthesis inhibitor cycloheximide and both the non-selective SK inhibitor, SKi and the selective SK1 inhibitor, PF543. Functionally, exposure of the blood vessels to hypoxia can lead to either vasodilation or vasoconstriction depending on the vascular bed, the animal species being studied and the experimental methodology. For example, several studies have shown that hypoxia causes dilation of the coronary arteries in isolated whole heart preparations (Daut et al., 1990, Gasser et al., 1998). However, results obtained from studies using isolated coronary arteries to examine the effect of hypoxia were not consistent, with some showing dilation (Kerkhof et al., 2002, Lynch et al., 2006) and others constriction (Graser and Vanhoutte, 1991, Muramatsu et al., 1992). Herrera and Walker provided evidence that hypoxia elicits vasorelaxation in endothelium-denuded rat aortic rings in part via inhibition of L-type Ca²⁺ channels (Herrera and Walker, 1998). A role for reduced Ca²⁺ efflux

through voltage gated calcium channels (VGCC) has also been shown in smooth muscle from porcine coronary, rabbit cerebral, femoral and coeliac arteries (Franco-Obregon and Lopez-Barneo, 1996, Smani et al., 2002). Moreover, several studies showed that hypoxia stimulates the release of relaxing factors by the endothelium such as prostacyclin in rat tail artery, rat skeletal muscle arterioles and canine femoral artery (Busse et al., 1983, Messina et al., 1992). Thus, the mechanisms for hypoxia-induced effects on the vasculature remain controversial.

Considering the aforementioned background, I hypothesized that hypoxia may increase SK1 in rat aortic endothelium. Once SK1 is activated, it is re-located from the cytosol to the plasma membrane where it is in close proximity to its substrate, sphingosine. Thus S1P is synthesised and exported through transporter proteins into the extracellular milieu where it acts as an agonist for S1P receptors or is retained within the cell to function as an intracellular messenger.

Moreover, it has been reported that the SK/S1P pathway may contribute to the regulation of blood pressure. Indeed, it has been demonstrated that administration of S1P or FTY720, S1P receptor agonist, to anaesthetised rats was associated with a transient decline of blood pressure followed by an equally rapid restoration of pressure (Forrest et al., 2004). The initial reduction in blood pressure is likely to be due to decreased heart rate. In contrast, continuous infusion of FTY720 was associated with an increase in blood pressure to conscious rats (Forrest et al., 2004). S1P has also been shown to regulate blood pressure, enhance histamine clearance and facilitate recovery from anaphylactic shock via S1P₂ receptors (Olivera et al., 2010). Recently, it has been shown that Nogo-B deficient mice were significantly hypotensive compared with wild type controls (Cantalupo et al., 2015). Nogo-B is a membrane protein of the endoplasmic reticulum that negatively regulates endothelial S1P formation by inhibiting serine palmitoyltransferase, a rate-limiting enzyme of *de novo* sphingolipid biosynthesis. In the same study, it was demonstrated that Nogo-B null mice are resistant to angiotensin II-induced hypertension through S1P/S1P₁ eNOS-dependent pathway. In contrast, genetic deletion of SK1 significantly inhibits both the acute hypertensive response to angiotensin II in anaesthetized mice and the sustained hypertensive response to continuous administration of angiotensin II in conscious mice, suggesting that S1P generated by SK1 may have a pro-hypertensive role (Wilson et al., 2015). The significant role of the SK/S1P signalling pathway has been shown not only in systemic hypertension but also in pulmonary arterial hypertension. Chen et al has reported that SK1 is upregulated in lung and PASMCs of patients with pulmonary arterial

hypertension (Chen et al., 2014). Deletion of SK1 or inhibition of SK1 by SK inhibitor or antagonising S1P₂ receptor using JTE013 in mice was protective against development of hypoxia-induced pulmonary hypertension and pulmonary vascular remodelling, suggesting a critical role for SK1/S1P/S1P₂ pathway in pathogenesis of pulmonary arterial hypertension (Chen et al., 2014). A recent study has also demonstrated that inhibiting SK1 activity using PF543, a selective SK1 inhibitor decreased right ventricular hypertrophy in a mouse hypoxic model of pulmonary arterial hypertension (MacRitchie et al., 2016). Thus, a full understanding of S1P-mediated regulation of blood pressure may offer novel therapeutic options in the treatment of hypertension and related cardiovascular diseases.

4.1.1 Aims

- Examine the effect of hypoxia on the contractile response to U44619 in endothelium-intact and denuded rat aortic rings.
- Study the distribution of SK1 and S1P receptors in rat aortic endothelium.
- Investigate the effect of hypoxia on SK1 expression in the presence and absence of SK inhibitors in rat aorta.
- Examine S1P-induced relaxation under normoxic and hypoxic conditions and elucidate the potential mechanisms of action underlying these effects.
- Investigate the S1P-induced relaxation in the presence and absence of hypoxia in rat aortic rings obtained from spontaneously hypertensive Wistar Kyoto rats.

4.2 Methods

4.2.1 Tissue preparation for immunofluorescent studies

Rat aortic sections were prepared, fixed and immunostained as detailed in Section 2.3.3. Briefly vessels from male Sprague-Dawley rats were dissected immediately after death and placed into 1ml Krebs-Henseleit solution previously gassed with 95% O₂, 5% CO₂ at 37 °C. The segments were then treated with specific inhibitory agents for 30 min (detailed below), then subjected to normoxia/hypoxia for 30 min and fixed by 3% paraformaldehyde overnight at 4 °C. The vessels were then labelled with specific primary and secondary antibodies, placed on a glass slide and the protein of interest visualized by confocal microscopy.

Hypoxia was induced by placing the artery in 1 ml Krebs' solution which had been constantly gassed with 95% N₂, 5% CO₂ for approximately 30 mins to reduce oxygen tension to 1-1.5% O₂ saturation (Kerkhof et al., 2002).

4.2.2 Experimental protocols

4.2.2.1 Protein synthesis inhibitor

A number of experiments were performed where the vessel was pre-treated with 10 µM cycloheximide, a protein synthesis inhibitor (Schneider-Poetsch et al., 2010), for 30 min prior to exposure to normoxia/hypoxia for 30 min.

4.2.2.2 SK inhibitors

A series of experiments was carried out where the vessels were preincubated with 10 µM SKi, a non-selective SK inhibitor (Mair et al., 2010) or 100 nM PF543, a selective SK1 inhibitor (Schnute et al., 2012) followed by exposure to normoxia/hypoxia for 30 min.

4.2.3 Image analysis

A Bio-Rad Radiance 2100 confocal microscope (x20 oil/water immersion lenses) was used to visualise the protein of interest as described in detail in Section 2.3.7.

4.2.4 Tissue preparation for myography

Rat aortic sections were prepared for functional studies as detailed in Section 2.4.1. Briefly, vessels from male Sprague-Dawley rats or spontaneously hypertensive Wistar Kyoto rats were dissected immediately after death and placed into Krebs-Henseleit solution previously gassed with 95% O₂, 5% CO₂ at room temperature. Then the thoracic aorta was carefully dissected free from adherent connective tissue and cut into 2-3 mm rings. Vessel rings were mounted in a wire myograph and maintained at 37 °C in oxygenated Krebs-Henseleit buffer (95% O₂, 5% CO₂). The vessels were allowed to equilibrate for 15-30 min. Thereafter, vessels were stretched until an optimum resting tension of 1gm was reached and allowed to equilibrate for 30 min prior to challenge with KCl (80 mM) to assess viability. Vascular endothelial function was assessed at the end of the experiment by adding 1 μM ACh. Relaxation to ACh > 50% was indicative of a vessel with an intact endothelium. Where indicated, endothelium was removed by gentle rubbing of the intimal surface with forceps. Relaxation to ACh < 10% denoted an endothelium-denuded vessel.

4.2.5 Experimental protocols for myography

The experimental and hypoxia protocol for myography were described in detail in Section 2.4.2 (see also Figure 4-1, page 100 and Figure 4-14, page 117).

4.2.5.1 U46619 protocol

To investigate the effect of hypoxia on contraction to the thromboxane A₂ mimetic U46619, endothelium-intact and denuded aortic rings were subjected to 30 min hypoxia then re-oxygenated and maintained under normoxic conditions. Then concentration response curves were generated by cumulative addition of U46619 (1×10⁻⁹-1×10⁻⁶M) with 10 min between each addition. In experiments where the effects of SKi, a non-selective sphingosine kinase inhibitor (10 μM), on U46619-induced contraction were to be investigated under hypoxia and normoxia, the SKi was added 15 min before exposure to hypoxia and was present during generation of the concentration-response curve.

4.2.5.2 Agonist comparison

After 30 min equilibration and challenge with KCl, aortic rings under normoxic conditions were submaximally contracted with U46619 (3×10⁻⁸M). When a stable level of contraction

was achieved, concentration-response curves were performed by cumulative addition of agonists. S1P, CYM5541, a selective S1P₃ receptor agonist, and SEW2871, a selective S1P₁ receptor agonist, (1×10^{-9} - 3×10^{-6} M) were added in half-log molar concentration in a cumulative manner with 5 min between each addition.

4.2.5.3 Antagonists and inhibitors protocol

In experiments where the effects of antagonists or inhibitors on agonist-induced relaxation were to be assessed, the antagonist of interest was added 30 min prior to contraction with U46619, and was present during generation of the concentration-response curve. A list of antagonists and inhibitors and their final bath concentrations used was provided in Chapter Two (Table 2-1).

4.2.5.4 Statistical analysis

Graph Pad Prism (version 5) software was used to analyse all the data. Contractile responses to U46619 were expressed as mean \pm standard error of the mean (S.E.M) in grams. All relaxation responses are reported as a percentage loss of the tone induced by U46619. Results are expressed as mean \pm SEM of n, where n is the number of arterial segments from different animals. Differences between concentration-response curves were tested by two way analysis of variance (ANOVA) with a Bonferroni's post-test. To compare the effect of enzyme inhibitors and antagonists on U44619-induced contraction in rat aorta, one-way ANOVA with a Dunnett's post was used. In all cases, statistical significance was accepted when $p < 0.05$.

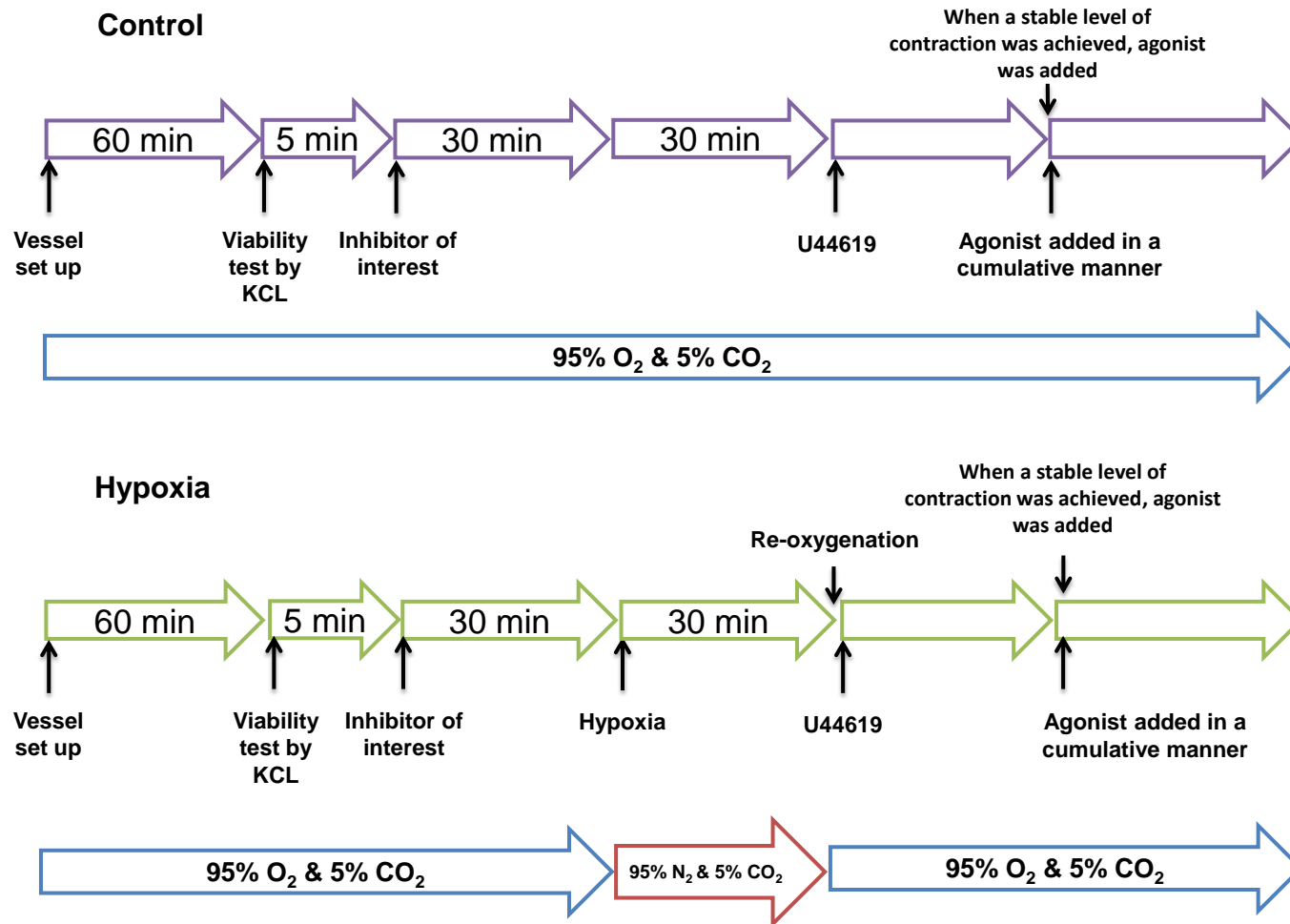


Figure 4-1 Diagram showing the experimental protocols used for the myography experiments.

4.3 Results

4.3.1 Effect of hypoxia on contractile responses to U46619 in rat aorta.

In order to determine whether hypoxia alters the contractile response to U46619, a series of experiments was carried out to examine the response of endothelium-intact and denuded rat thoracic aorta under control and hypoxic conditions. Addition of the thromboxane A₂ receptor agonist U46619 (1×10^{-9} - 1×10^{-6} M) to intact thoracic aorta in the presence or absence of hypoxia resulted in a concentration-dependent contraction. Previous exposure to 30 mins of hypoxia significantly enhanced the contractile response to U46619 (maximal contraction 1.7 ± 0.2 g, n=8; Figure 4-2A) compared with vessels under normoxic conditions (maximal contraction 1.2 ± 0.2 g, n=9; Figure 4-2A). The contractile response to U46619 was also significantly enhanced in denuded rat thoracic aorta subjected to 30 min hypoxia (maximal contraction 2.0 ± 0.3 g, n=9; Figure 4-2B) compared with denuded vessels under normoxic conditions (maximal contraction 1.4 ± 0.2 g, n=9; Figure 4-2B).

Pre-treatment of the vessels with the non-selective SK inhibitor SKi (10 μ M) did not significantly alter the contraction induced by U46619 in intact rat aorta (maximal contraction 1.4 ± 0.8 g, n=9; Figure 4-3A) or denuded rat aorta (maximal contraction 1.7 ± 0.3 g, n=10; Figure 4-3B) under control conditions. However, pre-treatment of the intact aorta with SKi prior to exposure to hypoxia significantly reduced the enhanced contraction ordinarily induced by hypoxia (maximal contraction 1.0 ± 0.2 g, n=8; Figure 4-4A). In contrast, SKi had no effect on hypoxia-enhanced contractile response to U46619 in denuded rat aorta (maximal contraction 1.8 ± 0.2 g, n=9; Figure 4-4B).

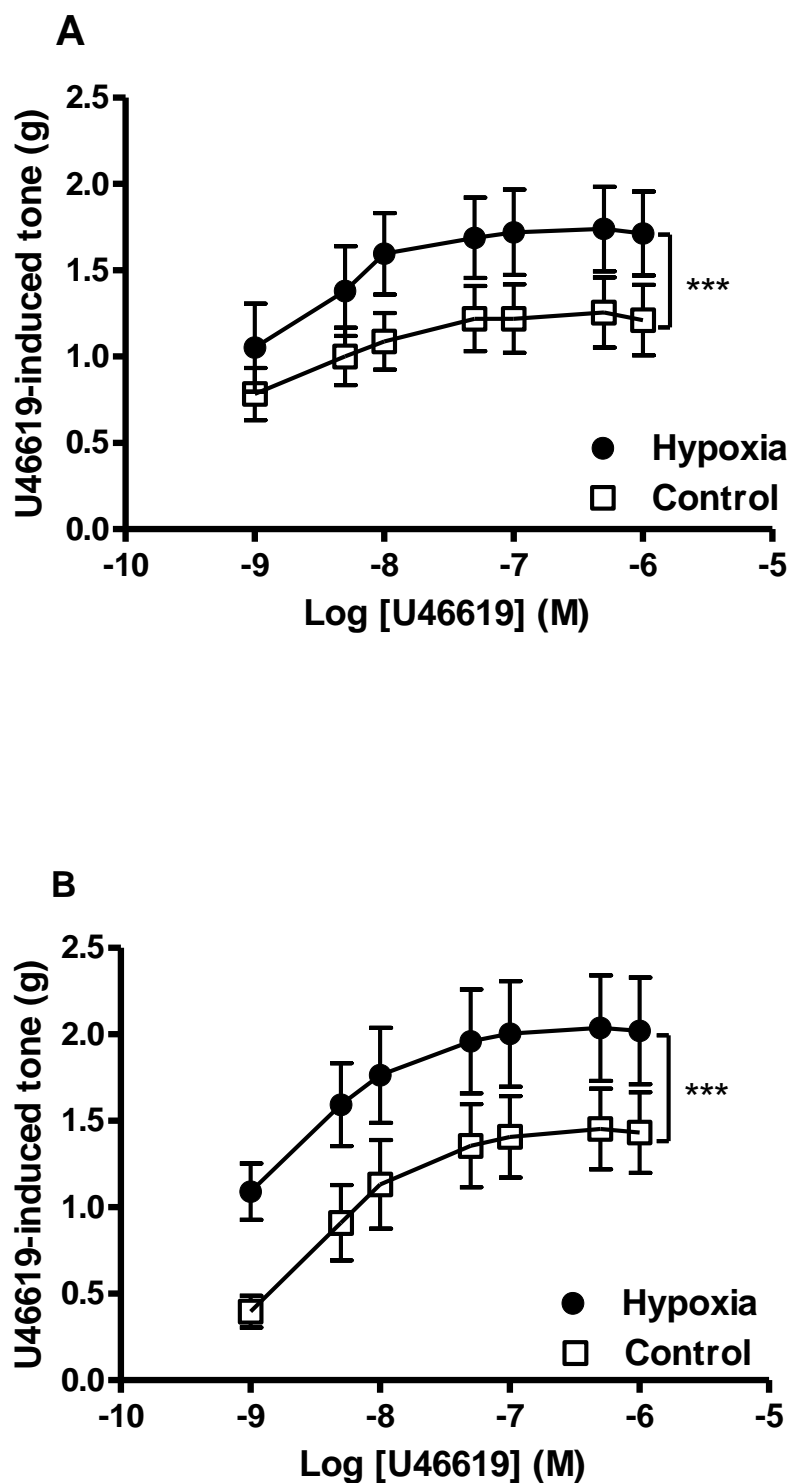


Figure 4-2 Contractile responses to U46619 in rat aorta under control and hypoxic conditions.

Concentration response curves for the contractile effect of U46619 (1×10^{-9} - 1×10^{-6} M) in (A) endothelium-intact rat aorta in presence (n=8) and absence (n=9) of hypoxia and (B) endothelium-denuded rat aorta in presence (n=9) and absence (n=9) of hypoxia. Data are expressed as U46619-induced tone (in grams) and shown as mean \pm SEM for n arteries from different animals. ***p<0.001 vs control as determined by two-way ANOVA.

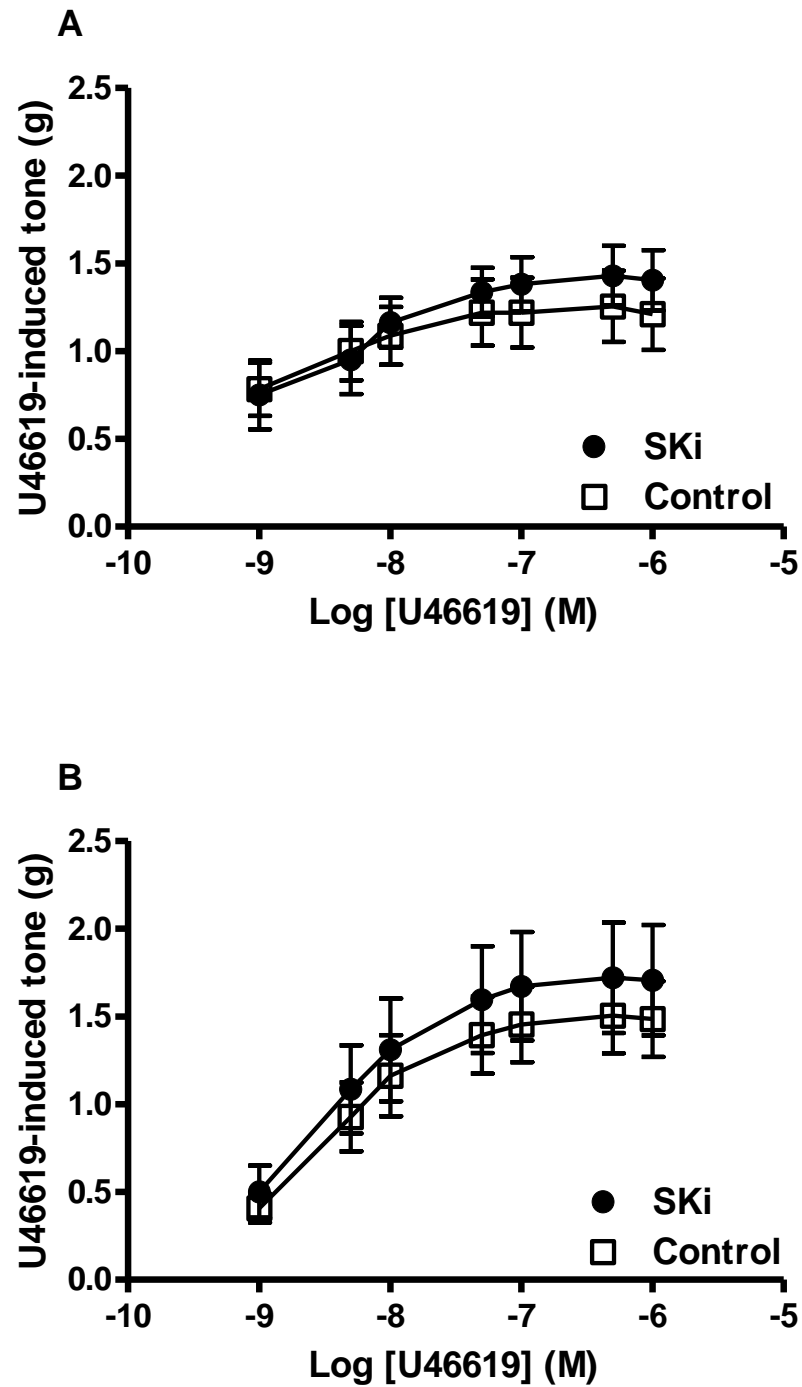


Figure 4-3 Contractile responses to U46619 in rat aorta in presence and absence of SKi under control conditions.

Concentration response curves for the contractile effect of U46619 (1×10^{-9} - 1×10^{-6} M) in (A) endothelium-intact rat aorta in presence (n=9) and absence (n=9) of SKi (10 μ M) and (B) endothelium-denuded rat aorta in presence (n=10) and absence (n=10) of SKi. Data are expressed as U46619-induced tone (in grams) and shown as mean \pm SEM for n arteries from different animals.

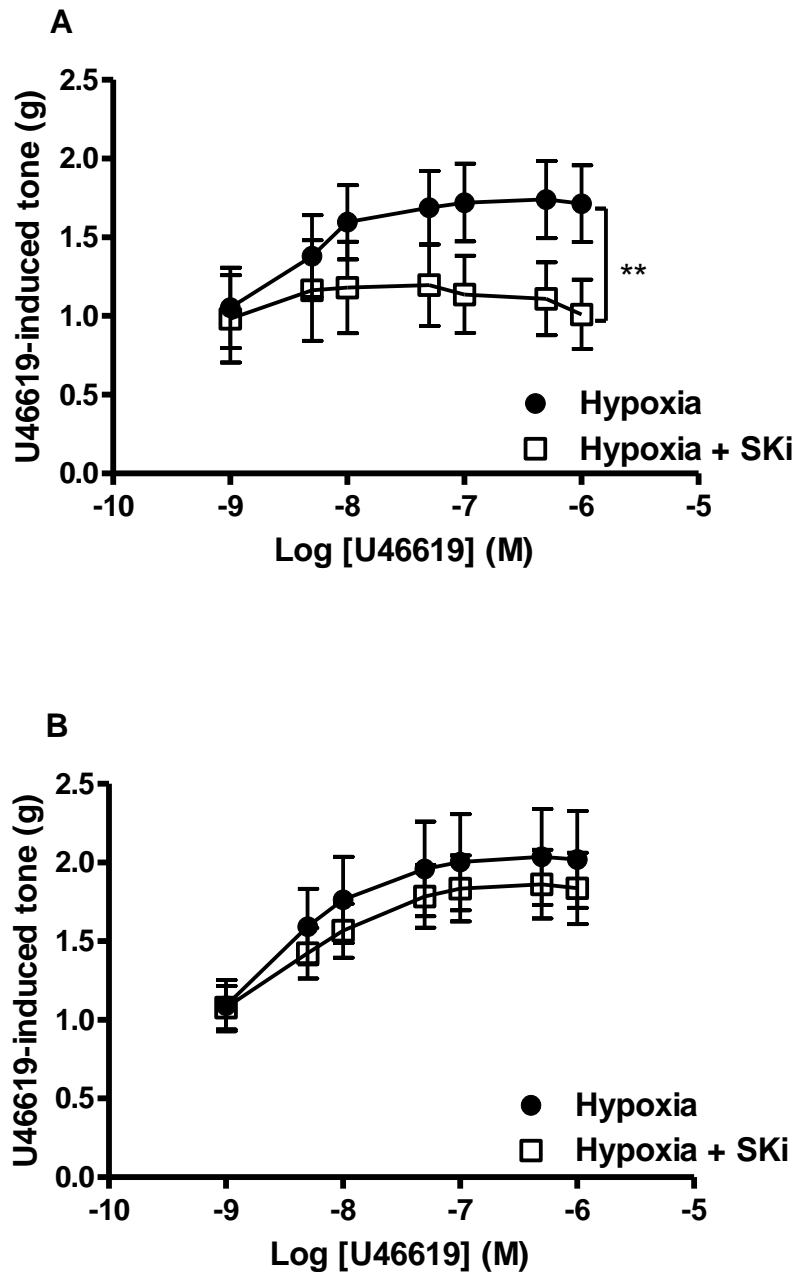


Figure 4-4 Contractile responses to U46619 in rat aorta in presence and absence of SKi and under hypoxic conditions.

Concentration response curves for the contractile effect of U46619 (1×10^{-9} - 1×10^{-6} M) in (A) endothelium-intact rat aorta in presence (n=8) and absence (n=8) of SKi (10 μ M) and (B) endothelium-denuded rat aorta in presence (n=9) and absence (n=9) of SKi. Data are expressed as U46619-induced tone (in grams) and shown as mean \pm SEM for n arteries from different animals. **p<0.01 vs control determined by two-way ANOVA.

4.3.2 S1P-induces vascular relaxation in an endothelium dependent manner.

To confirm that S1P has a relaxant effect on rat aorta, its effect on aortic tone was directly assessed. Addition of S1P (1 nM-30 μ M) to endothelium-intact thoracic aorta pre-contracted with U46619 resulted in a concentration-dependent, slowly developing relaxation, which reached a maximum of $22.5 \pm 3.3\%$, n=6; Figure 4-5A. A significant difference was observed between maximal relaxation induced by S1P and loss of contractile force in time control vessels (maximal relaxation $7.9 \pm 0.9\%$, n=6; Figure 4-5A). In aortic rings where the endothelial layer was removed, relaxation in response to S1P was abolished (maximal relaxation $7.8 \pm 1.7\%$, n=6; Figure 4-5B).

Furthermore, the expression of S1P₁ and S1P₃ receptors was examined in endothelium of rat aorta by immunofluorescent studies. It was found that both receptor subtypes are expressed widely in the endothelium (Figure 4-6 and 4-7 respectively). To determine whether S1P-stimulated vascular relaxation is mediated through S1P₁ and/or S1P₃ receptors, dose response curves for a selective S1P₁ agonist, SEW2871, and a selective S1P₃ agonist, CYM5541, were constructed in endothelium-intact U46619 pre-contracted arteries. SEW2871 did not induce significant vasorelaxation in rat aorta (maximal relaxation $5.9 \pm 2.0\%$, n=5; Figure 4-8A), whereas addition of CYM5541 (1 nM-30 μ M) induced a concentration-dependent relaxation of a similar magnitude to S1P itself (maximal relaxation $20.2 \pm 4.0\%$, n=6; Figure 4-8B). In aortic rings where the endothelial layer was removed, relaxation in response to CYM5541 was markedly attenuated (maximal response $11.1 \pm 2.9\%$, n=7; Figure 4-8C).

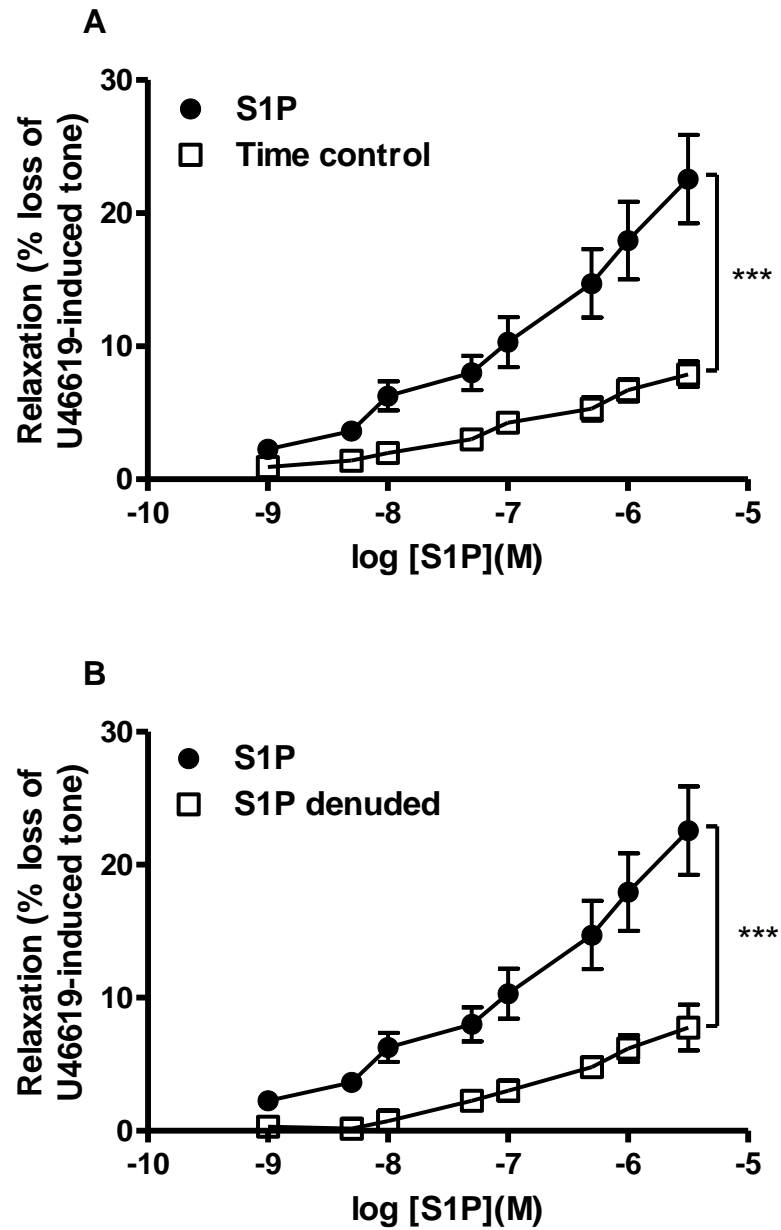


Figure 4-5 S1P stimulated aortic relaxation in an endothelium dependent manner.

(A) Concentration–response curves showing the vasorelaxant effect of S1P alone (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact aortic rings (n=6) compared to time control (n=6). (B) Vasorelaxant effect of S1P alone (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact (n=6) and endothelium-denuded (n=6) rat aorta. ***p<0.001 vs S1P alone as determined by two-way ANOVA.

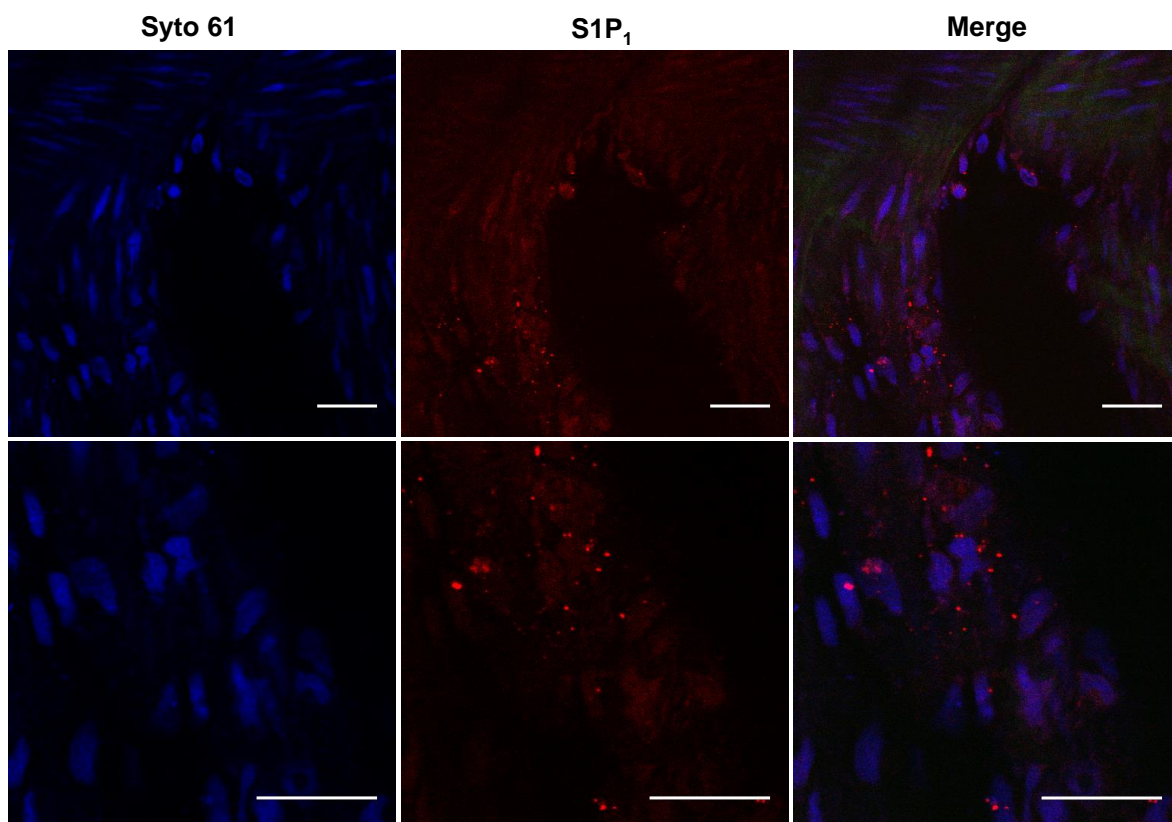


Figure 4-6 S1P₁ receptor expression in rat aortic endothelium.

Representative immunofluorescent images for S1P₁ receptors (red). Syto 61 nuclear stain was used to stain the endothelial and smooth muscle cell nuclei (blue). The green colour in the merged image represents the autofluorescence of the internal elastic lamina. Results are representative of 3 separate experiments. Scale bar is 30 μ m for all images.

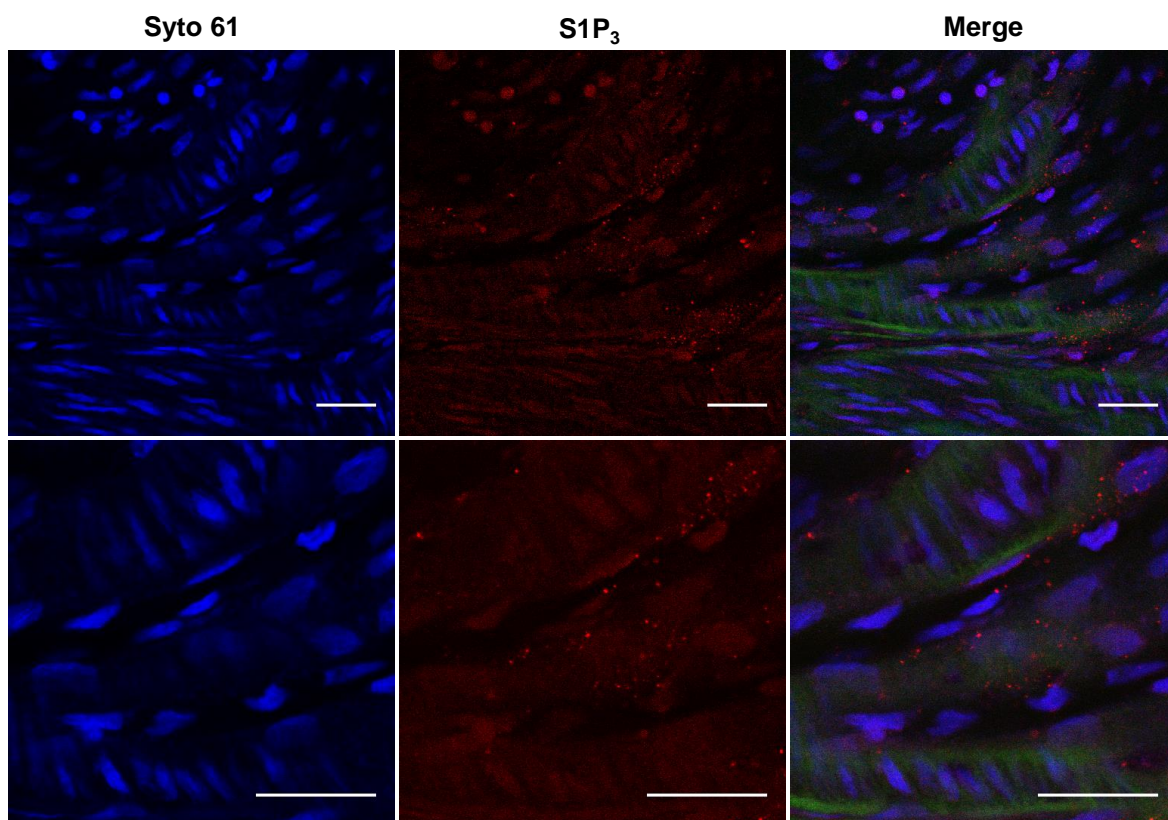


Figure 4-7 S1P₃ receptor expression in rat aortic endothelium.

Representative immunofluorescent images for S1P₃ receptors (red). Syto 61 nuclear stain was used to stain the endothelial and smooth muscle cell nuclei (blue). The green colour in the merged image represents the autofluorescence of the internal elastic lamina. Results are representative of 3 separate experiments. Scale bar is 30 μ m for all images.

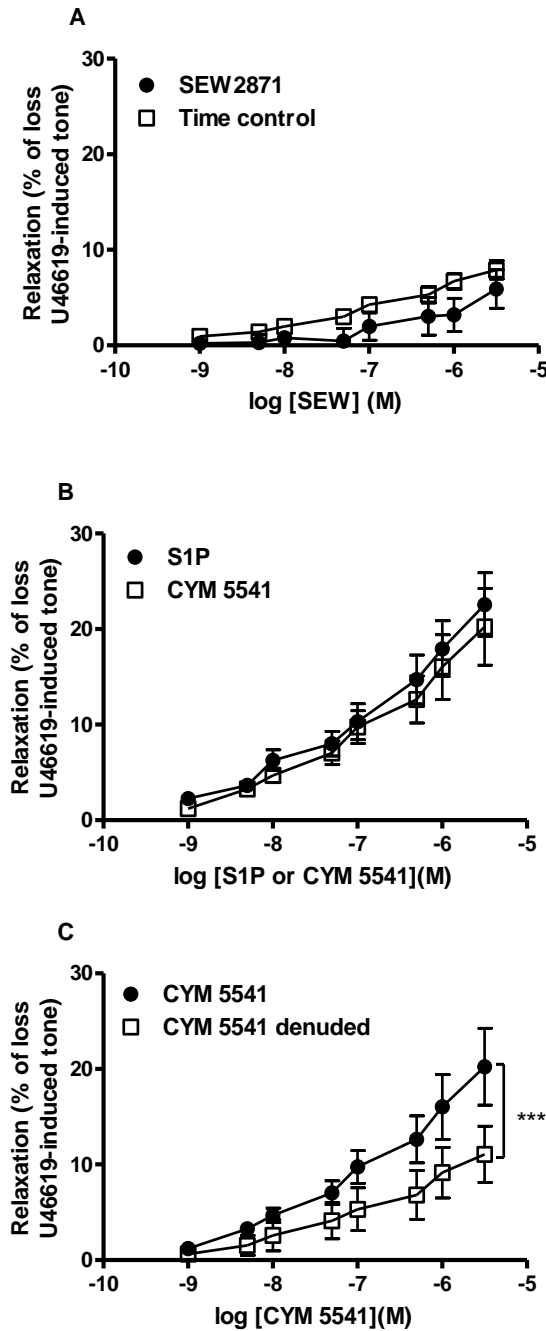


Figure 4-8 S1P and CYM 5541 induce aortic relaxation in an endothelium-dependent manner.

(A) Concentration–response curves showing the effect of SEW2871, a selective S1P₁ receptor agonist, (1 nM–30 μM) on U46619-pre-contracted endothelium-intact (n=5) rat aorta compared to time control (n=6). (B) Vasorelaxant effect of S1P and CYM 5541, a selective S1P₃ receptor agonist, (1 nM–30 μM, n=6) on endothelium-intact U46619 pre-contracted, rat aorta. (C) Vasorelaxant effect of CYM 5541, a selective S1P₃ receptor agonist, (1 nM–30 μM) on endothelium-intact (n=6) and endothelium denuded (n=7) U46619 pre-contracted, rat aorta. ***p<0.001 vs CYM 5541 (C) as determined by two-way ANOVA.

4.3.3 TRPV1 channels are involved in S1P-stimulated relaxation.

To assess whether TRPV1 channels contribute to S1P-induced relaxation, aortic rings were pre-treated with AMG 9810, a TRPV1 antagonist 30 min before addition of S1P. This led to a significant attenuation of S1P-mediated relaxation (maximal relaxation $9.7 \pm 0.8\%$, $n=6$, Figure 4-9).

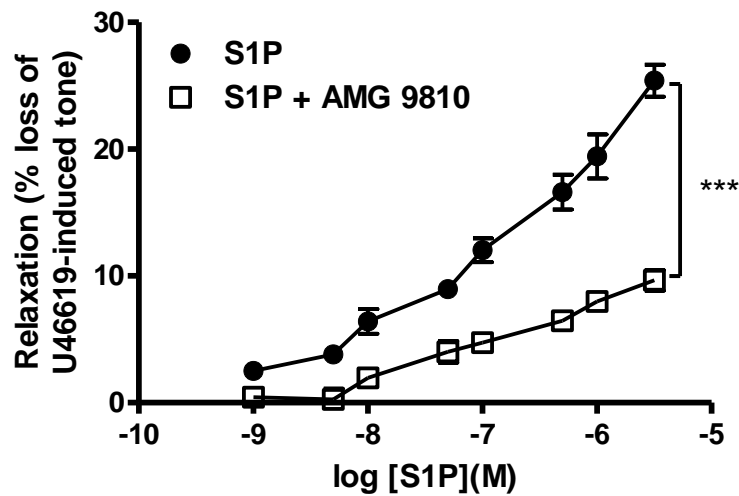


Figure 4-9 Role of TRPV1 channels in S1P-mediated relaxation.

Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in the presence and absence of AMG 9810, a TRPV1 antagonist (10 μ M, $n=6$). *** $p < 0.001$ vs S1P alone as determined by two-way ANOVA.

4.3.4 Effect of hypoxia on SK1 enzyme expression in rat aortic endothelium.

It was found that SK1 is prominently expressed in endothelial cells of rat aorta. Similar to rat CA, immunofluorescence staining for SK1 (red) in rat aorta appears as red punctate staining in the cytosol of the cells (Figure 4-10). Moreover, it was also demonstrated that exposure of rat aortic rings to hypoxia for 30 min resulted in a significant increase (61%) in integrated density ($2.6 \times 10^6 \pm 2.6 \times 10^5$ AU, n=4) corresponding to SK1 expression in comparison with arteries maintained under normoxic conditions ($1.0 \times 10^6 \pm 1.7 \times 10^5$ AU, n=4; Figure 4-11). In contrast, hypoxia had no effect on phosphorylated-SK1 (pSK1) integrated density ($1.1 \times 10^6 \pm 1.7 \times 10^5$ AU, n=3) compared with the corresponding levels in control vessels ($1.0 \times 10^6 \pm 1.2 \times 10^5$ AU, n=4; Figure 4-12).

4.3.5 Effect of cycloheximide on hypoxia-induced increase in SK1 expression.

Pre-treatment of aortic rings with cycloheximide for 30 min under normoxic conditions resulted in a small reduction in SK1 integrated density ($0.83 \times 10^6 \pm 1.0 \times 10^5$ AU, n=4) compared with controls ($1.0 \times 10^6 \pm 1.7 \times 10^5$ AU, n=4; Figure 4-13). However, in arteries preincubated with cycloheximide prior to exposure to hypoxia, there was a significant reduction (50%) in hypoxia-induced increase in integrated density of SK1 ($1.3 \times 10^6 \pm 2.5 \times 10^5$ AU, n=3) compared with controls ($2.6 \times 10^6 \pm 2.6 \times 10^5$ AU, n=5; Figure 4-13).

4.3.6 Effect of hypoxia on S1P-stimulated vascular relaxation

Addition of 5×10^{-9} M U46619 was used to constrict the aortic rings after exposure to hypoxia. This concentration was used as it generated contraction of a similar magnitude to 3×10^{-8} M U46619 under control conditions. Inhibitors used in these experiments did not affect the U46619-induced tone (Table 4-1 and 4-2). In experiments involving SKi, U46619 at 3×10^{-8} M was used under both control and hypoxic conditions since SKi had previously been found to attenuate contraction in hypoxic rings (Figure 4-4). The S1P-induced relaxation in rat aorta was markedly increased in those arteries subjected to 30 min hypoxia. The maximal relaxation in response to S1P under hypoxic conditions was $31.8 \pm 6.3\%$, n=10 compared to control vessels (maximal relaxation $19.3 \pm 3.1\%$, n=9; Figure 4-15A). This was also significantly greater than loss of tone in time control vessels after exposure to hypoxia (maximal relaxation $19.7 \pm 5.9\%$, n=10; Figure 4-15B). In aortic rings

where the endothelial layer was removed, the enhanced relaxation in response to S1P under hypoxic conditions was abolished (maximal relaxation $11.4 \pm 1.3\%$, $n=8$; Figure 4-15C).

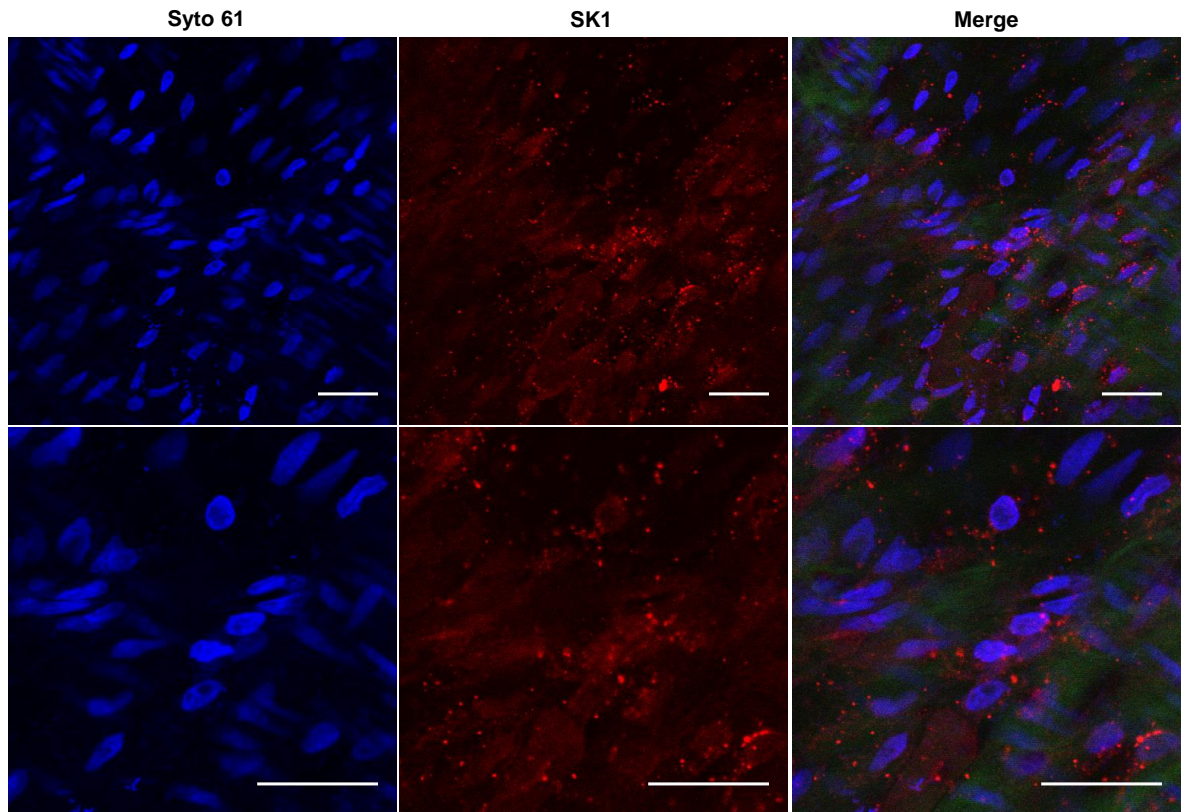


Figure 4-10 Expression of SK1 in endothelial cells of rat aorta.

Rat aortic sections were immunostained for SK1 (red) with anti-SK1 antibody. Syto 61 nuclear stain was used to stain the endothelial and smooth muscle cell nuclei (blue). The green colour in the merged image represents the autofluorescence of the internal elastic lamina. Scale bar is 30 μm in all images. Images are representative of at least 3 separate experiments.

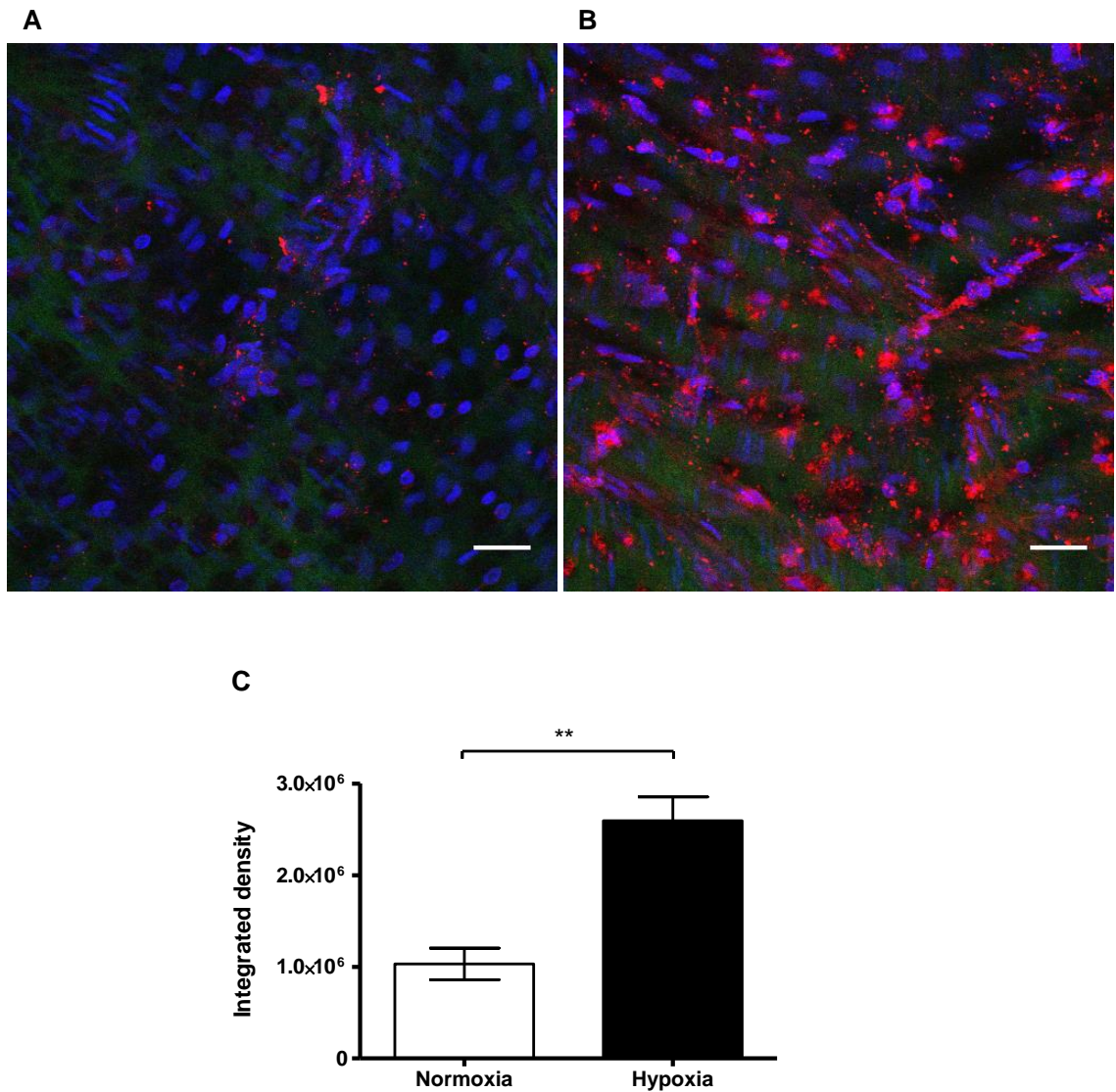


Figure 4-11 Effect of hypoxia on SK1 expression levels.

Representative immunofluorescent images showing expression of SK1 (red) in rat aortic endothelium under normoxic conditions (A) and hypoxic conditions (B). (C) Quantitative fluorescence measurement of SK1 under normoxia and hypoxia. Scale bar is $30 \mu\text{m}$ for both images. $**p < 0.01$ vs normoxia as determined by unpaired two tailed t-test. Results are representative of 4 separate experiments.

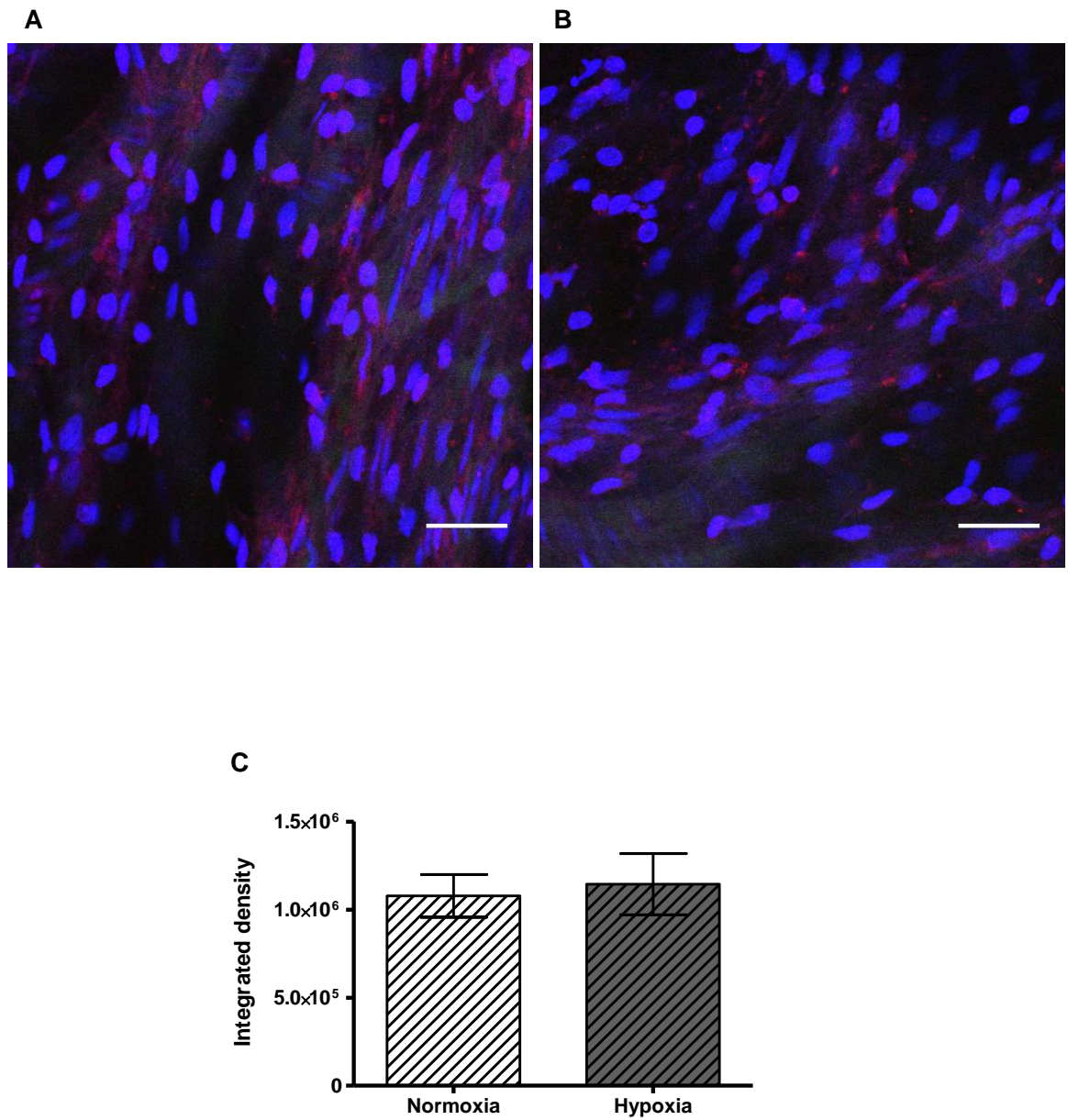


Figure 4-12 Effect of hypoxia on pSK1 levels.

Representative immunofluorescent images showing effect of hypoxia on pSK1 (red) level in rat aortic endothelium (B) compared with normoxia (A). (C) Quantitative fluorescence measurement of pSK1 under normoxia and hypoxia. Results are representative of 3-4 separate experiments. Scale bar is 30 μm for both images.

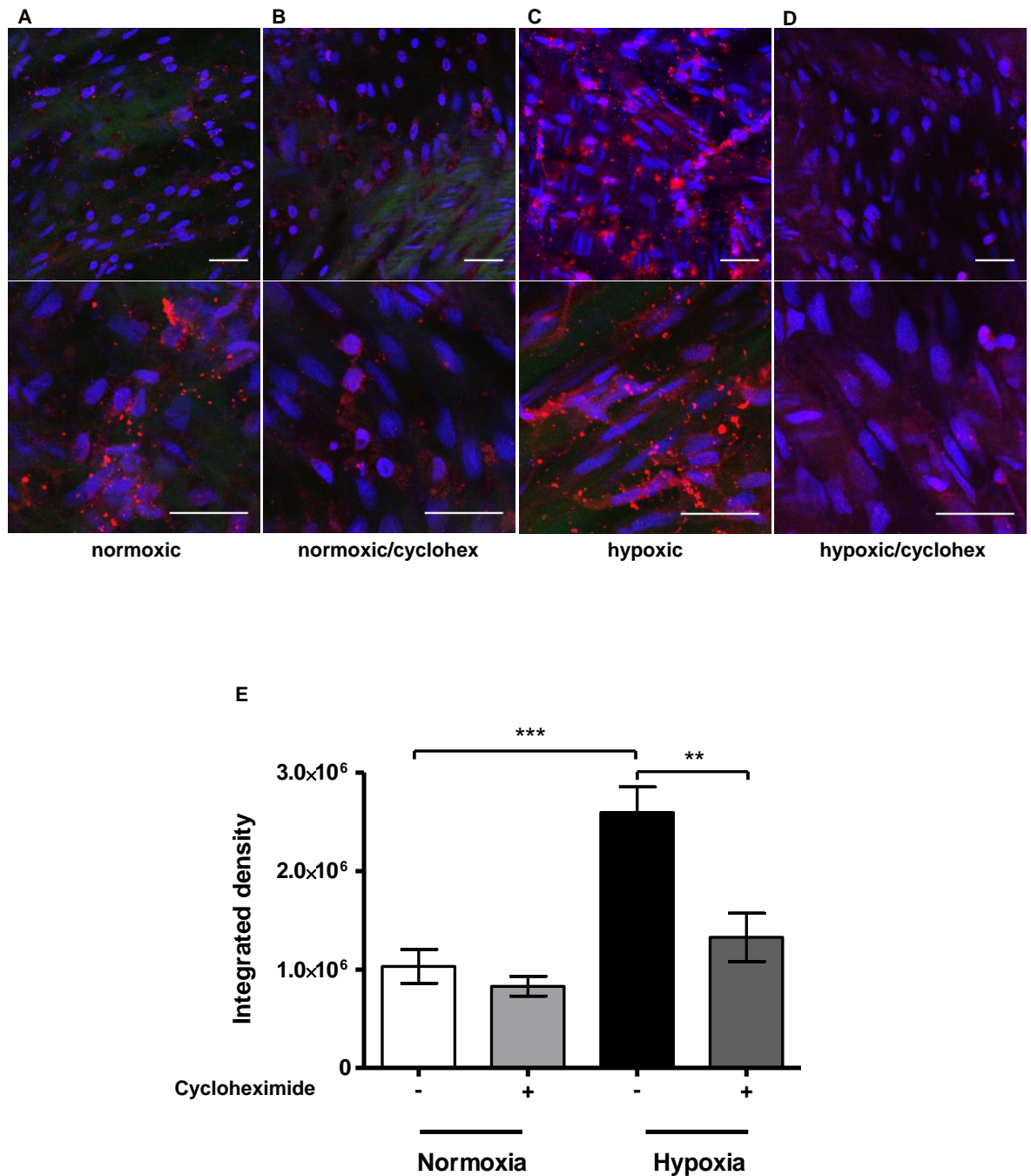


Figure 4-13 Effect of cycloheximide on hypoxia-induced increase in SK1 expression.

Representative immunofluorescent images show expression of SK1 (red) in rat aortic endothelium (A) and following pre-treatment with cycloheximide (10 μ M, 30 min) under normoxic conditions (B). Expression of SK1 in rat aorta subjected to 30 min hypoxia (C). Preincubation of rat aorta with cycloheximide reduced the hypoxia-induced increase in SK1 expression (D). (E) Quantitative fluorescence measurement of SK1 in the presence and absence of cycloheximide. **p < 0.01, ***p < 0.001 vs hypoxia alone as determined by one way ANOVA with Bonferroni's post-test. Results are representative of 3-4 separate experiments. Scale bar is 30 μ m for all images.

Table 4-1 Effect of enzyme inhibitors and antagonists (all used at 10 μ M concentration except PF543 which was used at 100 nM) on U46619-induced contraction (3×10^{-8} M) in rat aortae under control conditions.

	Mean U46619-induced contraction (g)	Number of arteries from different animals (n)
Control	1.7 ± 0.2	7
SKi	1.4 ± 0.1	8
PF543	1.9 ± 0.1	9
ROMe	1.6 ± 0.2	6
L-NNA	1.9 ± 0.2	7
CAY10444	1.5 ± 0.2	12

Data are expressed as mean \pm SEM for (n) number of arteries from different animals. Statistical analysis using one way ANOVA with Dunnett's post test revealed no significant differences.

Table 4-2 Effect of enzyme inhibitors and antagonists (all used at 10 μ M concentration except PF543 which was used at 100 nM) on U46619-induced contraction (5×10^{-9} M) in rat aortae under hypoxic conditions.

	Mean U46619-induced contraction (g)	Number of arteries from different animals (n)
Control	1.7 ± 0.1	6
SKi	1.6 ± 0.1	8
PF543	1.5 ± 0.2	7
ROMe	1.5 ± 0.1	6
L-NNA	1.5 ± 0.2	6
CAY10444	1.4 ± 0.3	10

Data are expressed as mean \pm SEM for (n) number of arteries from different animals. Statistical analysis using one way ANOVA with Dunnett's post test revealed no significant differences.

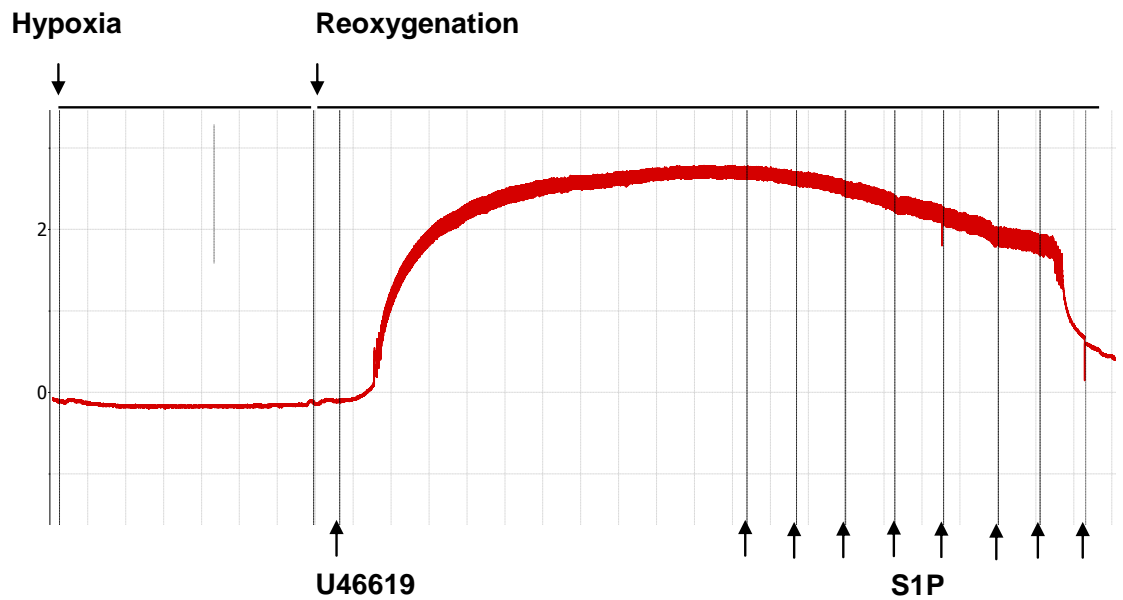


Figure 4-14 Representative trace showing the vasorelaxation response to S1P following exposure to hypoxia.

The vessels are exposed to hypoxia for 30 min, and then reoxygenated, pre-contracted by U46619 and S1P (1 nM–30 μ M) dose response curve is created.

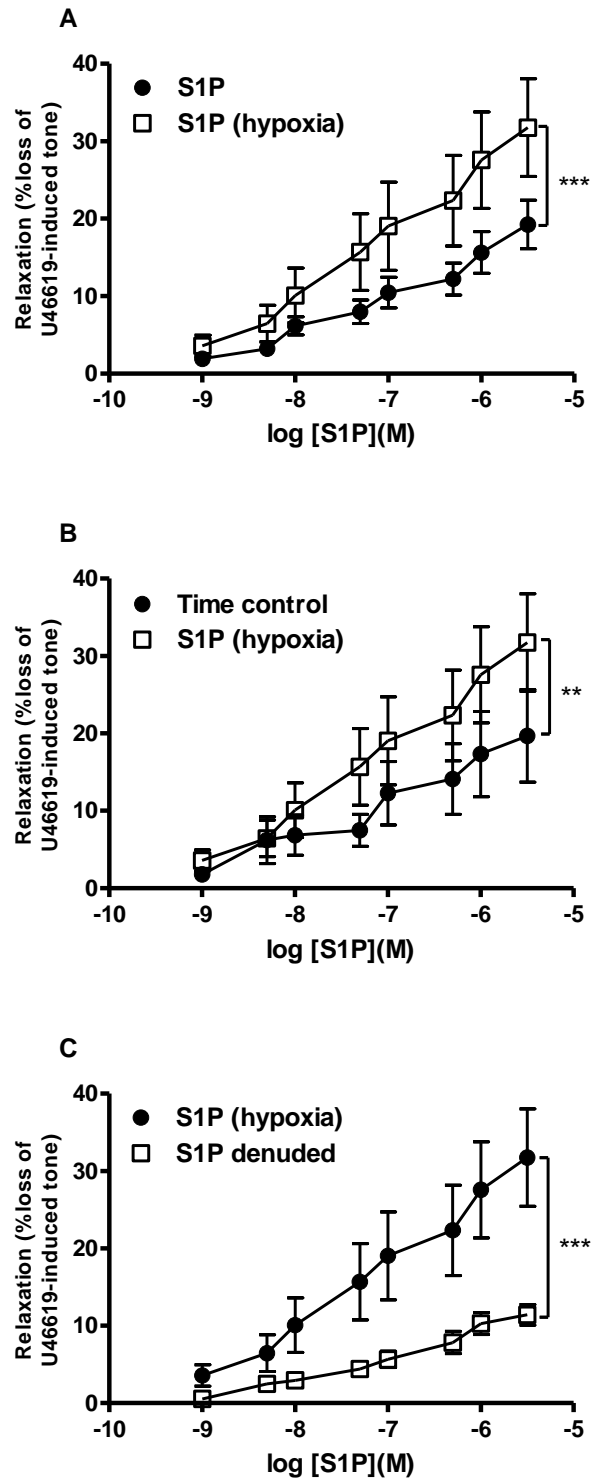


Figure 4-15 Effect of hypoxia on S1P-induced relaxation in rat aorta.

(A) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in the presence (n=10) and absence (n=9) of hypoxia. (B) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta (n=10) compared with time control (n=10) and under hypoxic conditions. (C) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact (n=10) and endothelium denuded (n=8) rat aorta under hypoxic conditions. ***p<0.001 vs S1P control (A), **p<0.01 vs time control (B), ***p<0.001 vs S1P (hypoxia, C) as determined by two-way ANOVA.

4.3.7 Effect of sphingosine kinase inhibitors on hypoxia-induced increase in SK1 expression.

Under normoxic conditions, pre-treatment of aortic rings with the non-selective SK inhibitor, SKi (10 μ M) or the selective SK1 inhibitor, PF543 (100 nM) led to an obvious reduction in SK1 immunostaining (red punctate staining in the images). However image J software yielded no significant differences in the integrated density of SK1 and this is likely because image J is also quantifying the background noise ($1.1 \times 10^6 \pm 2.9 \times 10^5$ AU, n=3; Figure 4-16 and $1.1 \times 10^6 \pm 1.8 \times 10^5$ AU, n=3; Figure 4-18 respectively). In contrast, addition of either SKi (Figure 4-16) or PF543 (Figure 4-18) markedly reduced (54%) the increase in SK1 expression caused by hypoxia when added 30 min prior to exposure to hypoxia ($1.2 \times 10^6 \pm 1.0 \times 10^5$ AU, n=3; Figure 4-16 and $1.2 \times 10^6 \pm 1.0 \times 10^5$ AU, n=3; Figure 4-18 respectively) compared with controls ($2.6 \times 10^6 \pm 2.6 \times 10^5$ AU, n=4).

4.3.8 Effect of inhibitors and antagonists on S1P-stimulated relaxation.

4.3.8.1 Effect of SK inhibitors on S1P-induced relaxation in endothelium-intact rat aorta

Under control conditions, preincubation of endothelium-intact rat aortic rings with the non-selective SK inhibitor, SKi (10 μ M) caused a significant attenuation of S1P-induced relaxation (maximal relaxation $9.6 \pm 1.3\%$, n=6) compared with control vessels (maximal relaxation $20.8 \pm 3.2\%$, n=10; Figure 4-17A). SKi also markedly reduced the enhanced S1P-induced relaxation caused by hypoxia (maximal relaxation $9.5 \pm 3.4\%$, n=7) compared with control vessels ($31.8 \pm 6.3\%$, n=10; Figure 4-17B). Moreover, the selective SK1 inhibitor, PF543 (100 nM) also significantly inhibited the S1P-stimulated relaxation in endothelium-intact vessels under control conditions (maximal relaxation $8.9 \pm 2.1\%$, n=9) compared to control vessels (maximal relaxation $13.6 \pm 1.8\%$, n=8; Figure 4-19A). The hypoxia-enhanced S1P-induced relaxation was also markedly decreased by pre-treatment with PF543 before exposure to hypoxia (maximal relaxation $13.6 \pm 4.1\%$, n=7) compared with control vessels (maximal relaxation $26.8 \pm 5.8\%$, n=7; 4-19B). However, responses to S1P were not significantly inhibited by pre-treatment with the selective SK2 inhibitor, ROME (10 μ M) under either control conditions (maximal relaxation $11.4 \pm 1.6\%$, n=5; Figure 4-20A) or hypoxic conditions (maximal relaxation $22.5 \pm 5.1\%$, n=6; Figure 4-20B).

4.3.8.2 Effect of L-NNA and CAY10444 on S1P-induced relaxation in endothelium-intact rat aorta.

30 min preincubation of endothelium-intact aortic rings with the nitric oxide synthase inhibitor L-NNA (10^{-4} M) led to inhibition of S1P-stimulated relaxation under control conditions (maximal relaxation $9.7 \pm 2.2\%$, $n=7$) compared with control vessels (maximal relaxation 23.1 ± 6.5 , $n=7$; Figure 4-21A). L-NNA also caused a marked attenuation in enhanced S1P-induced relaxation caused by hypoxia (maximal relaxation $13.8 \pm 8.2\%$, $n=6$) compared with control vessels (maximal relaxation $42.9 \pm 18.0\%$, $n=5$; Figure 4-21B). The selective S1P₃ receptor antagonist, CAY10444 (10 μ M, 30 min pre-treatment) did not significantly attenuate the responses to S1P under either control conditions (maximal relaxation $21.7 \pm 4.2\%$, $n=8$) compared with control vessels (maximal relaxation $18.1 \pm 3.3\%$, $n=6$; Figure 4-22A) or hypoxic conditions (maximal relaxation $38.1 \pm 7.8\%$, $n=6$) compared with control vessels ($29.1 \pm 5.9\%$, $n=5$; Figure 4-22B).

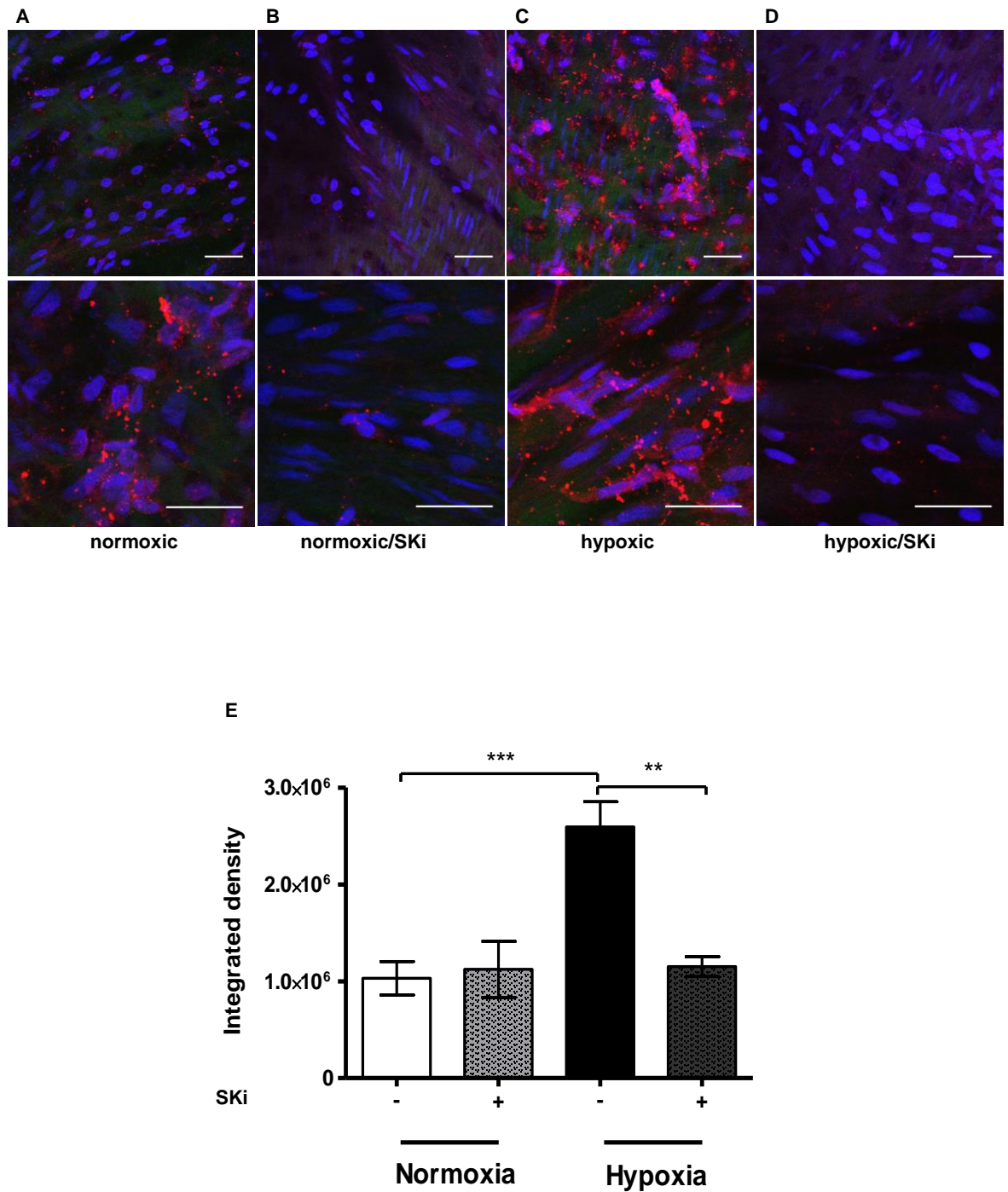


Figure 4-16 Effect of SKi on hypoxia-induced increase in SK1 expression.

Representative immunofluorescent images show expression of SK1 (red) in rat aortic endothelium (A) and following pre-treatment with SKi, a non-selective SK inhibitor, (10 μ M, 30 min) under normoxic conditions (B). Expression of SK1 in rat aorta subjected to 30 min hypoxia (C). Preincubation of rat aorta with SKi reduced the hypoxia-induced increase in SK1 expression (D). (E) Quantitative fluorescence measurement of SK1 in the presence and absence of SKi. **p < 0.01, ***p < 0.001 vs hypoxia alone as determined by one way ANOVA with Bonferroni's post-test. Results are representative of 3-4 separate experiments. Scale bar is 30 μ m for all images.

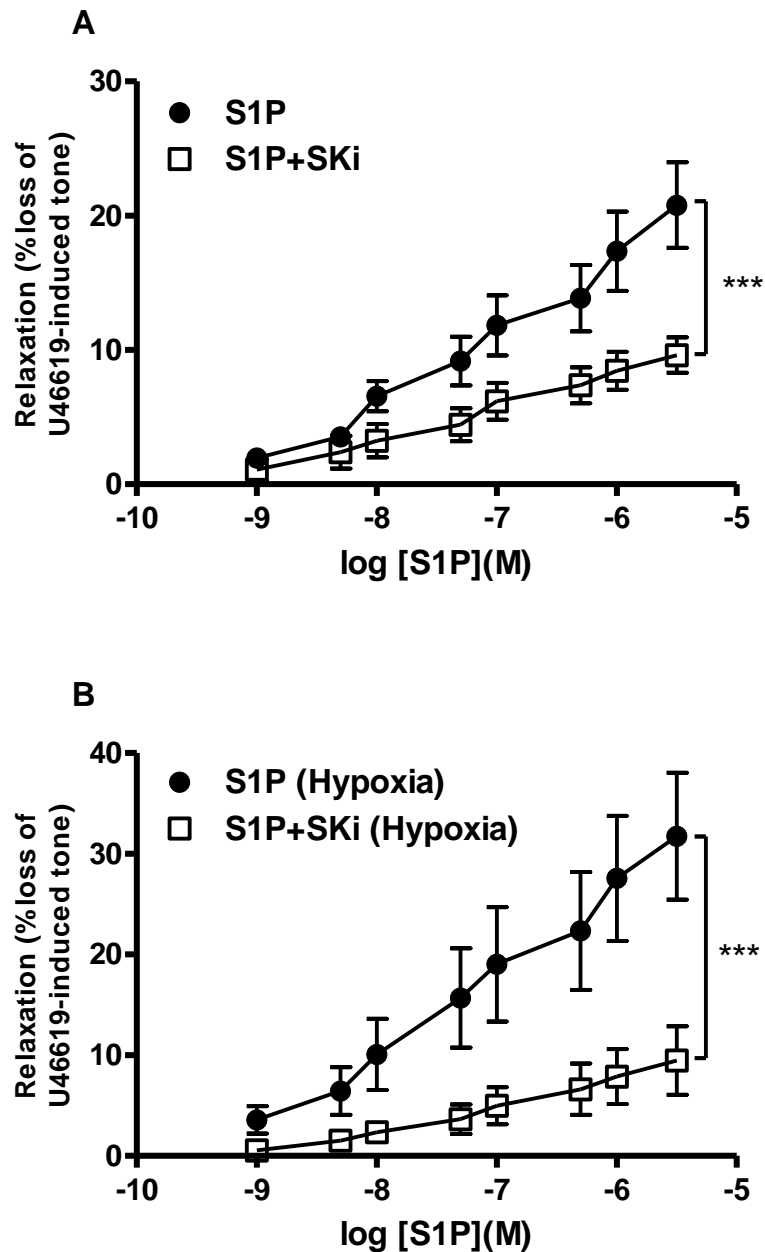


Figure 4-17 Effect of SKi on S1P-induced relaxation in rat aorta.

(A) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in presence (n=6) and absence (n=10) of SKi, a non-selective SK inhibitor, (10 μ M) under control conditions. (B) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in presence (n=7) and absence (n=10) of SKi (10 μ M) under hypoxic conditions. ***p<0.001 vs S1P alone as determined by two-way ANOVA.

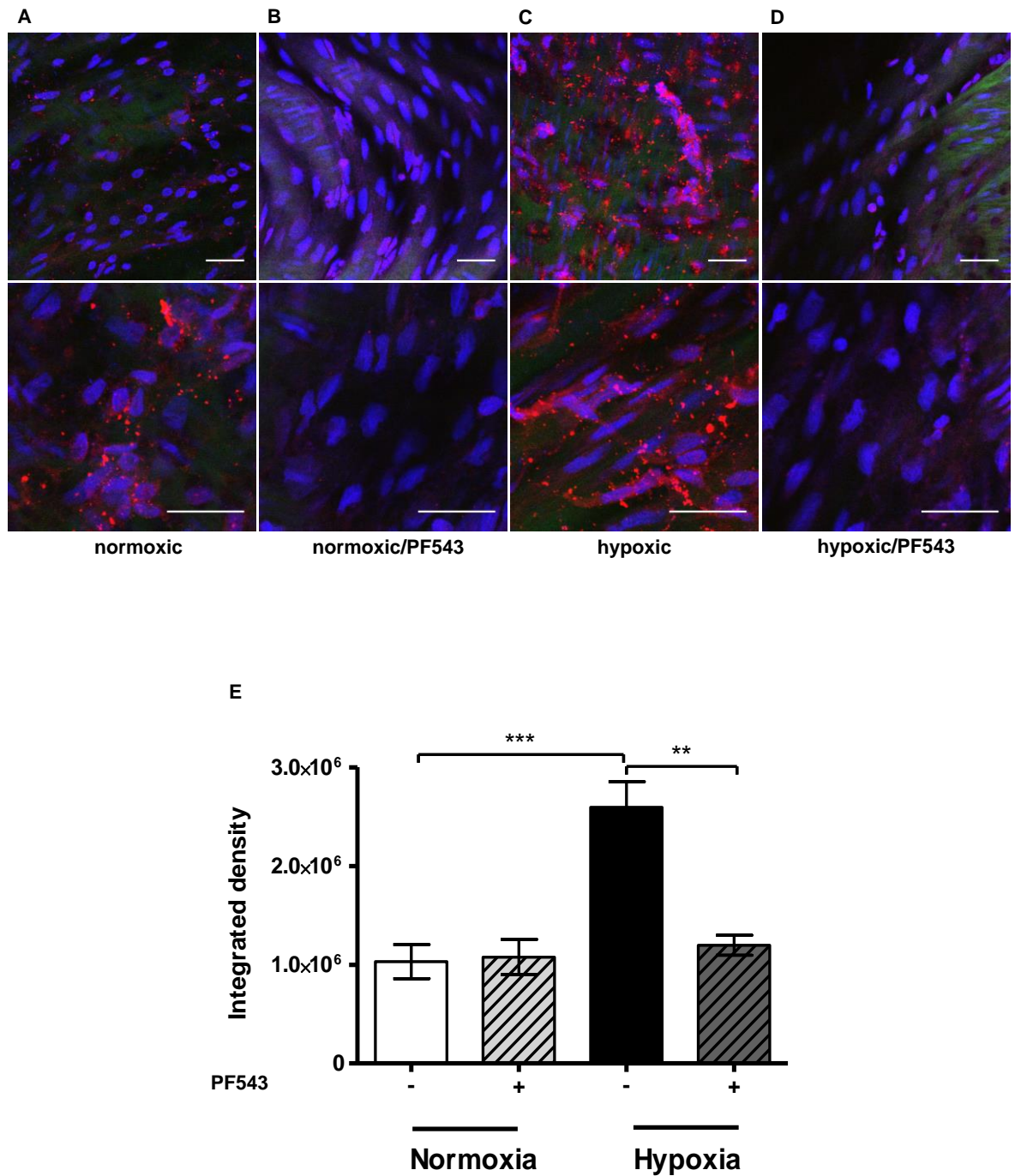


Figure 4-18 Effect of PF543 on hypoxia-induced increase in SK1 expression.

Representative immunofluorescent images show expression of SK1 (red) in rat aortic endothelium (A) and following pre-treatment with PF543, a selective SK1 inhibitor, (100 nM, 30 min) under normoxic conditions (B). Expression of SK1 in rat aorta subjected to 30 min hypoxia (C). Preincubation of rat aorta with PF543 reduced the hypoxia-induced increase in SK1 expression (D). (E) Quantitative fluorescence measurement of SK1 in the presence and absence of PF543. ** $p < 0.01$, *** $p < 0.001$ vs hypoxia alone as determined by one way ANOVA with Bonferroni's post-test. Results are representative of 3-4 separate experiments. Scale bar is 30 μm for all images.

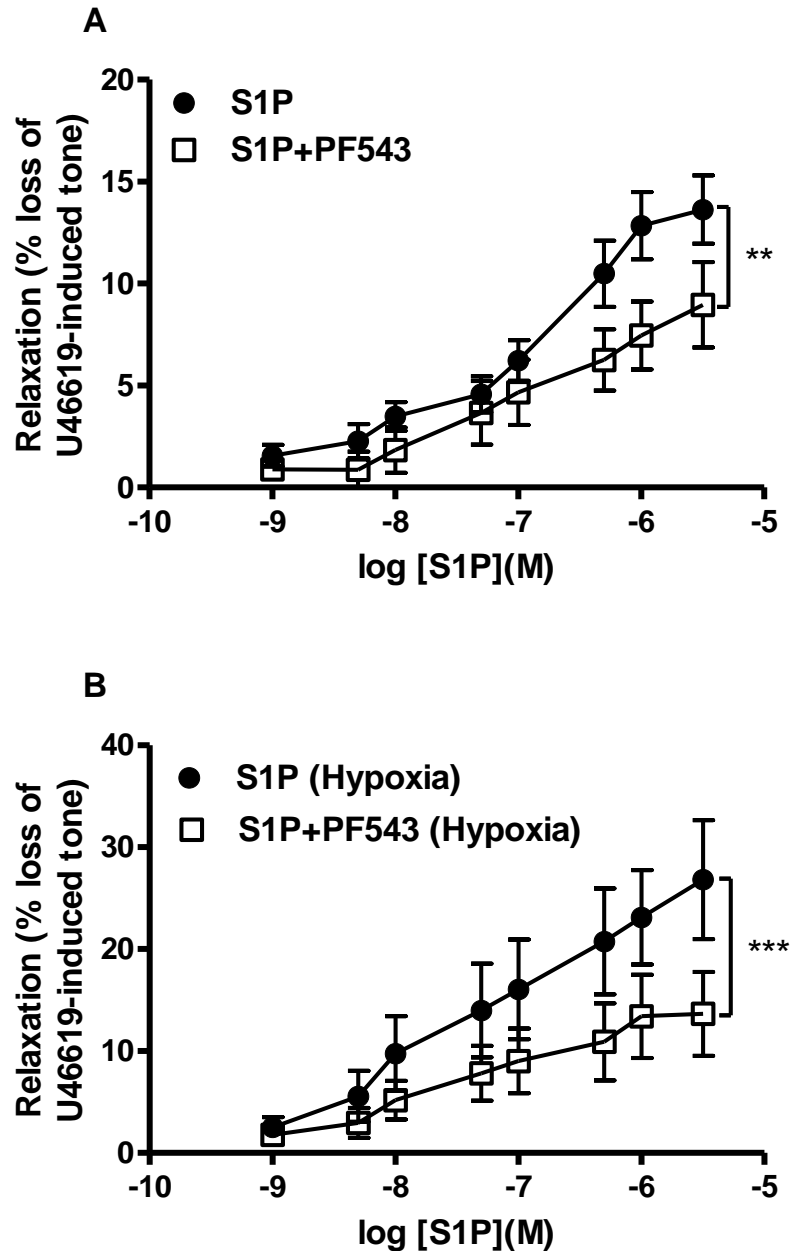


Figure 4-19 S1P-induced relaxation in rat aorta in presence and absence of PF543.

(A) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in presence (n=9) and absence (n=8) of PF543, a selective SK1 inhibitor, (100 nM) under control conditions. (B) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in the presence (n=7) and absence (n=7) of PF543 (100 nM) under hypoxic conditions. **p<0.01, ***p<0.001 vs S1P alone as determined by two-way ANOVA.

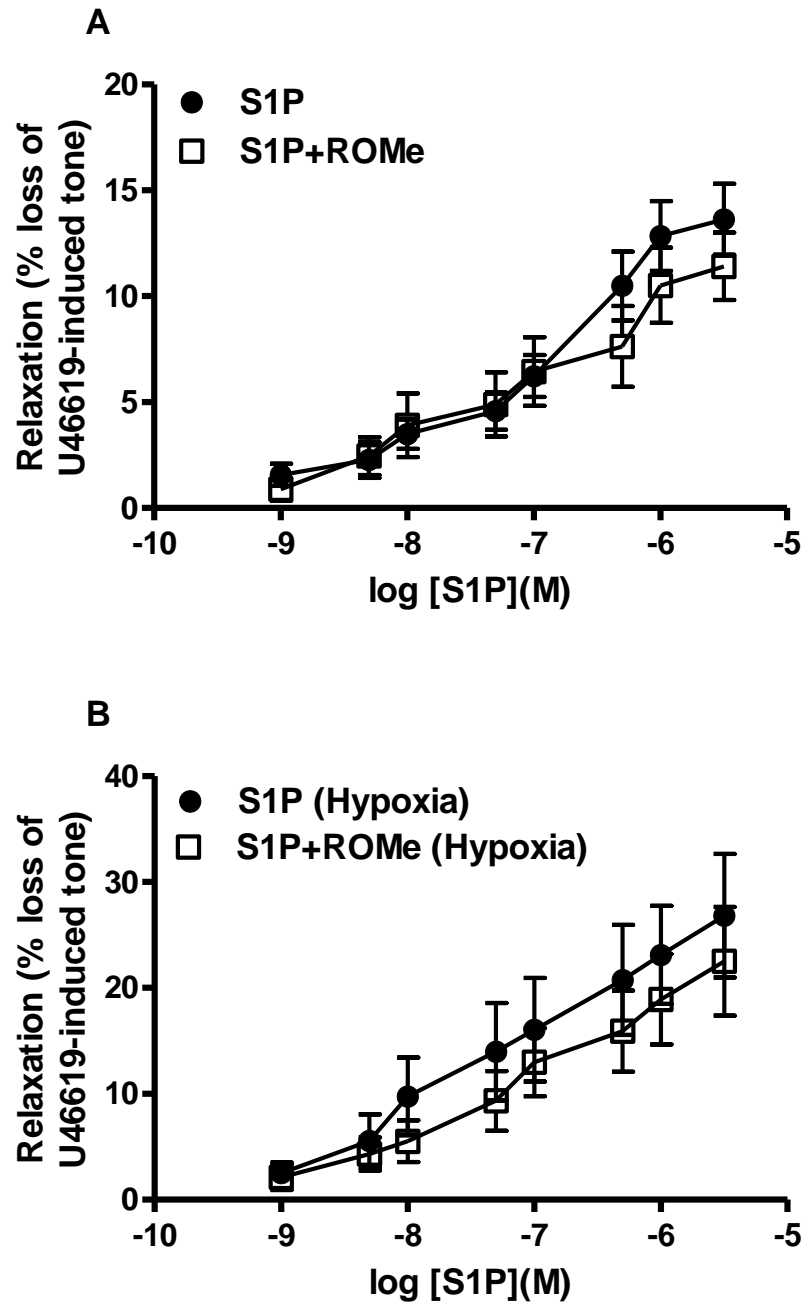


Figure 4-20 S1P-induced relaxation in rat aorta in presence and absence of ROme.

(A) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in presence (n=5) and absence (n=8) of ROme, a selective SK2 inhibitor, (10 μ M) under control conditions. (B) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in the presence (n=6) and absence (n=7) of ROme (10 μ M) under hypoxic conditions.

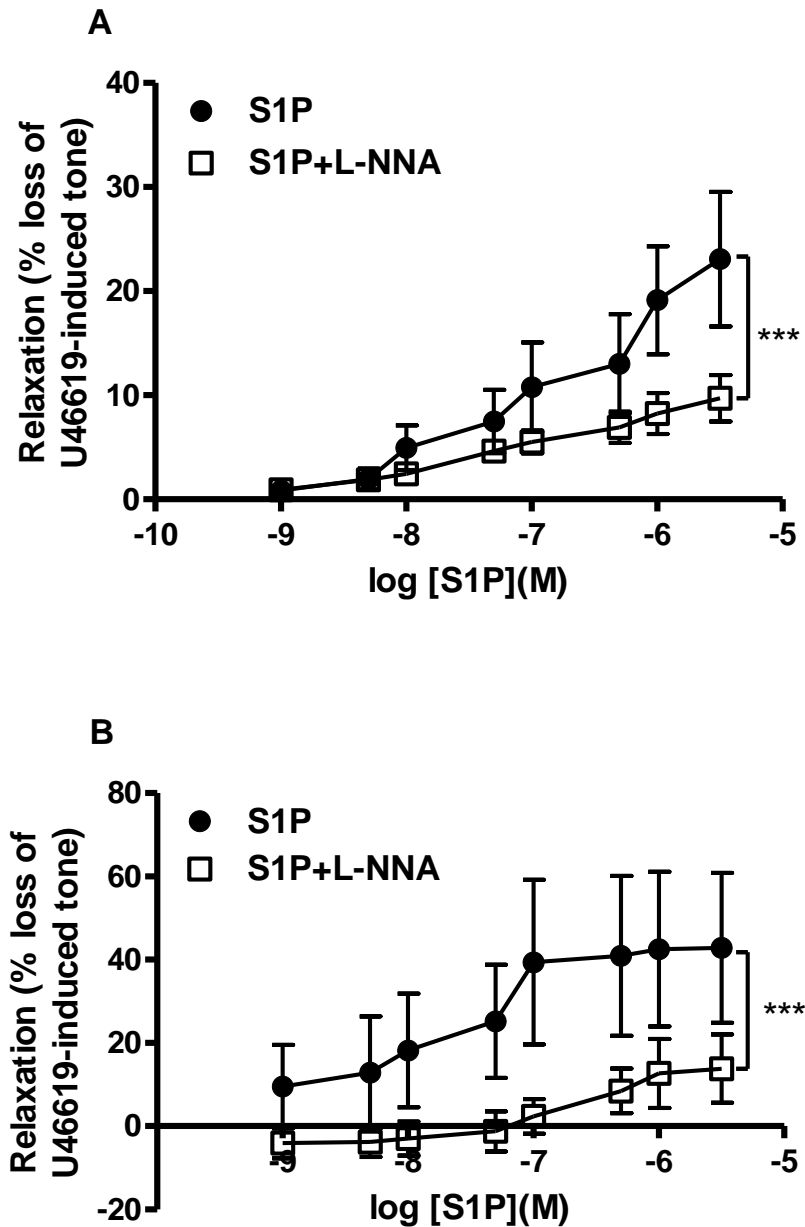


Figure 4-21 S1P-induced relaxation in rat aorta in presence and absence of L-NNA.

(A) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in the presence (n=7) and absence (n=7) of L-NNA, a nitric oxide synthase inhibitor, (10^{-4} M) under control conditions. (B) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in the presence (n=6) and absence (n=5) of L-NNA (10^{-4} M) under hypoxic conditions. ***p<0.001 vs S1P alone as determined by two-way ANOVA.

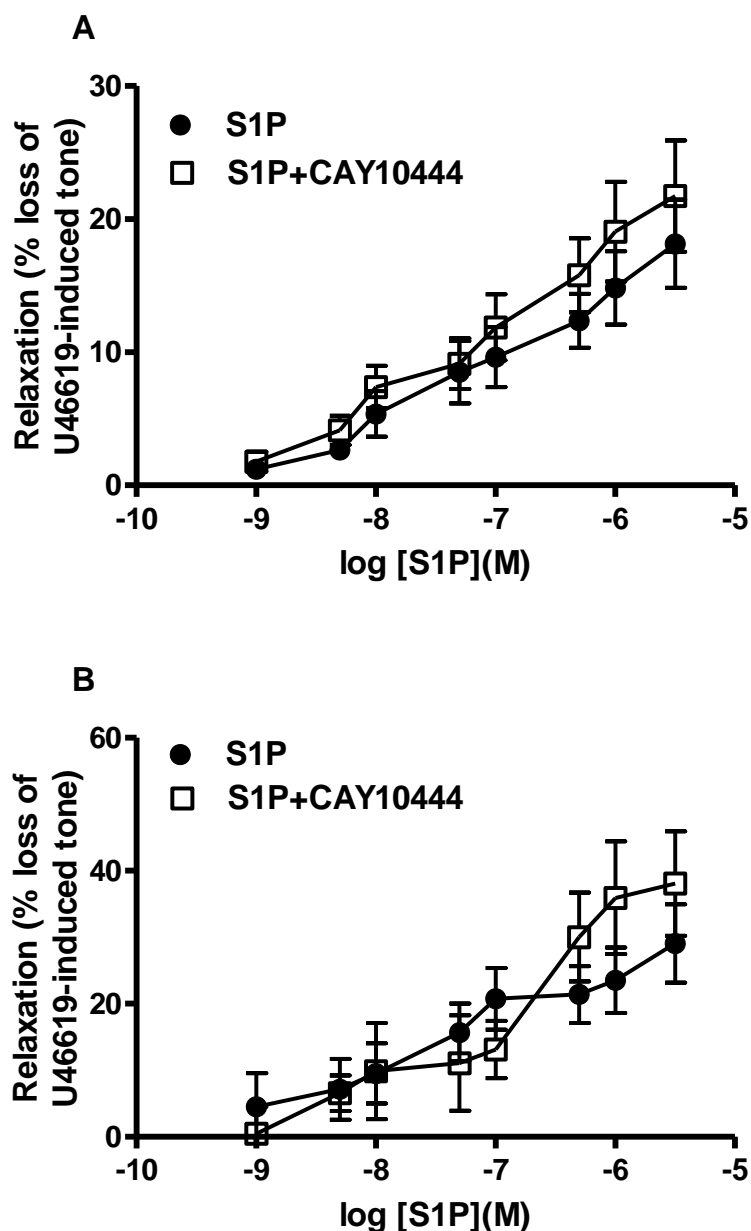


Figure 4-22 S1P-induced relaxation in rat aorta in presence and absence of CAY10444.

(A) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in the presence (n=8) and absence (n=6) of CAY10444, a selective S1P₃ antagonist, (10 μ M) under control conditions. (B) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aorta in the presence (n=6) and absence (n=5) of CAY10444 (10 μ M) under hypoxic conditions.

4.3.9 S1P-stimulated vascular relaxation in rat aorta from spontaneously hypertensive rats

A series of experiments was carried out to directly assess the vasorelaxant effect of S1P on endothelium-intact rat aortic rings obtained from spontaneously hypertensive Wistar Kyoto rats under control and hypoxic conditions. It was found that S1P-induced relaxation was significantly reduced in vessels obtained from hypertensive rats (maximal relaxation $6.1 \pm 5.1\%$, $n=6$) compared with control vessels obtained from normal Sprague-Dawley rats (maximal relaxation $13.6 \pm 1.7\%$, $n=6$; Figure 4-23A) under control conditions. In contrast, there was no significant difference between hypoxia-enhanced S1P-induced relaxation in vessels obtained from hypertensive rats (maximal relaxation $20.7 \pm 6.8\%$, $n=6$) and normal Sprague-Dawley rats (maximal relaxation $26.8 \pm 5.8\%$, $n=7$; Figure 4-23B). Thus, S1P was still able to stimulate relaxation in hypertensive vessels after exposure to hypoxia (maximal relaxation $20.7 \pm 6.8\%$, $n=6$, $6.1 \pm 5.1\%$, $n=6$ respectively; Figure 4-24A). Moreover, pre-treatment of rat aortic rings obtained from hypertensive rats with the selective SK1 inhibitor, PF543 (100 nM) before exposure to hypoxia significantly reduced the enhanced S1P-induced relaxation caused by hypoxia (maximal relaxation $9.7 \pm 6.7\%$, $n=6$; Figure 4-24B).

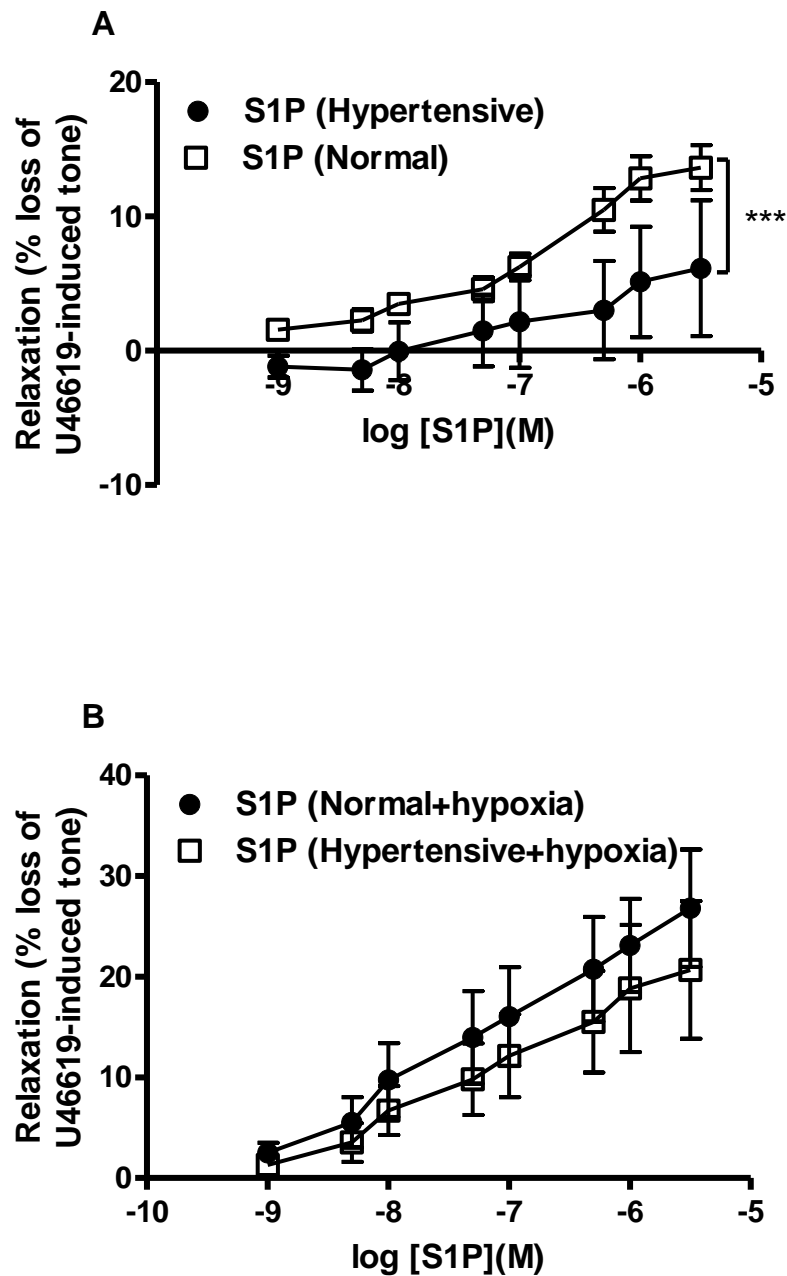


Figure 4-23 S1P-stimulated relaxation in rat aorta from normal and hypertensive rats.

(A) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aortic rings from hypertensive rats (n=6) and normal rats (n=6) under control conditions. (B) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aortic rings from hypertensive rats (n=6) and normal rats (n=7) under hypoxic conditions. $^{**}p<0.01$ vs S1P (control) as determined by two-way ANOVA.

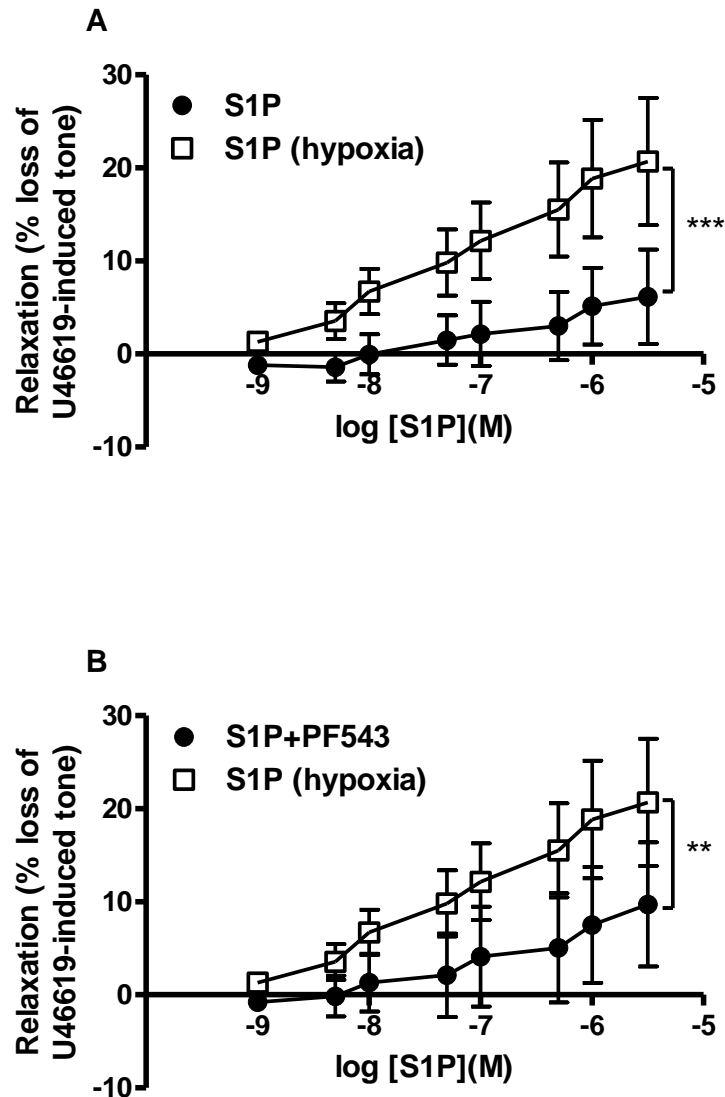


Figure 4-24 S1P-induced relaxation in rat aorta from hypertensive rats.

(A) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aortic rings from hypertensive rats in the presence (n=6) and absence (n=6) of hypoxia. (B) Concentration–response curves showing the vasorelaxant effect of S1P (1 nM–30 μ M) on U46619-pre-contracted endothelium-intact rat aortic rings from hypertensive rats in the presence (n=6) and absence (n=6) of PF543, a selective SK1 inhibitor (100 nM) under hypoxic conditions. **p<0.01 vs S1P (hypoxia), ***p<0.001 vs S1P alone as determined by two-way ANOVA.

4.4 Discussion

In chapter 3, it was found that short periods of hypoxia increased SK1 expression in isolated rat coronary artery endothelium. To determine whether the hypoxia-induced increase in SK1 may regulate vascular function through the generation of S1P, it was important to carry out functional experiments. However, isolation of rat coronary artery is a slow and difficult process due to its small size (200 μm) and position within the myocardial tissue. In addition, the amount of tissue that can be dissected would not be enough to conduct immunofluorescence and functional studies at the same time. Thus, rat aortic rings were used to separately examine whether hypoxia can similarly increase SK1 expression and whether this has an impact on vascular reactivity.

First, a series of experiments was carried out to investigate the effect of hypoxia on U46619-induced contraction in endothelium-intact and denuded rat aortic rings. A number of studies have reported that hypoxia augmented contractile sensitivity to U46619 in isolated cerebral artery (Sillau et al., 2002) and phenylephrine in isolated rat abdominal aorta (Gräser and Rubanyi, 1993). In the current study, it was found that exposure of endothelium-intact rat aortic rings to hypoxia increased the contractile sensitivity to U46619 compared with controls (Figure 4-2A). Removal of the endothelium had no effect on the enhanced contractile response to U46619 (Figure 4-2B). Moreover, pre-treatment with SKi significantly decreased the hypoxia-augmented contractile response to U46619 in endothelium-intact aortic rings. In contrast SKi did not reduce the hypoxia-enhanced contractile sensitivity to U46619 in endothelium-denuded aortic rings. These findings suggest that hypoxia increases sensitivity of smooth muscle cells to contractile agents. The effect of SKi in endothelium-intact but not denuded rings is more difficult to explain. It must be assumed that the effect of SKi is at the level of the endothelium and that the reduction in generation of S1P from the endothelium is what reduces contractility in response to U46619. Although, S1P has been demonstrated to stimulate relaxation of pre-contracted arterial preparations, S1P also exhibited a vasoconstrictor effect on basal arterial tone in isolated arteries (Coussin et al., 2002, Salomone et al., 2003). Therefore, it might be possible that the S1P generated in endothelial cells during hypoxia is transported out to activate S1P₂ in smooth muscle cells in a paracrine manner and this may explain why SKi attenuated the hypoxia-augmented contractile response to U46619 in endothelium-intact vessels. However, a direct effect of SKi on the smooth muscle under hypoxic conditions is

also a possibility which cannot be ruled out. Using a selective S1P₂ receptor antagonist and S1P transporter inhibitors may further elucidate this mechanism.

Several studies have previously shown that S1P induces endothelium-dependent vasorelaxation in rat aorta (Nofer et al., 2003, Roviezzo et al., 2006). Here, it was also shown that S1P stimulates relaxation in isolated rat aortic rings and this effect was almost entirely dependent on the presence of an intact endothelium, suggesting that S1P-induced relaxation is endothelium-dependent (Figure 4-5B). Moreover, various studies have reported that S1P-induced relaxation is mediated through its S1P₁ (Igarashi and Michel, 2000, Igarashi et al., 2001) and/or S1P₃ receptor (Nofer et al., 2003, Mair et al., 2010). Therefore, S1P receptor agonists were used to explore whether S1P-induced relaxation in rat aortae is mediated through S1P₁ and/or S1P₃ receptors. It was found that addition of CYM5541, an S1P₃ agonist, stimulated relaxation in endothelium-intact but not endothelium-denuded isolated rat aortic rings whereas SEW2871, an S1P₁ agonist had no effect, suggesting that S1P may elicit its effect via S1P₃ receptors (Figure 4-8 A & B). Similar to experiments performed in the rat coronary artery (chapter 3), polyclonal antibodies to SK1 enzyme, S1P₁ and S1P₃ receptors were used to examine the expression of SK1 enzyme, S1P₁ and S1P₃ receptor subtypes in rat aortic endothelium. It was found that the rat aortic endothelium exhibits an intense immunostaining for SK1 enzyme and S1P₁ and S1P₃ receptors (Figure 4-6, 4-7, 4-10). Unlike S1P and CYM5541, which induced relaxation in rat aortic rings, SEW2871 was without effect. This may suggest that S1P-stimulated relaxation is mediated through S1P₃ receptor. In line with this, it has also been reported that FTY720, an S1P analogue, stimulated endothelium-dependent relaxation in phenylephrine-pre-contracted aortic rings and deletion of either eNOS or S1P₃ inhibited the vasorelaxation response, suggesting that FTY720-induced relaxation is mediated by eNOS/NO pathway and S1P₃ receptors (Tolle et al., 2005). In the same study, it was also shown that eNOS activation was attenuated by pre-treatment with the PI3K inhibitor LY294002, confirming involvement of Akt in eNOS activation and also by the SK inhibitor DMS, demonstrating a critical role for SK in mediating FTY720-stimulated relaxation.

Recent studies have shown that TRPV1 is widely expressed in blood vessels such as rat skeletal muscle arteries, mesenteric, femoral, carotid and aorta (Lizanecz et al., 2006, Toth et al., 2014). Kark et al demonstrated that stimulation of TRPV1 in blood vessels may induce constriction or dilation depending on the vascular bed used (Kark et al., 2008). It was suggested that TRPV1-stimulated relaxation may be related to the perivascular sensory

neuronal terminals, which were thought to stimulate vasodilation via release of neurotransmitters such as CGRP and substance P (Zygmunt et al., 1999). Another study showed that TRPV1 stimulation induces porcine coronary vasodilation and this effect was inhibited by removal of endothelium, the TRPV1 antagonist capsazepine, the NOS inhibitor L-NAME as well as K^+ channel antagonists (Bratz et al., 2008). In contrast, stimulation of TRPV1 in arteriolar smooth muscle cells induced vasoconstriction through an increase in intracellular Ca^{2+} concentration (Cavanaugh et al., 2011, Czikora et al., 2012). The present study found that the TRPV1 antagonist, AMG 9810 significantly decreased the S1P-stimulated vasorelaxation in endothelium-intact rat aorta, suggesting that part of the S1P-induced relaxation may be mediated through activation of TRPV1 channels (Figure 4-9). Therefore, vascular TRPV1 may play an important role in regulation of vascular tone via a NO-dependant pathway. Stimulation of endothelial TRPV1 by S1P may represent a novel pharmacological target for treatment of vascular diseases.

In chapter 3, short-term hypoxia was shown to increase SK1 expression in rat coronary artery endothelium and this effect was significantly reduced by pre-treatment with cycloheximide, a protein synthesis inhibitor, SKi, a non-selective SK inhibitor or PF543, a selective SK1 inhibitor. Indeed, short term hypoxic stress has been shown to increase SK1 protein levels in glioma cell lines starting from 1 hr after commencing hypoxic stress and this was accompanied by an increase in expression of HIF-1 α and HIF-2 α (Anelli et al., 2008). SK1 but not SK2 mRNA was also increased with a maximal 2-fold enhancement occurring after 4 h of treatment with a hypoxia-mimicking agent. Another study demonstrated that short periods of hypoxia significantly increased SK1 immunofluorescence in rat amoeboid microglial cells and this increase in SK1 immunofluorescence started 15 min after exposure to hypoxia and reached a maximum level after 1 hr hypoxia (Lin et al., 2011). Therefore, one of the aims of this chapter was to determine whether hypoxia increases SK1 expression in rat aortic rings and how this may regulate vascular function through the generation of S1P. In agreement with data obtained from the rat coronary artery, exposure of rat aortic rings to short-term (30 min) hypoxia resulted in a marked increase in SK1 expression in rat aortic endothelium compared with controls (Figure 4-10). Using human anti-phospho-specific SK1 (Ser-225) antibody revealed no differences in pSK1 immunostaining under normoxic and hypoxic conditions (Figure 4-11). However, SK1 can also be activated via phosphorylation-independent mechanisms (ter Braak et al., 2009, Gault et al., 2012). It was also observed that pre-treatment with cycloheximide 30 min before exposure to hypoxia resulted in a significant reduction in the hypoxia-induced increase in SK1 expression (Figure 4-13). A small

decrease in SK1 expression under normoxic conditions with cycloheximide was also seen. This data suggests that hypoxia may increase SK1 synthesis in rat aortic rings. Furthermore, pre-treatment of rat aortae with SKi (Figure 4-16) or PF543 (Figure 4-18) visually appeared to reduce SK1 immunostaining (red punctate staining in the images) under normoxic conditions but image J software yielded no significant differences in the integrated density of SK1 and this is likely because image J is also quantifying the background noise in these images. Pre-treatment with SKi (Figure 4-16) or PF543 (Figure 4-18) prior to exposure to hypoxia resulted in a reversal of the hypoxia-induced increase in SK1 expression.

Various previous studies have demonstrated arterial relaxation in response to hypoxia in several arteries from different animal species including rat, and human coronary arteries precontracted with KCL or U46619 (Kerkhof et al., 2002, Lynch et al., 2006). Lynch et al showed that hypoxic dilation was reversed by retuning to normoxic conditions but addition of glibenclamide, a K_{ATP} channel blocker, had no effect (Lynch et al., 2006). Vasorelaxation in response to hypoxia was also reported in rabbit basilar and carotid arteries (Pearce et al., 1992) and porcine and rat aortae pre-contracted with phenylephrine (Gasser et al., 1993, Herrera and Walker, 1998). In endothelium-denuded rat aortae pre-contracted with phenylephrine, the L-type Ca^{2+} channel antagonist nifedipine abolished the vasorelaxation response to mild hypoxia and reduced the vasodilation response to moderate hypoxia whereas glibenclamide only diminished the response to moderate hypoxia (Herrera and Walker, 1998). Therefore the mechanism of hypoxic-induced vascular relaxation is still an area of controversy and further experiments are required to elucidate the underlying mechanism of vasodilation in response to hypoxia. In the present study, it was important to investigate whether the hypoxic-induced increase in SK1 may be involved in regulation of vascular activity. This study found that the S1P-induced relaxation in endothelium-intact aortic rings was significantly enhanced in those rings subjected to a short period of hypoxia compared with control vessels and time controls (Figure 4-15A & B). Moreover, the hypoxia-enhanced S1P-induced relaxation was abolished by removal of endothelium, specifically implicating the vascular endothelium in this response (Figure 4-15C). Mair et al (2010) showed that the relaxation response to anandamide, an endogenous cannabinoid, and S1P in the rat coronary artery was significantly attenuated by SKi. Therefore, SK inhibitors were used here to examine whether the increase in SK1 caused by hypoxia may mediate the augmented relaxation response to exogenous S1P. In the current study, it was found that pre-treatment of endothelium-intact rat aortic rings with either SKi (Figure 4-17A & B) or PF543 (Figure 4-

19A & B) prior to exposure to hypoxia inhibited the relaxation response to S1P in both control and hypoxic vessels. In contrast, addition of the selective SK2 inhibitor ROME 30 min before commencing hypoxia failed to inhibit either S1P-induced relaxation under control conditions or the hypoxic augmented relaxation response to S1P (Figure 4-20A & B). These findings suggest that the relaxation response of rat aortae to S1P under control and hypoxic conditions is partly-dependent on SK1 but not SK2. SK1 is likely to be activated by addition of S1P and translocated to the plasma membrane to generate more S1P from sphingosine and the generated S1P contributes to endothelium-dependent vasorelaxation through downstream activation of eNOS/NO signalling pathway (Figure 4-25). In agreement with this, a previous study has shown that intracellular generation of S1P mediates ACh-induced vasorelaxation in thoracic aortae via activation of NO pathway without transport of S1P extracellularly to activate cell surface S1P receptors, demonstrating a critical role of intracellular S1P in ACh-induced vasorelaxation (Roviezzo et al., 2006).

Various studies have shown that S1P is likely to elicit its relaxation response via S1P₃ receptor in rat thoracic aortae (Nofer et al., 2004) and coronary arteries (Mair et al., 2010). In line with this, it was found that the S1P₃ agonist, CYM5541, but not the S1P₁ agonist, SEW2871 induced a similar magnitude relaxation response in isolated rat aortae compared to S1P, suggesting that S1P₃ agonist-induced vasorelaxation is a G_q mediated effect which is regulated by S1P₃ but not S1P₁ receptors (Figure 4-25). Evidence for G_q mediated translocation of SK1 was reported in HEK-293 cells (ter Braak et al., 2009). Activation of G_q-coupled receptors stimulated a rapid and long lasting relocalisation of SK1 to the plasma membrane and this effect was independent of the two downstream effectors of G_q: protein kinase C (PKC) and elevated calcium (ter Braak et al., 2009). However, addition of CAY10444, a selective S1P₃ antagonist, prior to commencing hypoxia failed to inhibit the relaxation response to S1P (Figure 4-22B). CAY10444 is a low potency antagonist and therefore a proportion of S1P₃ receptors might be left unoccupied by CAY10444 (receptor reserve) in the aortic rings which could then be activated by exogenous S1P (Jongsma et al., 2006). Further studies utilising another S1P₃ antagonist are required to investigate the role of S1P₃ receptors fully. Moreover, stimulation of TRPV1 channels in response to S1P may provide an alternative mechanism by which S1P induces vasorelaxation in isolated arteries (Figure 4-8). However, the role of TRPV1 under hypoxic conditions in mediating the relaxation to S1P has still to be investigated.

Several studies have reported that the eNOS pathway may function as a downstream target for the biological responses to S1P including vasorelaxation (di Villa Bianca et al., 2006, Roviezzo et al., 2006). It has also been shown that stimulation of cultured vascular endothelial cells by S1P increases eNOS activity and subsequently enhances nitric oxide generation (Igarashi et al., 2001). Thus, increased NO production in vascular endothelium may play a key role in modulations of vascular tone. Strong evidence exists that S1P₁ and/or S1P₃ receptor subtypes play an important role in mediating S1P activation of eNOS (Nofer et al., 2004, Theilmeier et al., 2006, Tatematsu et al., 2013). Roviezzo et al showed that S1P induced endothelium-dependent vasorelaxation in rat aorta was inhibited by L-NAME. Therefore, a nitric oxide synthase inhibitor (L-NNA) was used to determine whether the hypoxia-enhanced relaxation response is mediated by NO. It was observed that pre-treatment of rat aortic rings with L-NNA, before exposure to hypoxia inhibited the vasorelaxation response to S1P (Figure 4-21B). The S1P-induced relaxation was also inhibited by L-NNA in control vessels (Figure 4-21A). This suggests that generation of NO is a critical downstream event in S1P-mediated vascular relaxation.

Another aim of this study was to determine the effect of hypertension on the S1P-mediated relaxation in rat aortic rings obtained from spontaneously hypertensive Wistar Kyoto rats under control and hypoxic conditions. It was found that the vasorelaxation response to S1P was significantly attenuated in aortic rings obtained from hypertensive rats under control conditions (Figure 4-23A). In contrast, there was no significant difference in hypoxia-enhanced vasorelaxation responses to S1P between aortic rings from hypertensive and control rats (control vessels obtained from male Sprague-Dawley rats) (Figure 4-23B). Moreover, pre-treatment of aortic rings that were obtained from hypertensive rats with PF543, a selective SK1 inhibitor, prior to exposure to hypoxia markedly attenuated the hypoxia-enhanced response to S1P, suggesting that hypoxia may increase SK1 levels and in turn generate more S1P (Figure 4-24B). In summary, although the vasorelaxation response to S1P was markedly attenuated under control conditions in aortic rings from hypertensive rats, the hypoxia-enhanced vasorelaxation response to S1P was maintained. More experiments are required to elucidate the role of SK1/S1P/S1P receptors signalling pathway in regulation of blood pressure and the pathogenesis of hypertension.

4.5 Conclusion

This study shows that hypoxia induced an increase in SK1 expression in rat aortic endothelium and enhanced vasorelaxation responses to S1P. S1P stimulated NO-dependent

vascular relaxation via the S1P₃ receptor. SK1 but not SK2 enzyme was involved in the S1P-induced relaxation under control and hypoxic conditions. The underlying mechanism of hypoxia-enhanced S1P-induced relaxation is likely related to the increase in SK1 expression induced by hypoxia. Furthermore, the SK1 mediated hypoxia-enhanced vasorelaxation response to S1P is maintained in hypertension. This is a novel area of investigation, which requires further development and may potentially provide further targets in the modulation of S1P cardiovascular effects.

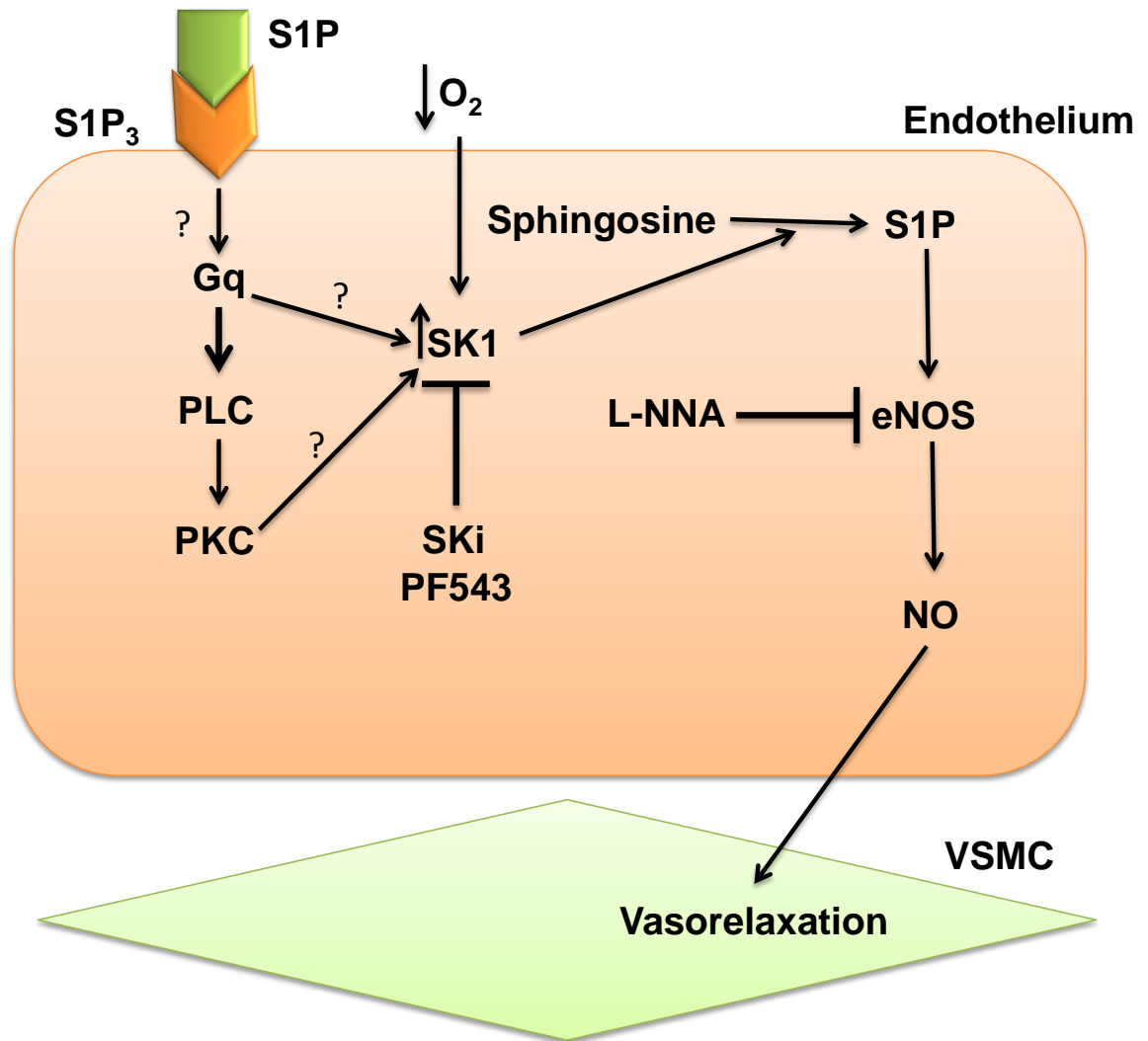


Figure 4-25 A schematic diagram showing the mechanism of S1P-induced relaxation.

Exogenous S1P acts as agonist for S1P₃ receptor which leads to activation of SK1 via G_q-mediated effect. The activated SK1 generates more S1P which functions as intracellular messenger and activates eNOS to produce NO. Hypoxia increases SK1 expression in endothelial cells and when activated through S1P/S1P₃/G_q signalling pathway, it generates more intracellular S1P that stimulates eNOS to generate more NO.

Chapter five

5 Influence of hypoxia on SK1 expression in cultured endothelial cells and cardiac tissue

5.1 Introduction

Three variants of SK1 exist that differ only at their N-terminus. SK1a has a molecular mass of 42.5 kDa, while SK1b has an 86 amino acid N-terminal extension and is a 51 kDa protein compared to SK1a (Pitson et al., 2000, Ota et al., 2004). SK1c possesses an additional 14 amino acids compared to SK1a and so has a molecular mass of 43.9 kDa. Several stimuli have been shown to regulate SK1 activity including agonists of GPCRs, tyrosine kinase receptors and immunoglobulin receptors, proinflammatory cytokines, calcium and protein kinase activators (Taha et al., 2006b, Alemany et al., 2007, Pyne et al., 2009). Decreased oxygen availability (hypoxia) has also been shown to increase SK1 expression in human pulmonary smooth muscle cells and the human endothelial cell line EA.hy926 (Ahmad et al., 2006, Schwalm et al., 2008). Moreover, it has become increasingly recognized that the SK/S1P signalling pathway plays a critical role in cardioprotection against conditions associated with oxidative stress, such as acute or chronic ischaemia or ischaemia/reperfusion injury. For instance, addition of S1P to adult mouse cardiomyocytes enhances survival during exposure to *in vitro* hypoxia (Zhang et al., 2007). The protective effect of exogenously applied S1P was also reported in isolated mouse hearts subjected to ischaemia/reperfusion injury (Jin et al., 2002, Lecour et al., 2002). Moreover, it is well established that ischaemic preconditioning followed by extended ischaemia and then reperfusion protects the heart against ischaemia/reperfusion injury although the precise mechanism remains elusive. Ischaemic preconditioning is associated with increased SK activity compared with non-preconditioned hearts, suggesting that SK may mediate the cardioprotection afforded by ischaemic preconditioning (Jin et al., 2004). Vessey et al demonstrated that exposure of non-preconditioned mouse hearts to 45 min ischaemia decreased SK activity more than 60% which did not recover during 45 min reperfusion (Vessey et al., 2006). 30 min ischaemia also led to a decline in SK activity but this partially recovered during reperfusion in the same study. In preconditioned hearts, SK activity decreased by only half of that seen in non-preconditioned hearts during ischaemia and upon reperfusion, activity then recovered to more than 90% of normal (Vessey et al., 2006). Therefore, SK1 is cardioprotective but more in depth insight into the regulation of this enzyme by hypoxia/ischaemia in vascular and cardiac cells is required in order to understand the mechanism by which SK1 elicits this effect.

In chapter 3 and 4, it has been demonstrated that hypoxia increases SK1 expression in rat coronary and aortic endothelium. Furthermore, the hypoxia-induced increase in SK1

expression was inhibited by pre-treatment with SK1 inhibitors. Therefore, a series of experiments was carried out to determine whether the hypoxia-induced increase in SK1 expression is limited to whole vessel preparations or can also be induced in a cultured human endothelial cell line and rat cardiac tissue.

5.1.1 Aims

The aims investigated in this chapter were:

- Study the effect of hypoxia on SK1 expression in HUVECs in the presence and absence of SK1 inhibitors.
- Study the effect of hypoxia on SK1 expression in cardiac tissue in the presence and absence of SKi, a non-selective SK inhibitor, PF543, a selective SK1 inhibitor, and cycloheximide, a protein synthesis inhibitor.
- To determine whether hypoxia induces phosphorylation of SK1 in cardiac tissue.

5.2 Methods

5.2.1 Cell culture

The HUVEC cell culture protocol was detailed in Section 2.5.1. Briefly, HUVECs were grown in 75 cm³ culture flasks and maintained in Endothelial Cell Growth Media MV / MV2 (PromoCell, Germany). The cells were kept incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The media was replaced every 48 hr. Cells were routinely sub-cultured when approximately 80-90% confluence was reached and used for experiments at passage 4 to 6.

5.2.2 Experimental protocols

HUVECs were seeded in 6 well plates and grown to approximately 90% confluence. The cells were then incubated for 24 hr with serum-free medium. Then the cells were treated with specific inhibitory agents as outlined below and subjected to 30 min normoxia/hypoxia. Hypoxia was induced by constant supply of the media with gas composed of 95% N₂, 5% CO₂ for approximately 30 mins to reduce oxygen tension to 5-7% O₂ saturation.

5.2.2.1 Inhibitory agents

A series of experiments was performed where the cells were pre-treated with cycloheximide, a protein synthesis inhibitor, SKi, a non-selective SK inhibitor, MG132, proteasomal inhibitor, CA-074ME, lysosomal inhibitor, or a combination of MG132 and CA-074ME (all at 10 µM concentration) for 30 min prior to exposure to normoxia/hypoxia for 30 min.

5.2.2.2 Western blotting

Following pre-treatment with the inhibitory agents, HUVEC lysates were prepared and samples were then loaded on NuPAGE® Novex® 4-12 % Bis-Tris mini gels at 10 µg of protein per well. Immunoblotting was then carried out with antibodies against SK1 and the loading control GAPDH (the dilutions used are found in Table 2-4).

5.2.3 Preparation of heart tissue

Heart tissue was removed and dissected immediately after death from Male Sprague-Dawley rats as described in Section 2.5.3. The right ventricular wall was cut into 8 pieces and each piece of tissue was pre-incubated with a specific inhibitory compound before exposure to hypoxia or normoxia for a particular length of time as outlined below.

5.2.3.1 The used inhibitory agents

A series of experiments was carried out where the tissue pieces were pre-incubated with SKi, a non-selective SK inhibitor (10 μ M), PF543, a selective SK1 inhibitor (100 nM) or cycloheximide, a protein synthesis inhibitor (10 μ M) for 30 min before exposure to normoxia/hypoxia for 30 min.

5.2.3.2 Western blotting

Following pre-treatment with the inhibitory agents and exposure to hypoxia, heart tissue was pulverised in liquid nitrogen into a fine powder by using a mortar and pestle and resuspended in ice-cold lysis buffer. The extract was transferred into ice-cold centrifuge tubes and centrifuged (8000 x g, 10 min) on a bench top centrifuge. The supernatants were then collected, prepared and protein estimation was carried out with addition of 30 μ g of protein per well. Immunoblotting was then carried out with antibodies against SK1, pSK1 and the loading control GAPDH (the dilutions used are found in Table 2-4).

5.2.4 Statistical analysis

Graph Pad Prism (version 5) software was used to analyse all the data. All results were expressed as mean \pm standard error of the mean (S.E.M) and n represents the number of independent experiments conducted. ANOVA with Dunnett's post-test was used. P-values were considered statistically significant when $p < 0.05$.

5.3 Results

5.3.1 Expression of SK1 in HUVECs in presence and absence of different inhibitory agents under normoxia and hypoxia

We first investigated the effect of different inhibitors on SK1 expression under normoxic and hypoxic conditions in HUVECs. The cells were pre-incubated with SKi, a non-selective SK inhibitor or cycloheximide, a protein synthesis inhibitor or MG132, a proteasomal inhibitor or CA-074ME, a lysosomal inhibitor or a combination of MG132 and CA-074ME for 30 min prior to exposure to normoxia/hypoxia. Cell samples were then Western blotted with anti-SK1 antibody. Band densities were quantified as the ratio of SK1 to GAPDH. The specificity of the anti-SK1 antibody was tested by comparison with a previously validated antibody.

The anti-SK1 antibody used in this study detected SK1b ($M_r = 51$ kDa) in HUVECs. No change in expression of SK1b was seen following exposure to 30 min hypoxia compared with normoxia in HUVECs (Figure 5-1). Under normoxia, there was no effect on the expression of SK1b with any of the inhibitory agents (Figure 5-2). Moreover, pre-treatment with CA-074ME or a combination of MG132 and CA-074ME before exposure to hypoxia led to a trend of increase in relative expression of SK1b compared to untreated hypoxic control. A small increase in SK1b expression was also observed when the cells were pre-treated with MG132 alone prior to exposure to hypoxia. The representative blot (Figure 5-3) demonstrates the trend of increase in SK1b expression following pre-incubation with MG132, CA-074ME or a combination of MG132 and CA-074ME relative to control. Pre-treatment with SKi or cycloheximide had no effect on SK1b expression (Figure 5-3).

5.3.2 Expression of SK1 in cardiac tissue following treatment with specific inhibitory agents under normoxia and hypoxia

To examine the influence of specific inhibitory drugs on the expression of SK1 in cardiac tissue under normoxic and hypoxic conditions, pieces of cardiac tissue were pre-treated with SKi, a non-selective SK inhibitor, PF543, a selective SK1 inhibitor or cycloheximide, protein synthesis inhibitor for 30 min before exposure to normoxia/hypoxia.

The anti-SK1 antibody used in these experiments detected SK1a in the heart tissue as the immunostaining corresponded to a band of ~ 42 kDa. Compared with normoxia, hypoxia had no significant effect on expression of SK1a in rat heart tissue (Figure 5-4). Under

normoxia, a significant reduction in SK1 expression was observed following pre-treatment with PF543 or cycloheximide relative to untreated control (Figure 5-5). Pre-treatment with SKi led to a small reduction in relative SK1 expression under normoxia as well. Unlike normoxia, pre-treatment with SKi or PF543 or cycloheximide was without effect on expression of SK1 following exposure to hypoxia compared with hypoxia alone (Figure 5-6). There was also no effect on relative expression of phosphorylated SK1 following exposure to hypoxia compared to normoxia in heart tissue (Figure 5-7).

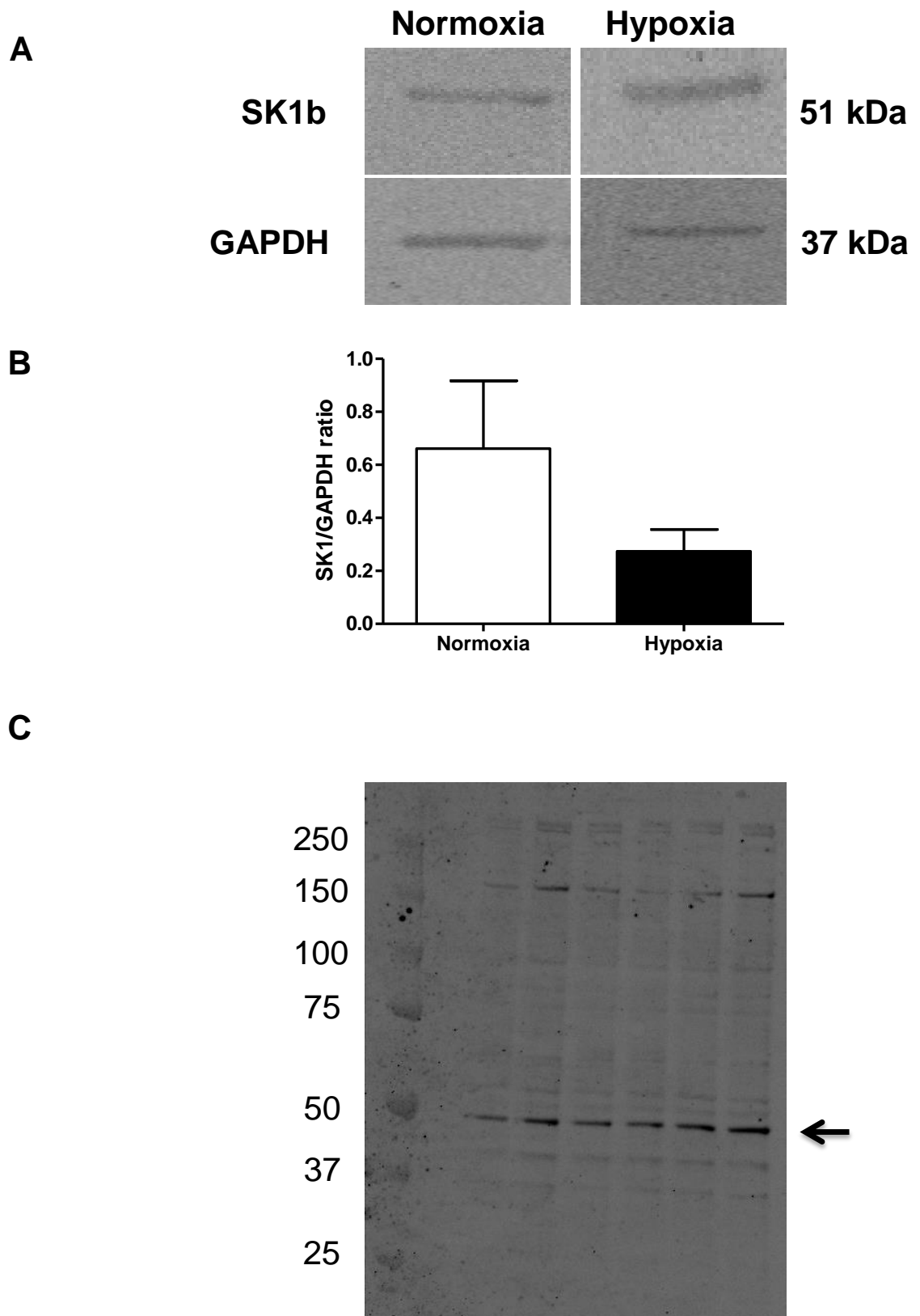


Figure 5-1 Expression of SK1b in HUVECs under normoxia and hypoxia.

(A) Representative immunoblot showing the SK1b expression under normoxic conditions and following the exposure to 30 min hypoxia. (B) Quantification of SK1 relative to GAPDH was determined by densitometric analysis. (C) A representative full-length blot shows which bands were used for analysis. The data are expressed as mean \pm SD for **two separate experiments**.

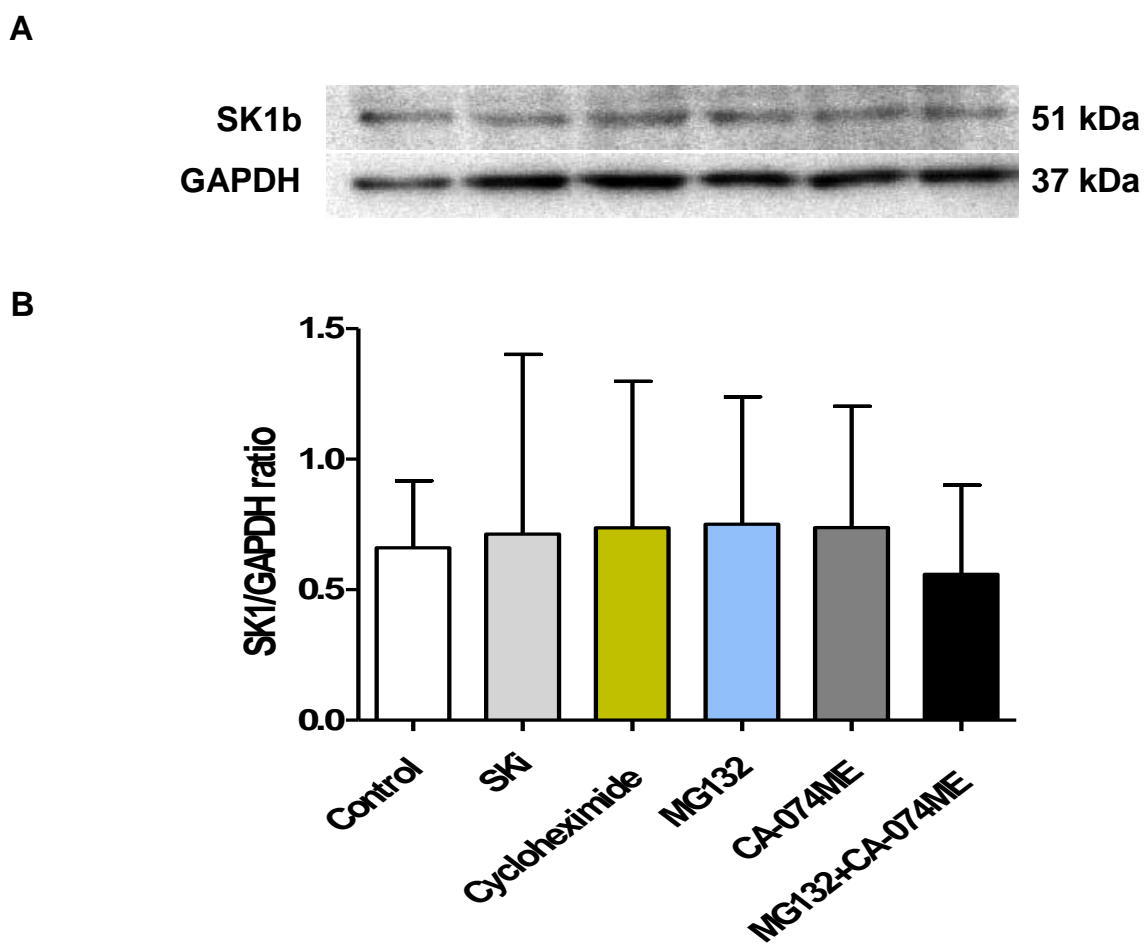
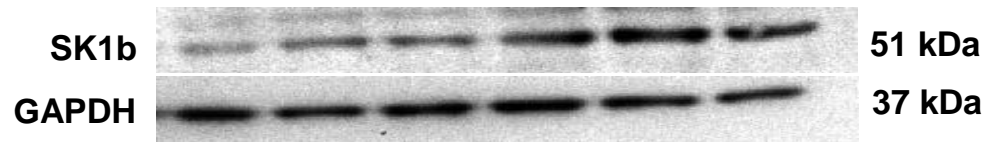


Figure 5-2 Effect of SKi, cycloheximide, MG132 and CA-074ME on SK1 in HUVECs under normoxia.

(A) Representative immunoblot showing the effect of SKi, a non-selective SK inhibitor (10 μ M, 30 min pre-treatment), cycloheximide, a protein synthesis inhibitor (10 μ M, 30 min pre-treatment), MG132, a proteasomal inhibitor (10 μ M, 30 min pre-treatment), CA-074ME, a lysosomal inhibitor (10 μ M, 30 min pre-treatment) and the effect of combined pre-treatment with MG132 and CA-074ME on expression of SK1 in HUVECs under normoxic conditions. (B) Quantification of SK1 relative to GAPDH was determined by densitometric analysis. The data are expressed as mean \pm SD for **two separate experiments**.

A



B

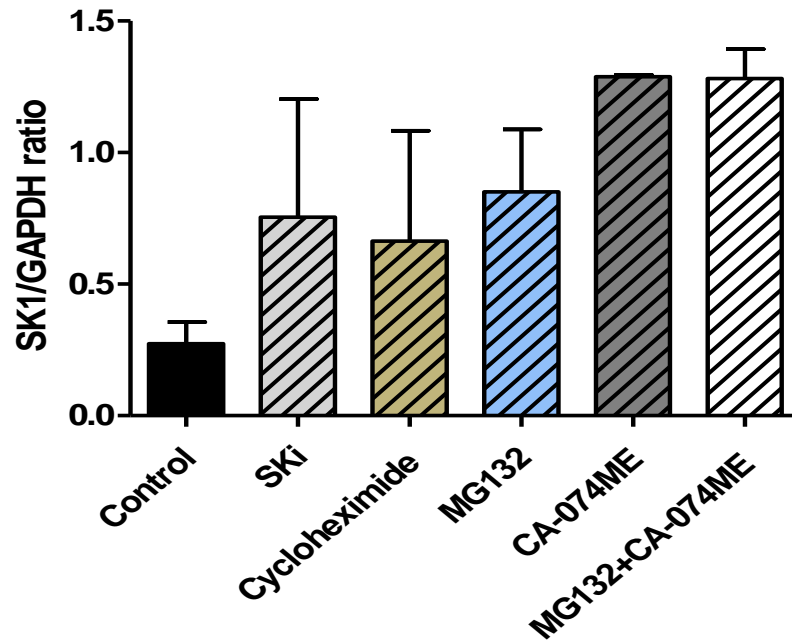


Figure 5-3 Effect of SKi, cycloheximide, MG132 and CA-074ME on SK1 in HUVECs under hypoxia.

(A) Representative immunoblot showing the effect of SKi, non-selective SK inhibitor (10 μ M, 30 min pre-treatment), cycloheximide, protein synthesis inhibitor (10 μ M, 30 min pre-treatment), MG132, proteasomal inhibitor (10 μ M, 30 min pre-treatment), CA-074ME, lysosomal inhibitor (10 μ M, 30 min pre-treatment) and the effect of combined pre-treatment with MG132 and CA-074ME on expression of SK1 in HUVECs under hypoxic conditions. (B) Quantification of SK1 relative to GAPDH was determined by densitometric analysis. The data are expressed as mean \pm SD for two separate experiments. * p <0.05 vs control as determined by ANOVA with Dunnett's post-test.

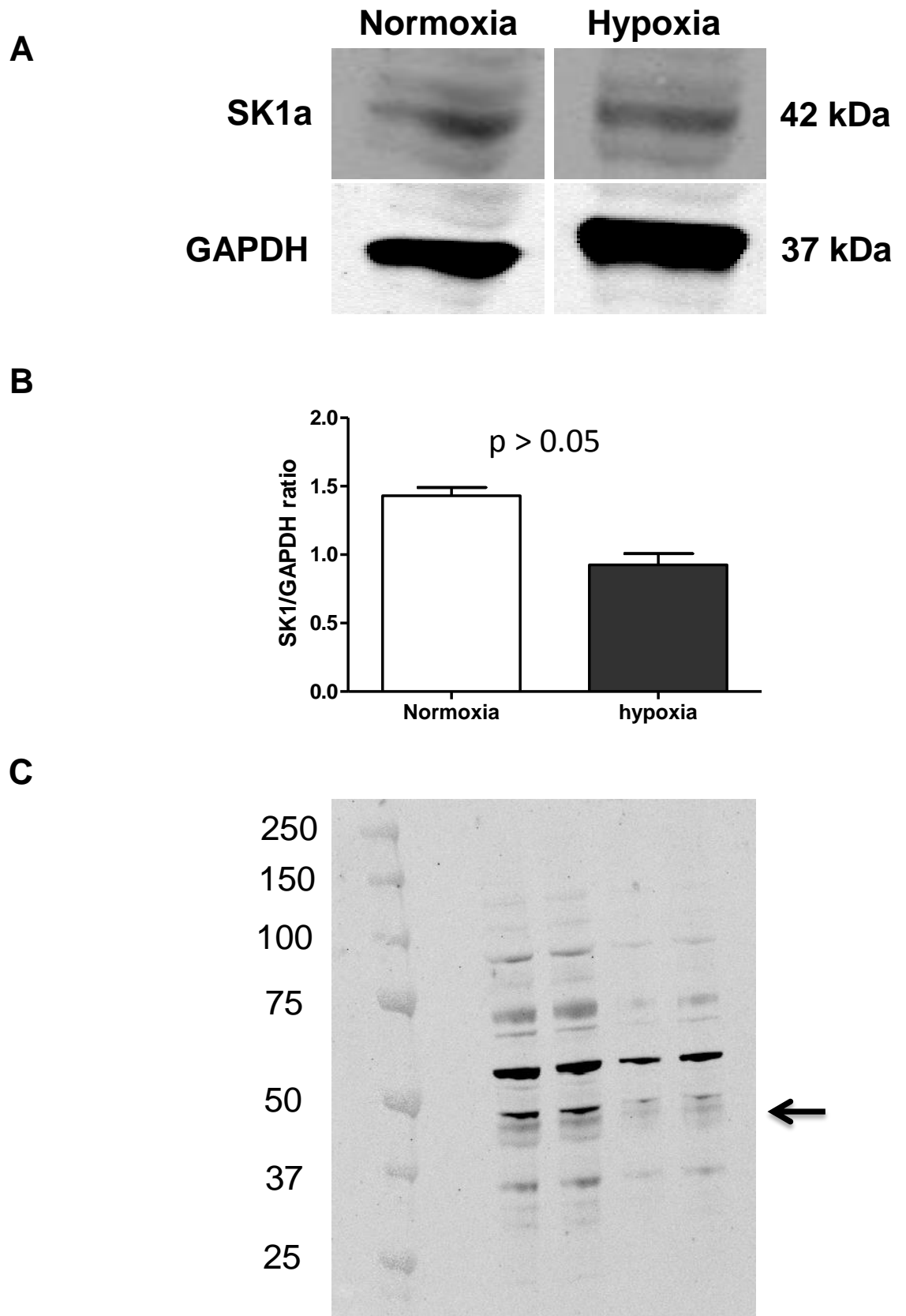
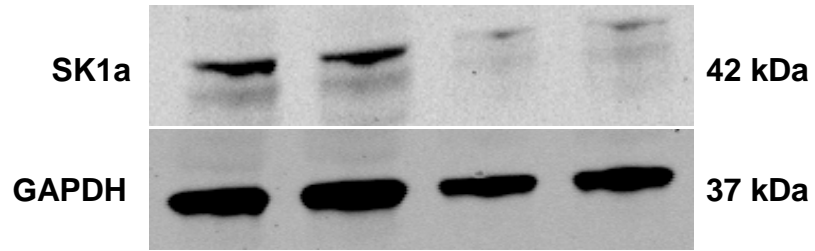


Figure 5-4 Expression of SK1a in rat heart tissue under normoxia and hypoxia.

(A) Representative immunoblot showing the SK1a expression under normoxic conditions and following the exposure to 30 min hypoxia. (B) Quantification of SK1 relative to GAPDH was determined by densitometric analysis. (C) A representative full-length blot shows which bands were used for analysis. The data are expressed as mean \pm SEM for three separate experiments.

A



B

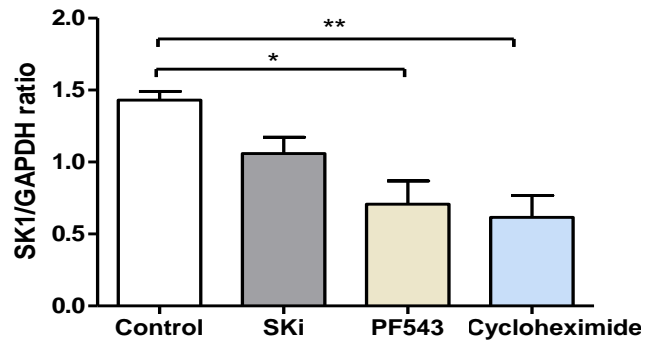


Figure 5-5 Effect of SKi, PF543 and cycloheximide on SK1 in rat cardiac tissue under normoxia.

(A) Representative immunoblot showing the effect of SKi, non-selective SK inhibitor (10 μ M, 30 min pre-treatment), PF543, a selective SK1 inhibitor (100 nM, 30 min pre-treatment) and cycloheximide, protein synthesis inhibitor (10 μ M, 30 min pre-treatment) on expression of SK1 in rat cardiac tissue under normoxic conditions. (B) Quantification of SK1 relative to GAPDH was determined by densitometric analysis. The data are expressed as mean \pm SEM for three separate experiments.

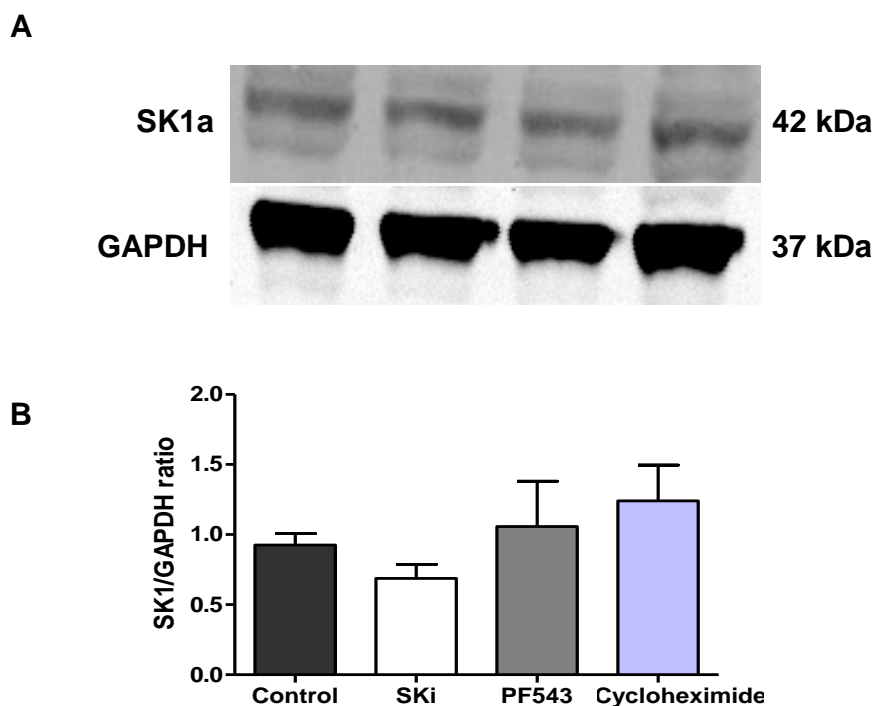


Figure 5-6 Effect of SKi, PF543 and cycloheximide on SK1 in rat cardiac tissue under hypoxia.

(A) Representative immunoblot showing the effect of SKi, non-selective SK inhibitor (10 μ M, 30 min pre-treatment), PF543, a selective SK1 inhibitor (100 nM, 30 min pre-treatment) and cycloheximide, protein synthesis inhibitor (10 μ M, 30 min pre-treatment) on expression of SK1 in rat cardiac tissue under hypoxic conditions. (B) Quantification of SK1 relative to GAPDH was determined by densitometric analysis. The data are expressed as mean \pm SEM for three separate experiments.

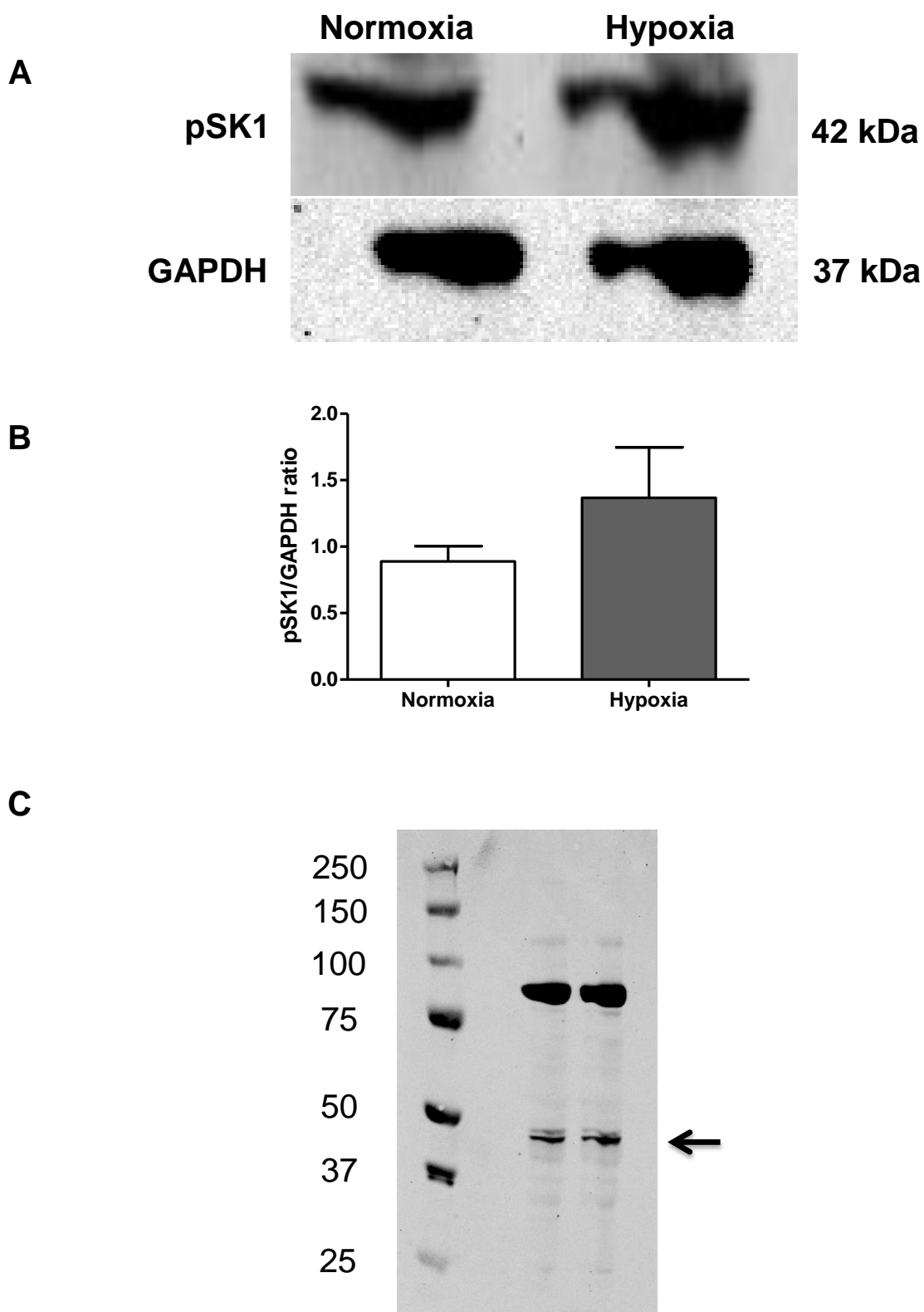


Figure 5-7 Effect of hypoxia on pSK1 in rat cardiac tissue.

(A) Representative western blot showing expression of pSK1 rat cardiac tissue following exposure to 30 min hypoxia. (B) Quantification of pSK1 relative to GAPDH was determined by densitometric analysis. (C) A representative full-length blot shows which bands were used for analysis. The data are expressed as mean \pm SEM for three separate experiments.

5.4 Discussion

In chapter 3 and 4, it was shown that short periods of hypoxia induced an increased SK1 expression in coronary and aortic endothelium. The hypoxia-induced increase in SK1 was inhibited by pre-treatment with SKi, PF543 and cycloheximide. Thus, one of this chapter's aims was to determine the effect of hypoxia on SK1 expression in human cultured endothelial cells, HUVECs. A number of studies have demonstrated that exposure of human endothelial cell line EA.hy926 and human pulmonary smooth muscle cells to hypoxia led to an increase in SK1 expression (Ahmad et al., 2006, Schwalm et al., 2008). Furthermore, Lin et al showed that exposure of rat amoeboid microglial cells to a short period of hypoxia resulted in a rapid increase in SK1 expression starting 15 min after exposure to hypoxia (Lin et al., 2011). In the present study, hypoxia did not change the expression of SK1 in HUVECs (Figure 5-1). Moreover, pre-treatment of HUVECs with SKi, cycloheximide, MG132, CA-074ME or a combination of MG132 and CA-074ME had no effect on the SK1 expression compared with untreated control under normoxic conditions (Figure 5-2). However, pre-treatment with CA-074ME or a combination of MG132 and CA-074ME led to a trend of increase in the SK1 expression compared with untreated control cells under hypoxic conditions (Figure 5-3). A small increase in SK1 expression was also observed in cells pre-treated with MG132. These data suggest that there is a more rapid turnover of SK1 under hypoxic conditions in HUVECs. Only two independent experiments were carried out and further experiments are required to draw firm conclusions. Although, SKi has been shown to induce lysosomal degradation in a number of cell lines including human endothelial cells EA.hy 926, human podocytes and mesangial cells (Ren et al., 2010) and proteasomal degradation in human pulmonary smooth muscle cells, breast cancer cells and androgen sensitive prostate cancer cells (Loveridge et al., 2010), in HUVECs, SKi failed to reduce SK1 expression under normoxia and hypoxia. In this study, the anti-SK1 antibody used identified SK1b (band at 51 kDa) in HUVECs and it is likely that hypoxia induces SK1 expression and therefore this compensates for the degradation of SK1 in response to SKi. Resistance of SK1b to SKi-induced degradation has been previously shown in androgen-independent prostate cancer (LNCaP-AI) cells (Loveridge et al., 2010). In LNCaP-AI, SK1 siRNA or cycloheximide was not effective in reducing SK1b expression and it is likely due to a slow turnover rate of SK1b. Thus, a combined treatment of LNCaP-AI with SK1 siRNA and SKi succeeded

in reducing the SK1b expression as the SKi-induced degradation was not opposed by de novo synthesis of the enzyme (Loveridge et al., 2010).

Another aim of this study was to investigate the effect of hypoxia on expression of SK1 in rat cardiac tissue with and without pre-treatment with specific inhibitory agents including SKi, PF543 and cycloheximide. It has been previously shown that deletion of SK1 or SK2 increased the extent of ischaemia/reperfusion-induced damage in mouse hearts and the protective effect of IPC was impaired in SK1 or SK2 null hearts compared with wild type (Jin et al., 2007, Vessey et al., 2011). Therefore, it has been proposed that the SK/S1P pathway may play an important role in cardiac protection against an ischaemic insult. Here, short-term hypoxia had no effect on expression of SK1 in rat heart tissue (Figure 5-4). Pre-treatment with PF543 or cycloheximide led to a significant reduction in the SK1 expression under normoxic conditions (Figure 5-5). A small decrease in SK1 expression was also seen with SKi pre-treatment. In contrast, no significant change in expression of SK1 was observed with SKi or PF543 or cycloheximide pre-treatment prior to exposure to hypoxia (Figure 5-6). These data support similar observations reported recently in pulmonary vessels and right ventricles where treatment with PF543 had no effect on expression of SK1 in mice subjected to chronic hypoxia for 21 days (MacRitchie et al., 2016). In the current study, it was also observed that hypoxia had no effect on ser225 phosphorylation of SK1 compared with normoxia (Figure 5-7). It is important to note that the anti-SK1 antibody used in these experiments identified SK1a (band at ~ 42 kDa) in rat heart tissue. Hypoxia obviously induces resistance to SK inhibitors in terms of degradation in rat heart tissue. The resistance of SK1a to SK inhibitors is likely due to the compensatory increase in SK1a expression and not a result of SK1 phosphorylation as hypoxia did not increase it. Collectively, reduction of SK1 expression and/or activity due to hypoxic/ischaemic insult may enhance cardiomyocyte apoptosis by shifting the 'rheostat' in favour of ceramide (a pro-apoptotic signal). Thus, maintenance of SK1 activity following hypoxia/ischaemia insult might be a promising therapeutic strategy in prevention of cardiomyocyte apoptosis and recovery of cardiac function.

5.5 Conclusion

In summary, I have shown that pre-incubation of endothelial cells, HUVECs, with lysosomal and/or proteasomal inhibitor prior to exposure to a short period of hypoxia apparently increases the SK1 expression in comparison with untreated control, suggesting that hypoxia increased turnover rate of SK1 in particular cell lines. However, hypoxia also

makes SK1 resistant to SKi-induced degradation which is likely due to a compensatory increase in SK1 expression. This is also the case in heart tissue where hypoxia-induced resistance to the effect of the SK inhibitor in causing degradation was likely through a compensatory upregulation of SK1 expression.

Chapter six

6 General discussion

6.1 General Discussion

A growing body of evidence exists for the significant contribution of the SK1/S1P pathway in cardioprotection against ischaemic insult. For instance, deletion of SK1 gene in ventricular cardiomyocytes led to increased cell death following exposure to hypoxia (Tao et al., 2007). Moreover, exogenous S1P was found to prevent hypoxic cell death in SK1 null cardiomyocytes. It has also been shown that monoganglioside GM-1, which increases intracellular S1P by activating SK1 was cytoprotective in wild type cardiomyocytes but not in SK1 null myocytes (Tao et al., 2007). Exogenous S1P also exhibited protective effects against IR injury in isolated adult mouse hearts (Jin et al., 2002, Lecour et al., 2002). However, little is known about the role of the SK/S1P signalling pathway at the level of coronary arteries during hypoxic/ischaemic event. This thesis has examined the effect of hypoxia on SK1 expression in several cell types, but with a focus on vascular endothelium and how this may regulate vascular tone through generation of S1P. The present study shows for first time that a short period of hypoxia causes an increase in SK1 expression in coronary and aortic endothelium. Although this has been demonstrated previously in human glioma cells and rat amoeboid microglial cells (Anelli et al., 2008, Lin et al., 2011), this observation in endothelial cells is novel. Examination of the mechanism responsible for the hypoxia-induced increase in SK1 revealed that hypoxia increases synthesis of SK1, as it was inhibited by cycloheximide, a protein synthesis inhibitor. Moreover, pre-treatment with a number of proteasomal or lysosomal degradation inhibitors did not increase SK1 expression under normoxic conditions or induce any further increase in SK1 level under hypoxia.

It was also demonstrated that SKi, a non-selective SK inhibitor, and PF543, a selective SK1 inhibitor not only inhibit the catalytic activity of SK1 but they also reduce SK1 expression in coronary and aortic endothelium under normoxic and hypoxic conditions. Previous work showed that SKi enhances proteasomal degradation in human pulmonary artery smooth muscle cells, prostate and breast cancer cells (Loveridge et al., 2010) and lysosomal degradation in human endothelial cells EA.hy 926, human podocytes and mesangial cells (Ren et al., 2010). In the present study SKi induced both proteasomal and lysosomal degradation of SK1 in coronary artery endothelium as pre-treatment with MG132, a proteasomal inhibitor, and/or CA-074ME reversed the SKi triggered degradation of SK1.

The increase in SK1 due to hypoxia/ischaemia in coronary and aortic endothelium may contribute significantly to generation of more S1P which acts as a cardioprotective lipid (Kennedy et al., 2009). To explore the importance of this hypoxia-induced increase in SK1 in regulation of vascular function, S1P-stimulated vasorelaxation was assessed in rat aorta under normoxic and hypoxic conditions. In the current study, S1P was found to induce vasodilatation in endothelium-intact but not endothelium-denuded rat aortae, indicating that it is an endothelium-dependent effect. Moreover, the vasorelaxation response to S1P was likely mediated through S1P₃ receptors as CYM5541, a selective S1P₃ agonist but not SEW2871, a selective S1P₁ receptor agonist induced relaxation of a similar magnitude to that induced by S1P itself. These data support similar observations reported in rat coronary artery and aorta where endothelial S1P₃ receptor mediated the vasorelaxation response to exogenous S1P (Nofer et al., 2004, Mair et al., 2010). In the present study, it was also shown that exposure of aortic rings to hypoxia led to enhancement of the vasorelaxation response to exogenous S1P. Removal of endothelium also inhibited the enhanced vasorelaxation response to S1P seen under hypoxic conditions. SK1, likely via generation of S1P, has previously been found to mediate vasorelaxation responses to anandamide and S1P in rat coronary artery under normoxic conditions (Mair et al., 2010). In the current study, it was also demonstrated that the vasorelaxation response to exogenous S1P was SK1-dependent under normoxia and hypoxia in endothelium-intact aortic rings. Collectively, these data indicate that S1P exerts its vasodilator effect via activation of S1P₃/SK1/S1P signalling pathway. Moreover, it is likely that S1P₃ agonist-induced vasorelaxation is a G_q-mediated effect which is regulated by S1P₃ but not S1P₁ receptors. This argument is supported by a previous work where it has been shown that activation of G_q-coupled receptors stimulated a rapid and long lasting relocalisation of SK1 to the plasma membrane in HEK-293 cells (ter Braak et al., 2009).

The eNOS/NO pathway has previously been demonstrated to function as a downstream target for the biological responses to S1P including vasorelaxation (di Villa Bianca et al., 2006, Roviezzo et al., 2006). Increased eNOS activity was also reported in cultured vascular endothelial cells stimulated by S1P (Igarashi et al., 2001). Moreover, Roviezzo et al (2006) has demonstrated that ACh-induced vasorelaxation in thoracic aortae was mediated by SK, S1P and NO, suggesting that the SK/S1P pathway is critical for ACh-induced vasorelaxation. In the present study, S1P was found to exert vasorelaxation in rat aorta via a NO-dependant mechanism. Therefore, NO is the downstream effector which induces relaxation of the aortic smooth muscle cells. Moreover, the present study has demonstrated a novel mechanism of S1P-stimulated vasorelaxation by activation of

TRPV1 channels in endothelium-intact aortae. It is therefore possible that S1P can exert its vasorelaxant effect through an alternative S1P receptor-independent mechanism. However, involvement of TRPV1 channels in S1P-induced relaxation was only tested under normoxia. Thus, further experiments are required to examine whether S1P stimulated relaxation is mediated by TRPV1 channels under hypoxic conditions.

The significant role of S1P in control of vascular tone is well recognised and S1P, therefore, may play an important role in regulation of blood pressure and in the pathogenesis of hypertension. It has recently been shown that increasing S1P levels via deletion/loss of Nogo B, a membrane protein of the endoplasmic reticulum that negatively controls endothelial S1P levels, exerted a protective role against angiotensin II-induced hypertension (Cantalupo et al., 2015). Moreover, it was also observed that eNOS activity is increased in Nogo-B-null mice and addition of an S1P₁ antagonist reversed the enhanced vasorelaxation seen in Nogo-B-null mice. However, another study reported that S1P may play a pro-hypertensive role as loss of SK1 led to inhibition of both the acute hypertensive response to angiotensin II in anaesthetised mice and the sustained hypertensive response to continuous infusion of angiotensin II in conscious mice (Wilson et al., 2015). Thus, the role of S1P in regulation of blood pressure is still requires more investigation to delineate its complex role. In the present study, it was observed that the vasorelaxation response to S1P is abolished in aortic rings obtained from spontaneously hypertensive rats compared with control rats. In contrast, S1P-induced vasorelaxation was not impaired in aortic rings obtained from spontaneously hypertensive rats following exposure to hypoxia compared with control. Furthermore, the hypoxia-enhanced vasorelaxation response to S1P was also mediated through SK1 in vessels dissected from hypertensive rats and this is consistent with increased SK1 expression. Obviously, more experiments are required to clarify the importance of SK/S1P pathway in regulation of blood pressure and the pathogenesis of hypertension.

The influence of hypoxia on SK1 expression was also investigated at the level of cultured endothelial cells (HUVECs). No change in SK1 expression was seen following exposure of HUVECs to hypoxia compared with normoxia. Presence of SKi or cycloheximide was also without effect on SK1 expression under normoxic and hypoxic conditions. In contrast, pre-treatment with MG132, CA-074ME or a combination of MG132 and CA-074ME apparently increases SK1 expression under hypoxic but not normoxic conditions, indicating that there is a more rapid turnover of SK1 under hypoxic conditions in HUVECs. The anti-SK1 antibody used in this study identified SK1b (band at 51 kDa) in

HUVECs and hypoxia appears to increase SK1 expression and this perhaps compensates for the SKi-induced degradation. Therefore, a compensatory increase in SK1b expression counteracts the SK1b downregulation induced by SKi and maintains no change in SK1b expression overall. This is in accordance with a previous study where it has been demonstrated that treatment with SK1 siRNA or SKi or cycloheximide was not effective in decreasing SK1b expression in androgen-independent prostate cancer (LNCaP-AI) cells (Loveridge et al., 2010). However, SK1 expression was reduced when the LNCaP-AI were treated with a combination of SKi and SK1 siRNA and this is because the SKi-induced degradation was not opposed by de novo synthesis of the enzyme (Loveridge et al., 2010). Thus, there is resistance to SKi in HUVECs because of a compensatory increase in SK1b expression. Similarly, in heart tissue, no significant difference in expression of SK1 was observed between normoxia and hypoxia. However, in the case of heart tissue, the anti-SK1 antibody detected SK1a (band at ~ 42 kDa). Under normoxic conditions, SK inhibitors or cycloheximide was effective in reducing expression of SK1a. Loveridge et al (2010) has also demonstrated that SK1a is more sensitive to SKi-induced degradation than SK1b in the LNCaP-AI. In contrast, SK inhibitors or cycloheximide failed to reduce SK1a expression under hypoxic conditions in the current study, suggesting that hypoxia induces resistance to SK inhibitors and cycloheximide. It is possible that hypoxia induces resistance to SK inhibitors in terms of degradation through an increase in SK1a expression and therefore, the SK1a-induced degradation by SK inhibitors is balanced by a compensatory upregulation of SK1 expression.

6.2 Limitations and future work

In this thesis, the use of confocal imaging provided very important information in investigating the effect of hypoxia on SK1 in the endothelium of isolated vascular preparations under conditions which are more physiological compared with cultured cell lines. However, the physiological relevance could be improved further in future with additional experiments using in vivo models. The influence of hypoxia/ischaemia on SK1 in isolated vascular preparations could be assessed in vivo by inducing ischaemia via ligation of the coronary artery or exposure of animals to hypoxia and then dissecting the artery in the ischaemic area to assess SK1 expression. It would also be important to investigate the effect of ischaemia/reperfusion on SK1 in isolated arteries and in vivo to establish the effect of reoxygenation/reperfusion itself on SK1 expression. Moreover, in the present study it was shown that pre-treatment with PF543, a potent selective SK1 inhibitor reduced SK1 expression in coronary and aortic endothelium. Further experiments

are required to interrogate the mechanism of PF543-induced reduction in SK1. Previous work has demonstrated that PF543 enhances proteasomal degradation of SK1 in human pulmonary artery smooth muscle cells and prostate cancer cells (Byun et al., 2013, McNaughton et al., 2016). Thus, the vascular rings could be pre-treated with proteasomal, lysosomal, or a combination of both proteasomal and lysosomal inhibitor prior to addition of PF543 to assess SK1 expression in presence of the degradation inhibitors.

The specificity of the antibody used to detect SK1 was tested by using a blocking peptide and by comparison with a previously validated antibody. Further experiments can be carried out to test antibody specificity by using negative controls. For instance, a cell line or tissue that is known not to express the SK1 enzyme can provide a better negative control. SK1 siRNA can also be used to knockdown SK1 and thus provide reliable negative controls. It is also important to carry out a number of experiments in order to examine whether the diluents for the used inhibitors affect the SK1 expression or not. Different diluents were used to dilute the used inhibitors including distilled water, BSA/PBS, DMSO and ethanol. Therefore, effect of the diluents on results obtained from immunofluorescent and functional studies should be tested. Normoxia is another issue that should be further evaluated. Currently exposure of cultured cells or isolated tissue to atmospheric normoxic (21 %) oxygen concentrations is widely used technique. It is, however, not considering the fact that oxygen levels within the human body are much lower than that of the atmosphere (McKeown, 2014). For instance, the oxygen concentration in arteries, lungs, and liver is about 10-13% and 7 % in the bone marrow. Further evaluation, therefore, should be considered for using normoxic (20 % oxygen) solutions for in vitro experiments.

Hypoxia-induced increase in expression of SK1 has previously been shown in vascular smooth muscle cells (Ahmad et al., 2006). However, it was not possible to examine the influence of hypoxia on SK1 expression in vascular smooth muscle cells in the current study due to poor penetration of the antibodies deep to smooth muscle layers. It was expected that antibodies may pass through the opening of internal and external lamina but it seems they act as a barrier. Theoretically, it might be possible to use a digestive enzyme (very low concentration) to induce larger openings in the internal and external lamina without losing the whole vessel structure. This may help the antibodies to penetrate deep towards smooth muscle cells.

Dissection of rat coronary artery is another issue in the present study as it is very slow and tricky. I have often been able to dissect two small rings one from right and the other from left coronary artery. This amount of tissue was not enough to carry out functional and immunofluorescent studies at the same time. Thus, I used rat aorta to conduct functional experiments. However, using large vessels such as aorta may not always reflect vascular reactivity of smaller arteries such as coronary, cerebral and renal arteries. However, although use of the aorta as a model to study vascular responses to S1P under normoxic and hypoxic conditions might be a limitation of this study, immunofluorescent studies showed that the response of SK1 to hypoxia in the aorta was similar to the coronary artery. Furthermore, previous work showed that S1P induces vasorelaxation in rat aorta (Nofer et al., 2004, Roviezzo et al., 2006) and exposure of aortic rings to hypoxia was also reported to induce vasodilation (Herrera and Walker, 1998). Therefore, understanding how hypoxia affects the SK/S1P pathway in vascular tissue such as aorta may help to clarify how hypoxia/ischaemia alters cardiovascular function in the whole animal.

Moreover, it has been shown in chapter five that hypoxia induces resistance to SK inhibitors or cycloheximide-triggered reduction in SK1 expression in HUVECs and rat heart tissue. It is likely that hypoxia increases SK1 expression and this is outweighing the degradation induced by SK inhibitors. Using a combined treatment with SK1 siRNA or cycloheximide and SK inhibitors may clarify the mechanism of hypoxia-induced SK1 resistance to SK inhibitors. Measurement of SK1 mRNA could also provide additional evidence for the compensatory hypoxia-induced increase in SK1 expression in HUVECs and heart tissue.

6.3 Clinical Relevance

Several studies have demonstrated that the S1P/SK pathway exhibited protective effects against hypoxic injuries in cardiomyocytes (Karlner et al., 2001, Tao et al., 2007). However, little is known about whether S1P/SK pathway play a role in protection of coronary arteries against hypoxic/ischaemic and reperfusion insult. Restoration of blood supply is critical to provide oxygen and nutrients to the starved myocardium and to minimise the size of the infarcted zone. However, reperfusion itself is also associated with microvascular dysfunction, inflammation, and oxidative damage. This vascular injury, in turn, leads to inadequate myocardial perfusion (the “no-reflow” phenomenon). It was shown that 30% of patients had this phenomenon which is associated with higher incidence of death (Ahmad et al., 2006, Niccoli et al., 2009). Prevention of microvascular damage

during ischaemia and reperfusion may enhance myocardial flow and therefore represents a promising therapeutic strategy to improve outcome. Recent studies demonstrated that pre-treatment with FTY720, a S1P receptor agonist, and SEW2871, a S1P₁ receptor agonist relieved coronary and mesenteric vascular dysfunction induced by cardiopulmonary bypass (Samarska et al., 2014). Galvani et al reported that HDL-bound S1P exhibited anti-inflammatory properties in mouse aorta through its function as a biased S1P₁ agonist (Galvani et al., 2015). Moreover, it has been shown that anandamide, an endogenous cannabinoid, induces coronary vasodilation through activation of SK1/S1P/S1P₃ signalling pathway (Mair et al., 2010). Therefore, targeting SK/S1P/S1P receptor pathway in vascular endothelium perhaps offers the potential for a more clinically useful treatment to limit vascular damage during an ischaemic event. This may include administration of S1P, its analogues or SK1 activators during the ischaemic attack which could limit microvascular dysfunction and allow for adequate cardiac perfusion and recovery.

6.4 Conclusion

In summary, this thesis has demonstrated for the first time that short periods of hypoxia induces an increase in SK1 expression in coronary and aortic vascular endothelium. Moreover, the current study has found that the enhanced vasorelaxation response to S1P under hypoxic conditions is mediated by SK1/S1P₃ signalling pathway. The thesis has also generated novel data on an alternative mechanism for the S1P stimulated vasorelaxation through activation of TRPV1 channels in aorta. Another important novel observation is that the SK1-mediated hypoxia-enhanced vasorelaxation response to S1P is maintained in hypertension. Furthermore, the present study has highlighted that hypoxia induces resistance of SK1 to SK inhibitors in terms of degradation in HUVECs and rat heart tissue. Additional experiments are required to clarify the mechanism of hypoxia-induced resistance to SK inhibitors which is likely through a compensatory SK1 upregulation. Considering the findings of the current study, targeting the SK1/S1P pathway could provide a novel therapeutic strategy to ameliorate the clinical signs associated with vascular complications caused by myocardial infarction.

Chapter seven

7 References

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