The role of mitochondria and K_{ATP} channels in the vasodilatation response to simvastatin. Comparison with the effects of simvastatin in pancreatic β-cells

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Thesis submitted to the University of Nottingham

For the degree of Doctor of Philosophy

April 2015

Abstract

Clinical trials have established the efficacy and safety of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) in lowering cardiovascular morbidity and mortality in patients with and without coronary artery diseases. Traditionally, the beneficial effects of statins have been ascribed entirely to their ability to lower serum cholesterol. However, evidence indicates that statins may exert cholesterol-independent or pleiotropic effects. As well as reducing plasma cholesterol levels, statins induce acute vasorelaxation which may contribute to the overall benefits of statins in the treatment of cardiovascular disease. The mechanism underlying this relaxation is unknown. Statins have been shown to alter mitochondrial function. Therefore, the aim of this study was to determine the role of the mitochondria in the relaxation to statins. Changes in rhodamine 123 fluorescence showed that simvastatin, but not pravastatin, depolarized the membrane potential of mitochondria in both isolated smooth muscle cells and intact blood vessels. As simvastatin, but not pravastatin, causes relaxation of the porcine coronary artery, this could be due to this effect on mitochondria. Mitochondria are known as the energy generating centre of the cells. However, there is growing consensus that mitochondria actively participate in intracellular signalling, such as production of reactive oxygen species (ROS) and regulation of the intracellular Ca^{2+} concentration. Moreover, ROS could play an important supportive role in a variety of vascular cell signalling processes, including activation of nitric oxide synthase (NOS), modulation of intracellular Ca^{2+}, and AMP kinase activation. Therefore, this study investigated whether the relaxation to the lipophilic statin simvastatin is due to an effect on the mitochondria. Relaxation of porcine coronary artery segments by statins was measured

using isolated tissue baths. Simvastatin, but not pravastatin, produced a slow relaxation of the coronary artery, which was independent of K^+ channel activation, nitric oxide, cyclo-oxygenase, or the endothelium. The relaxation was attenuated by the mitochondrial complex I inhibitor rotenone and the complex III inhibitor myxothiazol, or a combination of the two. Simvastatin inhibited calcium-induced contractile responses, and this inhibition was partially reversed by incubation with the complex I inhibitor rotenone suggesting that mitochondrial function is required for the effect of simvastatin on calcium influx. The effect of mitochondrial complex III inhibitor, antimycin A, was examined as a comparison with simvastatin. Antimycin A induced porcine coronary relaxation and inhibited Ca^{2+} influx in isolated porcine coronary smooth muscle cells.

Evidence from a number of clinical trials highlights a potential association between treatment with lipophilic statins and increased risk of development of diabetes. The close connection between energy metabolism and insulin secretion in pancreatic β-cells suggests that the glycaemic effects of simvastatin may also result from a direct mitochondrial action with reduction in insulin secretion and, hence, result in a reduced control of plasma glucose levels. Although simvastatin depolarized mitochondria in pancreatic *β*-cells, it also directly inhibited K_{ATP} channels. Pravastatin, on the other hand, had no effect on either measurement, suggesting that these phenomena relate to the lipophilicity of the compounds. The inhibition of K_{ATP} channels by simvastatin is likely to underlie the increase in insulin secretion observed within days of simvastatin treatment. On the other hand, the effects on mitochondrial membrane potential may be detrimental, particularly with chronic treatment, although further studies are required in order to determine whether this plays a role in the increased risk of diabetes observed with lipophilic statins.

Overall, our results demonstrated that simvastatin alters mitochondrial membrane potential in vascular smooth muscle cells and pancreatic β-cells. The relaxation to simvastatin in the porcine coronary artery is dependent, in part, upon mitochondrial activity. Alteration of mitochondrial membrane potential by simvastatin may lead to inhibition of calcium influx, hence stimulation of relaxation. On the other hand, the effects on mitochondrial membrane potential in pancreatic β-cell may be detrimental, particularly with chronic treatment due to the increased risk of diabetes observed with lipophilic statins.

Conference presentations

1. HM Almukhtar, PA Smith, RE Roberts (2012). Effects of mitochondrial complex inhibitors on porcine coronary tone: role of reactive oxygen species. BPS Winter Meeting, London, Queen Elizabeth II Conference Centre. Poster presentation. http://www.pa2online.org/abstracts/vol10issue4abst077p.pdf

2. HM Almukhtar, PA Smith, RE Roberts (2012). Role of mitochondria in the effect of simvastatin in isolated porcine coronary artery. 62nd Meeting of the British Microcirculation Society, Keble College, Oxford. Poster presentation. http://onlinelibrary.wiley.com/doi/10.1111/micc.12018/epdf

3. HM Almukhtar, RE Roberts, PA Smith (2013). Effect of statins on mitochondrial membrane potential and K_{ATP} activity in mouse primary β-cells. BPS Winter Meeting, London, Queen Elizabeth II Conference Centre. Poster presentation. http://www.pa2online.org/abstracts/vol11issue3abst138p.pdf

4. HM Almukhtar, PA Smith, RE Roberts (2013). Role of mitochondria in the relaxation to simvastatin in porcine isolated coronary artery. BPS Winter Meeting, London, Queen Elizabeth II Conference Centre. Poster presentation. http://www.pa2online.org/abstracts/vol11issue3abst126p.pdf

5. HM Almukhtar, PA Smith, RE Roberts (2014). Simvastatin Induced Porcine Coronary Artery Relaxation: Role of Ca^{2+} Influx and Mitochondrial Function. BPS Winter Meeting, London, Queen Elizabeth II Conference Centre. Poster presentation. http://www.pa2online.org/abstracts/vol12issue3abst271p.pdf

6. HM Almukhtar, PA Smith, RE Roberts (2014). Mechanism of Relaxation to Antimycin A, Mitochondrial Complex III inhibitor, in the Porcine Coronary Artery. BPS Winter Meeting, London, Queen Elizabeth II Conference Centre. Poster presentation. http://www.pa2online.org/abstracts/vol12issue3abst178p.pdf

Acknowledgements

"O My Lord, increase me in knowledge" Quran 20:114

First and above all, I praise ALLAH the Most Gracious, the Most Merciful for providing me with the opportunity and the capability to proceed with my research.

I would like to express my sincere gratitude to my supervisors Dr. Richard Roberts and Dr. Paul Smith for the continuous support throughout my Ph.D study, for their patience, motivation, enthusiasm, and immense knowledge. Their guidance helped me all the time of research and during the writing of the thesis. I could not have imagined having better supervisors for my Ph.D study. My deepest gratitude is also to Dr. Michael Garle, Liaque Latif, Emeka and Jagdish Heer, for being very helpful and for their great advice.

I would like to thank my fellow lab mates in E34 laboratory: I would thank the opportunity that allows me to meet such friends: Thank you to all friends I have made in Nottingham.

I would like to acknowledge all staff and members in the University of Nottingham, School of Life Sciences.

I would also like to thank Islamic Development Bank IDB for granting me a scholarship to pursue my higher education at the University of Nottingham.

I wish to thank my beloved mother, father and brothers for their continuous encouragement and help who were all patients during our long absence abroad. A big thanks to my wife, my little children. I will never be able to pay my debt of gratitude in full.

Lastly, but most importantly, May Allah's peace and blessing be upon our Beloved Prophet Muhammad who said, *"Allah makes the way to Jannah easy for him who treads the path in search of knowledge."*

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Abbreviations

3-NP	3-nitropropionic acid
AA	Arachidonic acid
ADP	adenosine diphosphate
AICAR	5-aminoimidizole-4-carboxamide riboside
AKt	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AOAA	amino-oxyacetic acid
ATP	adenosine triphosphate
BAECs	bovine aortic endothelial cells
BK_{Ca}	large conductance Ca^{2+}-activated K^+ channel
BSA	bovine serum albumin
Ca2+_CaM	Ca2+/calmodulin-dependent protein kinase
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
cGMP	Cyclic guanosine monophosphate
CICR	calcium-induced calcium release
Complex I	NADH–ubiquinone oxidoreductase
Complex II	Succinate–ubiquinone oxidoreductase
Complex III	Ubiquinone–cytochrome c oxidase
Complex IV	Cytochrome c oxidase
COX1	Cyclooxygenase
CPA	cyclopiazonic acid
Cu/ZnSOD	copper/zinc superoxide dismutase
DAG	diacylglycerol
DCF	2,7-dichlorofluorescein
DETCA	diethylthiocarbamate
DHETs	dihydroxyeicosatrienoic acids
DMEM	Dulbecco Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDHF	Endothelium-derived hyperpolarizing factor
EDTA	Ethylenediaminetetraacetic acid
EETs	epoxyeicosatrienoic acids
EGTA	ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
ET-1	endothelin
ETC	Electron transport chain
FADH	reduced flavin adenine dinucleotide
FADH2	Flavin adenine dinucleotide
FCCP	Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
H_2O_2	hydrogen peroxide
HDL	High-density lipoprotein
HETEs	monohydroxyeicosatetraenoic acids
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
IK_{Ca}	intermediate conductance calcium activated K^+ channel
IP3	inositol 1,4,5-trisphosphate
IP_3R	inositol-1,4,5-triphosphate receptor
K_{ATP}	ATP-sensitive K^+ channels
KCl	Potassium chloride
K_{ir}	inward rectifier K^+
Kv	Voltage-gated potassium channel
LDL	Low density lipoprotein
L-NAME	L-nitroarginine methyl ester

MLC kinase	myosin light chain kinase
MLCP	MLC phosphatase
MnSOD	manganese superoxide dismutase
MYPT1	myosin phosphatase target subunit 1
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NCX	sodium Na^+/Ca^{2+} exchanger
NOS	nitric oxide synthase
Nox	nicotinamide adenine dinucleotide phosphate-oxidase
NPo	channel activity
$O_2^{\cdot -}$	superoxide anion
ONOO	peroxynitrite
ox-pho	oxidative phosphorylation
PBS	Phosphate buffered saline
PCA	Porcine coronary artery
PEG-catalase	Catalase−polyethylene glycol
PGI_2	prostacyclin
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PKG	protein kinase G
PMCA	plasma membrane Ca^{2+}-adenosine 5′ triphosphatase
PPG	propargylglycine
QH	Quinone
QH2	Quinol
Rh123	Rhodamine 123
RNS	reactive nitrogen species
ROCCs	receptor-operated Ca^{2+} channels
ROS	reactive oxygen species
sGC	soluble guanylyl cyclase
SK_{Ca}	small conductance Ca^{2+} activated K^+ channel
SOCCs	store-operated Ca^{2+} channels
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
SUR	sulphonylurea receptors
SV	simvastatin
TEA	Tetraethylammonium chloride
TRP	transient receptor potential
TTFA	4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione
TXA2	thromboxane A2
U46619	9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α
VDAC	voltage dependant anion channel
VDCCs	voltage-dependent Ca^{2+} channels
VSMC	vascular smooth muscle cell
$\Delta\Psi_m$	membrane potential

CHAPTER I: GENERAL INTRODUCTION

1.1. Ca^{2+}-dependent contraction of smooth muscle

Vascular tone is regulated by contraction of the smooth muscle cells. Smooth muscle cells are the major constituent of the blood vessel wall. Vascular smooth muscle contractility is determined by the free intracellular Ca^{2+} and Ca^{2+} sensitivity of contractile proteins. The entry of Ca^{2+} from the extracellular space, through Ca^{2+} channels, as well as Ca^{2+} release from intracellular stores results in Ca^{2+} binding to the acidic protein, calmodulin, forming a Ca^{2+}/calmodulin complex. The complex in turn activates myosin light chain kinase (MLC kinase) with subsequent phosphorylation of the myosin light chains (Gao et al., 2013), which enables cross-bridge formation with actin. The latter is a key event in smooth muscle contraction (Webb, 2003) (Figure 1.1).

Agonists such as endothelin-1 act on receptors coupled to Gq proteins which activate phospholipase C enzyme (a membrane-bound enzyme). The membrane enzyme then catalyses the phosphatidylinositol cascade with the formation of two second messengers: inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 diffuses through the cytoplasm and binds to receptors on the sarcoplasmic reticulum (SR), causing release of Ca^{2+} into the cytosol (Berridge and Irvine, 1989, Northcott et al., 2002, Sata and Nagai, 2002). Mitochondrial uptake of Ca^{2+} may regulate the local Ca^{2+} near the IP3 receptor, so maintaining the sensitivity of the IP3 receptor to release Ca^{2+} from the SR (McCarron and Muir, 1999, Taylor et al., 1989). Indeed, IP3-induced Ca^{2+} release is inhibited by mitochondrial depolarization (Collins et al., 2000). DAG, along with Ca^{2+}, activates protein kinase C (PKC), which has contraction-promoting effects such as phosphorylation of L-type Ca^{2+} channels (Schuhmann and Groschner, 1994).

Smooth muscle relaxation occurs by decreasing the intracellular Ca^{2+} concentration (Morgan and Morgan, 1984) and increasing MLC phosphatase activity. Two mechanisms participate in removing Ca^{2+} from the cell to the external environment. Firstly, the membrane Ca^{2+} ATPase pumps Ca^{2+} from the intracellular into the extracellular space. Secondly, the sarcoplasmic reticulum Ca^{2+} ATPase pumps Ca^{2+} into the SR. Ca^{2+} uptake into the sarcoplasmic reticulum is dependent on ATP hydrolysis; in fact, mitochondria ensure efficient sarcoplasmic reticulum refilling (Poburko et al., 2009). The plasma membrane also contains a Na^{+}/Ca^{2+} exchanger which provides an additional mechanism for reducing intracellular Ca^{2+} (Campbell and Paul, 1992, Barron et al., 2000, Marin et al., 1999). In addition to the Ca^{2+}-dependent activation of MLCK, the state of MLC phosphorylation is further regulated by MLC phosphatase (Koga and Ikebe, 2008), which removes the high-energy phosphate from the MLC, thus promote smooth muscle relaxation (Webb, 2003). There are several reports of relaxation of smooth muscle that occur independent of changes in myosin light chain phosphorylation, small heat shock proteins (HSP) e.g. HSP20, HSP22 and HSP27, often termed as stress proteins, are likely candidates (Brophy et al., 1997, Knoepp et al., 2000, Salinthone et al., 2008). Phosphorylated HSP20 is thought to inhibit smooth muscle contraction as part of the mechanism of action of nitrovasodilators and β-adrenergic agonists (Rembold et al., 2000). On the other hand cellular stress which leads to increases in the phosphorylation of HSP27 inhibits phosphorylation of HSP20 (Fuchs et al., 2000). Several vasoactive hormones enhance expression of HSP27 in vascular smooth muscle, e.g. vasopressin, thrombin and sphingosine 1-phosphate, by a MAPK-dependent mechanism (Salinthone et al., 2008).

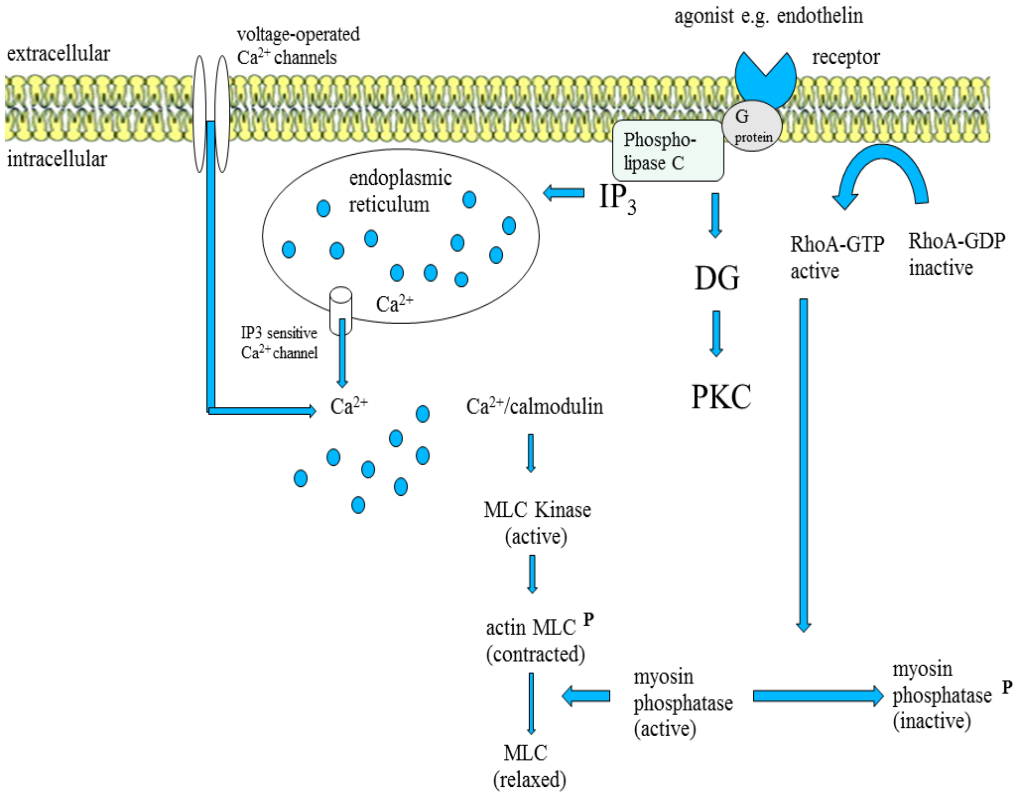


Figure 1.1. Regulation of smooth muscle contraction. Agonists like endothelin bind to a specific receptor and increased phospholipase C activity through G protein. Phospholipase C produces two potent second messengers: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP3). IP3 binds to specific receptors on the sarcoplasmic reticulum, causing release of calcium (Ca^{2+}). DG along with Ca^{2+} activates PKC, which has contraction-promoting effects such as phosphorylation of Ca^{2+} channels. Ca^{2+} binding to calmodulin leads to activation of myosin light chain kinase (MLC kinase). The latter phosphorylates the light chain of myosin, and, in conjunction with actin, cross-bridge cycling occurs, initiating shortening of the smooth muscle cell, the contractile response is maintained by a Ca^{2+}-sensitizing mechanism brought about by the inhibition of myosin phosphatase activity by Rho kinase.

1.2. Ca^{2+} - sensitization mechanism

The state of MLC phosphorylation and smooth muscle contraction is maintained independently of Ca^{2+} concentration by a Ca^{2+} sensitizing mechanism; this is brought about by Rho kinase, also known as ROCK. MLC phosphatase (MLCP), also known as myosin phosphatase, removes the high-energy phosphate from the MLC to promote smooth muscle relaxation. MLCP is a heterotrimeric protein consisting of a catalytic subunit and a regulatory subunit, MYPT1. Inhibition of MLCP is ascribed to

phosphorylation of MYPT1. Rho kinase plays an important role in the regulation of MYPT1 phosphorylation. Rho kinase, by inactivating MLC phosphatase, increases the level of phosphorylated MLC and thereby prolongs smooth muscle contraction (Uehata et al., 1997, Nobe and Paul, 2001, Sakurada et al., 2003, Pearson et al., 2013). Pharmacological inhibitors of Rho kinase, such as fasudil and Y-27632, block its activity and induce relaxation of isolated smooth muscle segments (Budzyn et al., 2006, Suzuki et al., 2012). The degree of myosin light chain phosphorylation and, hence, smooth muscle tone is determined by the relative activities of these two opposing enzymes, MLCK and MLCP (Webb, 2003).

1.3. Ca^{2+} -transport mechanisms across the plasma membrane

Ca^{2+} entry from the extracellular medium occurs through plasma membrane ion channels in response to a wide variety of stimuli, including membrane depolarization (Pesic et al., 2004), stretch, extracellular agonists (Ruegg et al., 1989), and the depletion of intracellular stores (Berridge et al., 2003, Weirich et al., 2005). Based on the mechanism of activation, there are three main routes by which Ca^{2+} enters cells across the plasma membrane: voltage-dependent Ca^{2+} channels (VDCCs), receptor-operated Ca^{2+} channels (ROCCs), and store-operated Ca^{2+} channels (SOCCs) (Gibson et al., 1998, Bootman et al., 2001, Brueggemann et al., 2006). On the other hand the removal of Ca^{2+} from the cytosol into the extracellular space occurs via plasma membrane Ca^{2+}-adenosine 5’ triphosphatase (ATPase) (PMCA) and sodium Na^{+}/Ca^{2+} exchanger (NCX) or intracellular pumps, which recycle Ca^{2+} back into organelles (Berridge et al., 2003). Interplay between these opposing processes is responsible for the maintenance of cytosolic Ca^{2+} at ~100 nM

whereas the extracellular medium is more than 1 mM (Rizzuto and Pozzan, 2006).

1.3.1. Voltage-gated Ca^{2+} channels

Voltage-gated Ca^{2+} channels (VDCCs) play a central role in the regulation of vascular tone and blood pressure regulation (van Meel et al., 1983, Moosmang et al., 2003). Activation of Ca^{2+} channels by membrane depolarization, leads to Ca^{2+} influx, Ca^{2+} elevation, and vasoconstriction (Berridge et al., 2000), whereas membrane potential hyperpolarization closes voltage-gated Ca^{2+} channels and leads to vasodilatation (Yanagisawa et al., 1993). Findings revealed that Ca^{2+} influx could also stimulate Ca^{2+} release from intracellular stores (Fernandez-Tenorio et al., 2011). L-type Ca^{2+} channel blockers are widely used to treat hypertension because of their ability to reduce arterial smooth muscle cell contractility (Saida and van Breemen, 1983, Kalsner, 1997). There are five distinct subtypes of voltage-gated calcium channels: L, T, N, P and R. Voltage-gated calcium channels are protein complex of 5 subunits: the central pore-forming subunit (termed α1) is associated with other accessory subunits that regulate channel gating and trafficking like the intracellular (β) subunit and the disulfide-linked glycoprotein subunits (α2 and γ). The fifth subunit is the transmembrane (δ) subunit (Catterall, 2011). In general L-type Ca^{2+} channels are particularly important in regulating contraction of cardiac and vascular smooth muscles. T and N channels mediate Ca^{2+} entry into neurons and involved in neurotransmitter release (Kuga et al., 1990, Todorovic and Jevtovic-Todorovic, 2011). Results have demonstrated the existence of two different types of Ca^{2+} channels, L-type and T-type, in smooth muscle cells from coronary arteries of guinea-pigs (Ganitkevich and Isenberg, 1990). However, the contribution of T-channels to the peripheral resistance still has not been ruled out (Cribbs, 2001,

Cribbs, 2006). The α1 subunits of the L-type channels contain binding sites for dihydropyridine Ca^{2+} channel blockers (Campbell et al., 1988). Clinically, Ca^{2+} channel antagonists like nifedipine, verapamil and diltiazem are commonly used for their cardiovascular effects e.g. blood pressure control (Klein, 1987, Kalsner, 1997). On the other hand, vasodilators like nitroprusside, by acting through cGMP and protein kinase G, could regulate the open-probability of Ca^{2+} channels thereby inhibiting Ca^{2+} influx. By contrast, vasoconstrictors act through the protein kinase C pathway, with subsequent activation of Ca^{2+} channels (Ishikawa et al., 1993, Taguchi et al., 1997, Chaplin and Amberg, 2012)

Data have shown that mitochondrial reactive oxygen species generation at complex III result in cardiac L-type Ca^{2+} channel inhibition (Scragg et al., 2008). Similarly, superoxide anion impairs Ca^{2+} channel function in arterial smooth muscle cells (Fusi et al., 2001). Conversely, hydrogen peroxide (H_2O_2) has been shown to stimulate arterial smooth muscle L-type Ca^{2+} channels by a mechanism involving oxidative activation of PKC (Tabet et al., 2004, Chaplin and Amberg, 2012).

1.3.2. Store-operated Ca^{2+} channels

Store-operated Ca^{2+} channels (SOCCs) are plasma membrane, non-selective, cation channels. They are activated to allow Ca^{2+} entry when the endoplasmic reticulum (ER) stores are depleted (Parekh, 2003). SOCCs are distinct from other membrane calcium channels. Many of them are speculated to belong to the family of transient receptor potential (TRP) channels (Golovina et al., 2001, Xu and Beech, 2001). It is unclear what kind of linkage couples them to the ER. However, two major theories have been proposed. First, SR Ca^{2+} depletion stimulates a second messenger, which activates plasma membrane Ca^{2+} channels (Bootman et al., 2001). Second, SOCCs are activated by either a conformational coupling between

Ca^{2+} channels on the SR and those on the plasma membrane; The protein STIM1 is the Ca^{2+} store sensor located in the membrane of the ER (Liou et al., 2005), and the PM protein Orai1 is the SOC channel (Yang et al., 2012). Cultured proliferative VSMCs display increased SOCE and high levels of STIM1 and Orai1 compared with their quiescent contractile freshly isolated counterparts (Potier et al., 2009). Store-operated Calcium entry does not appear to play a significant role in smooth muscle or cardiac contractility. In contrast, skeletal muscle contractility appears to depend on SOCE (Trebak, 2012). However, others have suggested that SOCCs play a role in Ca^{2+} entry and the control of vascular contractility (Zhang et al., 2002, Brueggemann et al., 2006). Like the SR channels, the SOCCs serve to amplify the rise in intracellular Ca^{2+} (Park et al., 2008). So far, only experimental compounds are known to block these channels. However, efforts are being made to develop specific blocking agents for therapeutic use as smooth muscle relaxants (Tosun et al., 1998a, Leung et al., 2008).

1.3.3. Receptor-operated Ca^{2+} channels

Receptor-operated Ca^{2+} channels (ROCCs) refers to Ca^{2+} permeable nonselective cation channels. These channels are opened independently of membrane potential or intracellular Ca^{2+} stores (Leung et al., 2008). Furthermore, ROCCs are not inhibited by organic Ca^{2+} channel antagonists. In fact, ROCCs are activated by a wide variety of agonists such as acetylcholine, angiotensin II, endothelin-1, noradrenaline, 5-HT and vasopressin that stimulate Ca^{2+} entry into the SMCs (Leung et al., 2008). The Ca^{2+} entry through ROCCs could be blocked by the multivalent cations La^{2+}, Cd^{2+}, Mn^{2+}, Ni^{2+} and Mg^{2+} (Ruegg et al., 1989). ROCCs are thought to be involved in guinea pig aorta contraction induced by noradrenaline, since the contraction was only partially blocked by Ca^{2+} channel blockers (Gouw et al., 1990). Similarly, in rat cultured aortic smooth muscle cells,

ATP, angiotensin II, and vasopressin caused Ca^{2+} influx that was independent of membrane depolarization and unaffected by Ca^{2+} channel antagonists, while significantly reduced by phospholipase C inhibitors (Ruegg et al., 1989).

1.4. K^+ channels mediated vasodilatation

Potassium channels are the dominant ion conductive pathways in electrically excitable cells, including those found in vascular muscle cells. As such, they contribute to the regulation of the membrane potential and vascular tone (Nelson et al., 1990). Opening of K^+ channels in the cell membrane increases K^+ efflux with membrane potential hyperpolarization. This effect is followed by the closure of voltage-dependent Ca^{2+} channels, with subsequent reduction in Ca^{2+} entry and vasodilation (Yanagisawa et al., 1993). Closure of K^+ channels has the opposite effect i.e. K^+ channels inhibition contributes to membrane depolarization and vasoconstriction (Post et al., 1992, Kirschstein et al., 2009). Thus, the presence of a physiological or pharmacological agent that alters membrane potential, for example, the K_{ATP} channel opener cromakalim, should cause a significant change in blood vessel diameter and eventually result in reduction in blood pressure (Yanagisawa et al., 1993). In the microcirculation, as in other vascular muscles, four distinct types of K^+ channel in arterial smooth muscle have been identified: voltage-dependent K^+ (K_V) channels, Ca^{2+}-activated K^+ (BK_{Ca}) channels, ATP-sensitive K^+ (K_{ATP}) channels, and inward rectifier K^+ (K_{ir}) channels (Standen and Quayle, 1998).

1.4.1. Voltage-activated K^+ channels

Voltage-activated K^+ channels (K_v) are a class of K^+ channels expressed by the vascular smooth muscle cells. These channels are activated by membrane depolarization with threshold potentials of ~-30 mV to allow an

efflux of K^+ resulting in repolarization and return to the resting membrane potential (Jackson, 1998). Therefore, they are thought to serve an important buffering function against depolarization and vasoconstriction. 4-aminopyridine is widely used as a pharmacological blocker of K_v channels (Heaps and Bowles, 2002). The concentration for half-maximal inhibition of K_v channel function has been shown to be ~ 1 mM (Okabe et al., 1987, Smirnov and Aaronson, 1992, Gelband and Hume, 1992). Studies from hypertensive animal models indicated decreased expression of functional K_v channels in vascular muscle cells (Jepps et al., 2011). K_v channel activity was also reduced in animal models of diabetes, *in vivo,* and similarly when vessels were incubated in high glucose solutions, *in vitro* (Bubolz et al., 2005, Liu et al., 2001, Li et al., 2004). Furthermore, 4-aminopyridine incubation causes arterial depolarization and constriction, suggesting that K_v channel activity exists in some blood vessels under basal conditions in health and disease (Jackson, 1998, Heaps and Bowles, 2002). Prostacyclin, through K_v activation, may contribute to endothelium-dependent relaxation in rabbit cerebral arteries (Dong et al., 1998). Vasodilators that act via nitric oxide, may also directly activate K_v channels (Irvine et al., 2003). Similarly, reactive oxygen species are also considered as modulators of K_v activity (Fike et al., 2013). On the other hand, vasoconstrictors may inhibit K_v by mechanisms that involve protein kinase C activation (Clement-Chomienne et al., 1996).

1.4.2. Calcium-Activated K^+ Channels

Calcium-activated K^+ channels (K_{ca}) are particularly abundant in vascular smooth muscle cells and appear to play a fundamental role in regulating resting membrane potential and vascular tone (O'Rourke, 1996, Ledoux et al., 2006, Dalsgaard et al., 2009). K_{ca} channel activity in vascular smooth muscle cells (VSMCs) is correlated with the occurrence of spontaneous

transient outward K^+ currents (STOCs). Localized calcium release events from ryanodine receptors, termed calcium sparks, activate nearby calcium-activated potassium channels, causing a hyperpolarizing current i.e. STOCs to oppose vasoconstriction (Zhuge et al., 2004). Therefore arterial tone results from the interplay of opposing calcium-dependent processes: constriction, which is driven by global increases in calcium induce vasoconstriction while localized calcium concentrations induce vasorelaxation. The K_{Ca} channel family consists of small conductance (SK_{Ca}), intermediate conductance (IK_{Ca},) and large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) subtypes. In the microcirculation, physiological activation of vascular BK_{Ca} channels may be an important negative feedback mechanism to counteract vessel depolarization and active vasoconstriction in response to some vasoconstrictors, for example, the thromboxane A2 (TXA2) mimetic (U46619) (Knot et al., 1998), ET-1 and angiotensin II (Scornik and Toro, 1992, Minami et al., 1995). K_{ca} thus represent an important pharmacological target. Pharmacological agents commonly used to inhibit BK_{Ca} channels include tetraethylammonium ion (1 mmol/L), charybdotoxin, and iberiotoxin, whereas NS-1619 has been shown to activate BK_{ca} channels (O'Rourke, 1996, Heaps and Bowles, 2002). Vasodilators that act through the cGMP and cAMP cascades may result in K_{ca} channel activation (Minami et al., 1993b). Results have also suggested that both PKG and PKA activate BK_{ca} channels (Song and Simard, 1995). Reactive oxygen species have been reported as modulators of BK_{ca} activity in porcine coronary artery smooth muscle cells (Barlow and White, 1998, Tang et al., 2004). In fact, the effects range from channel activation to inhibition depending on the type of ROS involved and local ROS concentration (Barlow and White, 1998, Soto et al., 2002, Gutterman et al., 2005).

1.4.3. ATP-sensitive K^+ channels

ATP-sensitive K^+ channels (K_{ATP}) were first identified in guinea pig cardiac myocytes (Suzuki et al., 2001, Noma, 1983). From then on, similar channel currents have been identified in pancreatic β-cells (Tarasov et al., 2004), smooth and skeletal muscles (Davies, 1990, Yokoshiki et al., 1998), central nervous system neurones (Ashcroft, 1988, Bernardi et al., 1988), and renal tubular cells (Mauerer et al., 1998, Loutzenhiser and Parker, 1994). The activity of K_{ATP} channels is controlled by intracellular ATP (see below); an increase in the ATP/ADP ratio closes the K_{ATP} channels (Cook and Hales, 1984). Cardiac K_{ATP} channels have a physiological role in modulating cardiac function under conditions of metabolic stress when ATP is reduced. During ischaemia, opening of cardiac K_{ATP} channels is thought to protect against myocardial damage. Increasing K^+ efflux and shortening of the action potential duration will decrease Ca^{2+} influx and the subsequent contraction, the latter eventually result in reducing cardiac work and conserving ATP (Gross and Peart, 2003, Yang et al., 2007, Yao and Gross, 1994). In fact, the reduction in infarct size by preconditioning was blocked completely by intracoronary 5-hydroxydecanoate (a mitochondria K_{ATP} channel blocker) or glibenclamide (a plasma membrane K_{ATP} channel blocker) (Auchampach et al., 1992, Foster et al., 2012). Moreover, ischaemia, hypoxia and metabolic inhibition-induced vasodilation in the coronary and cerebral circulation can be prevented by glibenclamide. Studies in dogs (Samaha et al., 1992) and humans (Farouque et al., 2002) have shown that *in vivo* application of glibenclamide increases coronary tone. Such evidence points to a functional role for K_{ATP} channels under physiological conditions (Daut et al., 1994). However, in porcine isolated coronary arteries K_{ATP} channels inhibition caused no change in basal contractility (O'Rourke, 1996).

In pancreatic β-cells, when the plasma glucose level rises, the concentration of ATP increases, the latter results in closure of K_{ATP} channels (Cook and Hales, 1984, Kakei et al., 1986). Closure of K_{ATP} channels in the plasma membrane allows the cells to depolarize, subsequently triggering calcium entry and insulin release (Miki et al., 1999, Henquin, 2000). K_{ATP} channels are targets for several clinically important groups of drugs, including the sulphonylureas, commonly used to treat type II diabetes (Nagashima et al., 2004), and the K_{ATP} channel openers, used to treat angina and hypertension (Arena and Kass, 1989, Gollasch et al., 1995).

1.4.3.1. Cellular mechanisms that regulate K_{ATP} channels

K_{ATP} channels are sensitive to the intracellular ATP/ADP ratio. In pancreatic β-cells, K_{ATP} channels are rapidly inhibited by ATP (Cook and Hales, 1984) and activated by ADP e.g., MgADP (Gribble et al., 1998). By sensing the ATP and ADP levels, these K_{ATP} channels are able to couple the cellular metabolism to membrane excitability (Tarasov et al., 2004). Beech et al. suggested that K_{ATP} channels in vascular smooth muscle may not respond primarily to changes in ATP nucleotide, instead diphosphates may play a primary role in the regulation of these channels under physiological conditions (Beech et al., 1993). Vasodilators like, isosorbide dinitrate, increase cGMP in the vascular smooth muscle cells. The increased levels of cellular cGMP result in protein kinase G (PKG) activation. In turn vascular K_{ATP} channels are opened by vasodilators that activate PKA and PKG. Vasodilators coupled to PKA could also activate K_{ca} (Song and Simard, 1995) and K_v (Aiello et al., 1995) to inhibit VDCC (Orlov et al., 1996). On the other hand, K_{ATP} are closed by vasoconstrictors that activate PKC (Chrissobolis and Sobey, 2002). In fact, vasoconstrictors coupled to PKC also inhibit Ca^{2+}-activated K^+ channels (Minami et al., 1993a) and voltage-dependent K^+ channels (Clement-Chomienne et al., 1996).

Inhibition of cellular metabolism with oxidative phosphorylation inhibitors e.g. cyanide or dinitrophenol, or with glycolysis inhibitors e.g. 2-deoxglucose or iodoacetic acid caused activation of K_{ATP} currents in smooth muscle cells (Silberberg and van Breemen, 1992, Teramoto and Brading, 1996). Several vasoconstrictors, for example, noradrenaline, histamine, 5-HT (Bonev and Nelson, 1996), endothelin-1 (Miyoshi et al., 1992), vasopressin (Wakatsuki et al., 1992) and angiotensin II (Miyoshi and Nakaya, 1991, Sampson et al., 2007) have been shown to inhibit vascular K_{ATP} channels in pig coronary artery. Using patch clamp, results have shown that H_2O_2 activates K_{ATP} current in smooth muscle cells isolated from mice aortas (Sharifi-Sanjani et al., 2013). Similarly, in guinea-pig ventricular myocytes, exposure of the patch membrane to ROS, $O_2^{\bullet-}$ and H_2O_2, increased the opening of K_{ATP} channels. Such effect of ROS was prevented by perfusion with glibenclamide (Tokube et al., 1998). On the other hand, H_2O_2 suppressed pinacidil-induced K_{ATP} channel activation in cultured aortic VSMCs (Yasui et al., 2012). Moreover, exposure of isolated mesenteric rings to H_2O_2 impaired the K_{ATP} channel-mediated vascular dilation (Yang et al., 2010).

1.4.3.2. Structure

The K_{ATP} channel is a hetero-octamer composed of two subunits, K^+ channel (Kir6.x) pore forming subunits and regulatory SUR subunits arranged in stoichiometry of 4:4. Two types are currently known: Kir6.1 which acts as the pore-forming subunit in vascular smooth muscle and Kir6.2 which forms the K_{ATP} channel pore in pancreatic β-cells, cardiac and skeletal muscles, and neurones. On the other hand, the sulphonylurea receptors (SUR): SUR1 and SUR2 (SUR2A, SUR2B) function as regulatory subunit, conferring sensitivities of the channel to several K_{ATP} channel

activators and inhibitors like sulfonylureas, MgADP and some channel openers. Different combinations of Kir and SUR subunits generate tissue-specific K_{ATP} channel subtypes, table 1.1 (Miki et al., 1999).

Table 1.1 K_{ATP} channel subunits distributions in different tissues

Tissue type	***Major Subunit composition***	
Pancreatic β-cell type	Kir6.2	SUR1
Neuron	Kir6.2	SUR1
Cardiac and skeletal muscle type	Kir6.2	SUR2A
Vascular smooth muscle type	Kir6.1	SUR2B

In 1991, Inoue et al. identified the K_{ATP} channel in rat liver mitochondrial inner membrane (mitoK_{ATP}) (Inoue et al., 1991). It has been proposed that mitoK_{ATP} may also consist of Kir and SUR subunits. Subsequent work revealed a possibly unique pharmacology in that mitoK_{ATP} was inhibited by 5-hydroxydecanoate and activated by diazoxide. Work on the mitoK_{ATP} channel has mainly been focused on ischemic preconditioning (Pain et al., 2000).

1.5. The vascular endothelium

The vascular endothelium is the inner lining of blood vessels. In the past, the endothelium was considered to be a passive, semipermeable barrier. In 1980, studies confirmed the necessity of an intact vascular endothelium in order to obtain a vasodilatation induced by acetylcholine (Furchgott, 1999, Furchgott and Zawadzki, 1980). It is now becoming clear that the endothelium actively participates in vascular homeostasis regulation through the release of potent vasoactive agents that elicit either contraction or relaxation of vascular smooth muscle (Pearson and Vanhoutte, 1993). The most important endothelial-derived substances are:

nitric oxide (NO) and prostacyclin (PGI_2) that act as vasodilators (Ignarro et al., 1987, Luckhoff et al., 1988, Mitchell et al., 2008), while thromboxane (TXA_2) and endothelin (ET-1) act as vasoconstrictors (Hill et al., 2000, Wilson et al., 2005, Loomis et al., 2005).

1.5.1. Nitric oxide:

In vascular endothelial cells, nitric oxide (NO) is formed from the cationic amino acid L-arginine during the conversion to L-citrulline. The reaction is catalysed by the endothelial isoform of NO synthase (eNOS) (Guterbaum et al., 2013) which is constitutively expressed. NOS is activated via changes in intracellular calcium and calmodulin in response to changes in shear forces or via a receptor-mediated process (White and Martin, 1989). Released NO is able to diffuse to adjacent smooth muscle cells where it activates soluble guanylyl cyclase (sGC) (Arnold et al., 1977, Rapoport and Murad, 1983). The latter converts GTP to cyclic-3,5-guanosine monophosphate. Accumulation of cGMP activates protein kinase G (PKG), which mediates the effects of NO/cGMP in the smooth muscle (Archer et al., 1994, Pfeifer et al., 1998). cGMP inhibits IP_3 levels which explains the findings that cGMP inhibits cytosolic calcium, through both inhibition of calcium influx and reduction of intracellular calcium release (Collins et al., 1986, Luckhoff et al., 1988). This in turn decreases the phosphorylation of myosin light chains, causing smooth muscle vasodilatation. NO could also directly activate K_{ca} channels of the vascular smooth muscle (Bolotina et al., 1994). Thus, nitric oxide plays a pivotal role in regulating vessel wall homeostasis.

1.5.2. Arachidonic acid (AA) metabolites:

Arachidonic acid metabolites play a key role in the regulation of diverse processes in both physiological and pathological states. Their vasoactive properties are evident from the finding that AA itself, when applied to blood vessels, produces relaxation (Rosolowsky and Campbell, 1993).

1.5.3. Cyclooxygenase (COX)-derived metabolites of AA:

Prostacyclin (PGI_2) has long been recognised as the chief AA metabolite in blood vessels, where it acts to mediate vasodilatation (Pratt et al., 1996). Prostacyclin is normally produced by cyclooxygenase, COX, from arachidonic acid (Caughey et al., 2001). COX-1 is present constitutively in most tissues. COX-2 is also present constitutively in the vascular endothelium and kidney (Dogne et al., 2005). PGI_2 diffuses to vascular muscle where it activates adenylyl cyclase, the latter in turn increases the production of adenosine 3,5-cyclic monophosphate (cAMP) which results in vascular relaxation. (Kukovetz et al., 1979).

1.5.4. Cytochrome P450-derived metabolites of AA.

The products of AA metabolism by the cytochrome P450 system are epoxyeicosatrienoic acids (EETs), monohydroxyeicosatetraenoic acids (HETEs) and dihydroxyeicosatrienoic acids (DHETs) (Zeldin, 2001). Of these, EETs are most relevant in blood vessels. They have been shown to mediate relaxation of vascular muscle by activating large-conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channels (Dong et al., 1997, Hayabuchi et al., 1998, Fisslthaler et al., 1999, Campbell et al., 2001, Gauthier et al., 2005, Huang et al., 2005).

1.5.5. Endothelium-derived hyperpolarising factor:

Considerable evidence shows that several receptor-dependent agonists, such as acetylcholine, bradykinin, histamine and substance P, release endothelium-derived hyperpolarizing factor (EDHF) that causes vascular smooth muscle hyperpolarization (Dong et al., 1997). In general, EDHF may be of greater importance in resistance than in large conduit arteries (Garland et al., 1995). The contribution of EDHF to endothelium dependent relaxation appears to be dependent on tissue and the type of the agonist (Waldron et al., 1996, Graier et al., 1996). EDHF diffuses to vascular smooth muscle where it activates potassium (K^+) channels activity with subsequent hyperpolarization and relaxation of the vascular muscle (Hayabuchi et al., 1998). In fact, the identity of EDHF remains to be established. The classical EDHF involves K^+ that effluxes via K^+ channels on endothelial cells. Spread of hyperpolarizing current from the endothelial cells, via myoendothelial gap junctions, to the smooth muscle cells hyperpolarizes and relaxes adjacent smooth-muscle cells by activating K^+ channels and Na^+/K^+ ATPase (Edwards et al., 1998). It could be a product of AA metabolism, EETs. The latter hyperpolarize and relax vascular smooth muscle cells (SMCs) by activating calcium-sensitive potassium (K_{Ca}) channels. Another possible mediator of EDHF- induced smooth-muscle relaxation is by activating of Na^+/K^+ ATPase (Edwards et al., 1998). H_2O_2 also considered as an endogenous EDHF and plays an important role in coronary autoregulation in co-operation with NO (Yada et al., 2003, Matoba et al., 2003, Liu et al., 2011).

1.6. HMG-CoA reductase inhibitors

Atherosclerosis, a pathologic and progressive process affecting the arterial walls, is characterized by the accumulation of lipid particles and immune

cells in subendothelial regions, and by the proliferation of vascular smooth muscle (VSM) cells, leading to a narrowing of the arterial lumen, following plaque rupture and thrombosis. An endothelial dysfunction, which is the first step in the disease, can be induced by a variety of risk factors such as elevated levels of LDL-cholesterol (Badimon and Vilahur, 2012). 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, known as statins, are some of the most effective and best tolerated lipid modifying agents for the treatment of hyperlipidemia (Schaefer et al., 2004, Cheung et al., 2004, Jorge et al., 2005). Many are presently approved for clinical use. Some are fungal –derived metabolites, like simvastatin, lovastatin and pravastatin. Others are totally synthetic like atorvastatin, cerivastatin fluvastatin and pitavastatin, but all share the HMG-CoA like moiety on their polycyclic backbone (Figure 1.2) (Schachter, 2005a).

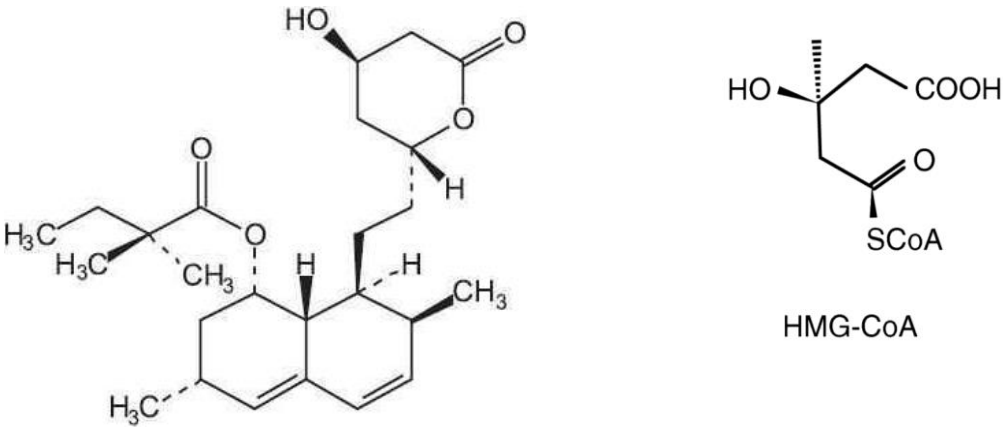


Figure 1.2. Chemical structure of simvastatin: simvastatin (left) have a lactone ring similar to HMG-CoA biologically active moiety (right). Adapted from (Wang and Asgharnejad, 2000).

1.6.1. Pharmacokinetics:

Simvastatin and lovastatin are administered orally as a lactone prodrug while pravastatin is administered as the active open ring form. Liver enzymes hydrolyse the prodrugs, simvastatin and lovastatin, to the active

hydroxyl acid derivatives. Gastrointestinal absorption of statins varies from 30% to 80%, with almost complete absorption for fluvastatin (Scripture and Pieper, 2001). The oral bioavailability of simvastatin and lovastatin is low, largely owing to their cytochrome P450 (CYP3A)-mediated first-pass metabolism (Neuvonen et al., 1998), whereas the bioavailability of fluvastatin and pravastatin is higher than simvastatin. All undergo first pass metabolism, either oxidized by CYP3A4 in the liver, or hydrolysed to several metabolites. Most of the absorbed dose is excreted in the bile. The lipophilic statins, simvastatin and lovastatin, enter the liver passively (Komai et al., 1992, Schachter, 2005a), whereas the water soluble agents, e.g. pravastatin, are taken up by an active transport (Komai et al., 1992). Thus in terms of tissue selectivity, pravastatin exhibits liver selectivity in comparison with other lipophilic statins (Koga et al., 1992). Plasma half-life of the parent compound ranges from 1 to 3 hours except for atorvastatin may reach up to 14 hours and rosuvastatin about 20 hours (Garcia *et al*., 2003).

1.6.2. Adverse effects

Statins are well tolerated. However, mild unwanted effects have been reported like myalgia, gastrointestinal disturbance, raised plasma level of liver enzymes, insomnia and rashes (Bitzur et al., 2013). Recently, increased risk of glucose intolerance has been reported especially with lipophilic statins (Byrne and Wild, 2011, Bellia et al., 2012, Wang et al., 2012, Carter et al., 2013, Dormuth et al., 2014, Turan et al., 2014, Macedo et al., 2014). More serious adverse effects are rare, but include severe myositis (Simsek Ozek et al., 2014). Myositis, rhabdomyolysis, is a class effect of statins, occurs also with other lipid-lowering drugs (especially fibrates) and it is dose-related.

1.6.3. Lipophilicity

Simvastatin and lovastatin are both examples of lipophilic statins that can cross the plasma membrane. On the other hand, the hydrophilic statins like pravastatin remain associated with the polar surface of the membrane (Koga *et al.*, 1992). The lipophilic statins can enter the cell membrane passively with a large volume of distribution among tissues (Ohtawa et al., 1999), while the water soluble agents are liver specific and an essential carrier mechanism is required for their uptake (Lennernas and Fager, 1997). Under physiological pH conditions the relative lipophilicity of various statins was: *simvastatin* = *cerivastatin* > *lovastatin* = *fluvastatin* = *atorvastatin* >> *pravastatin* (Joshi *et al*., 1999).

1.6.4. Mechanism of action as hypolipidaemic agents:

Statins act by reversible inhibition of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (HMG- CoA reductase), the enzyme responsible for HMG-CoA conversion to mevalonate (Stancu and Sima, 2001) . The structural similarity between the statins side chain and the enzyme substrate, HMG-CoA, renders them effective competitive blockers of the rate determining step in mevalonate biosynthesis (Schachter, 2005a) (Fig.2). Reducing intracellular cholesterol leads to upregulation of low density lipoprotein (LDL) receptor expression on the hepatocyte surface, resulting in an increase in the catabolism of LDL by increasing LDL extraction from the blood. Statins also have been shown to reduce, to a lesser extent, the level of triglyceride (Branchi *et al.*, 1999) and increase high density lipoprotein (Asztalos *et al.*, 2002). However, simvastatin might also exert cholesterol independent effects. Several landmark clinical trials have shown that simvastatin reduces mortality and morbidity in

patients both with and without hypercholesterolaemia (Fichtlscherer *et al*., 2006).

1.6.5. The pleiotropic effects of statins.

Several clinical and experimental studies have demonstrated that statins exert a variety of clinically useful outcomes in patients with a wide range of cholesterol levels, which cannot be attributed to their cholesterol lowering effects alone. Such outcomes are collectively known as pleiotropic effects of statins (Sadowitz et al., 2010a, Sadowitz et al., 2010b). These are unrelated, or indirectly related, to their effect on plasma cholesterol and are usually unanticipated. Understanding such effects is important to optimize statin use in the treatment and prevention of cardiovascular disease (Bonetti et al., 2003). The pleiotropic effects have been appreciated in cardiovascular disease (CVD). Some of the added benefits in CVD include: blood pressure-lowering effect with normalized vasomotion (Correa et al., 2014), improved endothelial cell function (Wolfrum et al., 2003), attenuation of endothelin-1 synthesis, down regulation of angiotensin receptor synthesis (Ichiki et al., 2001), improved bioavailability of NO (Hernandez-Perera et al., 1998), attenuation of vascular inflammation (Wassmann et al., 2002), decreased vascular smooth muscle cell (VSMC) proliferation and migration (Corpataux et al., 2005b), stabilization of atherosclerotic plaque, and inhibition of platelet aggregation (Libby, 2001).

1.6.6. Mechanism of action as a pleiotropic agent:

Statins are potent inhibitors of the rate-limiting step of the mevalonate pathway. They have a high affinity for HMG-CoA reductase and are able to significantly block the production of mevalonate, a necessary precursor for cholesterol synthesis. Furthermore, mevalonate is the building block for a

variety of other important isoprenoid intermediates, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate (Corpataux et al., 2005b, McTaggart, 2006). These lipophilic isoprenyl groups permit small G-proteins to anchor to cell membranes, thus enabling sub-cellular localisation, and intracellular trafficking of membrane-associated proteins (Lane and Beese, 2006). Ras translocation is dependent on farnesylation, whereas Rho translocation is dependent on geranylgeranylation (Noguchi et al., 1998), both of which exert important effects on vascular smooth muscle cells (Brown et al., 2006). Statins, by inhibiting isoprenylation, effectively lower membrane levels and activity of Ras/Rho proteins as a likely mechanism mediating some of the pleiotropic effects of statins and thus improve vascular function (Figure 1.3) (Nohria et al., 2009).

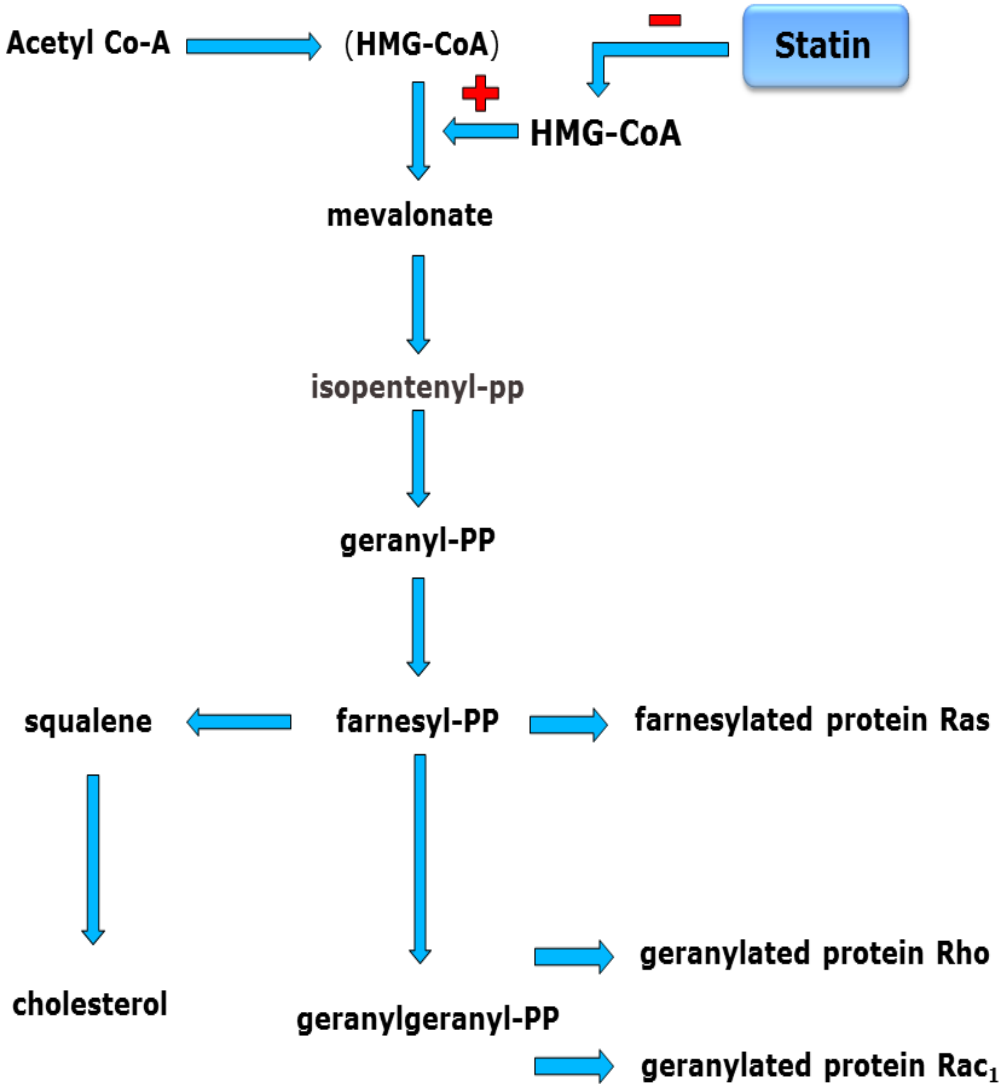


Figure 1.3. Cholesterol biosynthesis pathway and site of statins action, Statins inhibits HMG-CoA reductase. Thus, statins decrease isoprenoid intermediates such as farnesylpyrophosphate (F-PP) and geranylgeranyl-pryophosphate (geranylgeranyl-PP), with subsequent inhibition of isoprenylation of the small GTPases such as Ras, Rho, Rac1.

1.6.6.1. Effects of statins on vascular tone.

The role of HMG-CoA reductase inhibitors has been demonstrated to go beyond cholesterol biosynthesis, such as regulation of smooth muscle tone. Lipophilic statins such as cerivastatin, simvastatin, atorvastatin and lovastatin, but not the hydrophilic pravastatin induced coronary

vasodilation in the isolated bovine coronary arteries (Lorkowska and Chlopicki, 2005). Simvastatin also produced relaxation of both aorta and small mesenteric artery, with and without functional endothelium (Alvarez De Sotomayor et al., 2000). The relaxation of rat aorta was observed in rings precontracted with noradrenaline, endothelin-1 and KCl (60mM) (Mraiche et al., 2005). Similarly, lipophilicity is an important factor behind the effects of statins on vascular tone in the cerebral circulation (Beretta et al., 2011). On the other hand, pravastatin might induce vascular relaxation which appears to be endothelium-dependent. This could be due to differential expression in endothelial cells and smooth muscle cells of organic anion transporters required for cellular uptake of hydrophilic statins (Hasegawa et al., 2002). Clinically, in patients with type II diabetes, simvastatin improved the lipoprotein profile and significantly improved flow-mediated dilator response (Koh et al., 2005). Furthermore, simvastatin lowers blood pressure in patients with hypertension (Juncos et al., 2012), particularly in the presence of high levels of cholesterol. Thus, statins might work synergistically with antihypertensive drugs to significantly improve inadequately controlled hypertension (Mangat et al., 2007, Juncos et al., 2012, Xing et al., 2007, Correa et al., 2014). Similarly, lovastatin reduced vascular reactivity in kidneys of spontaneously hypertensive rats and attenuated the development of hypertension (Jiang and Roman, 1997). Lastly, statin therapy is associated with improved survival in patients suffering from peripheral vascular disease (Schillinger et al., 2004).

1.6.6.2. Statin and NO bioavailability:

A considerable number of studies have demonstrated a key role of endothelial nitric oxide synthase (eNOS) in statin-associated cardiovascular protection (Chen et al., 2000, Kalinowski et al., 2002, Kuhlmann et al.,

2004, Brandes, 2005, de Sotomayor et al., 2005, Rikitake and Liao, 2005). Results revealed that simvastatin acutely enhances NOS phosphorylation via the phosphatidylinositol -3 kinase protein kinase/Akt pathway. (Nakata et al., 2007, Ghaffari et al., 2011). Inhibition of Rho isoprenylation is one of the best examined pleiotropic effects of statins (Rashid et al., 2009, Rattan, 2010). It is well known that Rho/Rho kinase pathway negatively regulates eNOS phosphorylation through inhibition of protein kinase B (Akt) (Ming et al., 2002). Thus, long-term use of statins results in enhanced NO bioavailability in part due to blocking isoprenoid synthesis.

Caveolin, which acts as an inhibitor of eNOS activation, impairs NO release in the endothelial cells. It is interesting to consider that long-term statin therapy could improve endothelial function via down-regulation of caveolin, with subsequent activation of eNOS. On the other hand, Rossoni *et al*., demonstrated that short-term incubation with simvastatin result in phosphorylation of AMP kinase (Rossoni *et al.*, 2011). AMPK plays a role in the regulation of energy homoeostasis. However, it is now evident that AMPK is engaged in eNOS phosphorylation and NO production (Sun *et al*., 2006). Thus, short term simvastatin therapy could directly modulate small vessel contractility via a NO-dependent mechanism that is associated with AMPK phosphorylation (Rossoni *et al.*, 2011).

1.6.6.3. Statin and Prostaglandin bioavailability:

Cyclooxygenase (COX) enzymes are involved in the metabolism of arachidonic acid. Two major products, thromboxane A2 (TXA_2) and prostacyclin (PGI_2), are produced from arachidonic acid via COX. TXA_2 participates in vascular contraction, whereas PGI_2 acts as a vasodilator (Dogne et al., 2005). Results reveal the involvement of vasodilatory products from COX in the endothelium-dependent relaxation to statins in the aorta and small mesenteric artery (Alvarez De Sotomayor et al., 2000).

In fact, statins have been reported to stimulate the production of prostacyclin (Degraeve et al., 2001, Lorkowska B, 2005, Birnbaum et al., 2005). Degraeve *et al*. suggested that geranylgeranylated proteins negatively regulate COX-2 expression (Degraeve et al., 2001). Thus, statin therapy might enhance PGI_2 formation in a COX-2-dependent manner through inhibition of Rho (Degraeve et al., 2001). Conversely, data have shown that lovastatin inhibited prostacyclin release in cultured vein endothelial cells by reducing activity of COX-1 independent of mevalonate (Zhou et al., 2009). On the other hand, Alvarez De Sotomayor *et al*. demonstrated that simvastatin had no effect on prostacyclin release. Instead it might inhibit thromboxane TXA_2 formation from cyclooxygenase (de Sotomayor et al., 2005).

1.6.6.4. Statin and Ca^{2+} distribution:

Experimental observation revealed that simvastatin incubation suppressed the contraction induced by Bay K8644, an activator of voltage- dependent Ca^{2+} channel (VDCC) in rat aortic rings (Kang et al., 2014). Similarly, simvastatin suppressed the contraction mediated by high potassium-induced membrane depolarization, with subsequent opening of VDCC (Kang et al., 2014). Both results suggested that voltage-dependent Ca^{2+} entry might be altered in response to simvastatin. Alvarez de Sotomayor *et al*. demonstrated that simvastatin effects in the vascular smooth muscle might involve both Ca^{2+} release from intracellular pools with concurrent blockade of extracellular Ca^{2+} entry (Alvarez de Sotomayor et al., 2001). However, the data also show that the relaxant effect induced by simvastatin was not counteracted by the addition of $CaCl_2$, thus simvastatin might indirectly inhibit Ca^{2+} influx. Such a mechanism could be related to the inhibition of isoprenoid synthesis, the latter affects G-proteins function which is involved in communication among intracellular

Ca^{2+} pools and capacitative Ca^{2+} entry (Alvarez de Sotomayor et al., 2001). Similarly L-type Ca^{2+} channel blockade with a concomitant reduction in intracellular free Ca^{2+} has been proposed as a mechanism for lovastatin-induced relaxation in the cerebral circulation, independently of cholesterol and isoprenoid synthesis (Bergdahl *et al*., 2003). In the same study of Bergdahl *et al*., the hydrophilic pravastatin had no effect on contraction; The authors explained that cellular penetration by the lipophilic statin, lovastatin, was prerequisite for induction of vascular relaxation (Bergdahl et al., 2003). Similarly, in rat pancreatic β-cells the lipophilic simvastatin, but not by the hydrophilic pravastatin, inhibited Ca^{2+} influx through voltage gated Ca^{2+} channel (Yada *et al.*, 1999).

On the other hand, simvastatin induced an increase in cytosolic calcium in endothelial cells, by releasing Ca^{2+} from intracellular stores and by increasing Ca^{2+} entry through mevalonate-dependent pathway (Alvarez de Sotomayor and Andriantsitohaina, 2001). In skeletal muscle, acute application of simvastatin resulted in alteration of Ca^{2+} homeostasis with increase in cytoplasmic Ca^{2+}. The latter was a downstream result of an early alteration of mitochondrial membrane potential (Sirvent et al., 2005b).

1.6.6.5. Statin and K^+ channels

Another possible explanation for the simvastatin vasorelaxant effects is a direct modulation of K^+ channel activity. Lopez *et al*. suggested the K_{ca} subfamily as a potential target for rosuvastatin relaxation in rat aortic rings (Lopez et al., 2008). Furthermore, Lopez-Canales *et al*. reported that the methyl ester derivative of rosuvastatin mediated relaxation by activation of K^+ channels as a complementary mechanism for the relaxation induced by the NO pathway (Lopez-Canales *et al*., 2011). Similarly, Kuhlmann *et al.* demonstrated that cerivastatin activates calcium-activated K^+ channels in

endothelial cells isolated from human umbilical cord veins. The latter mechanism was suggested as a class effect of statins since the channel activation was completely reversed by the addition of mevalonate (Kuhlmann et al., 2004). Cerivastatin and fluvastatin, but not pravastatin, relaxed rat aorta and mesenteric arteries via multiple mechanisms that involve endothelium-dependent relaxations through nitric oxide and endothelium-independent relaxations via K^+ channel–mediated hyperpolarizations. The latter were abolished by 118 mM KCl and the K^+ channel inhibitors, 4-AP (K_v channel blocker) and TEA (nonselective inhibitor of K^+ channels) (Mukai et al., 2003). Another study by Miller *et al*. in insulin-resistant rats showed that statins lower cardiovascular morbidity and improve coronary and cerebral relaxation by restoring K_{ca} channel function to the normal level (Miller *et al*., 2004). Inadequate myocardial re-perfusion therapy, myocardial 'no-reflow', after ischaemia and reperfusion of the acute myocardial infarction has been suggested to be due to suppression of K_{ATP} channel opening. It is possible that simvastatin has beneficial effects in reducing myocardial no-reflow via activation of K_{ATP} channels (Zhao et al., 2006, Yang et al., 2007). In another study, glibenclamide (K_{ATP} channel blocker) significantly inhibited cerivastatin relaxation in rat aortic rings, suggesting involvement of K_{ATP} channel activation in cerivastatin relaxation (Sonmez Uydes-Dogan et al., 2005). Conversely, in porcine vascular coronary myocytes, simvastatin inhibited cromakalim- and pinacidil-evoked K_{ATP} channel openings (Seto et al., 2013). The same authors also reported that the lipophilic simvastatin causes inhibition of K_{ca} in coronary myocytes while the hydrophilic salt form of simvastatin has no effect on channel amplitude (Seto et al., 2007). Furthermore, simvastatin and lovastatin, but not pravastatin, inhibited pig coronary artery relaxations to K_{ATP} channel opener pinacidil, (Uhiara et al., 2012). Findings of Seto *et al*., and Uhiara *et al*., suggested that

simvastatin induced porcine coronary relaxation by K^+-channel independent mechanism.

1.6.6.6. Statin and Reactive Oxygen Species generation

Alvarez De Sotomayor *et al*. demonstrated that endothelium-dependent relaxation induced by simvastatin was significantly inhibited with superoxide dismutase (SOD) incubation in rat aorta. Such an observation highlighted the participation of free radicals in simvastatin-induced relaxation. On the other hand, acetylcholine-induced aortic relaxation was inhibited in simvastatin or atorvastatin-dosed rats (Parker et al., 2003). The latter study suggested an increased reactive oxygen species generation with the lipophilic simvastatin or atorvastatin, but not the hydrophilic pravastatin. Other reports have also demonstrated impaired endothelial relaxation to acetylcholine following lovastatin treatment (Roullet et al., 1993, Roullet et al., 1995). Furthermore simvastatin treatment resulted in AMPK activation through reactive nitrogen species (Choi *et al.*, 2008). As simvastatin has been shown to inhibit different mitochondrial complexes in the respiratory chain (Nadanaciva et al., 2007b), it is possible that simvastatin could stimulate mitochondrial reactive oxygen species (ROS) generation which in turn might regulate vascular tone. Interestingly, endothelium-dependent coronary vasodilatation operates through NADPH oxidase-derived ROS to activate Akt-eNOS axis (Feng et al., 2010). NADPH oxidase–mediated ROS release is associated with increased Rac1 activity (Sohn et al., 2000, Wassmann et al., 2002, Maack et al., 2003). The critical process in the activation of NADPH oxidase is the isoprenylation of Rac1 via geranylgeranylation, with subsequent translocation to the cell membrane (Seshiah et al., 2002). Thus, simvastatin treatment may contribute to inhibition of NADPH oxidase-mediated superoxide generation via targeting of Rac1 activation

(Erdos et al., 2006). Taking these published data into account, it is plausible that simvastatin could modulate coronary vascular tone via ROS. Such properties of statins might partly account for their early benefits in cardiovascular events.

1.7. Mitochondria

The main function of mitochondria is thought to be the synthesis of ATP which is used as an energy source by many cellular processes (Buttgereit and Brand, 1995). Mitochondrial oxidative phosphorylation employs the energy released by the oxidative transfer of electrons, from chemically-reduced substrates, to oxygen in order to generate transmembrane electrochemical gradient, the mitochondrial proton motive force (ΔP), The latter is subsequently used to power the phosphorylation of ADP via ATP synthase (Hatefi, 1985).

1.7.1. Mitochondrial oxidative phosphorylation

Mitochondria are intracellular organelles found in all human cells responsible for aerobic metabolism by oxidative phosphorylation. They are composed mainly of two lipid bi-layer membranes. The two membranes differ considerably in their permeability. The outer membrane is rich with cholesterol and permeable to ions, a property attributed to voltage dependent anion-selective channels (VADC) (Liu and Colombini, 1992). By contrast, the inner membrane is devoid of cholesterol and impermeable to ions. The latter is folded to form a shelf-like structure called "cristae" which are filled with enzymes that extract energy from nutrients. Embedded in the inner membrane are the proteins that are involved in the process of electron transport (Murphy and Smith, 2000). The space between the two membranes is called the intermembrane space. The electron carrier

proteins are arranged side by side like a chain. Three of these proteins are ion pumps. Most of the energy harvested is held in energy carriers such as NADH and FADH2 (Figure 1.4). The first step in NADH oxidation by complex I, or NADH-ubiquinone oxidoreductase, is the transfer of two electrons from NADH to the flavin mononucleotide (FMN) within the complex. Subsequently, these two electrons are passed from FMN to ubiquinone (Q), these electrons reduce ubiquinone (UQ) to ubiquinol (QH_2) which diffuses through the membrane to complex III. Energy from the electrons are also used to pump H^+ ions from the matrix into the intermembrane space (Hirst, 2010), The transfer of electrons can be blocked by the inhibitor rotenone (Friedrich et al., 1994). Complex II, succinate ubiquinone oxidoreductase is a second entry to the electron transport chain starting from FADH2, produced through the citric acid cycle, UQ is reduced to QH_2 through the oxidation of $FADH_2$ to FAD. This step can be competitively inhibited by malonate (Thorn, 1953). Electron transfer from succinate to ubiquinol does not release sufficient free energy for proton translocation, and so, unlike complex I, complex II does not pump protons to the intermembrane space. Therefore, complex II does not contribute the generation of ΔP. Electrons continue to travel down their electrochemical gradient to complex III, ubiquinol cytochrome-c oxidoreductase. Cytochrome-bc_1 in complex III transfers electrons from QH_2 to cytochrome-c. QH_2 is oxidized back to UQ which is now able to return to transport another pair of electrons, with the protons also being transferred across the membrane (Fernie *et al.*, 2004). The electron transfer at complex III can be inhibited by antimycin A (Gao et al. 2003), and this ultimately prevents the oxidation of ubisemiquinone molecules bound at the Qo site by complex III. The electrons shift down to the last protein of the chain, complex IV or cytochrome-c oxidase complex, with more H^+ ions being pumped into the intermembrane space (Verkhovsky et

al., 1999). Complex IV then transfers electrons from cytochrome-c to oxygen, the terminal electron acceptor, and water is formed as the final product. The inner mitochondrial membrane is impermeable to H^+ ions, thus the intermembrane space generates a proton motive force across the inner membrane of the mitochondria due to H^+ gradient. Subsequently, the H^+ ions diffuse back to the matrix through special channel protein called ATP synthase, complex V. The energy derived from the movement of these protons is used to synthesize ATP from ADP and phosphate. Formation of ATP by this mechanism is referred to as oxidative phosphorylation. Mitochondria are characterized by an alkaline and negatively charged matrix. Lipophilic cations can easily pass through the lipophilic barrier. As a consequence mitochondria can trap lipophilic compounds, like simvastatin, efficiently (Horobin *et al*., 2007).

1.7.2. Mitochondria and ROS generation in the vascular smooth muscle cells

The main enzymatic sources of superoxide production within the vascular wall are mitochondrial electron transport chain (Freed et al., 2014), NAD(P)H oxidase (Feng et al., 2010), cytochrome P450 Xanthine oxido-reductase (Fleming et al., 2001), nitric oxide synthases (Wang et al., 2000) and cyclooxygenases and lipoxygenases (Gunasekar et al., 1998). In addition to the generation of cellular energy, mitochondria also play an important role in a wide range of cellular activities like cellular signalling (Zhang and Gutterman, 2007). Mitochondria seem to be an important redox signalling node because of the ROS generated by the respiratory chain within the mitochondria. The site of the $O_2^{\bullet-}$ production is mainly thought to be complex III (Chandel et al., 2000, Guzy et al., 2005). In the matrix, the $O_2^{\bullet-}$ is converted to H_2O_2 by manganese superoxide dismutase (Mn-SOD). The latter is present in high concentrations within the matrix and it reacts very rapidly with $O_2^{\bullet-}$ (figure 1.4). The formed H_2O_2 can pass

easily through mitochondrial membranes and acts as a redox signal from the mitochondria to the rest of the cell. Complex III can also release $O_2^{\bullet -}$ into the intermembrane space (St-Pierre et al., 2002, Muller et al., 2004, Han et al., 2001). $O_2^{\bullet -}$, within the intermembrane space, can either be converted to H_2O_2 by an intermembrane space Cu,Zn-SOD or diffuse the outer membrane to the cytosol through the voltage-dependent anion channel. Although $O_2^{\bullet -}$ is shorter lived and less diffusible than H_2O_2. It can act as a redox signal (Zhou et al., 2010).

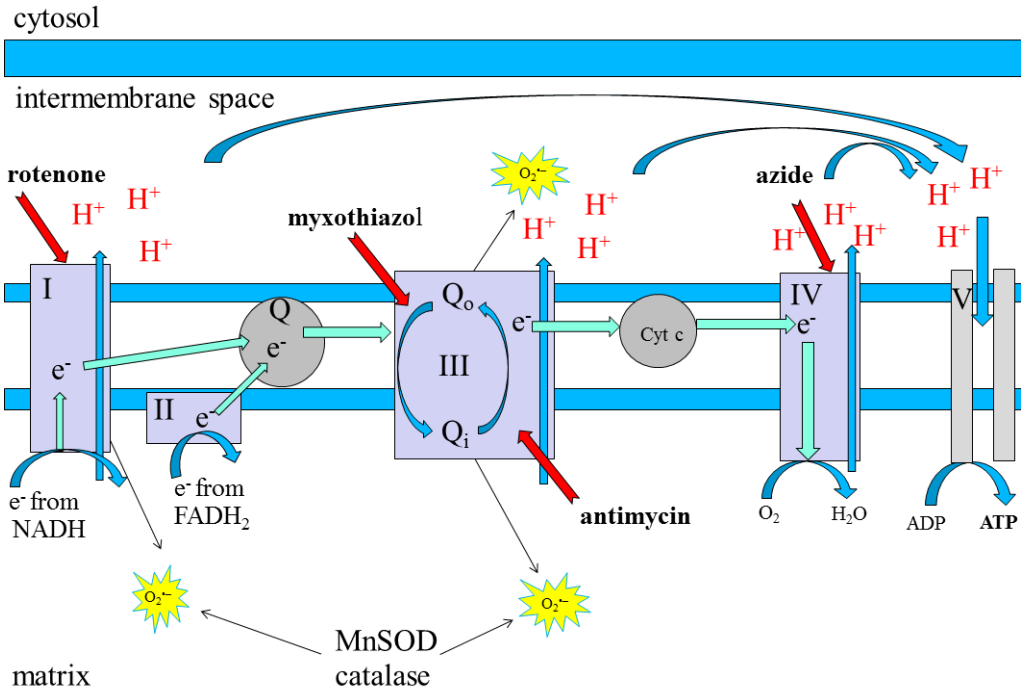


Figure 1.4. The mitochondrial electron transport chain complexes I and II reduce coenzyme Q via NADH and $FADH_2$ electrons. Coenzyme Q in turn shuttles the electrons to complex III, with subsequent transfer to cytochrome c. Complex IV reduce molecular oxygen to water via electrons from cytochrome c. The action of complexes I, III, and IV generates a proton motive force across the inner membrane of the mitochondria, which is used to phosphorylate ADP at ATP synthase. Complexes I and II generate superoxide into the mitochondrial matrix through the incomplete reduction of oxygen to superoxide, whereas complex III produces superoxide into both the matrix and the intermembrane space. The mitochondrial membrane potential (ΔΨm) is the electrochemical gradient that is present across the inner mitochondrial membrane and is generated by pumping H^+ by complexes I,III,IV of the respiratory chain. This potential is used to drive ATP synthesis via complex V.

1.7.3. Mitochondria and ROS in the regulation of vascular tone:

ROS generation is traditionally considered as a pathological response since free radicals in blood vessels quench NO produced by the endothelium with resultant impairment in vasomotor function. However, experimental data have shown that NADPH-mediated ROS, within the physiological levels, are necessary to activate the phosphatidylinositol 3-kinase–Akt–endothelial NO synthase axis. Subsequent endothelial NO synthesis mediates endothelial-dependent coronary relaxation (Feng *et al*., 2010). H_2O_2 also mediates coronary vessels relaxation via BK_{ca} channel activation (Barlow et al., 2000, Thengchaisri and Kuo, 2003). Alternatively, H_2O_2 increased coronary blood flow by acting directly on vascular smooth muscle via K_v channel (Rogers et al., 2006). H_2O_2 might also act as EDHF in human mesenteric arteries (Matoba et al., 2002).

In the large vessels, superoxide and hydrogen peroxide-activate calcium sparks in the endothelial cells. The Ca^{2+} spark could raise local Ca^{2+} concentrations sufficiently to participate in eNOS activation, thereby inducing endothelium-dependent relaxation (Gutterman, 2005) In fact, H_2O_2 exposure could increase eNOS mRNA expression (Drummond *et al.,* 2000). On the other hand, in the small coronary arterioles, mitochondrial respiratory chain-derived H_2O_2 was found to be responsible for flow-mediated vasodilatation that was independent of NO (Cai, 2005). The majority of ROS produced by the cells originates from mitochondrial metabolism. Thus mitochondria could play a key role in cell signalling (Xi et al., 2005, Zhang and Gutterman, 2007). In general, mild mitochondrial depolarization or electron transport blockade induced ROS elevation with increased calcium spark frequency, the latter stimulating K_{Ca} channels with resultant cerebral artery vasodilation (Xi *et al.*, 2005). By contrast, marked depolarizations of the mitochondria using FCCP or rotenone reduced

calcium spark activity, thereby reducing opening of BK_{Ca} channels. In human coronary arterioles, complex I and III-derived H_2O_2 was responsible for flow-mediated vasodilation (Liu *et al.*, 2003). Moreover, mitochondrial depolarization with subsequent activation of eNOS by ROS –independent mechanisms had been suggested as a mechanism for cerebral artery vasodilation (Katakam et al., 2013). On the other hand, ROS generation by smooth muscle mitochondria could act as the initial stimulus for cold-induced cutaneous artery vasoconstriction through RhoA activation (Bailey *et al*., 2005). Similarly, experiments under hypoxic conditions revealed that increased mitochondrial peroxide generation might attenuate bovine coronary arterial relaxation (Gao *et al*., 2009).

1.7.4. Interplay between mitochondria and cytosolic Ca^{2+} signals

Mitochondria are now have an acknowledged importance in the control of physiological Ca^{2+} signals in various cell types, including smooth muscle. The mitochondrial proton gradient and ΔΨM drives Ca^{2+} uptake into the organelle. Therefore, mitochondria may take up and sequester a large amount of Ca^{2+} from the cytoplasm over a wide range of concentrations, ~200 nM to >10 μM, and modulate the time course and amplitude of Ca^{2+} signals (McCarron et al., 2012). Mitochondria also supply ATP for ion pumps within the smooth muscle cell, thereby regulating Ca^{2+} efflux (Drummond and Fay, 1996). The mitochondria appeared to be located close to influx and release sites to modulate Ca^{2+} signals (McCarron et al., 2012) and the results suggest a new role for mitochondrial Ca^{2+} influx in regulating calcium release activated channel (CRAC) activity in intact cells (Glitsch et al., 2002, Malli et al., 2003). Results also suggest mitochondrial ROS can modulate voltage-gated Ca^{2+} channel (Fearon, 2006, Scragg et al., 2008).

1.7.5. Mitochondria as a target for statin therapy:

Considering the complexity of mitochondria, it is not surprising that they could be a target for the statin-induced pleiotropic effects. Results have shown that fluvastatin produces mitochondrial membrane depolarization in human hepatocellular carcinoma cells (Zhang *et al.*, 2010). Similarly, simvastatin, atorvastatin and lovastatin, caused a dose-dependent cytotoxicity of rat hepatocytes with subsequent ROS formation and mitochondrial depolarization (Abdoli et al., 2013). It is well established that the mevalonate pathway is involved not only in the biosynthesis of cholesterol but also in the biosynthesis of ubiquinone, coenzyme Q10. The latter is an obligatory coenzyme for mitochondrial enzyme complexes in oxidative phosphorylation for the production of ATP. As such, HMG-CoA reductase inhibitors block the cellular production of both cholesterol and of coenzyme Q10 leading to impairment in mitochondrial bioenergetics, e.g. simvastatin-induced CoQ10 deficiency has been proposed as a reason for hepatotoxicity (Tavintharan et al., 2007). Similarly, simvastatin, but not pravastatin, caused worsening of myocardial mitochondrial respiration during ischaemia by reducing myocardial coenzyme Q10 levels (Satoh et al., 1995). In skeletal muscle, statins induce toxicity which is related to alterations in mitochondrial respiration calcium homeostasis (Kwak et al., 2012, Galtier et al., 2012). In fact, the results have shown that simvastatin and lovastatin inhibit complex I, II + III, IV and V in heart and liver mitochondria and they may even act as mitochondrial uncoupler (Nadanaciva et al., 2007a, Nadanaciva et al., 2007b). By contrast, pravastatin has no observable effect on any of the complexes, which could be related to the structural difference of the latter i.e. β-hydroxy acid in pravastatin in comparison to lactone ring in simvastatin (Nadanaciva et al., 2007a, Nadanaciva et al., 2007b).

Conversely, simvastatin attenuated mitochondrial membrane depolarization in the cardiac myocytes after exposure to oxidative stress (Jones et al., 2003b). Similarly, in isolated rat liver mitochondria, simvastatin and atorvastatin have antioxidative properties by lowering intra-mitochondrial ionized calcium (Parihar *et al.*, 2011). Interestingly, opposite effects of simvastatin on mitochondria from cardiac and skeletal muscles have been suggested i.e improvement of antioxidant capacities in heart, while conversely the ROS production by the skeletal muscle induced mitochondrial impairments (Bouitbir et al., 2012). Collectively, these data confirm mitochondrial modulation by simvastatin; the mechanism needs to be carefully considered.

1.8. Starting hypothesis:

As well as reducing plasma cholesterol levels, statins induce acute vasorelaxation which may contribute to the overall benefits of statins in the treatment of cardiovascular disease. The mechanism underlying this relaxation is unknown. Statins have been shown to alter mitochondrial function. Therefore, this study investigated the role of mitochondria in the relaxation to simvastatin.

CHAPTER II: ACUTE EFFECTS OF SIMVASTATIN ON PORCINE CORONARY TONE: ROLE OF K_{ATP} AND MITOCHONDRIA

2.1. INTRODUCTION

HMG-CoA reductase inhibitors, or statins, are the drug of first choice for treatment of hypercholesterolemia (Dansette et al., 2000, Pedersen et al., 2004, Gupta et al., 2010). Treatment with statins reduces cardiovascular risk and, more specifically, has a beneficial effect on coronary artery disease (Waters et al., 1994). However, these beneficial effects cannot be explained fully through reductions in plasma cholesterol levels (Omori et al., 2002). A number of studies have demonstrated an effect of statins on vascular tone (McGirt et al., 2002, Mukai et al., 2003, Lorkowska and Chlopicki, 2005). These direct effects on vascular smooth muscle tone are thought to underlie some of the improvements in cardiovascular outcomes in patients on statins. Treatment with statins has also been shown to improve endothelial function in disease states (Treasure et al., 1995, Dupuis et al., 1999, Alvarez De Sotomayor et al., 2000, Tiefenbacher et al., 2004). Indeed, statins have been shown to produce both endothelium-dependent and –independent relaxations of blood vessels. For example simvastatin, atorvastatin, cerivastatin, but not pravastatin, display endothelium-dependent vasorelaxation, suggested to be mediated by nitric oxide and prostanoids (Alvarez De Sotomayor et al., 2000, Sonmez Uydes-Dogan et al., 2005, Lorkowska and Chlopicki, 2005). Previous clinical investigations demonstrated that the effects of statin on vascular coronary tone occur within hours (Iida et al., 2007, Kono et al., 2013). Data from the isolated heart preparation showed that simvastatin caused a concentration-dependent coronary vasodilatation within minutes (Gryglewski et al., 2001). Similarly, simvastatin elicits dilation of the isolated porcine retinal arteriole within a few minutes (Nagaoka et al., 2007).

NO with PGI_2 are essential for the maintenance of cardiovascular health (Mitchell et al., 2008). NO has numerous beneficial effects including the regulation of arterial tone and thus blood flow (Sausbier et al., 2000, Drouin et al., 2007). Kureishi *et al* reported that simvastatin and pravastatin treatment led to a dose-dependent increase in Akt phosphorylation which is implicated in NO regulation within 15 minutes, and peaked at about 1 hour (Kureishi et al., 2000). Moreover, lovastatin and pravastatin stimulate eNOS phosphorylation in bovine aortic endothelial cells as early as 1 min which reach maximal levels at 30 min in Akt-dependent manner (Harris et al., 2004). Similar evidence from bovine aortic endothelial cell culture using nitric oxide electrode measurement has demonstrated that simvastatin activates NO increase within 1 minute (Kaesemeyer et al., 1999). In rat mesenteric artery, simvastatin acutely phosphorylate eNOS and modulate vascular contractility via AMPK dependent phosphorylation (Rossoni et al., 2011). Activation of the soluble guanylyl cyclase/cGMP pathway is generally considered to be a major vasodilatory mechanism for NO (Sausbier et al., 2000). However, studies have demonstrated that NO also elicits vasodilation through cGMP-independent pathways by activating potassium channels (Bolotina et al., 1994, Deka and Brading, 2004).

The results regarding prostacyclin production are inconsistent. For example, atorvastatin increased the tissue concentration of COX2 as downstream to eNOS phosphorylation (Birnbaum et al., 2005). Others, like De Sotomayor et al. have suggested that simvastatin did not affect the release of prostacyclin but it inhibited the generation of thromboxane A_2 from the COX-2 isoform (de Sotomayor et al., 2005). On the other hand, lovastatin diminished prostacyclin production (Zhou et al., 2009). Atorvastatin also has been shown to reduce the expression of

cyclooxygenase-2 in cultured smooth muscles (Hernandez-Presa et al., 2002).

Interestingly, in studies in which the hydrophilic pravastatin produces a relaxation, the responses are dependent upon the presence of the endothelium (Jorge et al., 2005, Ghaffari et al., 2011), whereas endothelium-independent effects only seem to be observed by lipophilic statins (Tesfamariam et al., 1999, Bergdahl et al., 2003). This could be due to differential expression in endothelial cells and smooth muscle cells of organic anion transporters required for cellular uptake of hydrophilic statins (Hasegawa et al., 2002).

As well as stimulating vasodilatation, our laboratory has demonstrated that simvastatin inhibits β-adrenoceptor-mediated relaxation of porcine coronary artery (Uhiara et al., 2012). Previously, Seto *et al* have shown that simvastatin inhibits Ca^{2+}-activated K^+ channels in coronary artery smooth muscle (Seto et al., 2007), which may be responsible for the inhibition of β-adrenoceptor-mediated relaxations. Moreover, Seto *et al* provided evidence for K_{ATP} channel inhibition by simvastatin in porcine coronary artery myocytes (Seto et al., 2013). On the other hand, Yang *et al* suggested that simvastatin increased coronary blood flow after myocardial infarction via activation of the K_{ATP} channels (Yang et al., 2007). Similarly, cerivastatin-induced rat aortic ring relaxation was significantly reduced in the presence of glibenclamide, a K_{ATP} channel blocker, and ouabain, a Na^+-K^+ ATPase inhibitor (Sonmez Uydes-Dogan et al., 2005). Rosuvastatin has been proposed to improve Ca^{2+}-activated K^+ channel function (Miller et al., 2004). Indeed, rosuvastatin-induced relaxation of rat aorta may be due to opening of Ca^{2+}-activated K^+ channels (Lopez et al., 2008). In the last few years H_2S has been suggested to be other mediator of statins' relaxant effect via K_{ATP} channel activation (Wojcicka et al., 2011).

On the other hand, alteration of mitochondrial function could be proposed as the main mechanism by which statins regulate vascular smooth muscle tone (Liu et al., 2003). Clearly, simvastatin has been proved to cause mitochondrial depolarization in skeletal muscle (Sirvent et al., 2005b) and hepatocytes (Abdoli et al., 2013). In fact, simvastatin and lovastatin inhibit mitochondrial complexes I, II, III, IV and V, and may even act as respiratory uncouplers. By contrast, pravastatin has no observable effect (Nadanaciva et al., 2007a, Nadanaciva et al., 2007b). Similarly, fluvastatin has been shown to produce depolarization of the mitochondrial membrane (Zhang et al., 2010). Inhibition of mitochondrial activity could lead to an increase in production of reactive oxygen species (ROS). An *in vitro* study using NO and superoxide sensor provided direct evidence that vascular endothelial cells released NO with concurrent release of superoxide within seconds after statin addition (Dobrucki et al., 2001). Interestingly, Alvarez de Sotomayor *et al* reported that the relaxation to simvastatin was sensitive to superoxide dismutase incubation, thus superoxide anion might contribute towards the actions of simvastatin on vascular tone (Alvarez De Sotomayor et al., 2000). ROS from mitochondria can activate Ca^{2+}-activated K^+ channels through generation of short, calcium sparks, leading to vasodilatation (Xi et al., 2005). Acute stimulation of mitochondrial superoxide generation in some cases is required for eNOS activation (Rowlands et al., 2011). Moreover, Katakam *et al* provided evidence for eNOS activation via pharmacological depolarization of the endothelial mitochondria (Katakam et al., 2013). On the other hand, data suggest a cross talk between mitochondria and NADPH oxidases (Doughan et al., 2008). For example, the complex I inhibitor, rotenone, or complex III inhibitor, myxothiazol, inhibit mitochondrial ROS production and prevent activation of NADPH oxidase (Lee et al., 2006, Rathore et al., 2008). Furthermore, inhibition of mitochondrial respiration might depress

intracellular ATP levels with subsequent K_{ATP} channels activation and smooth muscle relaxation (Yang et al., 2007, Tavackoli et al., 2004).

The aim of this study was to determine the effect of simvastatin on porcine coronary tone, and to determine whether the effects induced by simvastatin were mediated by NO, PGI_2 and/or K_{ATP} channels, and to examine the role of mitochondria as a possible target for simvastatin-mediated effects.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Simvastatin, pravastatin, lovastatin and the sodium salt of simvastatin were obtained from Tocris Cookson, Bristol UK. Rotenone, antimycin A, 3-nitropropionic acid (3-NP), 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TTFA), FCCP, oligomycin, TEA, 4-aminopyridine, glibenclamide, ebselen, PEG-catalase, DETCA, AOAA, PPG, U46619, rhodamine 123, nifedipine and indomethacin were obtained from Sigma-Aldrich, Poole, UK. Krebs-Henseleit buffer (NaCl 118, KCl 4.8, $CaCl_2.H_2O$ 1.3, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4.7H_2O$, glucose 11.1 in mM). Mitochondrial isolation buffer: Mannitol 210 mM, Sucrose 70mM, EDTA 1mM, Trizma 50mM (pH 7.4). Mitochondrial respiration buffer (potassium chloride 100 mM, mannitol 75 mM, sucrose 25mM, Trizma 10 mM, EDTA 0.1 mM, potassium dihydrogen phosphate 10 mM, magnesium sulphate 1 mM pH 7.1) at 37^{o}C. Medium 231 (Invitrogen) is a basal medium for the growth of human vascular smooth muscle cells. This medium is HEPES and bicarbonate buffered and is designed for use in an incubator with an atmosphere of 5% CO_2/95% air. Medium 231 is supplemented with smooth muscle growth supplement to induce differentiation of normal human vascular smooth muscle cells: fetal bovine serum 5% v/v, recombinant human basic fibroblast growth factor 2 ng/ml, recombinant human epidermal growth factor 0.5 ng/ml, and insulin 2 µg/ml. Hanks buffer containing (in mM): 5.6 KCl, 138 NaCl, 4.2 $NaHCO_3$, 1.2 NaH_2PO_4, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose and 10 HEPES [pH 7.4].

2.2.2 .Tissue Preparation

Porcine coronary arteries were used to characterize the effects of simvastatin (Sahni et al., 2008, Suzuki et al., 2011). Porcine hearts from pigs of both sexes were obtained from a local abattoir and transported back to the laboratory in an ice box containing Krebs-Henseleit buffer gassed with a mixture of 95% O_2 and 5% CO_2. A crude dissection was conducted to isolate the anterior proximal descending branch of the coronary artery. The artery was dissected out and cleaned of fat and connective tissues as previously described (Uhiara et al., 2012). The vessels were stored overnight at 4ºC in Krebs–Henseleit solution pre-gassed with 95% O_2: 5% CO_2. Previous experiments have demonstrated that these conditions have negligible effect on contractile and vasodilator function in isolated blood vessels (Lot and Wilson, 1994). On the next day, a fine dissection was performed to remove any excess connective tissues, fibers and fat. The artery segments were cut into rings of approximately 5 mm in length and these pieces then were ready to use in the experiment. Each coronary ring was suspended between two stainless steel metal hooks in 5 ml organ baths. The upper hook was connected to a force transducer for isometric tension recording while the other was connected to a glass support. Each bath was filled with 5 ml of Krebs-Henseleit solution and was connected to a thermostat to maintain the temperature at 37^{o}C and constantly gassed with carbogen (95% O_2, 5% CO_2). The whole setup was linked to Powerlab data acquisition system via an amplifier (AD Instruments). The transducers were calibrated with a weight of 10 grams.

2.2.3 Isometric tension recording

Tissues were initially pre-tensioned to 8 g, determined from preliminary studies and then left to relax to baseline for approximately 45-60 minutes.

Once a stable baseline was reached, two consecutive KCl responses with bath concentration of 60 mM were obtained for standardization. After about 10-15 min the tissue was washed out with Kreb-Henseleit, and then left for 30 min for the tone to re-stabilize to baseline. To elicit reproducible responses, the process was then repeated with a second KCl addition to each ring segment. Once again, the tissue was washed out with Kreb-Henseleit solution, to allow the segment tone to return to baseline. After about 30 min minutes, cumulative addition of U46619 (20 nM to 30 nM), a thromboxane A_2-mimetic, was added to stimulate tissue contraction to about 50-70% of the second KCl response before addition of a single concentration of simvastatin (300 nM to 10 μM), sodium salt of simvastatin, lovastatin or pravastatin (all 10 μM) once an appropriate level of U46619 response had been achieved (figure 2.2). Control tissues contained vehicle only (0.1% v/v DMSO for simvastatin and lovastatin). The tone was measured for a further 2 hours. In separate experiments, the effect of simvastatin after pre-contraction with endothelin-1 (10-30 nM) or 40-60 mM KCl was determined. In all experiments, the concentration of contractile agent was adjusted to obtain a similar level of pre-contraction between tissues. The general experimental protocol described above was repeated for each treatment experiments.

2.2.3.1. The role of endothelial factors in the relaxation response to simvastatin

In order to determine the role of endothelial factors on the effects of statins, tissues were incubated with the nitric oxide synthase inhibitor, L-NAME (300 μM), the cyclooxygenase inhibitor indomethacin (10 μM) or a combination of the two for 30-60 minutes prior to pre-contraction with U46619. The role of the endothelium in the statin-induced relaxation was

determined by endothelium denudation, achieved by gently rubbing the innermost surface of the artery with a pair of fine forceps on a wet tissue paper before attaching it to the setup. Successful removal of endothelium was tested at the end of the experiment with substance P (10 nM). Endothelium denuded segments which relaxed to less than 10% of the U46619-induced contraction were considered acceptable.

2.2.3.2 The role of K^+ channels in the relaxation response to simvastatin

In some experiments, to evaluate the role of K^+ channels, the segments were incubated with the non-selective K^+ channel blocker tetraethylammonium (TEA; 10 mM), K_{ATP} channel blocker glibenclamide (3 μM) or the K_v channel blocker 4-aminopyridine (1 mM) for 60 minutes prior to addition of U46619. Such concentrations of K^+ channel blockers have been shown effective in previous studies. Glibenclamide (10 μM) inhibits coronary contraction significantly, while K_v blockers 4-AP (10 μM) increases basal tone more than 50%.

2.2.3.3 The role of Na^+-K^+ ATPase pump in the relaxation response to simvastatin

In order to determine the role of the Na^+-K^+ ATPase pump in the relaxation response, rings were pre-incubated with 10 μM ouabain. Such concentration has been used in a previous study (Sonmez Uydes-Dogan et al., 2005). In another set of experiments, a lower concentration of ouabain (100 nM) was shown to be effective in this tissue (see chapter IV).

2.2.3.4. The role of H_2S in the relaxation response to simvastatin

The possible involvement of H_2S in the relaxation response to simvastatin (Wojcicka et al., 2011) was examined by incubating the tissues with the

H_2S synthesizing enzyme inhibitors amino-oxyacetic acid, AOAA, (100 μM) and propargylglycine, PPG, (10 μM) prior to pre-contraction with U46619.

2.2.3.5. Role of reactive oxygen species in the relaxation response to simvastatin

The role of reactive oxygen species in the relaxation response to statins was investigated by pre-incubation with a combination of ebselen (10 μM), a scavenger of hydrogen peroxide, PEG-catalase (300 Uml^{-1}), which metabolises hydrogen peroxide, and PEG-superoxide dismutase (100 Uml^{-1}), which metabolises superoxide free radicals. In a separate set of experiments, tissues were incubated with diethylthiocarbamate (DETCA), which inhibits superoxide dismutase (10 mM).

2.2.3.6. The role of NADPH inhibition in the relaxation response to simvastatin:

To examine a possible link between simvastatin relaxation and NADPH oxidase inhibition, tissues were exposed to the NADPH oxidase inhibitor DPI (10 μM), prior to pre-contraction with U46619.

2.2.3.7. Role of mitochondria in the relaxation response to simvastatin

To determine the role of mitochondria in simvastatin-induced relaxation, rings were incubated for 30 min prior to addition of U46619 with rotenone 10 μM, a complex I inhibitor, 3-NP and TTFA, complex II inhibitors (both at 10 μM), antimycin A 10 μM, an inhibitor of complex III at Qi site, myxothiazol 10 μM, an inhibitor of complex III at Qo site. The effect of FCCP 1 μM, a respiratory chain uncoupler, and oligomycin 10 μM, a Fo/F1 ATP synthase inhibitor were also determined.

2.2.4. Assessment of Mitochondrial membrane potential using Rh123:

Mitochondria are involved in a large variety of cellular functions. A characteristic feature of mitochondria is the generation of a large transmembrane potential mediated by the H^+ transport through activity of the respiratory chain. Modulation of the mitochondrial potential has been implicated in many different pathological situations (Lu et al., 2010). Therefore, the determination of the mitochondrial potential is important for the understanding of cellular functions. Rhodamine 123 (Rh123) is used to measure changes in mitochondrial potential with time (Palmeira et al., 1996). When the concentration of Rh123 is above threshold, quenching of the indicator occurs. On mitochondrial depolarization, dye moves out from the organelles thus increasing the fluorescence signal (Johnson et al., 1981). On the other hand, mitochondrial membrane hyperpolarization will decrease fluorescence intensity due to further concentration of the dye in the mitochondria.

2.2.4.1 Effect of statins on mitochondrial function isolation from porcine hearts:

Initial studies to determine the effect of statins on mitochondrial membrane potential were carried out using mitochondria obtained from porcine hearts. Heart tissue was washed with isolation buffer (Mannitol 210 mM, Sucrose 70mM, EDTA 1mM, Trizma 50mM, pH 7.4) to remove excess blood and weighed. Then about 1 g of tissue was chopped with scissors and homogenised in 10 ml of ice-cold isolation buffer using an ultra-turax homogenizer at 150 rpm for 10 min. Mitochondria were isolated from the tissue as previously described with minor modification (Frezza et al., 2007). Homogenate then centrifuged at 1000xg for 5 min to remove nuclei and unhomogenised material. The supernatant was then spun at 5000xg

for 10 min. The resulting supernatant was discarded and the pellet re-suspended. This was re-centrifuged at 5000xg for 15 min. The resultant pellets were then re-suspended in a minimum volume of isolation buffer and kept ice cold (figure 2.1). Mitochondria were added to respiration buffer (potassium chloride 100 mM, mannitol 75 mM, sucrose 25mM, Trizma 10 mM, EDTA 0.1 mM, potassium dihydrogen phosphate 10 mM, magnesium sulphate 1 mM, pH 7.1) at 37^{o}C. Glutamic acid 10 mM, and malic acid 2.5 mM were added to provide respiratory substrates for the mitochondria. Then Rh123 (200 nM) was added to the mixture. Changes in fluorescence of Rh123 were measured at an excitation wavelength of 503 nm, and emission 527 nm using a fluorometer (Hitachi F-2500). After the addition of the Rh123 and establishment of a steady reading, mitochondrial addition resulted in rapid drop in the fluorescence reading caused by quenching of the dye in the mitochondria. After the fluorescence reached a new steady state, the compounds of interest, simvastatin, lovastatin, pravastatin (10 μM each) in a minimum volume of solvent were examined. Finally, FCCP 1 μM was added at the end to completely uncouple the mitochondria and cause the fluorescence to raise as a positive control.

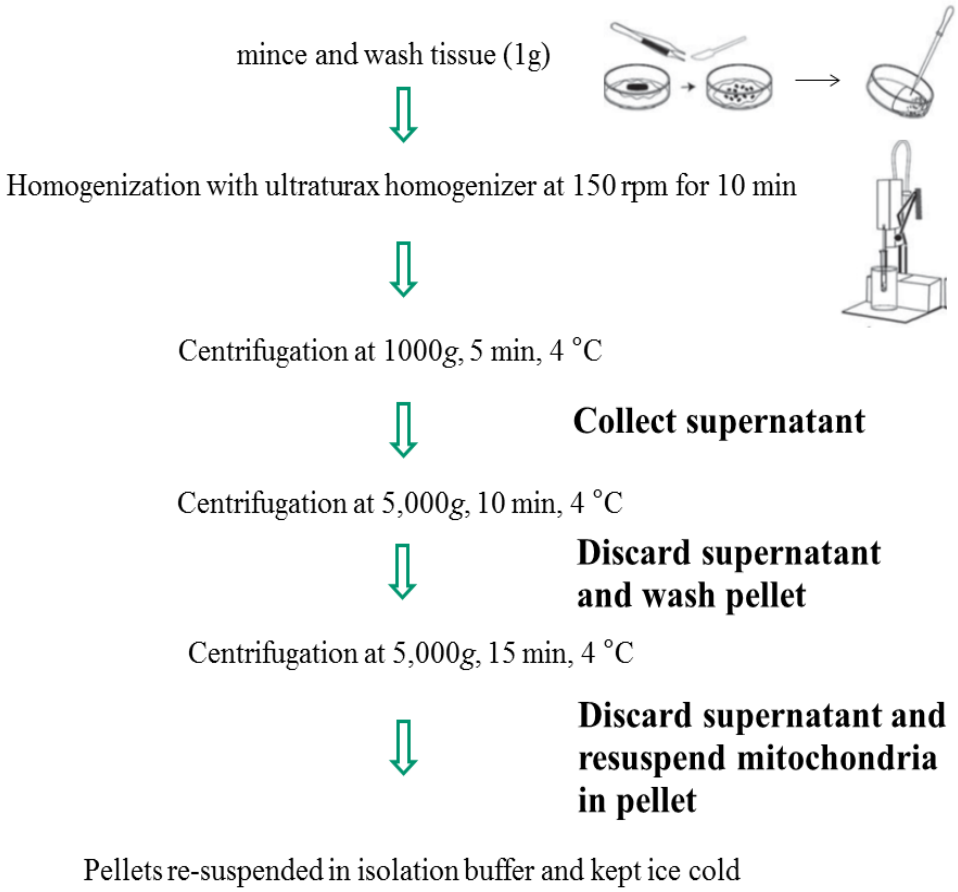


Figure 2.1: Isolation of mitochondria from pig heart.

2.2.4.2. Effect of statins on mitochondrial function in isolated vascular smooth muscle cells.

In order to determine whether statins alter mitochondrial membrane potential ΔΨm in intact cells, human aortic smooth muscle cells (Lonza; passages 7 to 9) were grown on sterilized glass cover slips were loaded with 20 µg ml^{-1} Rh 123 at 37°C for 10 min in Hanks buffer containing (in mM): 5.6 KCl, 138 NaCl, 4.2 $NaHCO_3$, 1.2 NaH_2PO_4, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose and 10 HEPES with 5 mmol/L glucose (pH 7.4). Cover slips were then washed with HANKS and placed in a superfusion system. Cells were continuously illuminated at an excitation wavelength of 485±10 nm at the lowest light intensity that gave a fluorescent signal. The emitted light was long pass filtered at 515 nm and visualised with a Zeiss

PlanNeofluar 40X/1.3 oil objective (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK) heated to 32°C with an Bioptechs objective heater (Intracel LTD, Royston, Herts, UK). Images were captured at a frequency of 1Hz with a Photonics ISIS CCD camera, DT3155 frame grabber (Data translation, UK) and Imaging workbench software (IW5.2 INDEC BioSystems, Santa Clara, CA, USA). Mitochondrial membrane potential during perfusion with simvastatin or pravastatin (both 10 μM) was monitored by the distribution of Rh123 across the inner mitochondrial membrane. Control cells were perfused with vehicle only (0.1% v/v DMSO). Images were analyzed using equation below:

$$(A-100)/ (B-100)*100$$

Where the basal fluorescence is considered to be 100 i.e. mitochondria is fully polarized. **A** value represents the increase in fluorescence induced by simvastatin, and **B** value represents the maximum fluorescence induced by FCCP perifusion.

2.2.4.3. Effect of statins on mitochondrial function in intact porcine coronary artery

In order to determine if statins alter mitochondrial membrane potential in intact blood vessels, coronary artery segments were incubated with Rh123 (10 μg/ml) at 37 °C for 10 min in Krebs-Henseleit buffer. The tissue was then washed twice with Krebs-Henseleit buffer and placed in a cuvette in a fluorometer (Hitachi F-2500) at 37 °C. Changes in fluorescence of Rh123 were measured at an excitation wavelength of 503 nm, and emission 527 nm. Fluorescence signals were allowed to stabilise before addition of simvastatin (10 μM), or DMSO (0.1% v/v) and fluorescence measured for about 20 min. Experiments were terminated by adding FCCP (1 μM) to uncouple mitochondrial respiration and establish the maximum release of Rh123.

2.3. Data Analysis

The relaxation responses were expressed as a percentage change from the agonist-induced pre-contraction over time. Values for all figures refer to mean ± SEM. Negative numbers indicate relaxation. Relaxation responses over time were compared by using two-way analysis of the variance (ANOVA) followed by a Bonferroni post-hoc test. Differences were considered to be significant when the P value was < 0.05. Changes in Rh123 fluorescence were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. *** indicates $p < 0.001$, ** indicates $p<0.01$, * indicates $p<0.05$ (2- way ANOVA followed by a Bonferroni post-hoc test) versus values obtained from the solvent control DMSO (0.1% v/v).

2.4. RESULTS

2.4.1. Acute effects of lipophilic versus hydrophilic statins on rings precontracted with U46619, ET-1 and KCl:

2.4.1.1. Effect of simvastatin and lovastatin:

As shown in figure 2.2 & 2.3, acute application of simvastatin elicited a concentration and time-dependent relaxation in pig proximal coronary artery pre-contracted submaximally with U46619. A lower concentration of simvastatin (300 nM) also produced a slow, time-dependent relaxation, but this did not achieve significance until 145 minutes (figure 2.4). A similar relaxation to simvastatin was obtained with coronary arteries pre-contracted submaximally with 10-30 nM endothelin-1 and 30-40 mM KCl (fig. 2.5 & 2.6).

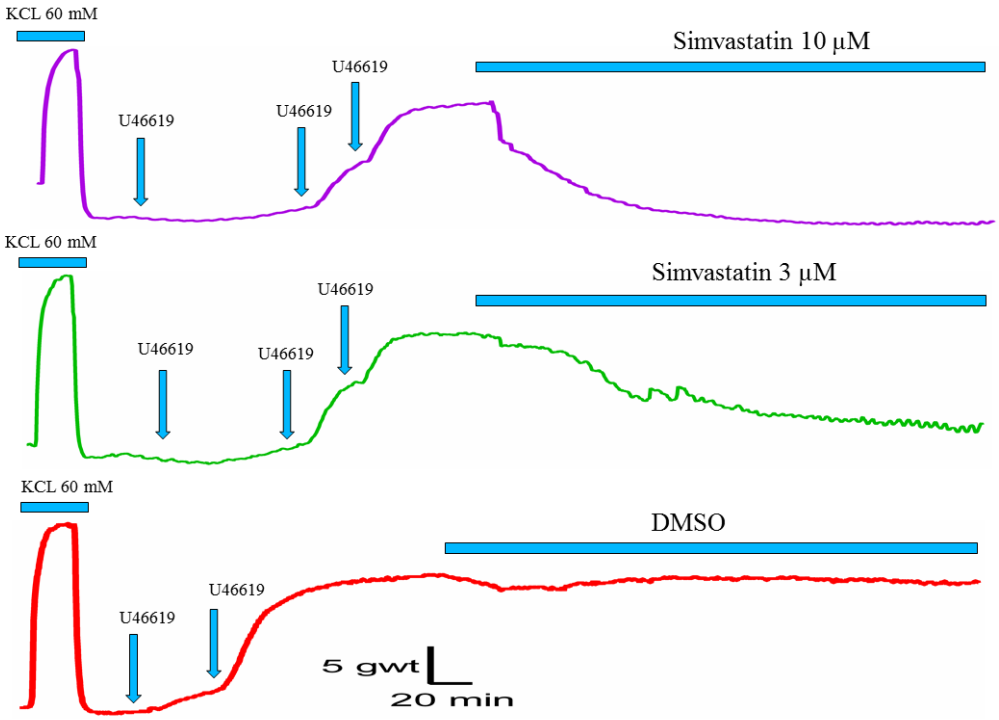


Figure 2.2. Original organ bath trace for simvastatin induced porcine coronary relaxation: KCl (60 mM) bath concentration were obtained for standardization, after about 10-15 min the tissue was washed out with Kreb-Henseleit, and then left to re-stabilize to baseline. Then cumulative addition of U46619 was added to stimulate tissue contraction to about 50-70% of KCl response before addition of a single concentration of simvastatin (3 µM or 10 µM). Control tissues contained vehicle only (0.1% v/v DMSO.

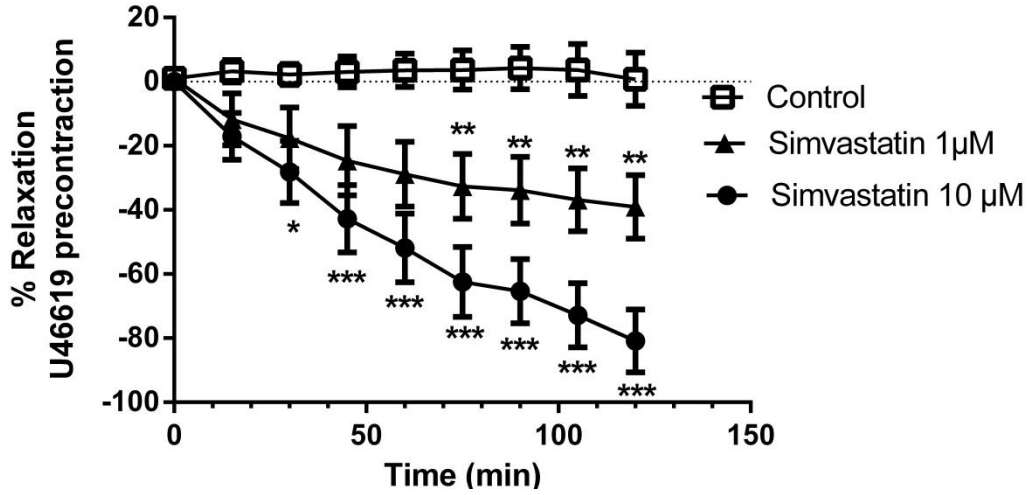


Figure 2.3. Effect of simvastatin (1 µM or 10 µM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6-9 different animals.

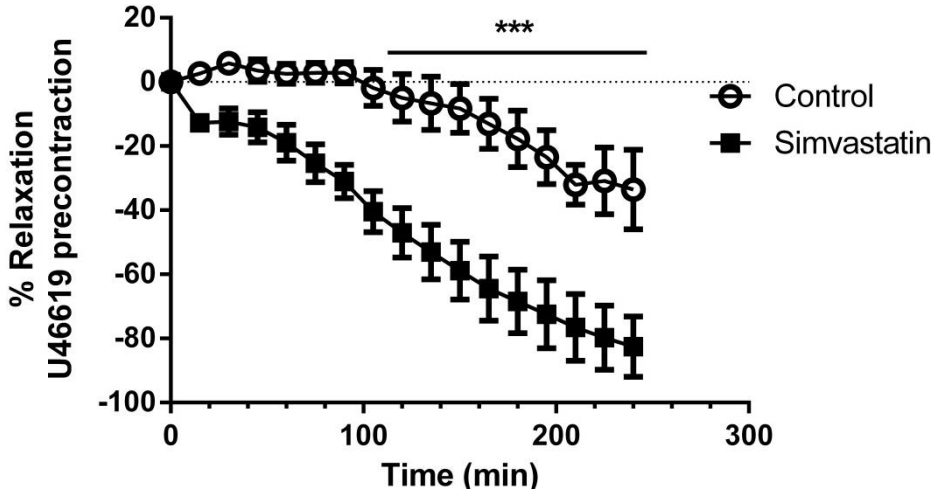


Figure 2.4. Effect of simvastatin (300 nM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 5 different animals.

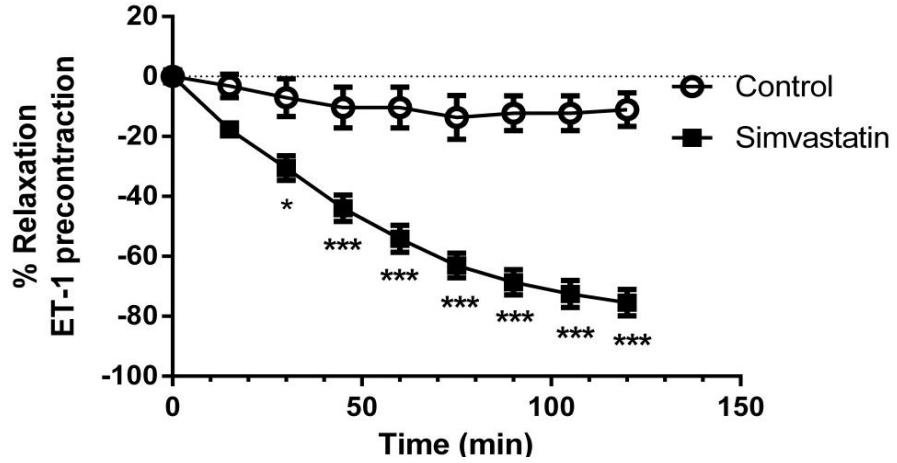


Figure 2.5. Effect of simvastatin (10 μM) in porcine coronary artery rings pre-contracted with 10-30 nM ET-1. Data are expressed as a percentage relaxation of the ET-1-induced contraction and are mean ± SEM from 9 different animals.

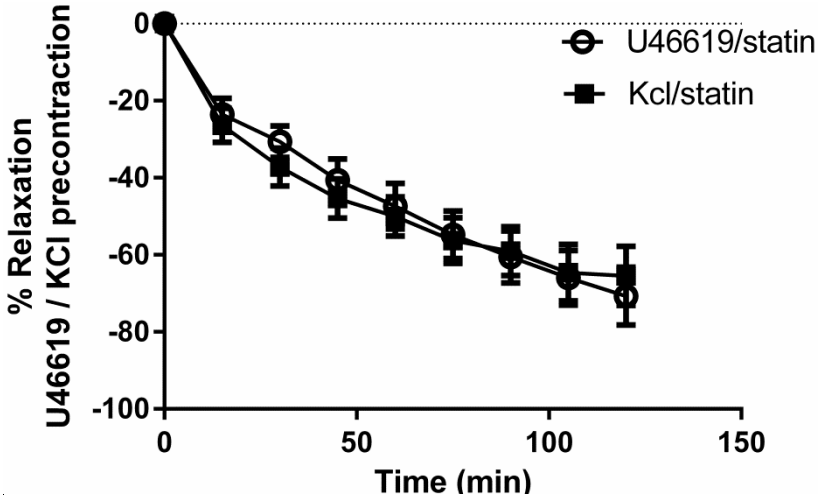


Figure 2.6. Effect of simvastatin (10 μM) in porcine coronary artery rings pre-contracted with 40-60 mM KCl compared to U46619 pre-contraction. Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 7 different animals.

The doses of simvastatin used in the study are supra-therapeutic compared (3-10 μM) to the doses used in hypercholesterolemia patients. However, the ranges of doses are very similar to those used in other *in vitro* studies of simvastatin (1–30 μM) (Luckhoff et al., 1988, Lorkowska et al., 2004), In fact, nonspecific tissue cytotoxicity was not involved with 10 μM simvastatin in vascular smooth muscle, as determined by another study (Kang et al., 2014). Moreover, coronary segments incubated with 10 μM simvastatin (1 h) have a comparable contractile response to DMSO-treated control segments (figure 2.7). Similarly, smooth muscle cells incubated with simvastatin 10 μM respond normally to 60 mM KCl depolarization after simvastatin washout (chapter III, figure 3.15).

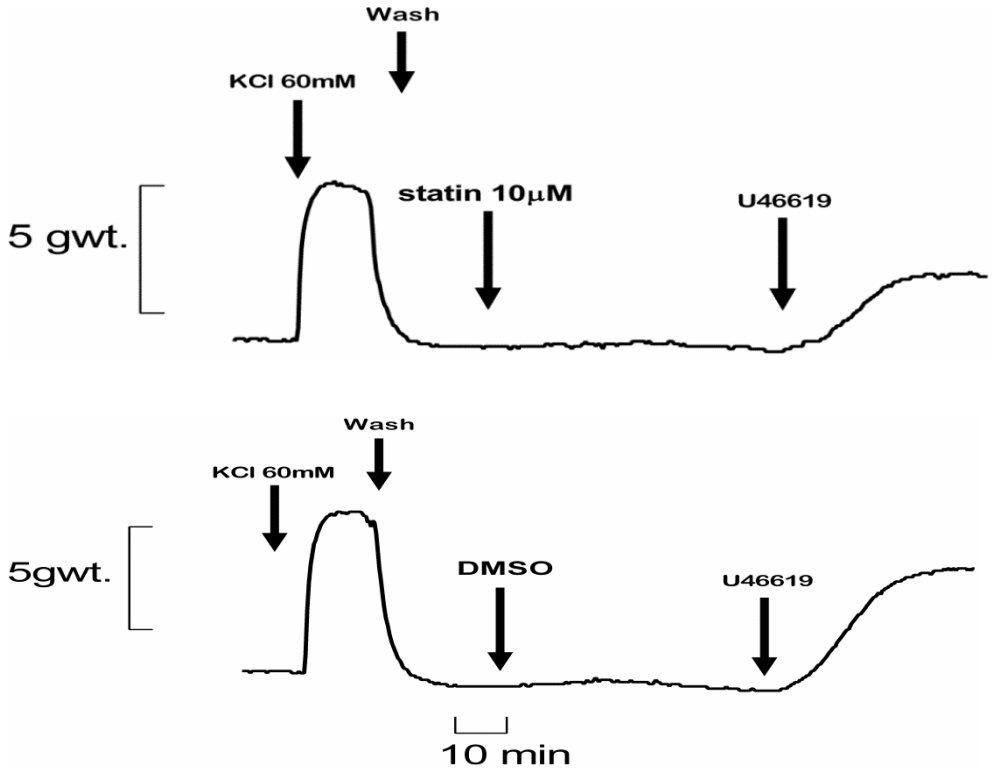


Figure 2.7. Contractile response of coronary segment treated with simvastatin. Coronary segments incubated with 10 μM simvastatin (statin) or DMSO as a solvent control for 1 h. Then U46619 (20 nM) was added to both segments to stimulate tissue contraction. As shown the statin treated segment had comparable response to DMSO treated segment.

Lovastatin (10 μM), another lipophilic HMG-CoA reductase inhibitor, also elicited a slow, time-dependent relaxation in a manner similar to simvastatin (figure 2.8).

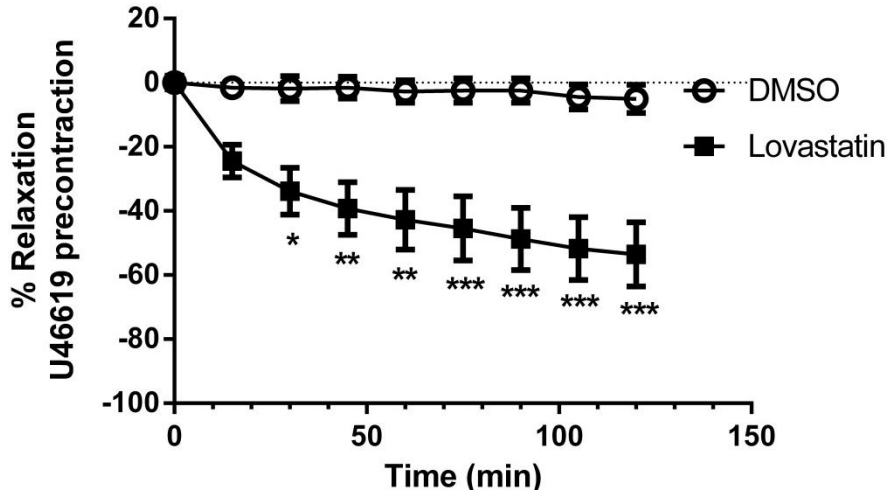


Figure 2.8. Effect of lovastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 8 different animals.

2.4.1.2. Effect of pravastatin and sodium salt of simvastatin:

On the other hand, neither pravastatin nor the sodium salt of simvastatin (figure 2.9 & 2.10) caused any significant relaxation in pig proximal coronary artery pre-contracted submaximally with U46619.

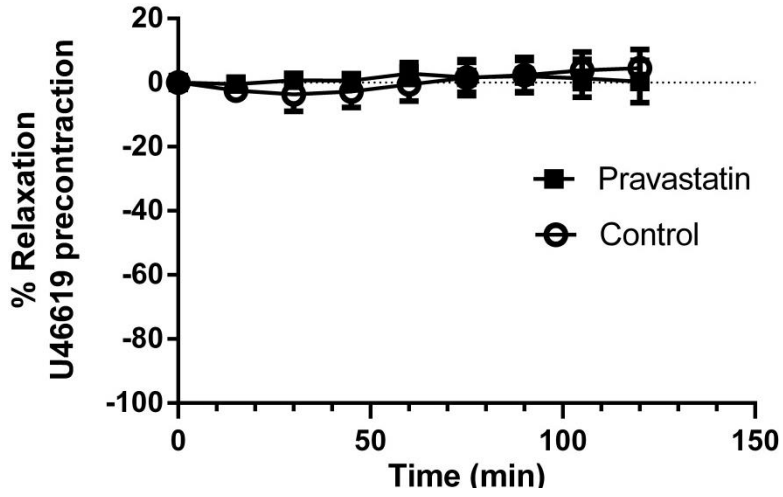


Figure 2.9. Effect of pravastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 8 different animals.

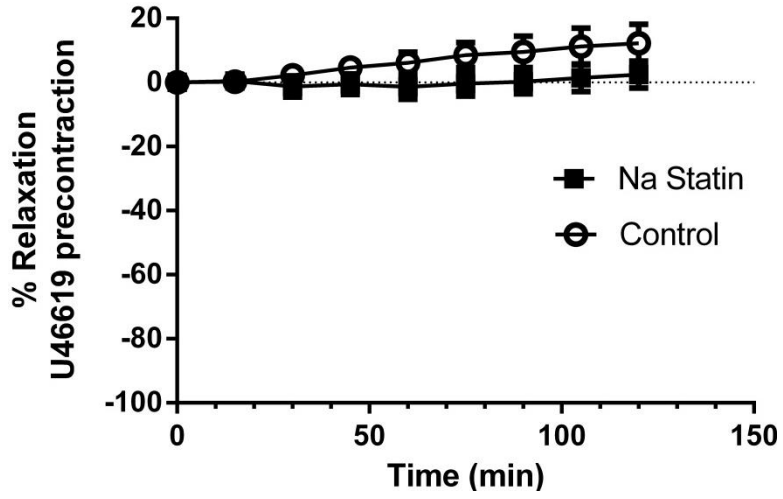
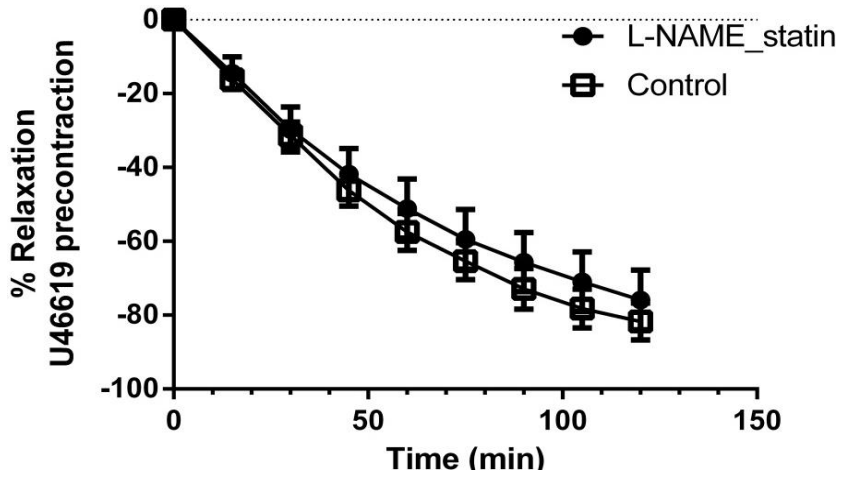


Figure 2.10. Effect of sodium salt of simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 8 different animals.

2.4.2. Role of endothelium-derived vasodilator factors in the relaxation response to simvastatin.

Pretreatment of the porcine coronary rings with a nitric oxide synthase inhibitor, L-NAME 300 μM had no significant effect on the relaxation to 10 μM & 3 μM simvastatin (figure 2.11A & 2.12 respectively). L-NAME 300 μM incubation increased basal tone of the isolated coronary segments by 10-20 % of the basal tone (figure 2.11B). Similarly, the cyclooxygenase inhibitor, indomethacin 10 μM (figure 2.13), or a combination of the two compounds (figure 2.14) had no significant effect on the relaxation to 10 μM simvastatin. Similar concentrations of L-NAME and indomethacin diminished simvastatin relaxation in bovine coronary artery in a previous study (Lorkowska and Chlopicki, 2005). Similarly, removal of the endothelium had no effect on the relaxation response to simvastatin (figure 2.15).

A-



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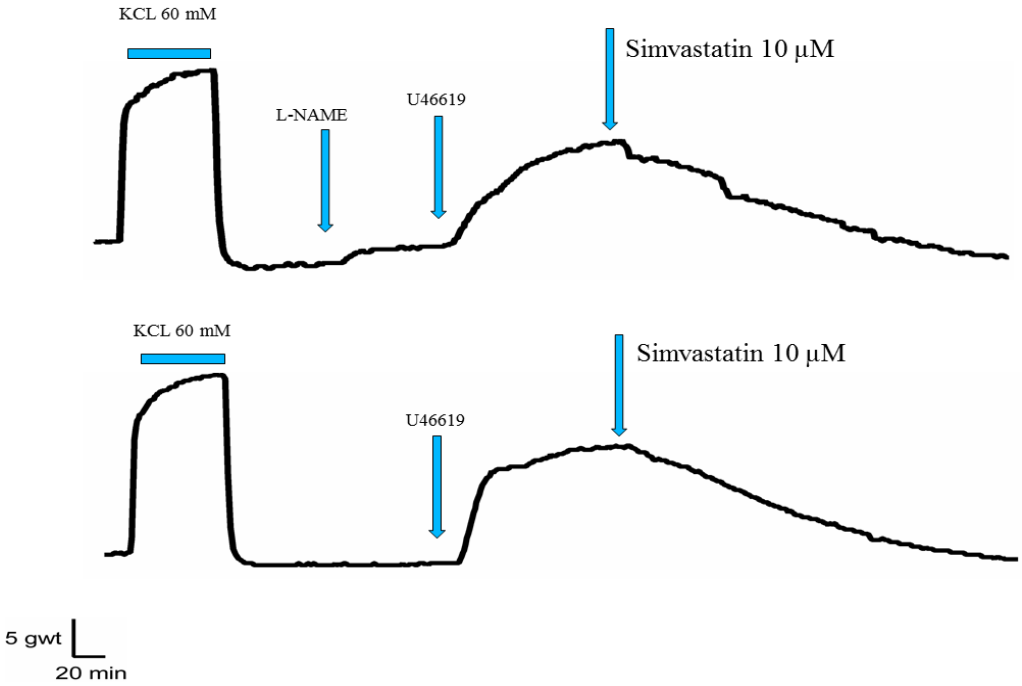


Figure 2.11. (A) Effect of L-NAME (300 µM) on relaxations to simvastatin (10 µM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 17 different animals. **(B)** Original organ bath trace for simvastatin induced porcine coronary relaxation in the presence of L-NAME: Contractions to KCl (60 mM) were used for standardization KCl, after about 10-15 min the tissue was washed out with Kreb-Henseleit, and then left to re-stabilize to baseline. L-NAME (300 µM) was added to one segment and compared with the control segment. As shown, L-NAME incubation increased basal tone of the coronary segment. Then a cumulative addition of U46619 was added to stimulate tissue contraction to about 50-70% of KCl contraction before the addition of a single concentration of simvastatin (10 µM). Control segments treated with simvastatin vehicle only (0.1% v/v DMSO).

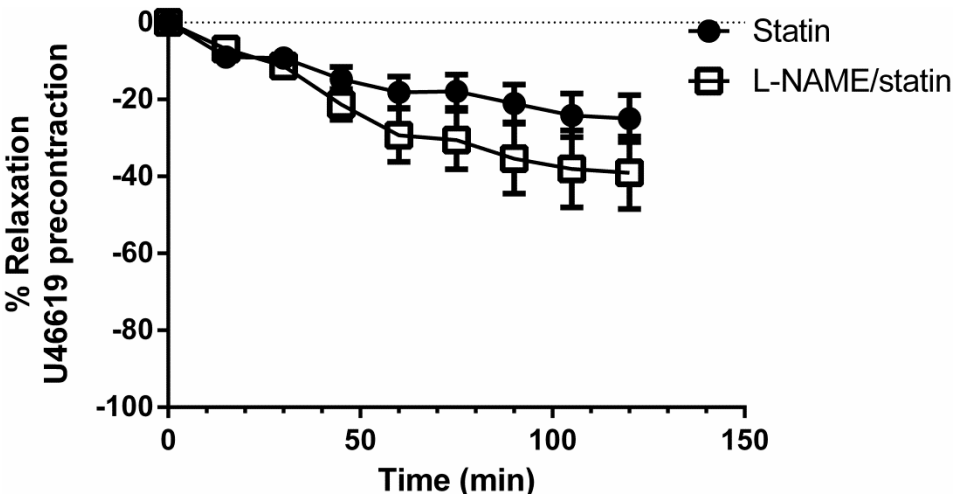


Figure 2.12. Effect of L-NAME (300 μM) on relaxations to simvastatin (3 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 5 different animals.

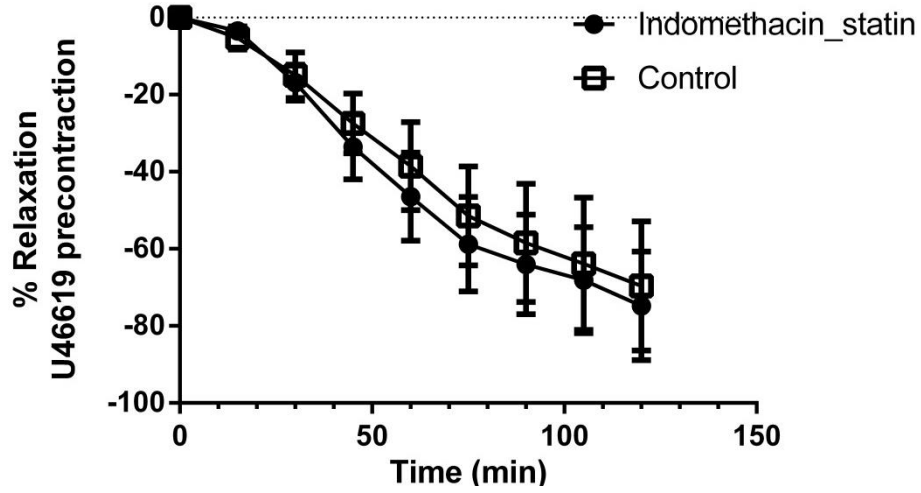


Figure 2.13. Effect of indomethacin (10 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals.

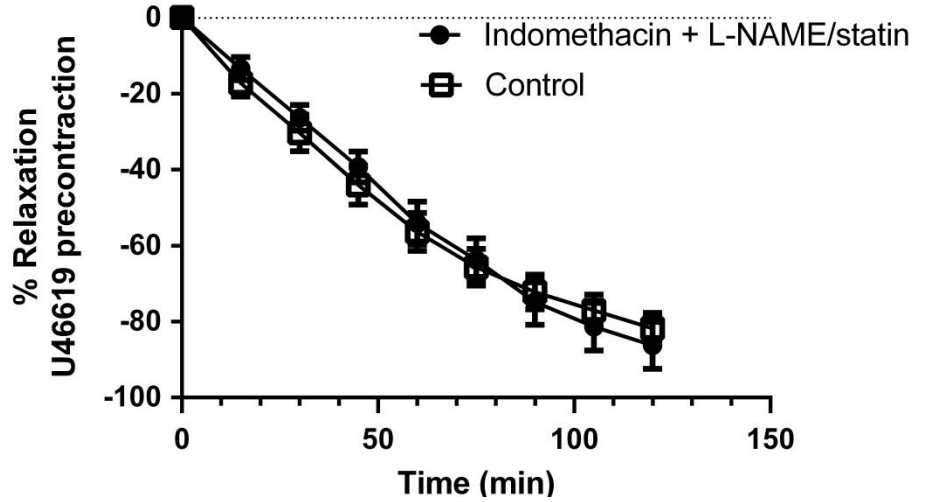


Figure 2.14. Effect of indomethacin (10 μM) and L-NAME (300 μM) combination on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals.

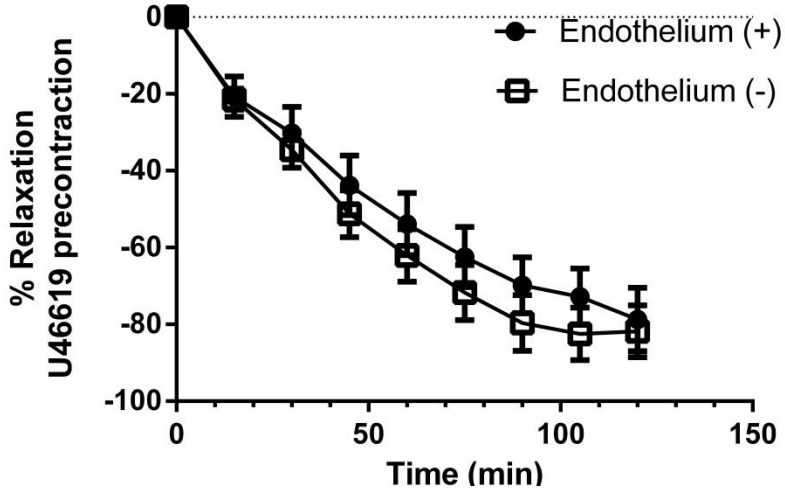


Figure 2.15. Effect of endothelium removal on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 12 different animals.

2.4.3. Role of AMPK in the relaxation response to simvastatin.

A previous study by Rossoni *et al* demonstrated that AMP kinase plays a role in the simvastatin mediated relaxation of isolated rat mesenteric resistance arteries (Rossoni et al., 2011). However, in the porcine coronary artery, incubation with the AMP kinase inhibitor dorsomorphin (10 μM) had no effect on simvastatin (10 μM) relaxation (figure 2.16).

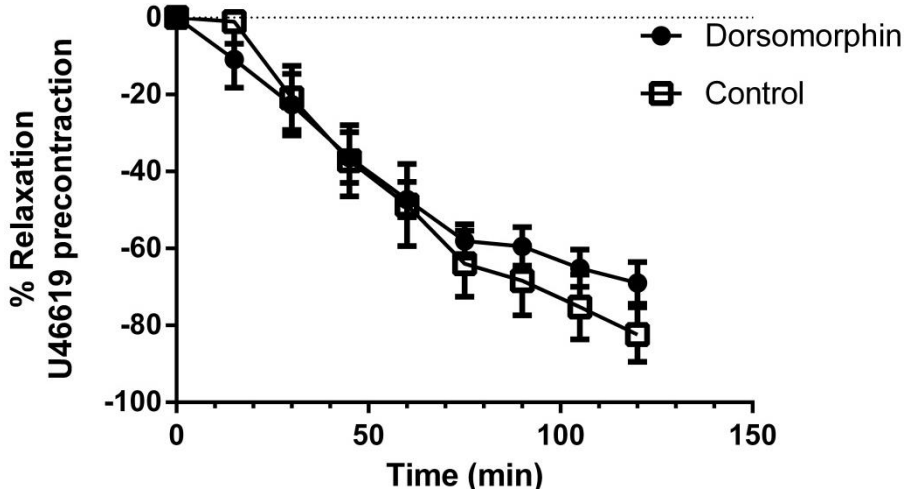


Figure 2.16. Effect of dorsomorphin (10 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 9 different animals.

2.4.4. Role of K^+ Channels in the relaxation response to simvastatin

The incubation of coronary rings with the non-selective K^+ channel blocker TEA (10 mM), the K_{ATP} channel blocker glibenclamide (3 μM), or the Kv blocker 4-aminopyridine (1 mM) had no effect on the relaxant responses to simvastatin (figure 2.17, 2.18 and 2.19 respectively). Since NO acts by activation of cGMP or directly by the activation of potassium channels (Bolotina et al., 1994), we have examined the combination of TEA (10 mM) and L-NAME (300 μM) on simvastatin relaxation. The combination as shown in (figure 2.17) had no effect on simvastatin relaxation in porcine coronary vessels. Previous studies have shown that TEA 1-10 mM is effective in K_{ca} block (White et al., 2001, Bratz et al., 2008). In this study, the maximum effective concentration has been used to examine the effect of K_{ca} channel on simvastatin 10 μM mediated relaxations. TEA 10 mM incubation increased contraction of the isolated coronary segments by 20-30 % of the basal tone (Heaps and Bowles, 2002).

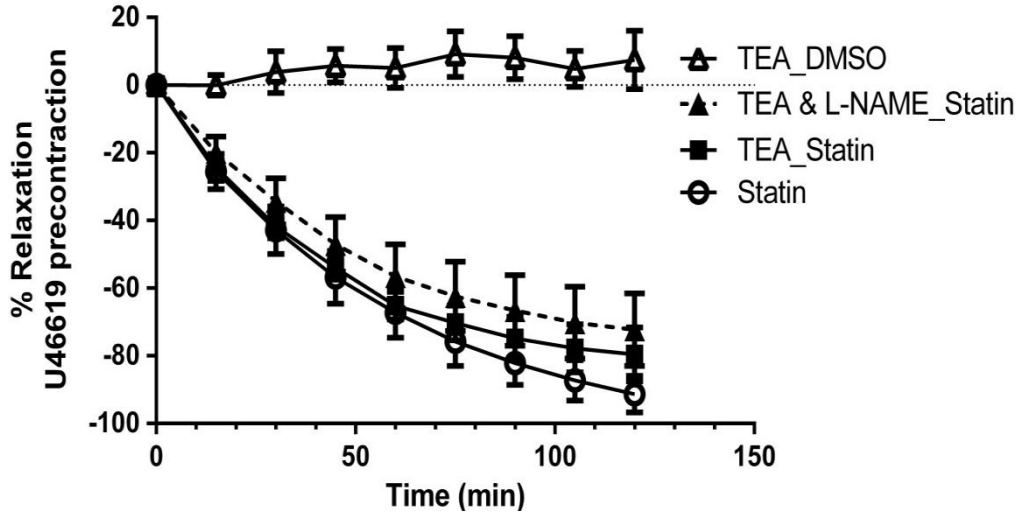


Figure 2.17. Effect of TEA (10 mM) alone or in combination with L-NAME (300 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 10 different experiments.

The effect of glibenclamide 10 μM was examined on simvastatin 10 μM relaxation. The concentrations of U46619 required to pre-contract coronary segments treated with glibenclamide was much higher than the concentration required to contract the control rings i.e. 40-50 nM for the treated rings compared to 10 nM for the control rings, thus glibenclamide 3 μM was chosen to examine the effects of K_{ATP} block on simvastatin relaxation. Such a concentration has been proved to effectively block K_{ATP} channels in porcine and human coronary arteries (Gollasch et al., 1995). In our experiments, glibenclamide incubation had no effect on basal tone of the porcine isolated coronary arteries in organ bath preparation. Similarly O'Rourke *et al* have shown that K_{ATP} channel block has no effect on porcine coronary tone (O'Rourke, 1996).

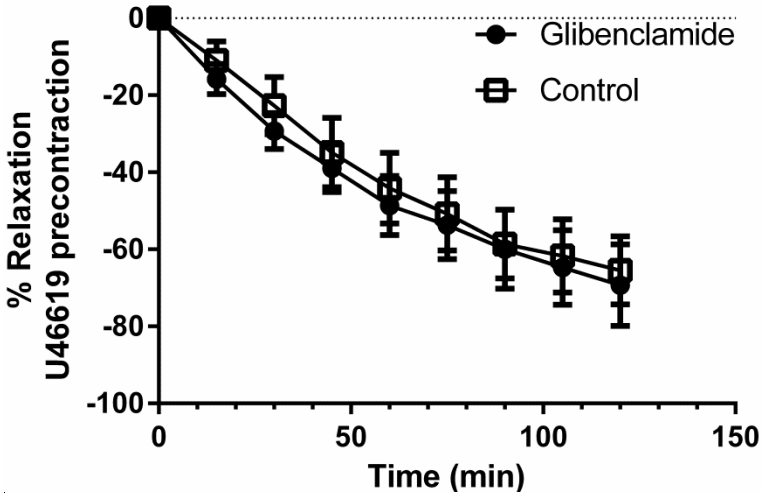


Figure 2.18. Effect of glibenclamide (3 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 7 different animals.

The role of K_v in simvastatin relaxation has been examined by incubation with K_v channel blocker, 4-AP, 10 μM. However such concentration of 4-AP increased the coronary segments tone up to 70-90 % of the basal tone. Previous study has shown that 4-AP increases coronary basal tone even more than TEA (Heaps and Bowles, 2002). Thus lower concentration of 4-

AP 1 mM have been used in our experiment to examine the effects of K_v block on simvastatin 10 μM relaxation, such concentration increased the basal tone by 10-20% only.

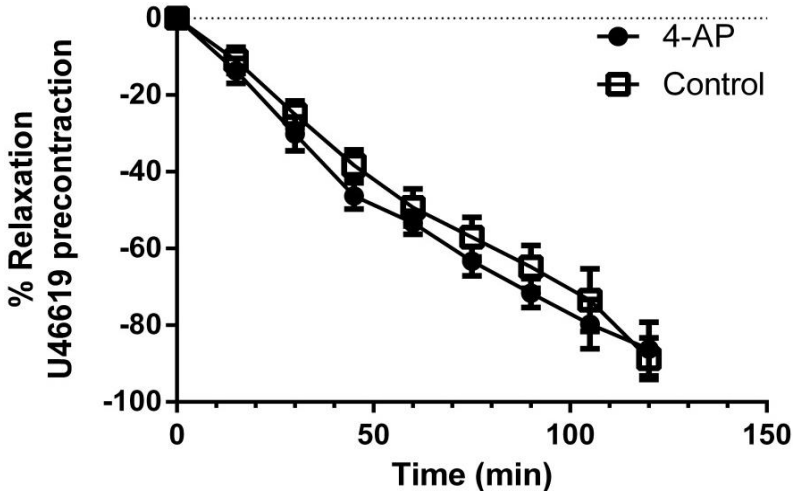


Figure 2.19. Effect of 4-AP (1 mM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals.

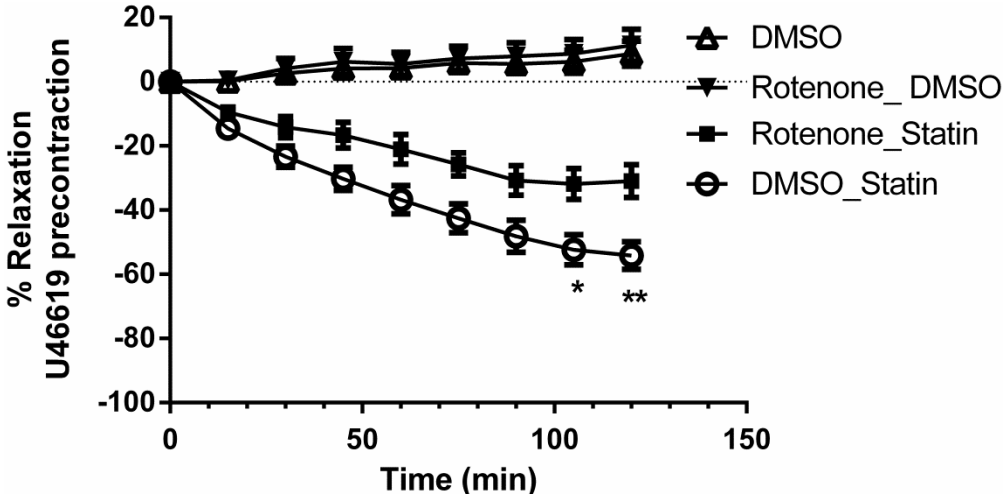
2.4.5. Role of the mitochondria in the relaxation response to simvastatin.

2.4.5.1 Effect of Mitochondrial Inhibitors on Simvastatin-induced relaxations.

Rotenone (10 μM), a complex I inhibitor, caused ~25% inhibition of the simvastatin relaxation in pig coronary rings pre-contracted with U46619 (figure 2.20 A&B & 2.23). Similarly myxothiazol (10 μM), a complex III inhibitor, partially inhibited simvastatin relaxation ~20% (figure 2.21 A&B). A combination of rotenone and myxothiazol caused further inhibition of the relaxation to simvastatin ~30% (figure 2.22 A&B & 2.23). On the other hand, antimycin A (10 μM), another complex III inhibitor did not modify the relaxant effect of simvastatin (figure 2.24 A&B). The complex II inhibitors 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TTFA), 3-nitropropionic acid (3-NP) (both 10 μM) and malonate 1 mM had no effect on the simvastatin response (figure 2.25, 2.26 & 2.27). Concentrations of

the inhibitors were chosen according to previous studies to achieve a complete inhibition of specific electron transport sites in each complex.

A-



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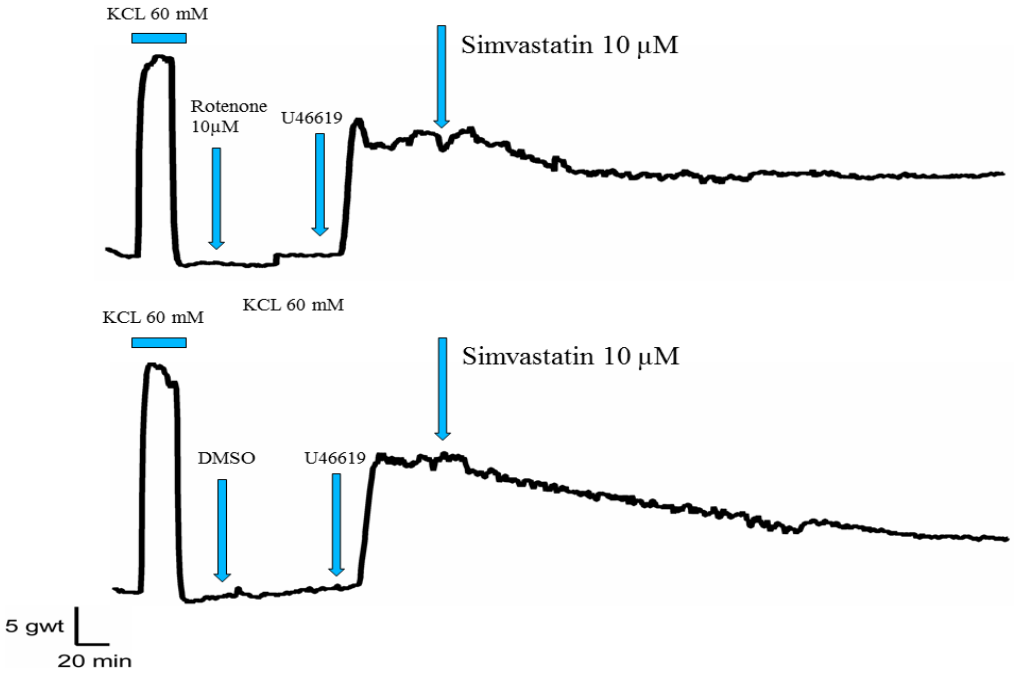

Figure 2.20. (A) Effect of rotenone (10 µM) on relaxations to simvastatin (10 µM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 10 different animals. **(B)** Original organ bath trace for simvastatin induced porcine coronary relaxation in the presence of rotenone (10 µM) : KCl (60 mM) bath concentration were obtained for standardization, after about 10-15 min the tissue was washed

out with Kreb-Henseleit, and then left to re-stabilize to baseline. rotenone (10 µM) was added to one segment and compared with the control segment (DMSO). Then a cumulative addition of U46619 was added to stimulate tissue contraction to about 50-70% of KCl contraction before the addition of a single concentration of simvastatin (10 µM).

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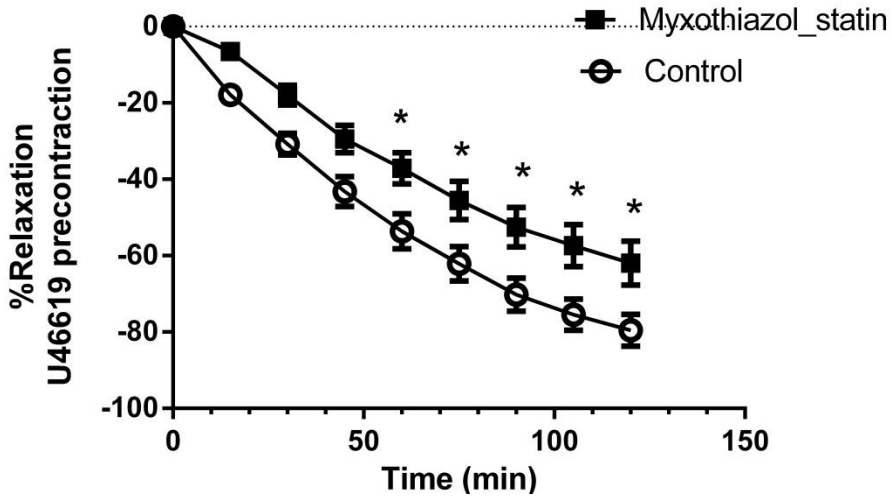

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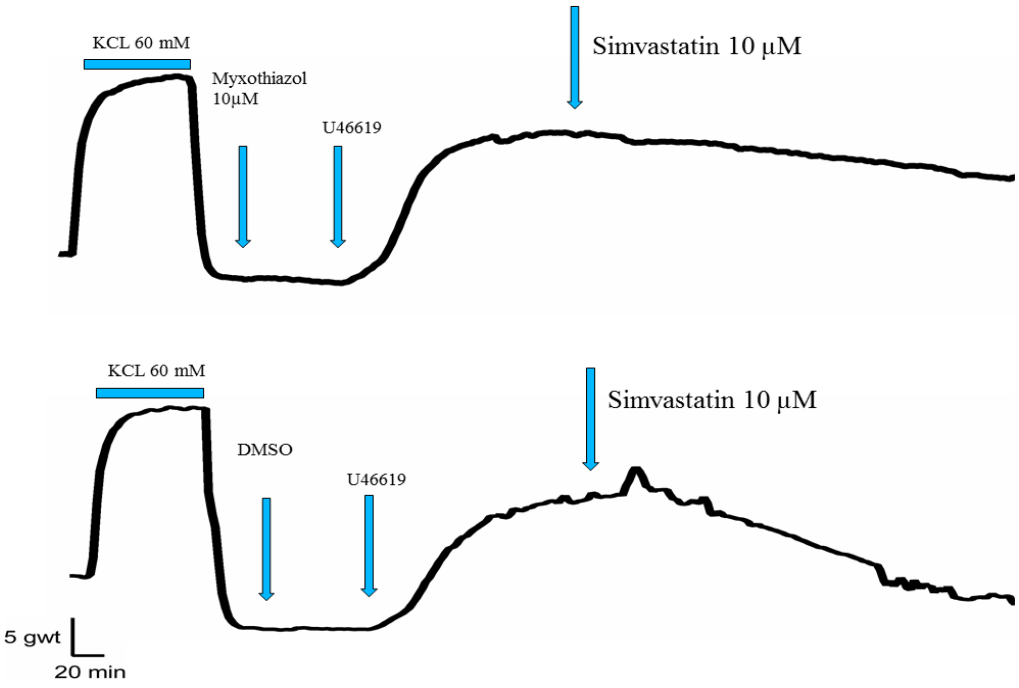

Figure 2.21. (A) Effect of myxothiazol (10 µM) on relaxations to simvastatin (10 µM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 12 different animals. **(B)** Original organ bath trace for simvastatin induced porcine coronary relaxation in the presence of myxothiazol (10 µM) : KCl (60 mM) bath concentration were obtained for standardization, after about 10-15 min the

tissue was washed out with Kreb-Henseleit, and then left to re-stabilize to baseline. myxothiazol (10 µM) was added to one segment and compared with the control segment (DMSO). Then a cumulative addition of U46619 was added to stimulate tissue contraction to about 50-70% of KCl contraction before the addition of a single concentration of simvastatin (10 µM).

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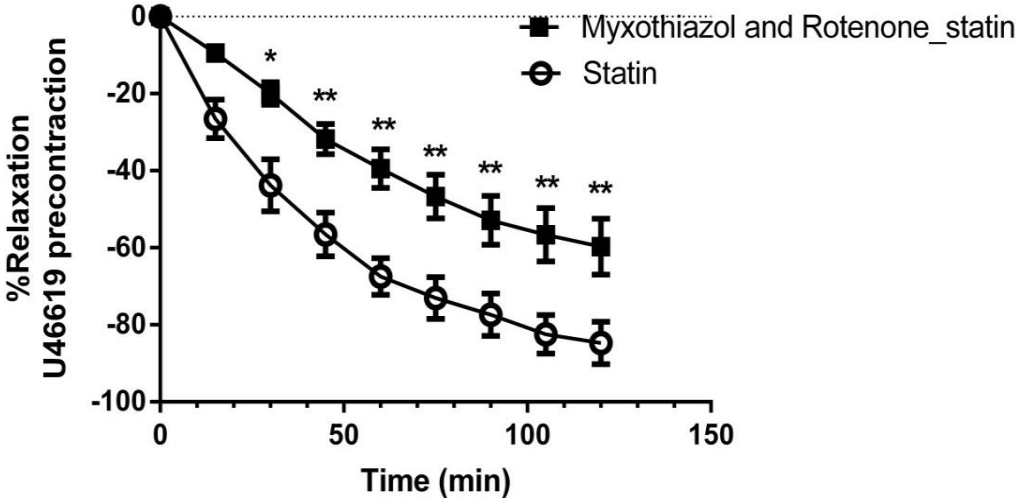

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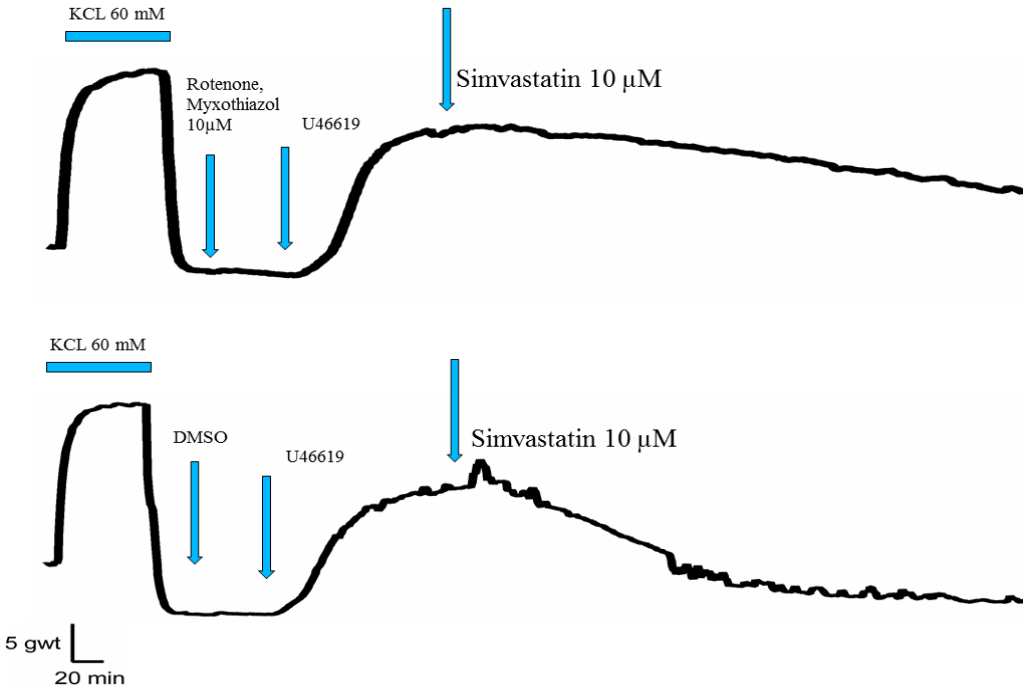


Figure 2.22. (A) Effect of rotenone (10 µM) and myxothiazol (10 µM) combination on relaxations to simvastatin (10 µM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ±

SEM from 7 different animals. **(B)** Original organ bath trace for simvastatin induced porcine coronary relaxation in the presence of rotenone and myxothiazol (10 μM each): KCl (60 mM) bath concentration were obtained for standardization, after about 10-15 min the tissue was washed out with Kreb-Henseleit, and then left to re-stabilize to baseline. rotenone and myxothiazol (10 μM each) were added to one segment and compared with the control segment (DMSO). Then a cumulative addition of U46619 was added to stimulate tissue contraction to about 50-70% of KCl contraction before the addition of a single concentration of simvastatin (10 μM).

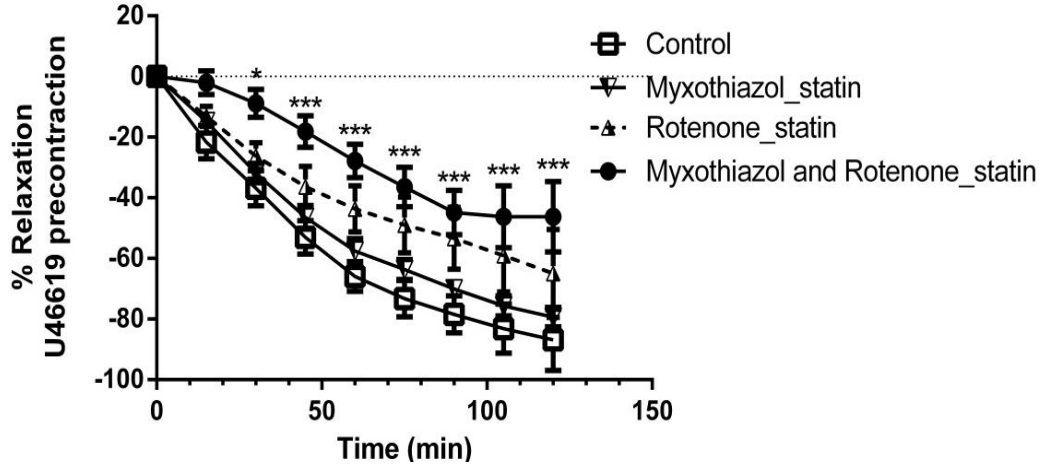
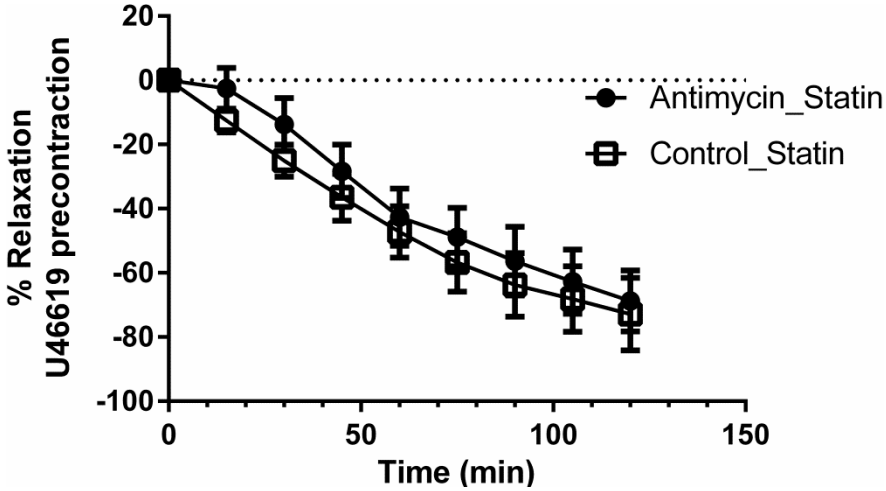


Figure 2.23. Effect of rotenone (10 μM) and myxothiazol (10 μM) combination on relaxations to simvastatin (10 μM) in porcine coronary artery rings, comparing the effect of the inhibitors in 4 segments from the same animal, pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals.

A-



B-

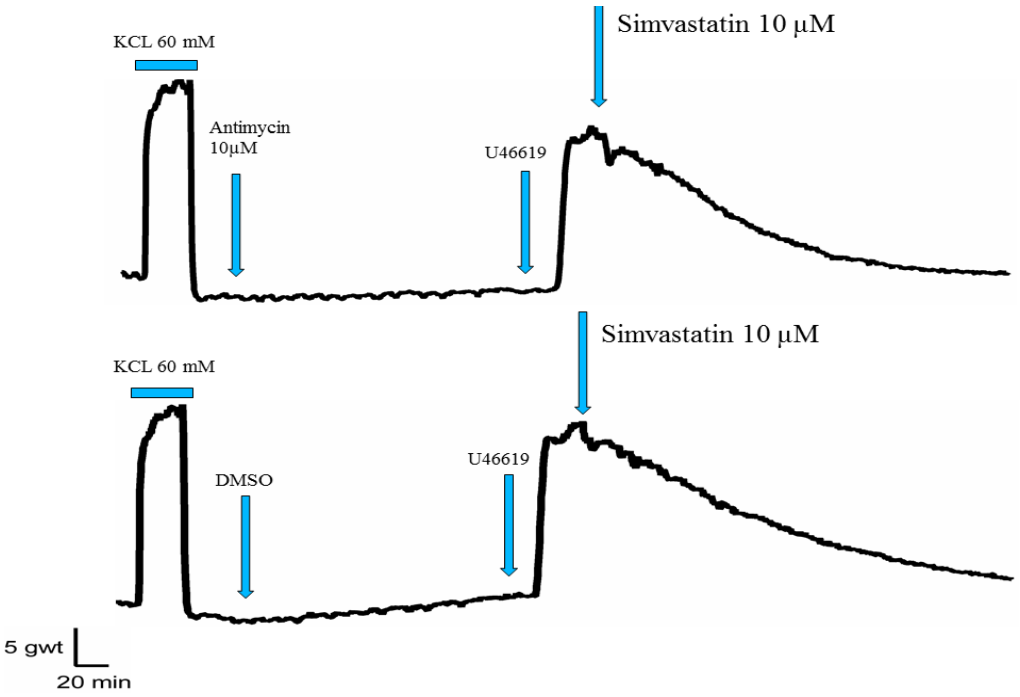


Figure 2.24. (A) Effect of antimycin A (10 µM) on relaxations to simvastatin (10 µM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals. **(B)** Original organ bath trace for simvastatin induced porcine coronary relaxation in the presence of antimycin (10 µM): KCl (60 mM) bath concentration were obtained for standardization, after about 10-15 min the tissue was washed out with Kreb-Henseleit, and then left to re-stabilize to baseline. antimycin (10 µM) was added to one segment and compared with the control segment (DMSO). Then a cumulative addition of U46619 was added to stimulate tissue contraction to about 50-70% of KCl contraction before the addition of a single concentration of simvastatin (10 µM).

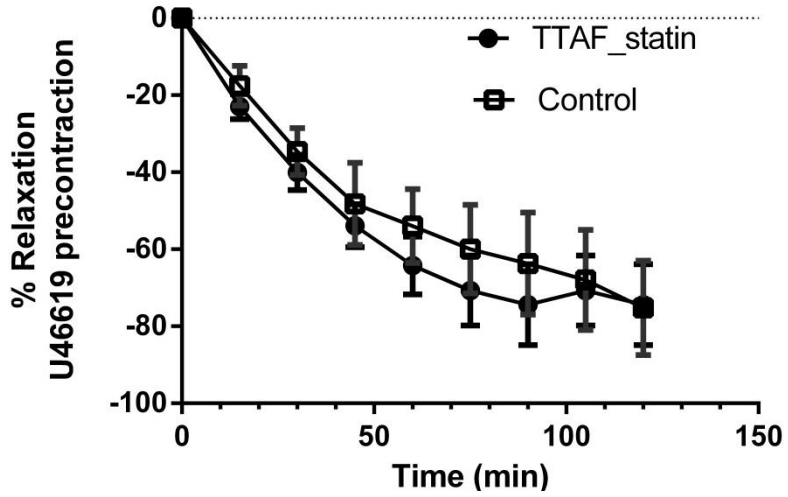


Figure 2.25. Effect of TTAF (10 µM) on relaxations to simvastatin (10 µM) in porcine coronary artery rings pre-contracted with U46619. Data are

expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 5 different animals.

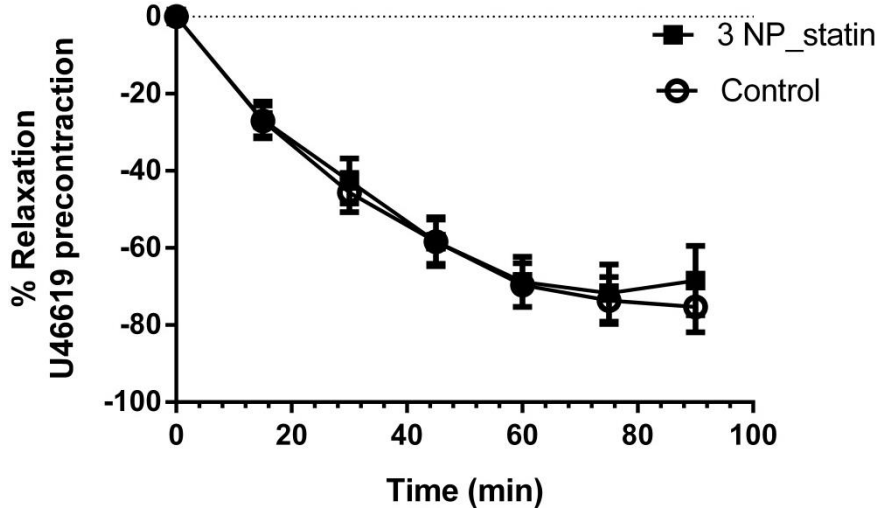


Figure 2.26. Effect of 3NP (10 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 11 different animals.

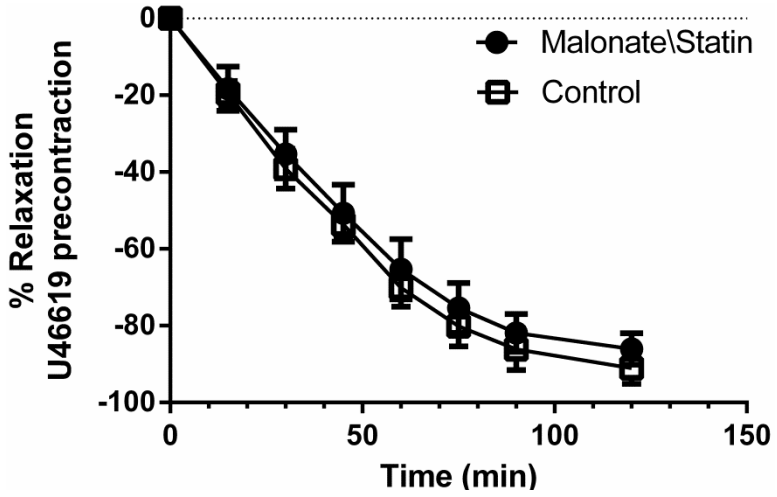
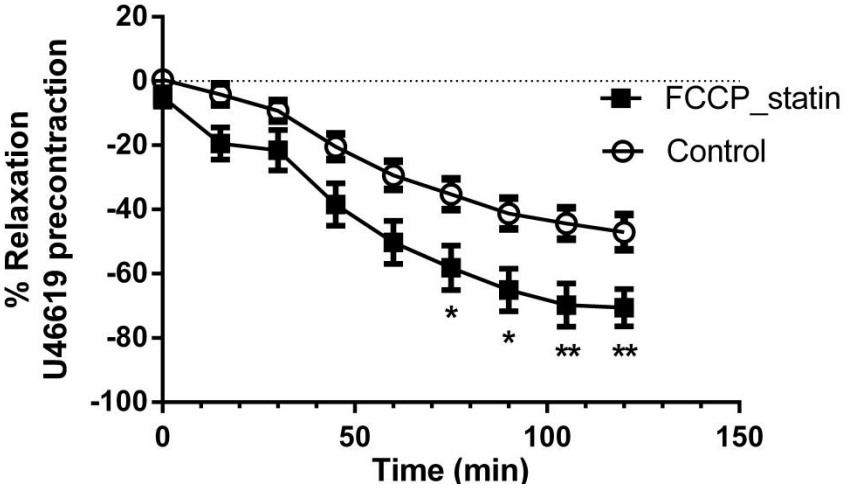


Figure 2.27. Effect of malonate (1 mM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 5 different animals.

2.4.5.2 Effect of Mitochondrial uncouplers on Simvastatin-induced relaxations.

The presence of the respiratory chain uncoupler FCCP (1 µM) enhanced the relaxation responses to simvastatin (figure 2.28 A&B).

A-



B-

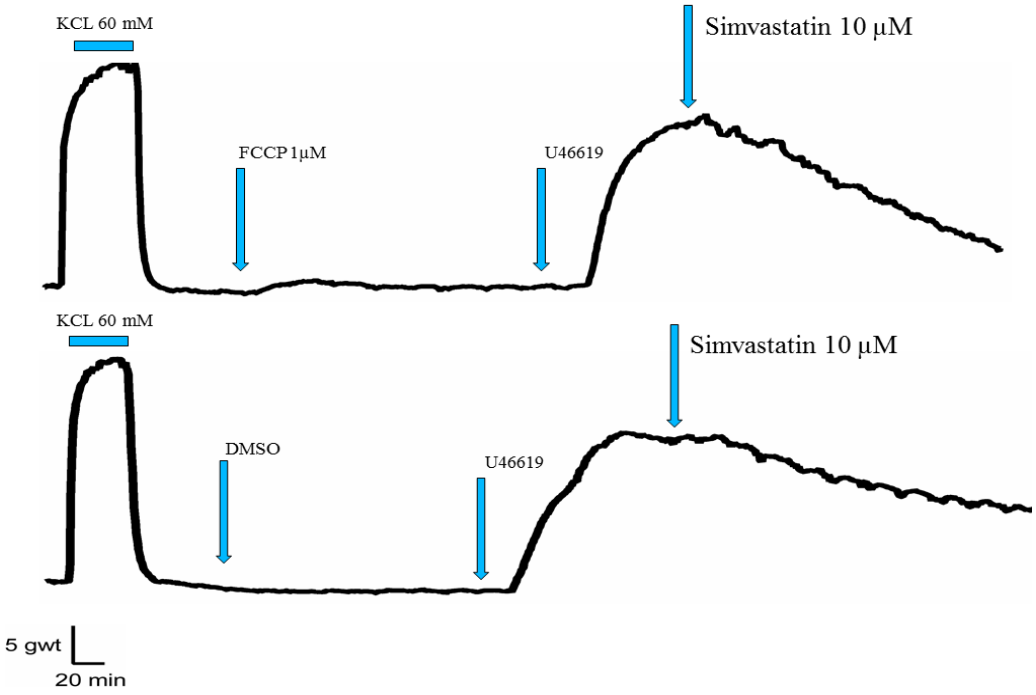


Figure 2.28. (A) Effect of FCCP (1 µM) on relaxations to simvastatin (10 µM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 15 different animals. **(B)** Original organ bath

trace for simvastatin induced porcine coronary relaxation in the presence of FCCP (1 μM): KCl (60 mM) bath concentration were obtained for standardization, after about 10-15 min the tissue was washed out with Kreb-Henseleit, and then left to re-stabilize to baseline. FCCP (1 μM) was added to one segment and compared with the control segment (DMSO). Then a cumulative addition of U46619 was added to stimulate tissue contraction to about 50-70% of KCl contraction before the addition of a single concentration of simvastatin (10 μM).

As a comparison with statins, the effects of myxothiazol and FCCP on relaxations to pioglitazone were determined. Myxothiazol and FCCP had no effect on pioglitazone relaxations (figure 2.29 & 2.30 respectively).

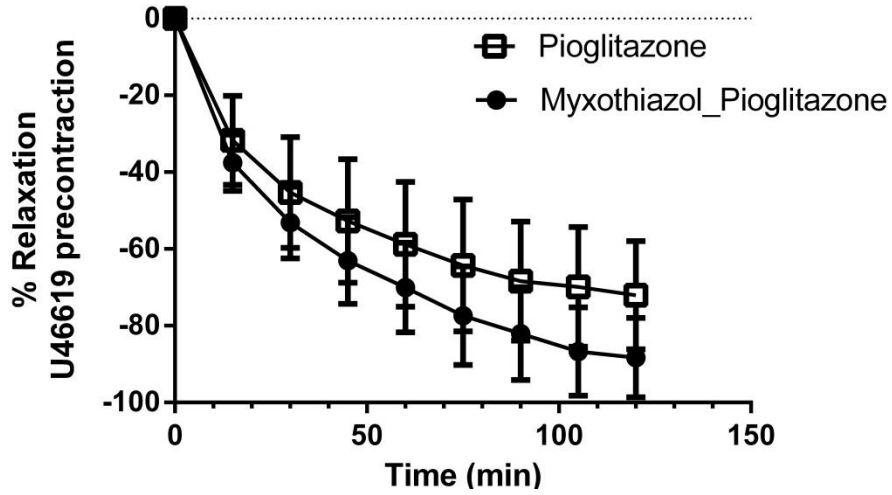


Figure 2.29. Effect of myxothiazol (10 μM) on relaxations to pioglitazone (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 5 different animals.

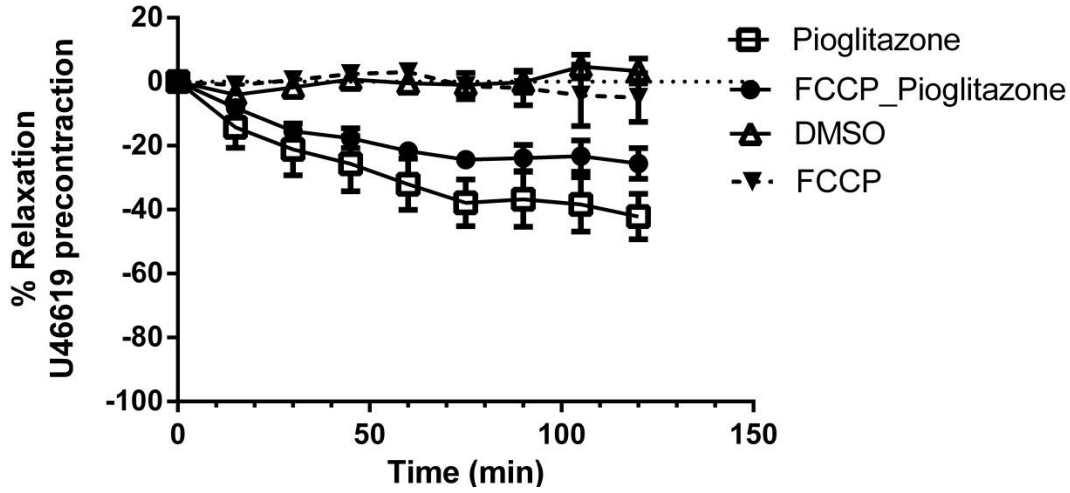


Figure 2.30. Effect of FCCP (1 μM) on relaxations to pioglitazone (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals.

2.4.5.3 Effect of Mitochondrial ATPase inhibitor on Simvastatin-induced relaxations.

The presence of the mitochondrial ATPase inhibitor oligomycin (10 μM; figure 2.31) did not alter the relaxation responses to simvastatin.

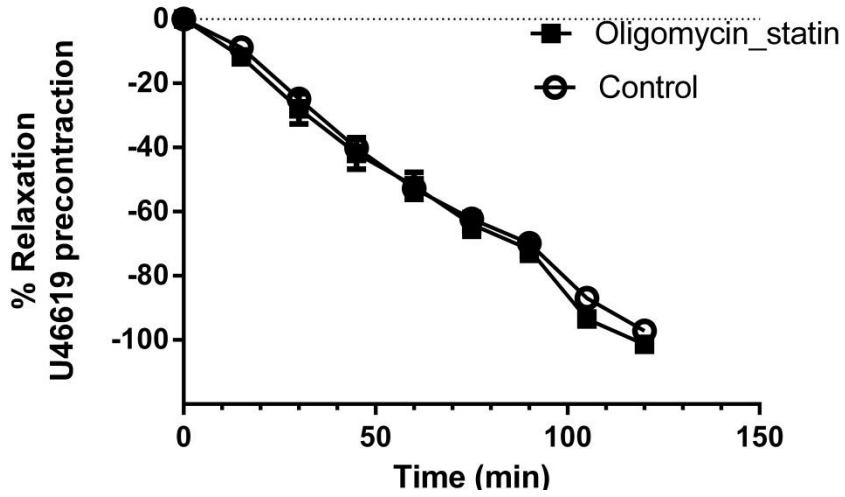


Figure 2.31. Effect of oligomycin (10 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 7 different animals.

2.4.5.4 Role of mitochondrial K_{ATP} channels:

Inhibition of mitochondrial K_{ATP} channels with 5-hydroxdecanoate 5-HD 1 mM had no effect on simvastatin-induce relaxations (figure 2.32). Such concentration has been shown effective in mitochondrial K_{ATP} blockade (Ghosh et al., 2000).

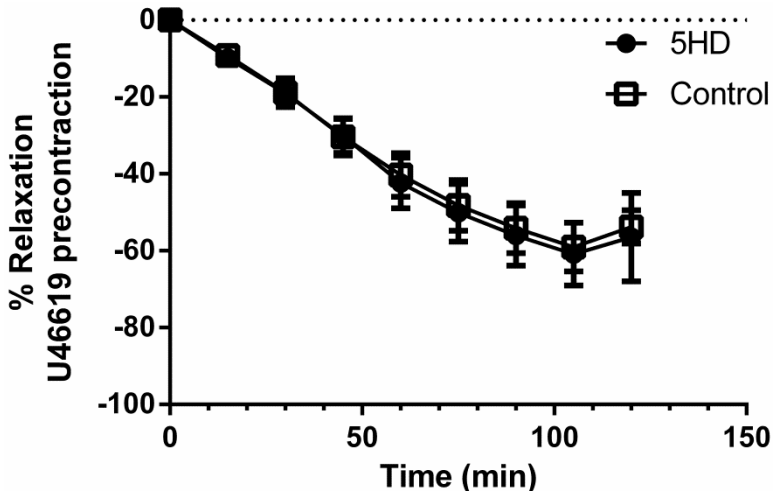


Figure 2.32. Effect of 5HD (1 mM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 7 different animals.

2.4.6 Role of Reactive Oxygen Species in the Relaxation Response to Simvastatin:

2.4.6.1 Reactive oxygen species of mitochondrial origin

Inhibition of mitochondrial electron transfer chain can lead to increases in production of reactive oxygen species, which may mediate the relaxation response. Indeed the simvastatin induced relaxation in aortic rings of rats was sensitive to superoxide dismutase and catalase (Alvarez De Sotomayor et al., 2000, de Sotomayor et al., 2005). Thus the effect of ebselen, hydrogen peroxide scavenger, (10 μM) alone or in combination with PEG-catalase (300 Uml^{-1}), which metabolises hydrogen peroxide, and PEG-superoxide dismutase (100 Uml^{-1}), which metabolises superoxide was examined. Neither ebselen on its own (figure 2.33 and 2.34), nor a combination of ebselen, PEG-catalase and PEG-superoxide dismutase had an effect on the relaxation response to simvastatin (10 μM) in the porcine coronary artery (figure 2.35).

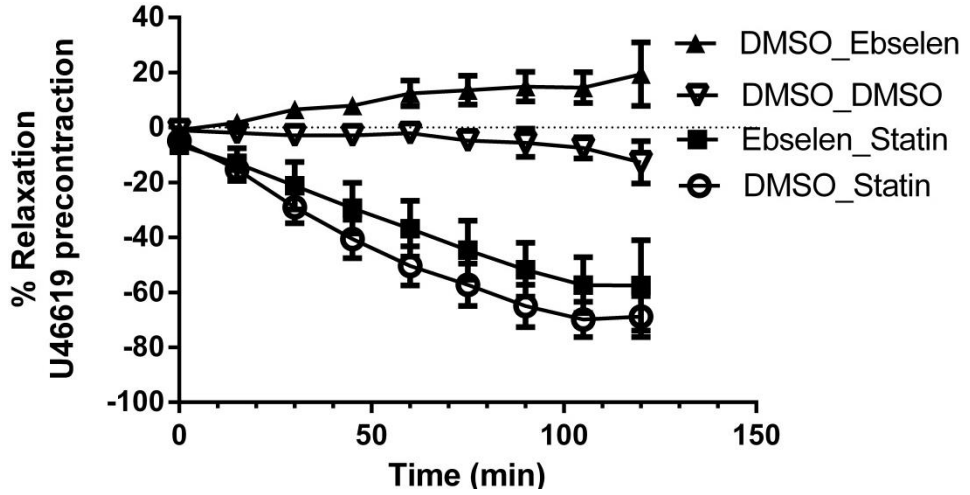


Figure 2.33. Effect of ebselen on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 8 different animals.

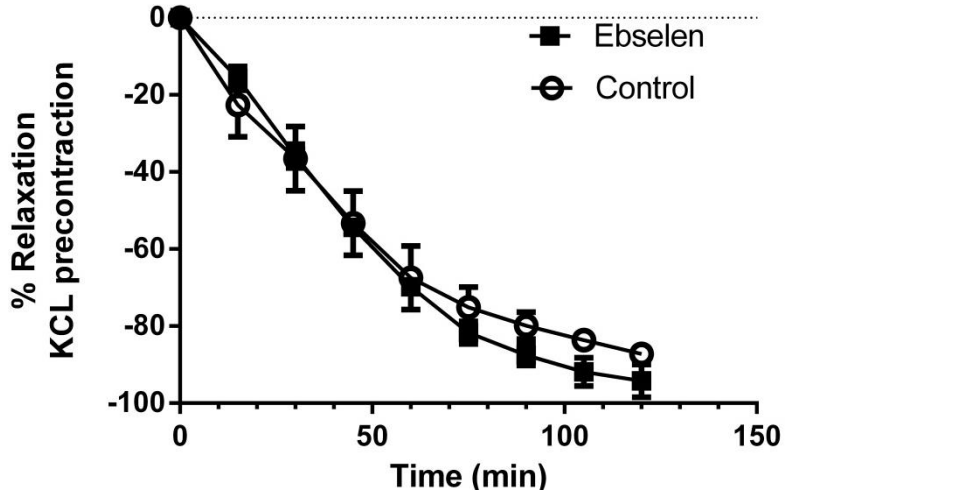


Figure 2.34. Effect of ebselen on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with KCl. Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 5 different animals.

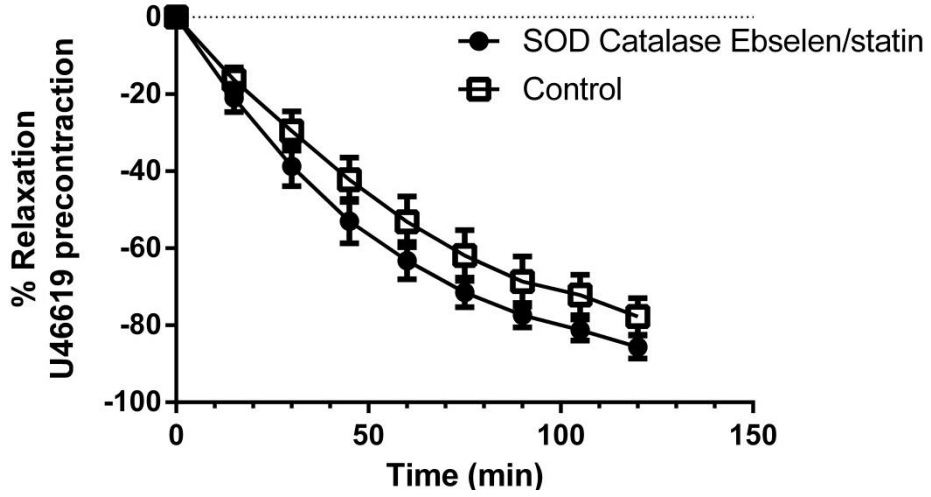


Figure 2.35. Effect of PEG-SOD, PEG catalase and Ebselen combination on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals.

Superoxide dismutase activity is necessary for NO-mediated vascular relaxation (Wambi-Kiesse and Katusic, 1999); by SOD inhibition the level of superoxide increased with resultant attenuation of vasorelaxation (Omar et al., 1991). In our study, the inhibition of SOD with 10 mM DETCA had no effect on the relaxation response to simvastatin 10 μM (figure 2.36).

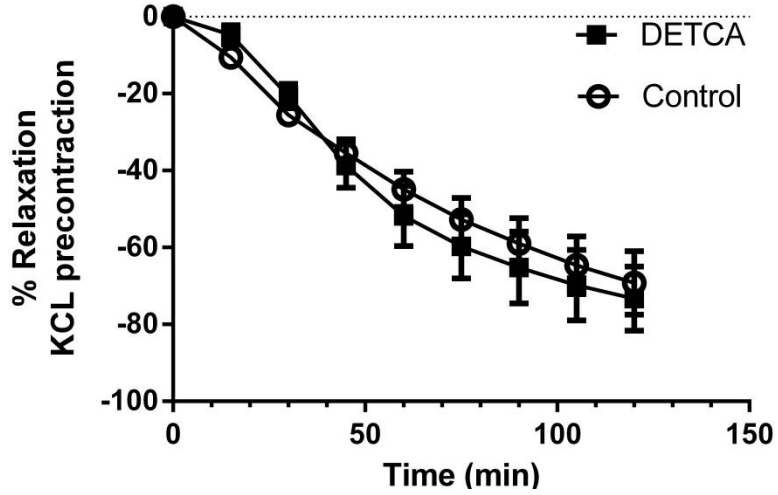


Figure 2.36. Effect of DETCA (10 mM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with KCl. Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 6 different animals.

2.4.6.2 Effect of NADPH oxidase inhibition on simvastatin mediated coronary relaxation:

Previous studies have suggested that statins regulate reactive oxygen species production through effects on NADPH oxidase (Wassmann et al., 2002, Erdos et al., 2006, Chen et al., 2008). Furthermore, studies have shown that regulation of coronary vascular tone may require NADPH oxidase-derived ROS to activate phosphatidylinositol 3-kinase-Akt-endothelial NO synthase axis (Larsen et al., 2009, Feng et al., 2010). Therefore, this present study determined whether inhibition of NADPH oxidase alters simvastatin-induced relaxations. Incubation with the non-selective NADPH oxidase inhibitor DPI (10 μM, a concentration used to inhibit vascular ROS production (Wind et al., 2010), had no effect on the simvastatin induced pig coronary relaxation (figure 2.37).

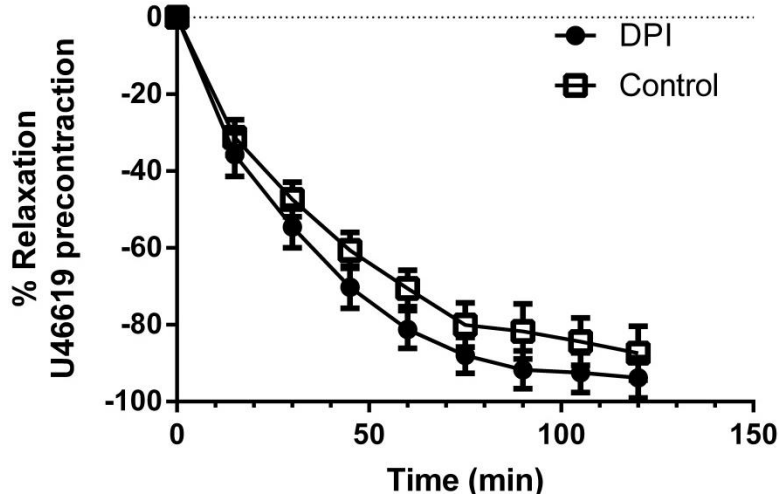


Figure 2.37. Effect of DPI (10 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 7 different animals.

2.4.6.3 Role of peroxynitrite

Superoxide reacts with NO and forms peroxynitrite, the latter produces dilatation in several vascular beds, including coronary vessels (Liu et al.,

1994). Therefore, this study examined the effect of uric acid (100 μM) as a peroxynitrite scavenger (Ohashi et al., 2005). As shown in figure 2.38, uric acid had no effect on the simvastatin relaxation in porcine coronary vessels.

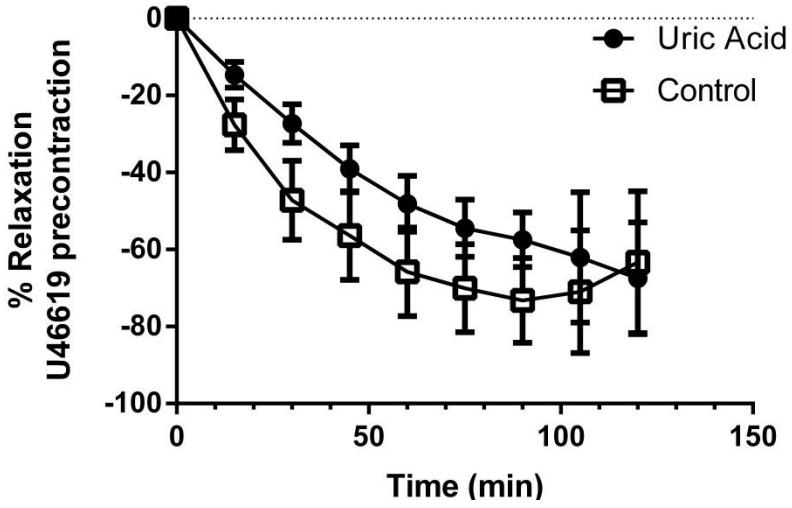


Figure 2.38. Effect of uric acid (100 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 7 different animals.

2.4.7. Effect of the antioxidant quercetin on simvastatin relaxation:

The flavonoid quercetin has anti-oxidant properties (Duarte et al., 2001). A previous study by Cristian *et al* has shown that quercetin (5–10 μM) could behave as a ‘‘coenzyme Q-mimetic’’ molecule, allowing a normal electron flow and protecting mitochondrial function (Sandoval-Acuna et al., 2012). Quercetin also protected mitochondrial function against the side effects of atorvastatin in rats (Bouitbir et al., 2012). Therefore, the effect of quercetin on the relaxation response to simvastatin in the porcine coronary artery was determined. As shown in figure 2.39, quercetin (10 μM) had no significant effect on simvastatin relaxation.

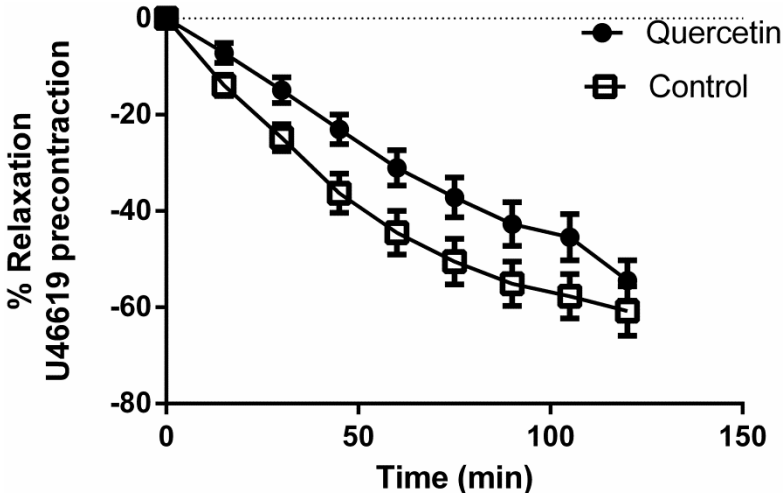


Figure 2.39. Effect of quercetin (10 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 14 different animals.

2.4.8 Effect of DTT on simvastatin relaxation:

A previous study has shown that thiol oxidation was observed to cause concentration-dependent relaxation of endothelium- removed bovine coronary arteries, such relaxation was reversed by a thiol reductant, 1 mM DTT, (Iesaki and Wolin, 2000). Results have also shown that NO has opposing effects on the motility of the gall bladder smooth muscle strips, it acts as a relaxant agent when NO˙is formed or a contractile agent when peroxynitrite species are formed (Alcon et al., 2001). However, our study shows that a reducing environment created by DTT has no effect on simvastatin-mediated porcine coronary relaxation (figure 2.40).

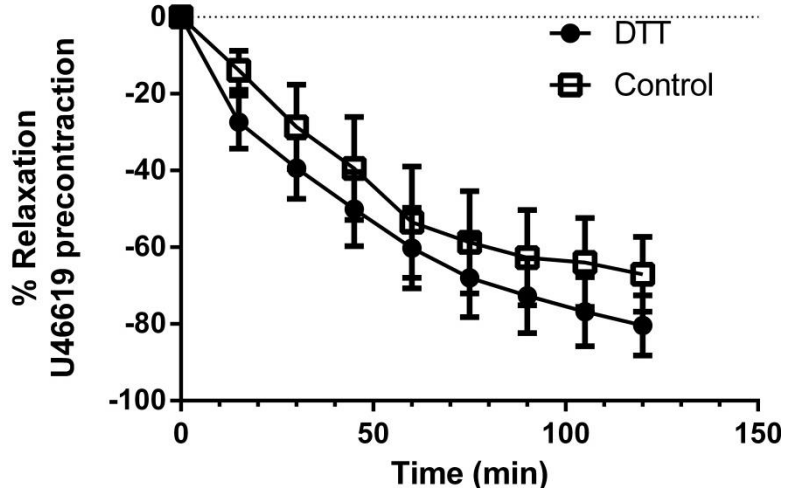


Figure 2.40. Effect of DDT (1 mM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 5 animals.

2.4.9. Role of H2S in simvastatin-induced coronary relaxation:

H_2S mediates relaxation of vascular smooth muscle. A previous study has demonstrated that simvastatin increases the release of H_2S from perivascular fat (Wojcicka et al., 2011). Therefore, in this study, the effect of the H_2S synthesis inhibitors amino-oxyacetic acid (AOAA, 100 μM) and propargylglycine (PPG, 10 μM) on the relaxation responses to simvastatin were determined. As shown in figure 2.41, H_2S inhibition had no significant effect on simvastatin 10 μM relaxation in PCA.

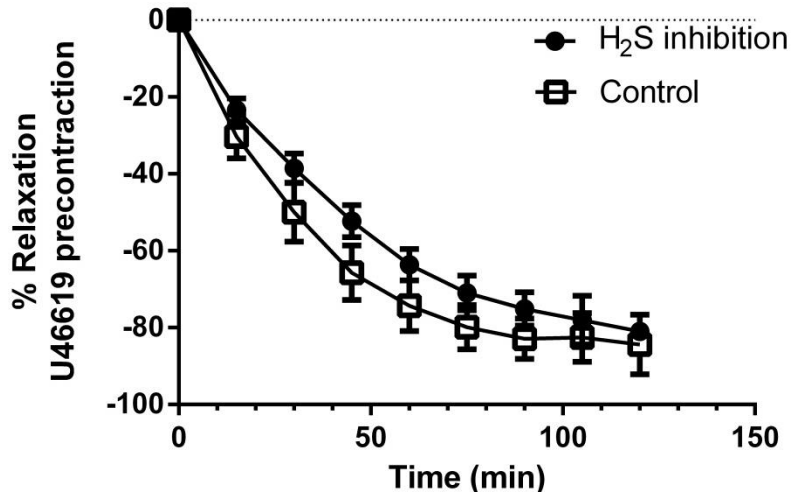


Figure 2.41. Effect of H_2S inhibition on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals.

2.4.10. Effect of Na^+/K^+ pump inhibition on Simvastatin-induced relaxations.

Na^+-K^+ ATPase pump activation have been suggested as mechanism for cerivastatin relaxation in rat aortic rings (Sonmez Uydes-Dogan et al., 2005). Therefore, this present study determined the effect of the Na^+-K^+ ATPase pump inhibitor ouabain on the simvastatin relaxations. Ouabain (100 nM), had no effect on simvastatin (10 μM) induced relaxation of porcine coronary artery (figure 2.42 & 2.43). Ouabain at 100 nM concentration has been shown to be effective at inhibiting the Na^+-K^+ pump block in our experiments (see chapter III).

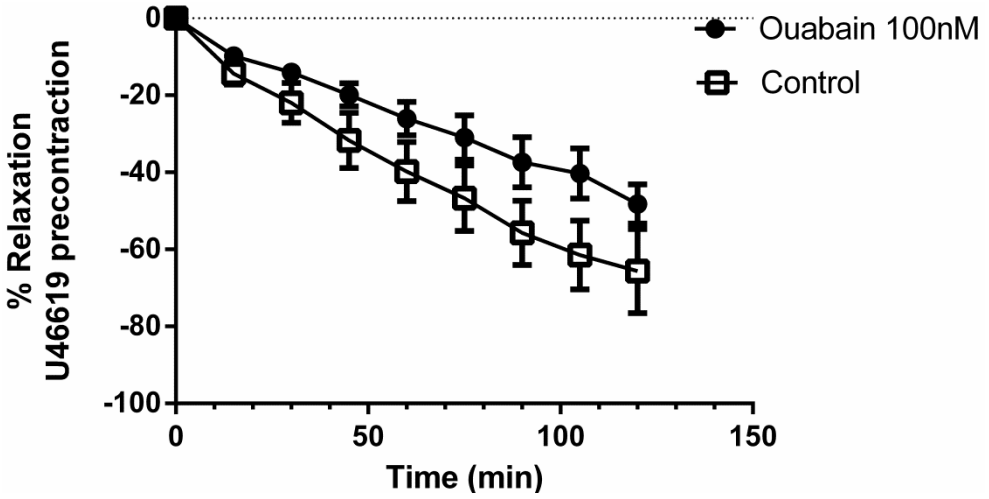


Figure 2.42. Effect of ouabain (100 nM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals.

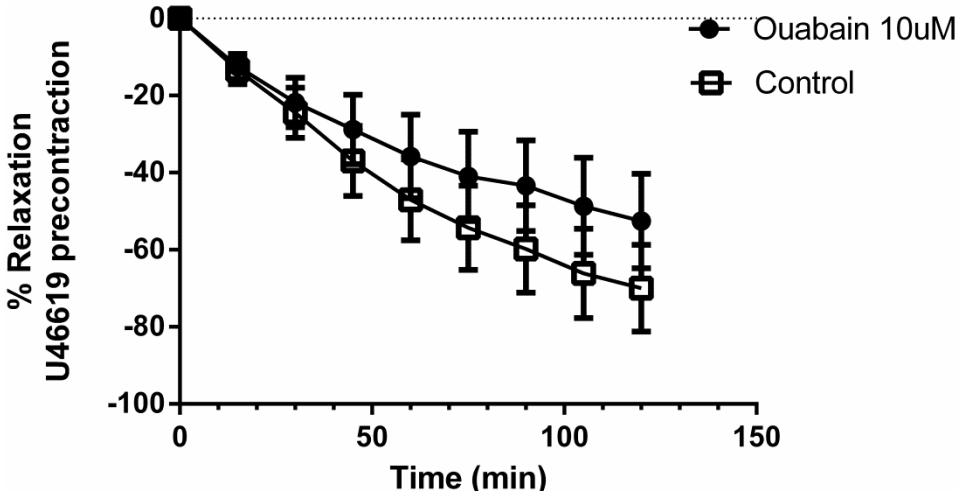


Figure 2.43. Effect of ouabain (10 μM) on relaxations to simvastatin (10 μM) in porcine coronary artery rings pre-contracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different animals.

2.4.11 Acute effects of Simvastatin on Mitochondrial Membrane Potential.

2.4.11.1 Effect of simvastatin on Membrane Potential of the isolated mitochondria

Previous studies in other cell types have demonstrated that simvastatin has an effect on the mitochondrial electron transport chain, altering mitochondrial membrane potential (Sirvent et al., 2005b, Dai et al., 2010, Tolosa et al., 2014). This study aimed to determine whether simvastatin has a direct effect on mitochondria isolated from a pig heart. Simvastatin and Lovastatin (10 μM each) produced an increase in total fluorescence in the isolated mitochondria from a pig heart loaded with Rh123 (figure 2.44 & 2.45 respectively). By contrast, pravastatin had no effect of the mitochondrial potential (figure 2.46).

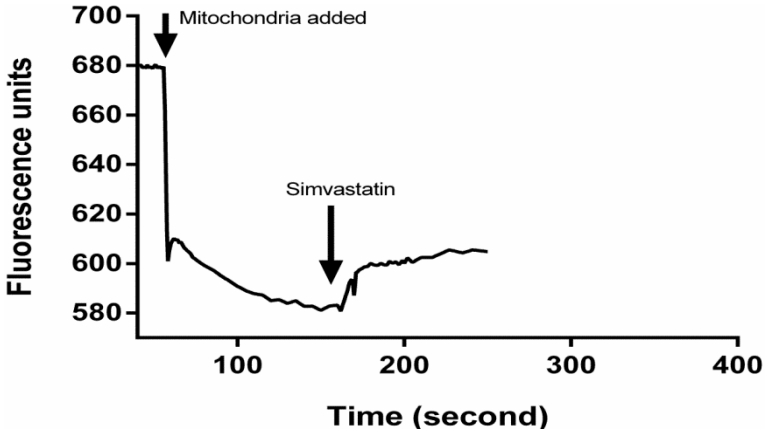


Figure 2.44. Typical trace showing the effect of simvastatin (10 μM) on Rh123 fluorescence in mitochondria isolated from pig hearts.

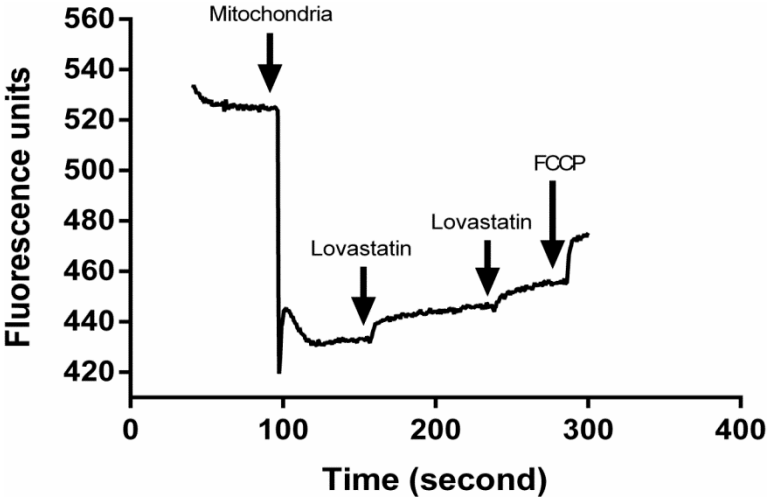


Figure 2.45. Typical trace showing the effect of lovastatin (10 μM) on Rh123 fluorescence in mitochondria isolated from pig hearts. At the end of the experiment FCCP was added to depolarize the mitochondria completely.

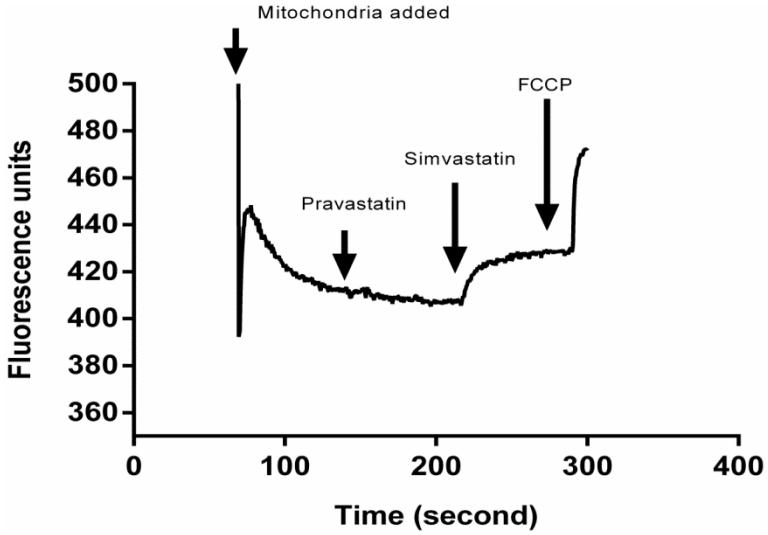
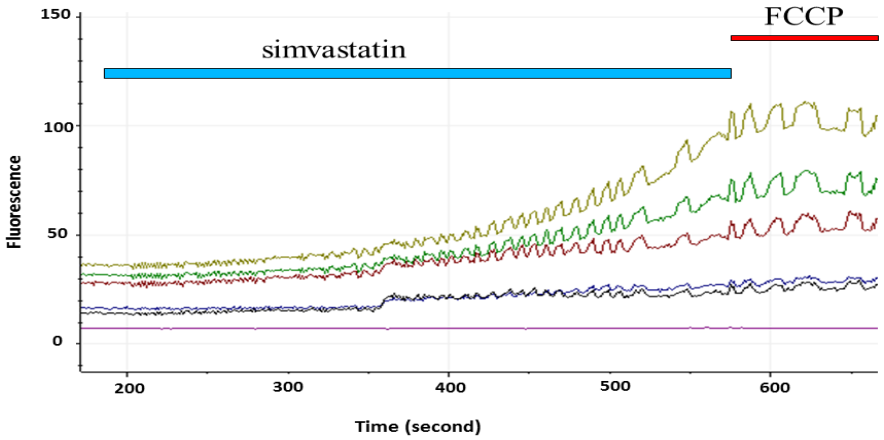


Figure 2.46. Typical trace showing the effect of pravastatin (10 μM) on Rh123 fluorescence in mitochondria isolated from pig hearts. Simvastatin (10 μM) was added after pravastatin for comparison. At the end FCCP (1 μM) added to get the maximum depolarization.

2.4.11.2. Acute effects of Simvastatin on Mitochondrial Membrane Potential in human aortic smooth muscle cells

In cultured human aortic vascular smooth muscle cells, 10 μM simvastatin, but not pravastatin, increased the fluorescence in Rh123-loaded cells (figure 2.47 a & b). In smooth muscle cells, a longer period of simvastatin perifusion was required to depolarize the mitochondria in comparison with pancreatic β-cell (chapter V). Because of the bleaching effect of the dye with continuous perfusion, results were collected with simvastatin (10 μM) (figure 2.48 & 2.49). Such a property could be due to the unusual Rh123 retention by the smooth muscle cells (Summerhayes et al., 1982).

A-



B-

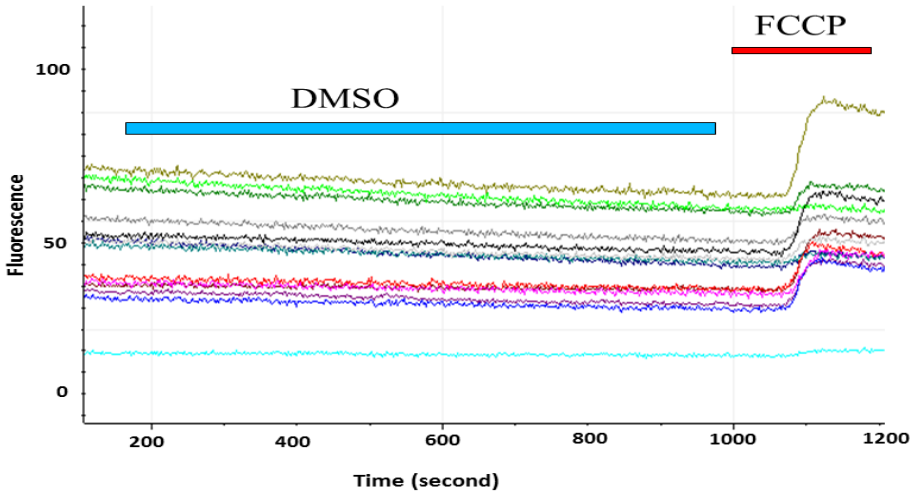
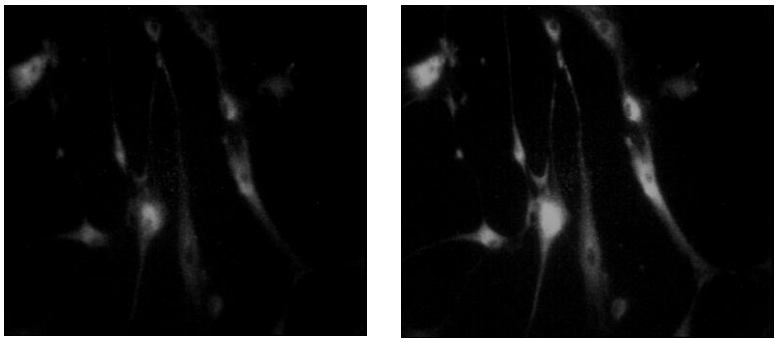


Figure 2.47. Representative effect of simvastatin on Rh 123 fluorescence: Typical trace showing the effect simvastatin and DMSO on Rh123 fluorescence in cultured human aortic smooth muscle. Simvastatin was applied for the duration of the bars. FCCP 1 µM was perfused as positive control to get the maximum fluorescence. A- Simvastatin (10 µM) B- DMSO as a solvent control.

A- B-



C-

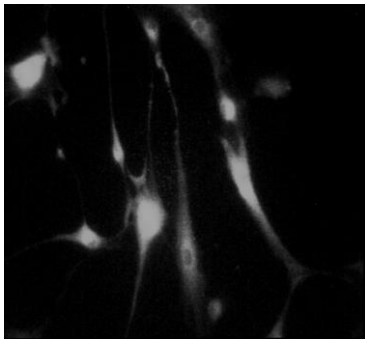


Figure 2.48. Typical fluorescence images from human aortic smooth muscle cells loaded with Rh123: A- in the absence (basal), B- presence of simvastatin (10 μM) or C- presence of FCCP (1 μM).

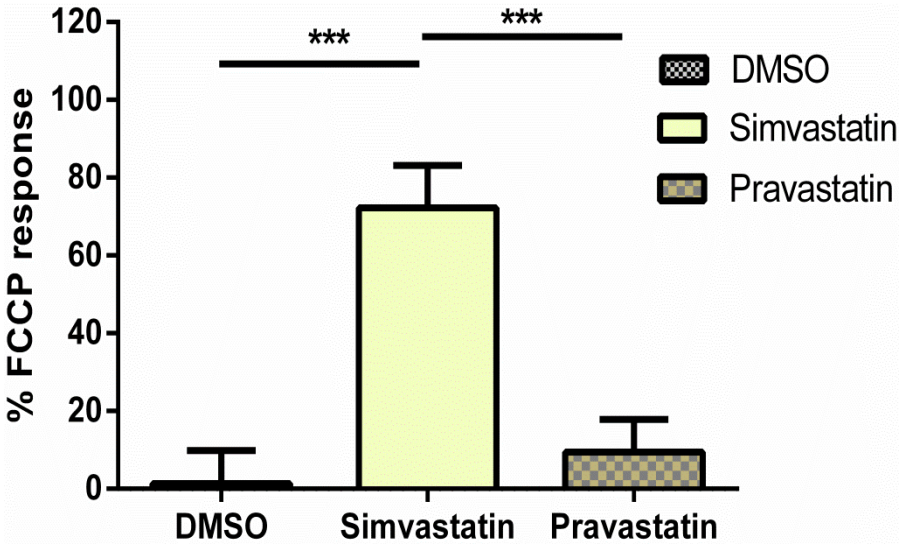


Figure 2.49. Cumulative fluorescence data from human aortic smooth muscle cells loaded with Rh123 in the presence of simvastatin (10 μM), pravastatin (10 μM), or vehicle control (0.1% v/v DMSO). Data are expressed as a percentage of the response to 1 μM FCCP in the same cells

and are mean ± SEM from n=15 separate experiments. *** indicates p<0.001, one-way ANOVA, followed by Tukey's multiple comparisons test.

2.4.11.3. Acute effects of Simvastatin on Mitochondrial Membrane Potential in segments of porcine coronary artery

In order to determine the effects of simvastatin on mitochondrial membrane potential in intact blood vessels. The tissue was incubated with Rh123 and placed in a cuvette in a fluorometer (Hitachi F-2500) at 37 °C as explained in the method. Fluorescence signals were allowed to stabilise before the addition of simvastatin (10 μM). As shown in (figure 2.50), a similar effect was observed in intact blood vessels, with an increase in Rh123 fluorescence.

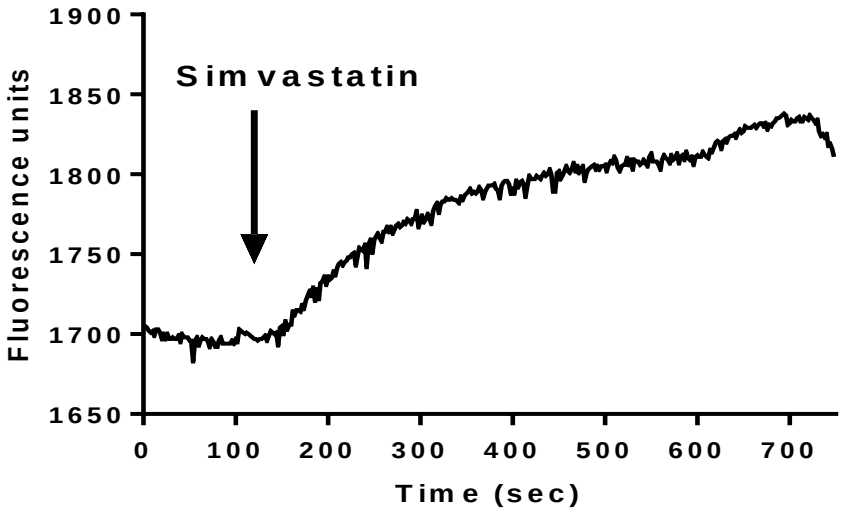


Figure 2.50. Typical trace showing the effect of simvastatin (10 μM) on Rh123 fluorescence in an intact segment of porcine coronary artery.

2.5 DISCUSSION

Statins inhibit HMG-CoA reductase, leading to a reduction in synthesis of mevalonate and hence cholesterol. As such they reduce plasma cholesterol levels. Statins also exhibit other beneficial effects within the cardiovascular system, including vasodilatation. Recent evidence suggests that the cardiovascular pleiotropic effects of statins are predominantly independent of reduction in plasma cholesterol (Liao and Laufs, 2005). A number of mechanisms have been put forward to explain the vasodilator effect of statins including an increase in NO bioavailability (Rikitake and Liao, 2005), direct effects on K^+ channels (Lopez et al., 2008), or inhibition of calcium channels (see chapter III) (Bergdahl et al., 2003). In this study we have demonstrated that simvastatin stimulates relaxation of the porcine coronary artery, and this effect is dependent, at least in part, on mitochondrial function.

Simvastatin produces a concentration dependent relaxation of the porcine coronary artery. The onset of the relaxation usually started within minutes after addition. However the relaxation was slow with a maximum response occurring after around 120 min. Indeed at lower concentrations (300 nM) the relaxation response did not become significant until around 120 min. The relaxation may be dependent upon the lipophilicity of statins. Simvastatin, which is 195 times more lipophilic than pravastatin produced a greater relaxation of the porcine coronary artery than slightly less-lipophilic lovastatin (85 times more lipophilic than pravastatin) (Joshi et al., 1999). On the other hand, the hydrophilic pravastatin, and the sodium salt of simvastatin failed to produce a relaxation. Similar effects have been shown in other studies, including the bovine coronary artery (Lorkowska and Chlopicki, 2005). However, previous studies have shown that the hydrophilic pravastatin induced endothelium-dependent relaxation in rat

aortic and mesenteric vessels (Kaesemeyer et al., 1999, Ghaffari et al., 2011). The difference in response to pravastatin might be due to differences in species (Sonmez Uydes-Dogan et al., 2005), the presence of a statin transporter on endothelial cells, which may be blood vessel specific (Ohtawa et al., 1999), or alternatively endothelium-dependent responses may only be seen in tissues expressing scavenging receptor SR-B1 with resultant endothelial nitric oxide synthase activation (Datar et al., 2010). Thus, the exact mechanism of pravastatin endothelium-dependent relaxation needs to be reassessed in further studies.

The relaxation to simvastatin was independent of the pre-contractile agent as a similar relaxation was obtained after pre-contraction with endothelin-1 or KCl. Previous studies have suggested that the relaxation response to statins was dependent upon NO (Rikitake and Liao, 2005, Lopez-Canales et al., 2011, Mukai et al., 2003, Mraiche et al., 2005). However, in the current study we have shown that NO is not involved in simvastatin relaxation; this is possibly related to differential tissue response to NO (Christie et al., 1989). Interestingly, results have shown that vasodilation in coronary arteries during increased myocardial oxygen demand is mediated by metabolic mechanisms rather than by endothelium-dependent production of NO (Lefroy et al., 1993, Nishikawa and Ogawa, 1997). A mechanism independent NO has been similarly suggested by Budzyn et al, (Budzyn et al., 2004).

The role of PGI_2 as a mediator of the simvastatin-induced relaxation was tested by incubation with the cyclooxygenase inhibitor indomethacin and the results clearly indicate that cyclooxygenase metabolites have no role in the coronary relaxation. Similar results have been reported by De Sotomayor et al (de Sotomayor et al., 2005). Indeed, lovastatin decreased basal prostacyclin production in cultured endothelial cells (Zhou et al., 2009). Conversely Lorkowska *et al* suggested a role for PGI_2 in simvastatin

mediated bovine coronary relaxation (Lorkowska and Chlopicki, 2005). Such differences may be related to species differences. The current study also demonstrates that the relaxation to simvastatin in the porcine coronary artery is endothelium-independent.

Previous studies have demonstrated that K^+ channel activation is involved in simvastatin relaxation. Specifically, K_{ATP} potassium channels and K_v potassium channels have been implicated in the relaxation response to cerivastatin in the rat aorta (Mukai et al., 2003, Sonmez Uydes-Dogan et al., 2005). Our results revealed that neither K_{ATP} channels nor K_v channels are involved in the relaxation to simvastatin in the porcine coronary artery. Indeed, neither TEA, a non-selective inhibitor of K^+ channels, nor high K^+ altered the simvastatin relaxation, suggesting activation of K^+ channels is not involved in the relaxation response. In fact, a previous study in our laboratory demonstrated that simvastatin inhibits K^+ channel-mediated relaxations in the porcine coronary artery (Uhiara et al., 2012).

Simvastatin has been shown to inhibit the Na^+-Ca^{2+} exchanger in the cardiomyocytes (Bastiaanse et al., 1994) and endothelial cells (Alvarez de Sotomayor et al., 1999). Sonmez *et al* (2005) also suggested activation of the Na^+-K^+ ATPase pump as a mechanism in cerivastatin-induced relaxation in isolated rat aorta. This study determined the effect of ouabain on the simvastatin relaxation. Our results demonstrated that ouabain had no effect on simvastatin relaxation, thus the Na^+-K^+ pump plays no role in the acute vascular relaxant effects of simvastatin in isolated porcine coronary artery.

H_2S has been shown to be produced endogenously from L-cysteine through the actions of cystathionine β-synthase and cystathionine γ lyase (Kimura, 2011). Previous studies have demonstrated that simvastatin increases the production of H_2S from perivascular tissue. However, this can be ruled out as a mechanism of the acute simvastatin induced relaxation in PCA since

inhibition of H_2S synthesis with AOAA and PPG had no effect on the relaxation response.

The findings presented here suggest that the relaxation in pig coronary artery segments is dependent upon the ability of the drug to penetrate across the plasma membrane of the smooth muscle cell and suggest that the site of action of simvastatin is not extracellular. Interestingly, an *in vitro* study by Skottheim *et al*. suggested that lactone forms of statins induce myotoxicity in human skeletal muscle cells more than the respective acid forms, such differences may be explained by lipophilicity since the lactone forms reach intracellular sites to different extent than the acid forms (Skottheim et al., 2008). These observations are consistent with previous data demonstrating that lipophilic statins, but not hydrophilic statins, like pravastatin, induce acute vasodilator effects (Mukai et al., 2003, Lorkowska B, 2005). One possible intracellular target for statins is the mitochondria. Previous studies have demonstrated that simvastatin at 20 times higher concentration than those used in this current study is able to alter mitochondrial membrane potential in intact hepatocytes, whereas a similar phenomenon was observed in permeabilized skeletal muscle cells with an EC50 of ~2 μM (Sirvent et al., 2005b, Abdoli et al., 2013). There is a growing consensus that mitochondria play an important role in the regulation of vascular tone. For example, flow-induced dilation involves generation of reactive oxygen species originating from mitochondria in endothelial cells (Bubolz et al., 2012). Therefore, we investigated whether the relaxation response was dependent upon mitochondrial activity. Inhibition of complex I with rotenone and complex III with myxothiazol caused significant inhibition of the simvastatin relaxation. A combination of the two produced a greater inhibition of the relaxation. On the other hand, the mitochondrial uncoupler FCCP enhanced the relaxation. Antimycin A, another complex III inhibitor, and inhibition of complex II had no effect.

Antimycin A and myxothiazol act at different parts of complex III, with myxothiazol acting at the Qo site, whereas antimycin A acts at the Qi site, which probably explains the different effects with these two compounds (Wu et al., 2010). The effects of rotenone and myxothiazol on the simvastatin relaxation are unlikely to be due to non-specific effects caused by inhibition of mitochondrial function since other complex inhibitors (antimycin A and complex II inhibitors), the ATP synthase inhibitor oligomycin, and the mitochondrial uncoupling agent FCCP had no inhibitory effect on the vasorelaxation.

Simvastatin produced an increase in Rh123 fluorescence in isolated mitochondria, which is indicative of a change in mitochondrial membrane potential ($\Delta\psi_m$). This was also seen in cultured aortic smooth muscle cells, demonstrating that simvastatin can depolarize the mitochondrial membrane potential in whole cells. By contrast pravastatin had no effect on the mitochondrial potential in smooth muscle cells, which correlates with the lack of relaxation with pravastatin. Similar effects on Rh123 fluorescence were found in intact blood vessels, suggesting that the effect of simvastatin on porcine coronary artery tone could be through effects on mitochondria. In combination with the relaxation studies, these experiments indicate that simvastatin does have an effect on vascular smooth muscle mitochondria. As the relaxation to simvastatin was reduced by complex I and complex III inhibition, but not complex II, these data indicate that the relaxation to simvastatin is dependent upon complex I and III activity.

Although studies demonstrate that simvastatin inhibits mitochondrial function, the mechanism by which it does this is unknown. Previous studies in skeletal muscle have suggested that simvastatin inhibits complex I (Sirvent et al., 2005a). Other studies have hypothesized that the effect on mitochondria is due to a reduction in the expression of co-enzyme Q10,

which is part of complex III (Vaughan et al., 2013, Tavintharan et al., 2007, Larsen et al., 2013). However, this is more likely to be due to long-term incubation with statins, rather than the acute exposure in this present study. Other studies have suggested that simvastatin increases mitochondrial K_{ATP} channel activity (Zhao et al., 2006, Jones et al., 2003a). However, the mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate had no effect on the relaxation, ruling this out as a mechanism.

Mitochondria are a major source of ROS within cells (Carreras et al., 2004). The major sources of ROS in the electron transport chain (ETC) are thought to be complex I and complex III (Carreras et al., 2004). Antimycin A and myxothiazol both increase production of ROS from complex III (Starkov and Fiskum, 2001, Mattiasson, 2004), and both compounds relaxed the porcine coronary artery (see Chapter IV). Release of ROS from the mitochondria into the cytosol can cause vasodilatation (Katakam et al., 2014) and can inhibit L-type calcium channels (Fearon, 2006, Scragg et al., 2008). Therefore, this study investigated whether ROS underlie the effect of simvastatin on vascular tone. A combination of the hydrogen peroxide scavenger ebselen, catalase, which metabolizes hydrogen peroxide, and superoxide dismutase, which metabolizes superoxide had no effect on the simvastatin relaxation. Furthermore, diethylthiocarbamate which inhibits superoxide dismutase, uric acid, peroxynitrite scavenger, and quercetin, flavonoid anti-oxidant (Wambi-Kiesse and Katusic, 1999) had no effect on the simvastatin relaxation. These data indicate that an increase in ROS production from the mitochondria does not play a role in the simvastatin-induced relaxation. NADPH oxidase (Nox) is a relevant source of H_2O_2 that mediates dilation of coronary arteries (Larsen et al., 2009) and mitochondrial superoxide could stimulate cytoplasmic Nox2, which has been linked to the development of endothelial oxidative stress and even hypertension (Nazarewicz et al., 2013). Our results with DPI, a

commonly used Nox enzyme inhibitor (Wind et al., 2010), suggest that Nox inhibition has no role in the simvastatin relaxation.

The effects of simvastatin on vascular tone in the porcine coronary artery occur at concentrations that are very close to the estimated therapeutic plasma concentration (from 0.01 to 1 μM in patients treated with a dosage range of 20 to 60 mg daily) (Laufs et al., 1998, Lilja et al., 1998, Kantola et al., 1998), although higher concentrations were required for maximum effect. We hypothesise that lipophilic statins accumulate within the mitochondria. Lipophilic compounds can easily pass through the mitochondrial membrane and, as the pH in the mitochondrial matrix is higher than the pH in the cytosol, these lipophilic compounds can become charged. As a consequence mitochondria can trap lipophilic compounds (Rashid and Horobin, 1991). Therefore, the concentration of statin within the mitochondria may be higher than that measured in the plasma. The time taken for uptake into the mitochondria would explain why the relaxation to simvastatin was slow; at the lowest concentration of simvastatin used (300 nM); the relaxation did not reach significance until around 145 minutes after addition.

In summary, this study has demonstrated that simvastatin alters mitochondrial membrane potential in vascular smooth muscle and the relaxation to simvastatin in the porcine coronary artery is dependent, in part, upon mitochondrial activity. The mechanism by which inhibition of mitochondria leads to relaxation of the porcine coronary artery is unknown. One possibility is that alteration of mitochondrial membrane potential by simvastatin may lead to inhibition of calcium influx, hence stimulation of relaxation, and this will be investigated in the next chapter.

CHAPTER III: EFFECT OF SIMVASTATIN ON Ca^{2+} INFLUX IN PORCINE CORONARY ARTERY

3.1. Introduction

Cytosolic free Ca^{2+} appears to play a fundamental part in the control of cell function (Hill-Eubanks et al., 2011). In vascular smooth muscle cells, Ca^{2+} has been implicated in cell proliferation and smooth muscle contraction (Karaki et al., 1997). When the intracellular Ca^{2+} increases, it combines with calmodulin. This complex activates myosin light chain kinase (MLCK) to phosphorylate the light chain of myosin. Smooth muscle relaxation is brought about by the removal of Ca^{2+}, which results in dissociation of the Ca^{2+}-calmodulin complex and dephosphorylation of myosin by myosin light chain phosphatase (Webb, 2003). The regulation of cytosolic Ca^{2+} is complex and depends, partly, on Ca^{2+} entry from the extracellular space through voltage-activated or receptor-operated Ca^{2+} channels, or both (Ren et al., 2010, Weirich et al., 2005). However a significant contributor to cytoplasmic free Ca^{2+} regulation in smooth muscle cell is the release of Ca^{2+} from internal stores by inositol 1,4,5-triphosphate (Yang et al., 2013, Yamamura et al., 2012). The regulation of these mechanisms is important for the maintenance of vascular tone (Marche and Stepien, 2000, Kamishima et al., 2000).

Simvastatin is a drug widely used in the treatment of hypercholesterolemia. This drug is an inhibitor of the rate-determining enzyme in the biosynthesis of cholesterol, 3-hydroxy-3-methylglutaryl-coenzyme A HMG-CoA reductase (Istvan and Deisenhofer, 2001, Istvan, 2002), and has proved useful in the reduction of plasma low-density lipoprotein LDL Level. Mounting clinical and experimental evidence suggests that the protective effects of statins on vascular disease relate not only to improving lipid profiles (Cheung et al., 2004, Davignon, 2004, Bonetti et al., 2003). In fact, HMG-CoA reductase provides the necessary building blocks for both cholesterol, and the isoprenoids farnesyl and

geranylgeranyl pyrophosphate, these are used for post-translational modification of Ras and Rho GTPases, respectively (Buhaescu and Izzedine, 2007). Reduced isoprenylation impairs Ras and Rho activation which play important roles in smooth muscle cell proliferation and contraction (Rattan, 2010, Corsini et al., 1991).

Not only chronic treatment, but also acute exposure to simvastatin is able to induce coronary relaxation (Wassmann et al., 2003, Takagi et al., 2006). There is increasing evidence that the actions of HMG CoA reductase inhibitors in the vasculature are attributable to more than their cholesterol-lowering function (Sadowitz et al., 2010a).

Simvastatin could affect Ca^{2+} release from intracellular pools, as described earlier in vascular smooth muscle cells (Tesfamariam et al., 1999, Escobales et al., 1996a), Capacitative Ca^{2+} entry is also affected as confirmed by the inhibition of contraction due to $CaCl_2$ after noradrenaline stimulation (Alvarez de Sotomayor et al., 2001).

The alteration of intracellular Ca^{2+} calcium homeostasis by statins was already discovered by other groups in different cell types; in cardiac cells, the activation of Ca^{2+} channels by high K^+ or Bay K8644 was completely abolished in mevinolin-treated cells (Renaud et al., 1986). Studies also have reported a blockade of L-type Ca^{2+} channels by simvastatin in rat islet β-cells (Yada et al., 1999) and rat glomerular mesangial cells (Ishikawa et al., 1995). Simvastatin could also inhibit catecholamine secretion induced by 56 mM K^+ through Ca^{2+} channels (Matsuda et al., 2008). Other HMG-CoA reductase inhibitors like fluvastatin, cerivastatin and pravastatin, markedly suppressed intracellular Ca^{2+} mobilization and nonselective cation current evoked by LPC in human aortic endothelial cells (Yokoyama et al., 2002). Rosuvastatin also attenuates the increase in intracellular free calcium and superoxide anion during L-glutamate application in cortical neurons (Domoki et al., 2010). On the other hand,

simvastatin induced an increase in cytosolic calcium Ca^{2+} in bovine aortic endothelial cells, by releasing Ca^{2+} from intracellular stores sensitive to thapsigargin and ryanodine, and increasing Ca^{2+} entry as a consequence of intracellular Ca^{2+} store depletion by a mechanism which does not occur via L-type Ca^{2+} channels (Alvarez de Sotomayor and Andriantsitohaina, 2001). In parallel, lovastatin exposure for more than 24 hours increased basal Ca^{2+} with increased vasoconstriction response induced by noradrenaline in isolated rat aortic and mesenteric arteries (Roullet et al., 1995, Roullet et al., 1993). Indeed it has been found that co-administration of Ca^{2+} channel blockers with statins has beneficial effects by inhibiting the progression of coronary atherosclerosis (Martin-Ventura et al., 2008, Bangalore et al., 2007, Clunn et al., 2010). However, a combination of nifedipine and statin may also result, in certain cases, in worsening of cardiac performance. This may suggest another mechanism of drug-drug interaction than the one based on liver metabolism i.e. nifedipine may interact with simvastatin by inhibiting cytochrome P-450 mediated biotransformation (Jasinska et al., 2006).

Mitochondria within the smooth muscle cell might be directly involved in taking up Ca^{2+} load from the cytosol following stimulation (Drummond and Fay, 1996). Recent studies are providing evidence that mitochondrial inhibition influences Ca^{2+} waves due to the spatial relationship between mitochondria and the sarcoplasmic reticulum (Sward et al., 2002). Ca^{2+} channel current could also be modulated by ROS generated at complex III of the mitochondrial electron transport chain (Fearon, 2006, Hsieh et al., 2001). We have shown in chapter 2 that simvastatin depolarizes mitochondria in smooth muscle cells. Thus, the aim of the present study was to examine the involvement of Ca^{2+} influx, as a potential mechanism, in simvastatin-induced relaxation of porcine coronary artery. Also, the effect of simvastatin on the Ca^{2+} influx induced by membrane

depolarization was studied in freshly isolated porcine coronary smooth muscle cells. Lastly, possible involvement of mitochondria in modulating Ca^{2+} influx was examined by incubation with mitochondrial respiratory chain inhibitors (Kamishima and Quayle, 2002, McCarron et al., 2013).

3.2 Materials and Methods

3.2.1 Materials

General laboratory reagents are listed in chapter 2, reagents specific to this chapter were purchased from the following sources: simvastatin was obtained from Tocris Cookson, Bristol UK. Rotenone, FCCP, U46619, cyclopiazonic acid and nifedipine were obtained from Sigma-Aldrich, Poole, UK. DABCO fluorescent mounting medium contains: 1-4-Diazabicyclo-2-2-2-octane (DABCO), Phosphate buffered saline (PBS) and glycerol. Dulbecco Modified Eagle Medium (DMEM) with D-valine and fetal bovine serum (SFBS). Antibiotic, antimycotic solution (sigma) at 10 mL/L which contains 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL.

3.2.2. Preparation and characterization of the smooth muscle cells (SMCs)

3.2.2.1. Preparation of the SMCs

3.2.2.1.1. Primary Vascular Smooth Muscle Cell Cultures (Explant Method)

Explant culture of medial tissue is the simplest and most reliable method of obtaining VSMC cultures. Porcine coronary arteries were obtained from a local abattoir as before. Arteries were dissected out of the heart. After connective tissue removal from around the artery, the vessel was washed with sterile saline. The artery was then placed on a sterile dissection board, in a hood, and covered with normal saline to keep it moist. The arteries were cut open longitudinally using sterile forceps and scissors, and the

luminal side of the vessel freed of endothelium by gently scraping the surface using forceps. The medial layers were placed in a separate Petri dish and cut carefully using a scalpel blade into approximately 1 × 1-cm pieces. These explants were then carefully placed into six-well plates. A coverslip was placed on top of the explants and covered with medium. The medium consisted of Dulbecco Modified Eagle Medium (DMEM) with D-valine and fetal bovine serum (SFBS). In addition the media contain antibiotic, antimycotic solution (sigma) at 10 mL/L which contains 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL. The segments were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2–95% air. Medium was changed after 24 h and then every 3–4 days. Cells started to grow out radially from the explants within 1 week of the culture.

3.2.2.1.2. Isolation of Smooth Muscle Cells by Enzyme dispersal method

Enzyme dispersion of the medial layer does not yield as many cells as the explant method, but the advantage is that the whole population of VSMCs is represented, rather than selecting for those capable of migrating from the tissue. In addition, the cells isolated by enzyme dispersion may represent a phenotype more closely related to that in the vessel wall because the time to isolation is much quicker 2 h as opposed to 2–3 weeks. The vessels were prepared as for the explant method. However, in this case, explants were placed in a sterile conical flask and incubated in serum-free digestion medium containing collagenase (2 mg/mL). Explants were incubated in a shaking water bath at 37°C for 30-45 minutes, with occasional dispersion using a wide-mouthed pipette. The dispersed cells were removed by centrifugation type (1,000 rpm for 5 min) and the remainder left to disperse further. The dispersed cells were centrifuged again and re-suspended in fresh Dulbecco Modified Eagle Medium (DMEM)

with D-valine and fetal bovine serum (SFBS) with antibiotic and antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 µg Amphotericin B per mL).

3.2.2.2. Immunohistochemistry: Characterization of the Smooth Muscle Cells

Confluent monolayers of vascular smooth muscle cells exhibited a characteristic 'hill and valley' morphology in culture. Isolated primary cells were identified by their positive reaction with antibodies against smooth muscle actin, which has been shown to be a specific marker for smooth muscle cells.

Cells were fixed using 4% paraformaldehyde for 20 min at room temperature. They were then permeabilized using PBS + 0.1% BSA + 0.15% Triton ×-100 (100 ml PBS + 100 mg BSA +15 µL Triton) at room temperature for 20 min. Cells were then blocked using dried powder milk (1.5 g in 30 ml) at room temperature. The blocking solution was then removed and replaced with the primary antibody (α-Actin primary mouse, sigma), (6 µL in 1200 µL), and incubated overnight in a humidifier at 4°C.

The following day, the primary antibody was removed and cells washed in PBS. After washes, cells were incubated with the secondary TRITC anti-mouse secondary antibody in PBS+0.1% BSA (6 µL in 1200 µL of PBS) and incubated at 37°C for 30 min

After this time, cells were washed with PBS. The nuclear stain DAPI (1 in 1000) was then added for 5 min, and cells washed again in PBS. Mounting solution was then added and a cover slip put on top of the cells. Micrographs were captured with DMRB Fluorescence Microscope Luca ^{EM}.

3. 3 Data Analysis

The relaxation responses were expressed as a percentage change from the agonist-induced pre-contraction over time. Values for all figures refer to mean ± SEM. Relaxation responses over time were compared by using two-way analysis of the variance (ANOVA) followed by a Bonferroni post-hoc test. Differences were considered to be significant when the P value was < 0.05. Changes in Fluo-4 fluorescence were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. 2-tailed unpaired t-test was used to determine statistical significance where $P<0.05$ was considered statistically significant. Where "n" indicates the number of independent experiments for each treatment. Asterisks indicate statistically differences from control.

3.4 Experimental protocol

3. 4.1 organ bath isometric studies

Porcine coronary arteries were set up in isolated tissue baths as previously described (Uhiara et al., 2012) see chapter II. Tissues were pre-contracted submaximally with KCl or U46619, in the presence of various inhibitors: L-NAME (300 μM) to inhibit nitric oxide synthase; cyclopiazonic acid (10 μM; CPA) to inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase; rotenone (10 μM) to inhibit complex I, FCCP (1 μM) to uncouple to mitochondria. Alternatively, tissues were exposed to calcium-free Krebs buffer containing (2 mM) EGTA. After the tissues had reached a steady contraction, 10 μM simvastatin was added and tone measured over 2 hours. In other experiments, tissues pre-contracted with KCl were exposed to nifedipine (1 μM) with or without simvastatin (10 μM).

Porcine coronary arteries were set up in isolated tissue baths as before. Tissues were exposed to Krebs-Henseleit buffer without calcium, and then exposed to 60 mM KCl. Concentration-response curves to $CaCl_2$ (1 μM to 3 mM) were then performed, in the absence or presence of simvastatin (3 to 10 μM). Control tissues were treated with DMSO as a solvent control. In some experiments, tissues were also exposed to mevalonate (10 mM) or rotenone (10 μM) prior to addition of simvastatin.

3.4.2. Effect of simvastatin on calcium influx in freshly isolated smooth muscle cells.

The concentration of intracellular calcium, $[Ca^{2+}]_i$, was monitored with Fluo-4 using standard epifluorescent imaging techniques similar to those described by (Smith et al., 1999). Smooth muscle cells were isolated from proximal coronary artery segments by incubation with type II collagenase (2 mg/mL) for 30 min, and then plated on glass coverslips. Cells were then loaded with the Ca^{2+}-sensitive fluorophore fluo-4 AM (5 μmol/L) in HEPES-buffered Hanks solution which contained (in mM): 138 NaCl, 4.2 $NaHCO_3$, 1.2 NaH_2PO_4, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with NaOH) with 5 mmol/L glucose for 1 hour at room temperature. The cells were then washed with Hanks buffer to remove extracellular dye before leaving them at room temperature for 15 minutes for complete de-esterification of the dye. Coverslips were then superfused with Hanks buffer at 37°C for 5 min for basal recording. The Hanks buffer was then replaced with high K^+ (50mM) Hanks containing (in mM) 50 KCl, 92.6 NaCl, 4.2 $NaHCO_3$, 1.2 NaH_2PO_4, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, and 10 HEPES [pH 7.4], which elicited a sustained increase in the fluorescence. Subsequently simvastatin (10 μM), or DMSO (0.1% v/v) as a control, was added to the perfusion media. Loaded cells were imaged using an inverted Axiovert 135TV microscope fitted with a 20x Fluo objective (Carl Zeiss Ltd, Welwyn Garden

City, UK). To monitor $[Ca^{2+}]_i$, cells were continuously illuminated at an excitation wavelength of 450-490 nm. The images were bandpass filtered at 515-565 nm, detected using a Photonics Science ISIS camera and captured at 1Hz with an 8-bit frame grabber (DT3155, Data Translation, Basingstoke, UK) and Imaging Workbench software (Indec Biosystems, Santa Clara, CA, USA). For image analysis, a region of interest (ROI) was drawn around each cell, the background fluorescence substracted, and the time course of its mean fluorescence intensity calculated. Data was analysed using Microcal ORIGIN 5.0 (Microcal Software Inc., Northampton, MA). Macros were created (see Appendices for the macro scripts).

3.5. RESULTS

3.5.1. Simvastatin inhibits KCl-induced contraction in porcine coronary arteries

As shown in figure 3.1, acute application of simvastatin elicited time dependent relaxation in pig proximal coronary artery pre-contracted submaximally with KCl (fig. 1).

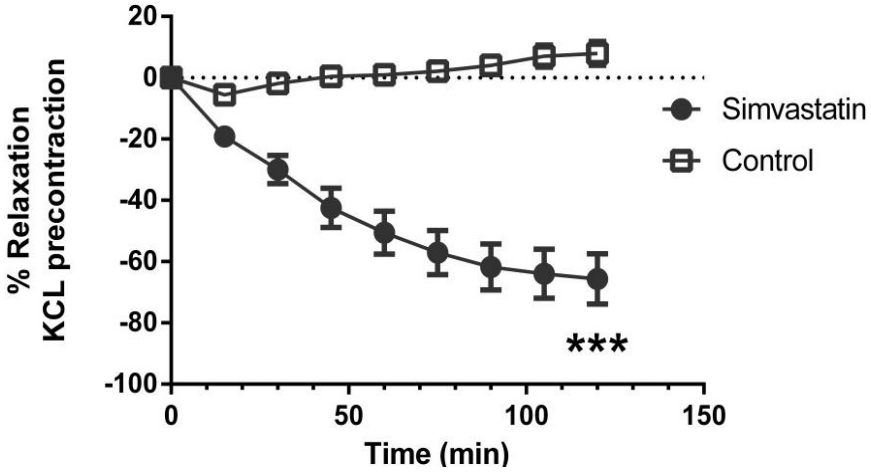


Figure 3.1. Acute effects of simvastatin (10 μM) in porcine coronary artery rings precontracted submaximally with KCl. Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 8 different experiments. *** indicates $p<0.001$, (2- way ANOVA followed by a Bonferroni post-hoc test) versus values obtained from the solvent control DMSO (0.1% v/v).

3.5.2. Role of endothelium-derived vasodilator factors in the relaxation response to simvastatin.

Pre-treatment of the porcine coronary rings with a nitric oxide synthase inhibitor, L-NAME (300 μM), had no significant effect on the relaxation to 3 μM simvastatin (figure 3.2). Similar results with L-NAME (300 μM) on simvastatin 10 μM have been shown in chapter II.

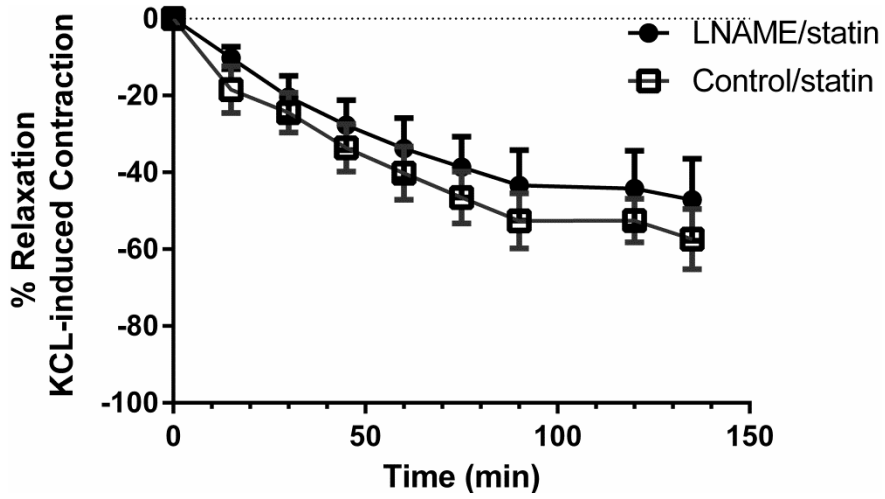


Figure 3.2. Acute effects of simvastatin (3 μM) in porcine coronary artery rings precontracted with KCl in the presence or absence L-NAME (300 μM). Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 5 different experiments. (2- way ANOVA followed by a Bonferroni post-hoc test).

3.5.3. Role of Sarcoplasmic reticulum Ca^{2+} -ATPase in acute simvastatin relaxation:

A possible mechanism of simvastatin relaxation could involve inhibition of the Ca^{2+}- ATPase and thus inhibition of SR calcium sequestration (Escobales et al., 1996a). CPA inhibits Ca^{2+}-ATPase. Thus, we examined CPA (10 μM) for its ability to block the relaxant effect of 10 μM simvastatin. Figure (3.3) clearly shows that CPA had no effect on simvastatin relaxation.

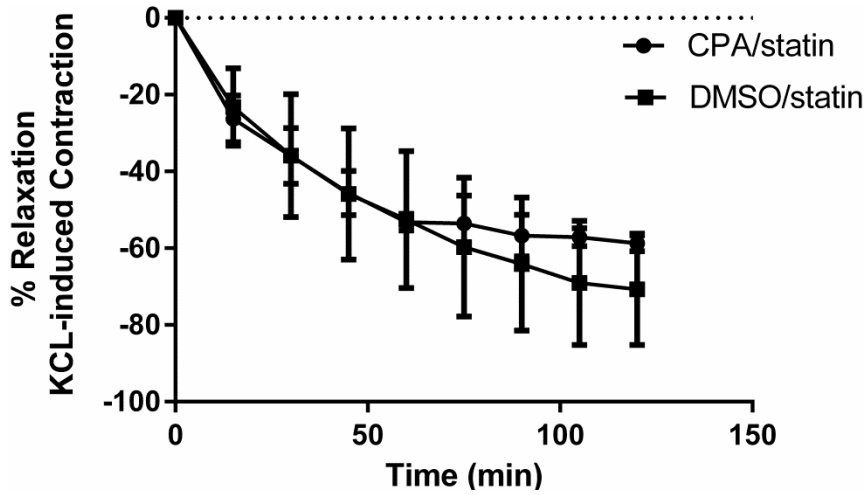


Figure 3.3. Acute effects of simvastatin (10 μM) in porcine coronary artery rings precontracted with KCl in the presence or absence cyclopiazonic acid (10 μM). Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 5 different experiments. (2- way ANOVA followed by a Bonferroni post-hoc test).

3.5.4. Simvastatin relaxation in Ca2+-free Krebs:

In separate experiments, tissues were incubated in calcium-free Krebs-Henseleit buffer. Under these conditions, the acute relaxation to simvastatin was significantly reduced, although not abolished completely (figure 3.4).

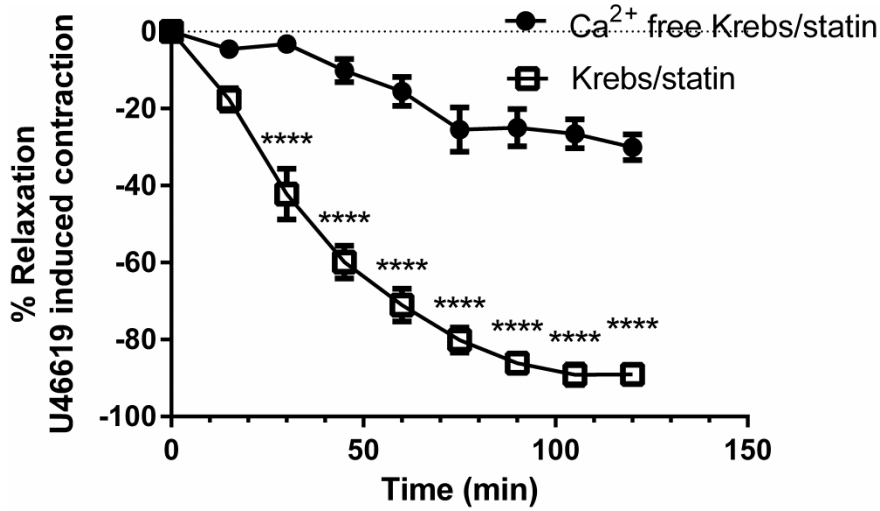


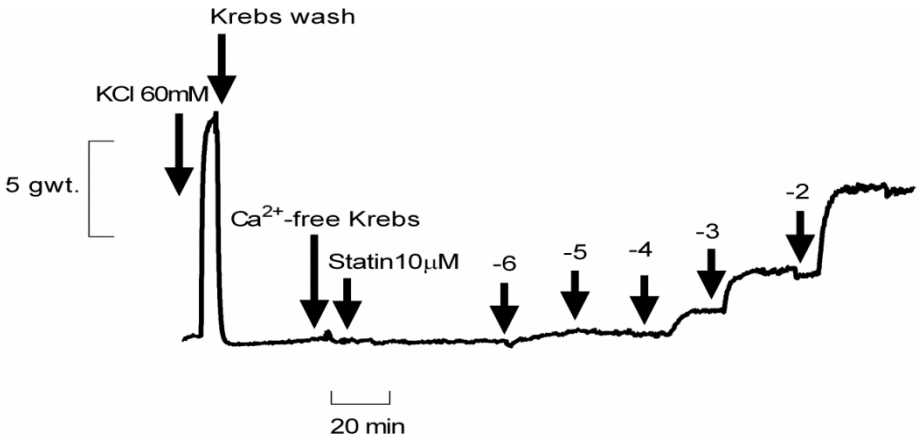
Figure 3.4. Acute effects of simvastatin (10 μM) in porcine coronary artery rings precontracted with U46619 in Ca^{2+}-free Krebs or normal Krebs. Data are expressed as a percentage relaxation of the U46619-induced

contraction and are mean ± SEM from 6 different experiments. (2-way ANOVA followed by a Bonferroni post-hoc test). **** indicates P ≤ 0.0001.

3.5.5. Simvastatin suppressed Ca^{2+}-induced contraction in coronary arteries:

In these experiments, simvastatin was added prior to contraction. The addition of simvastatin (10 μM) did not affect the base line tone in these arteries. In Ca^{2+}-free, 60 mM K^+-containing Krebs solution, $CaCl_2$ produced concentration-dependent contractions in porcine coronary arteries (Figure 3.5 & 3.6). Treatment with 3 μM or 10 μM simvastatin for 60 min suppressed $CaCl_2$-induced contractions (Figure 6).

A-



B-

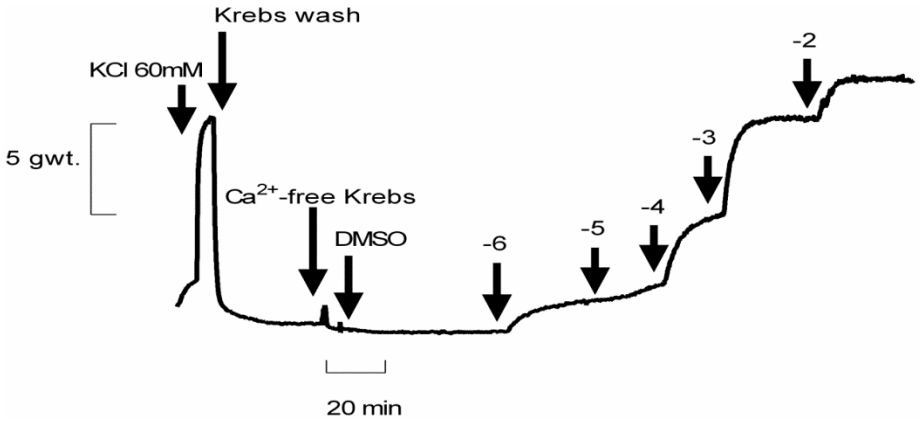


Figure 3.5. Representative organ bath tracing of contractile responses in segments of porcine coronary artery to Ca^{2+} re-introduction in a Ca^{2+} free,

high K^+ buffer in the presence of **A-** simvastatin 10 μM and **B-** DMSO as a vehicle control.

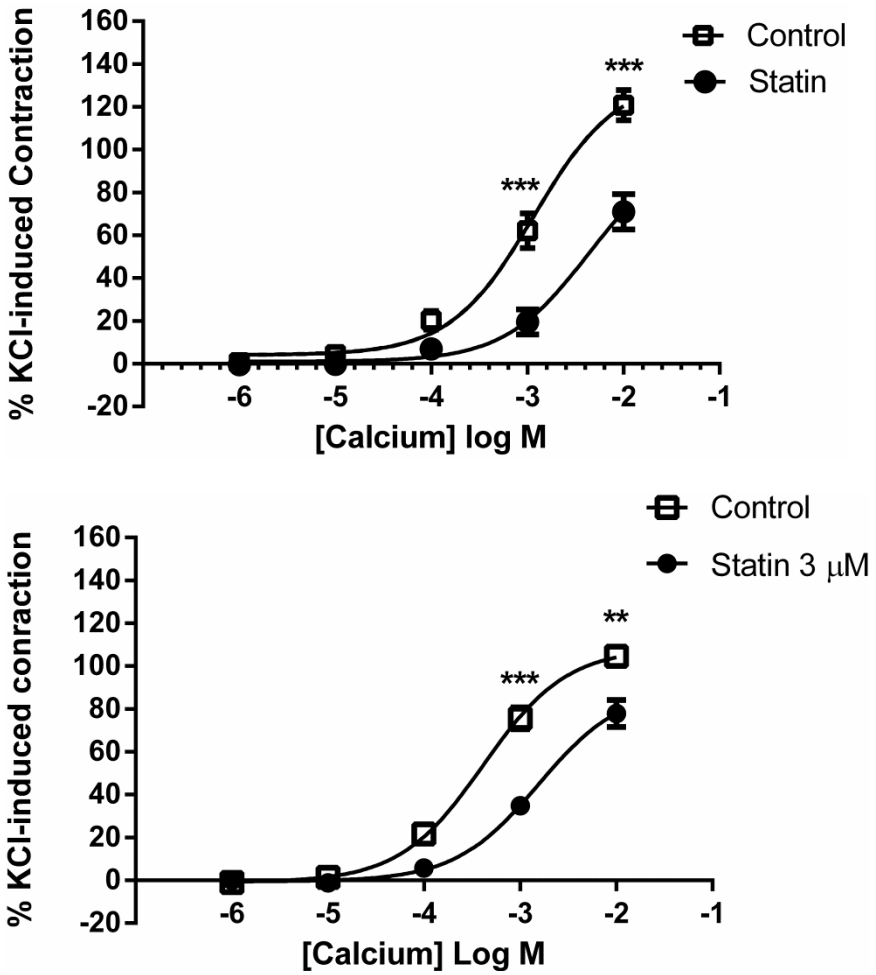


Figure 3.6. Contractile response to the reintroduction of calcium in a calcium free, high potassium Krebs' solution in the coronary artery in the presence of simvastatin for 1h (10 μM, n=9) and (3 μM, n=5). Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM. *** indicates $p < 0.001$ (2- way ANOVA followed by a Bonferroni post-hoc test).

Incubation of the porcine coronary rings with mevalonate 10 mM, the maximum concentration that has been used in previous studies to inhibit statin relaxation (Roullet et al., 1995, Alvarez de Sotomayor et al., 2001, Bergdahl et al., 2003), for 60 min had no effect on the simvastatin suppressant effect in $CaCl_2$-induced contractions (figure 3.7).

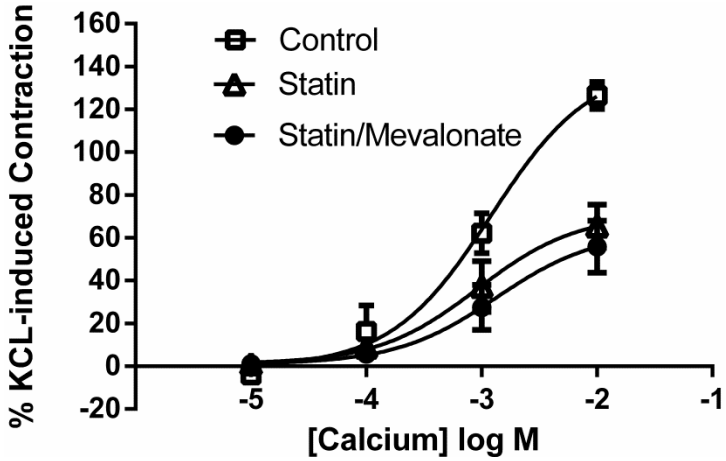


Figure 3.7. Contractile response to the reintroduction of calcium in a calcium free, high potassium Krebs' solution in the coronary artery in the presence of simvastatin with mevalonate (10 mM) both for 1h (n=5). Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM. (2- way ANOVA followed by a Bonferroni post-hoc test).

3.5.6. Role of voltage-gated Ca^{2+} channel in the acute simvastatin relaxation

The involvement of voltage-gated calcium channels in the relaxation to simvastatin was examined by incubation with nifedipine (1 μM) (Figure 3.8). Nifedipine on its own induced significant relaxation of the KCl precontracted coronary vessels within 10 min (figure 3.11). This was unaltered by the presence of rotenone (10 μM).

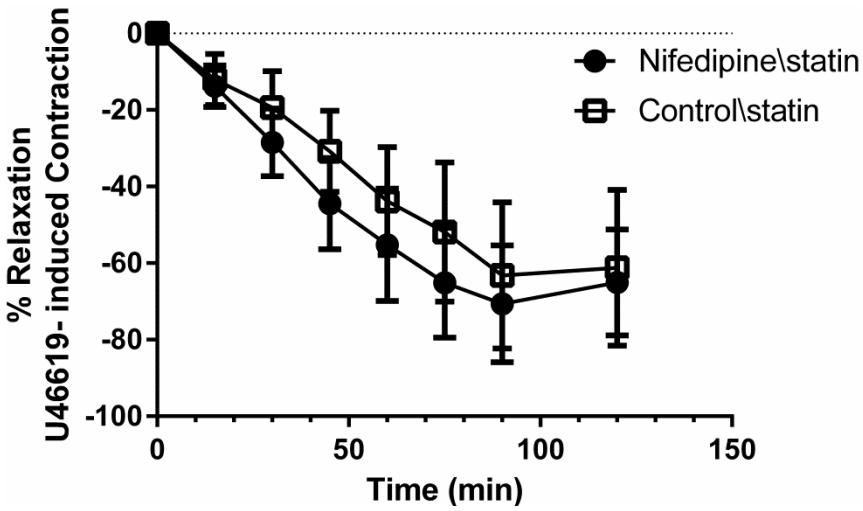
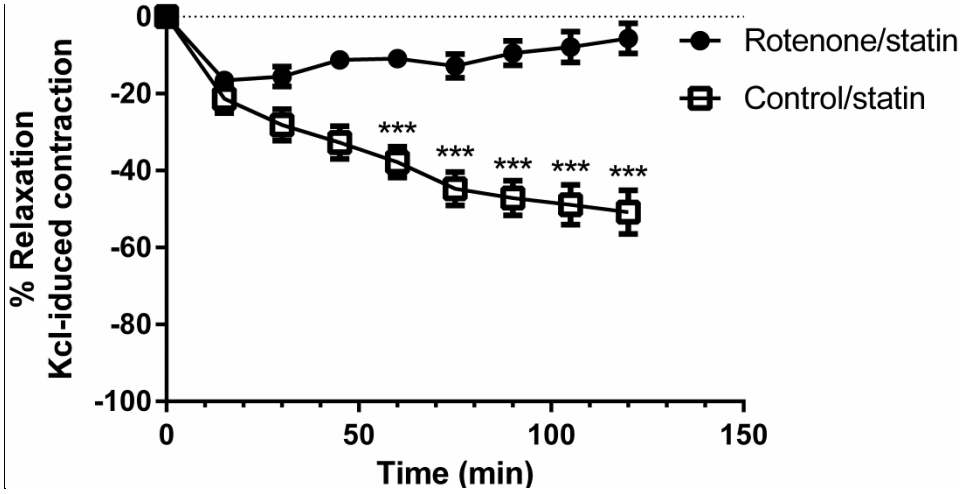


Figure 3.8. Acute effects of simvastatin (10 μM) in porcine coronary artery rings precontracted with U46619 in the presence and absence of nifedipine (1 μM). Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different experiments. (2-way ANOVA followed by a Bonferroni post-hoc test).

3.5.7. Effect of complex I mitochondrial inhibitor, rotenone, on simvastatin relaxation in porcine coronary segments precontracted submaximally with KCl:

The presence of rotenone 10 μM inhibited the relaxation to simvastatin after pre-contraction with KCl (figure 3.9 A&B). In comparison, FCCP 1 μM had no significant effect on the relaxation to simvastatin in the presence of KCl (figure 3.10). On the other hand, rotenone had no effect on the relaxation to nifedipine (figure 3.11), suggesting that rotenone is not acting directly at L-type calcium channels.

A-



B-

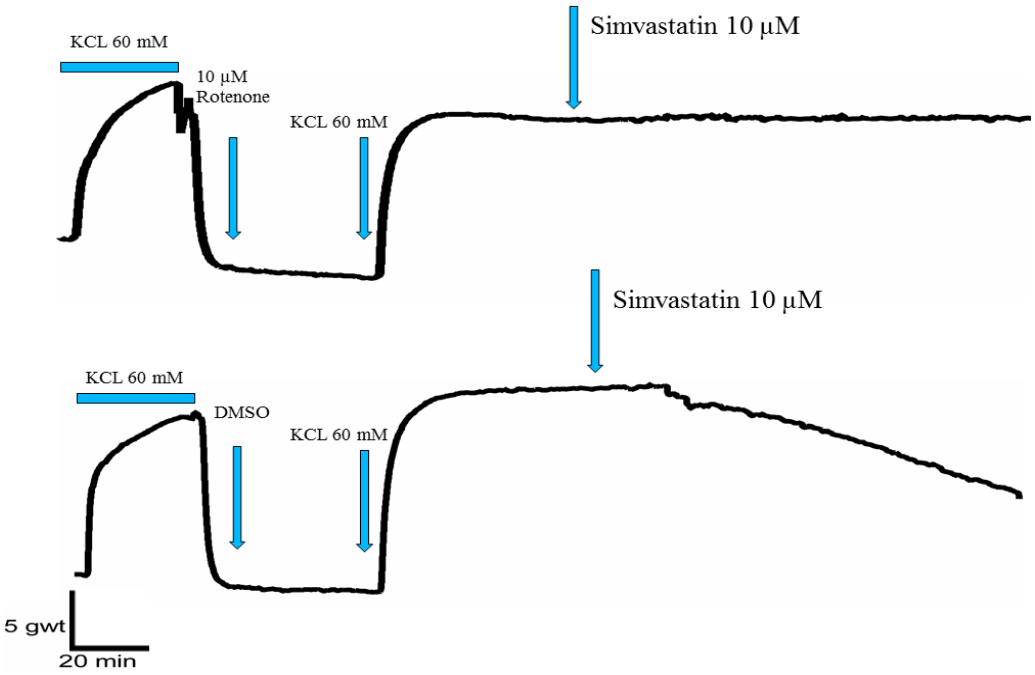


Figure 3.9. (A) Acute effects of simvastatin (10 µM) in porcine coronary artery rings precontracted submaximally with KCl in the presence or absence of rotenone (10 µM). Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 9 different experiments. *** indicates $p < 0.001$, (2- way ANOVA followed by a Bonferroni post-hoc test) versus values obtained from the solvent control DMSO (0.1% v/v). **(B)** Original organ bath trace for simvastatin induced porcine coronary relaxation in the presence of rotenone (10 µM): KCl (60 mM) bath concentration were obtained for standardization, after about 10-15 min the tissue was washed out with Kreb-Henseleit, and then left to re-stabilize to baseline. rotenone (10 µM) was added to one segment and compared with the control segment (DMSO). Then another addition of KCl (60 mM) was added to stimulate tissue contraction before the addition of a single concentration of simvastatin (10 µM).

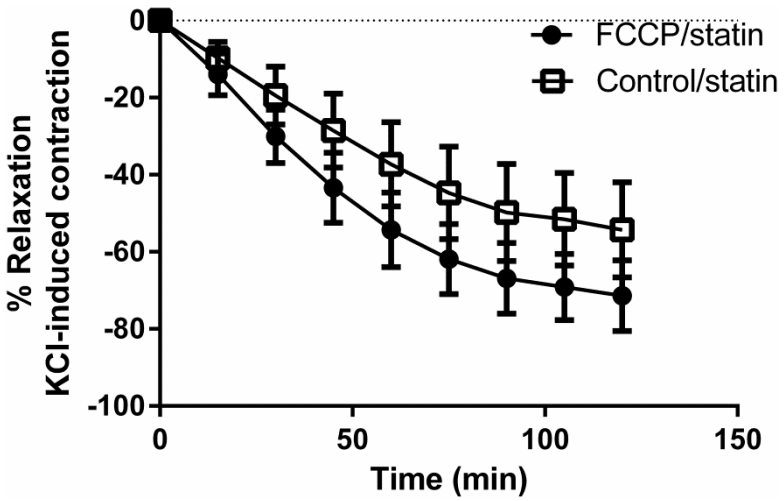


Figure 3.10. Acute effects of simvastatin (10 μM) in porcine coronary artery rings precontracted with KCl in the presence or absence of FCCP (1 μM). Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 4 different experiments. (2- way ANOVA followed by a Bonferroni post-hoc test).

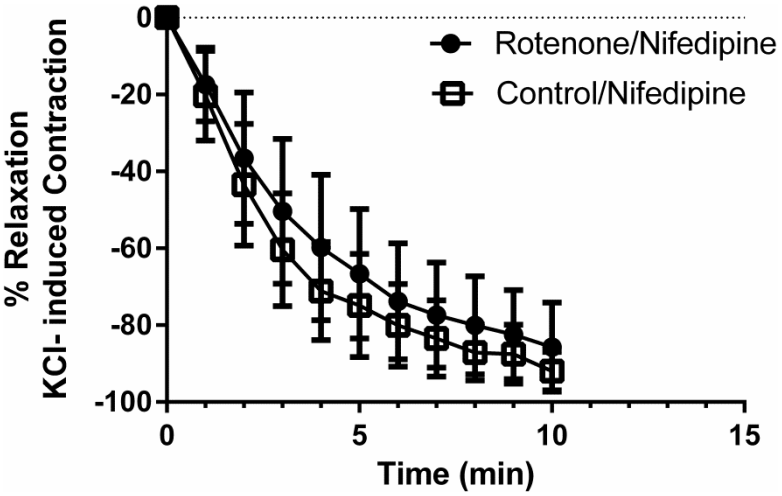


Figure 3.11. Effects of nifedipine (1 μM) in porcine coronary artery rings precontracted with KCl in the presence of (10 μM) rotenone. Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 5 different experiments. (2- way ANOVA followed by a Bonferroni post-hoc test).

Although the complex I inhibitor rotenone had no significant effect on the calcium-induced contraction in the porcine coronary artery (see chapter 4), it did partially prevent the inhibitory effect of simvastatin on the contractile responses in segments of porcine coronary artery to Ca^{2+} re-introduction in a Ca^{2+} free, high K^{+} (Figure 3.12).

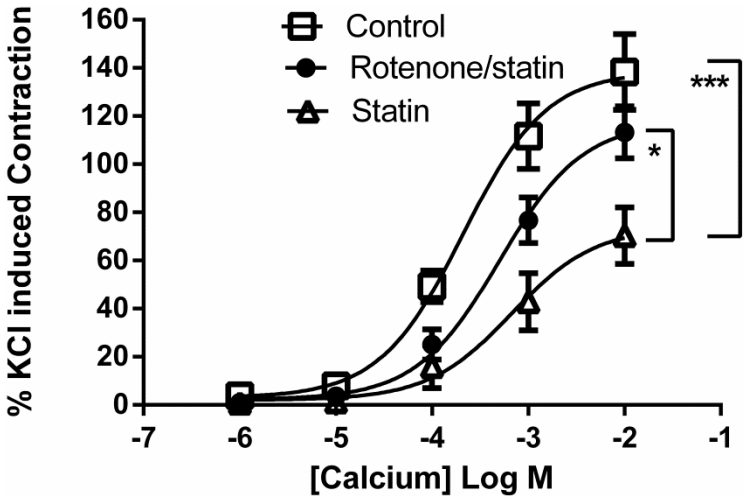


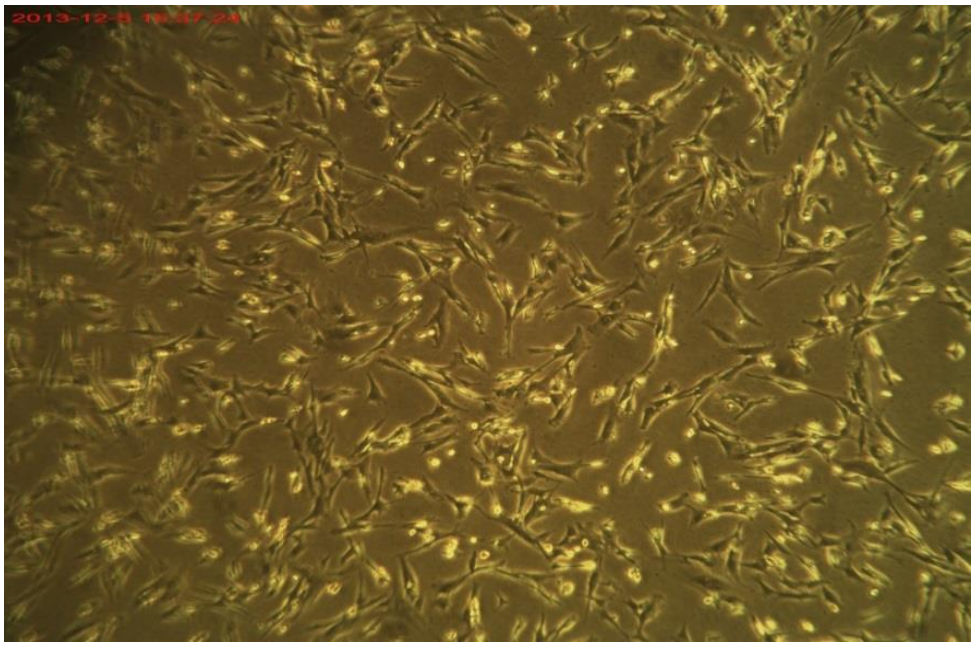
Figure 3.12. Contractile response to the reintroduction of calcium in a calcium free, high potassium Krebs' solution in the coronary artery in the presence of simvastatin and rotenone (10 μM) both for 1 h (n=6). Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM. * indicates P<0.05, *** indicates p < 0.001 (2- way ANOVA followed by a Bonferroni post-hoc test).

3.5.8. Simvastatin inhibited Ca^{2+} rise in isolated coronary porcine smooth muscle cells:

3.5.8.1 SMC in Cultures

Coronary smooth muscle cells grew like other smooth muscle cells in a 'hill-and-valley'-like pattern which is characteristic of smooth muscle cell growth (Figure 3.13). As soon as the cells became confluent, they were characterized by immunofluorescence staining for α-smooth muscle actin to differentiate smooth muscle cells from fibroblasts. The cells reacted intensely and the staining was exclusively in long, straight fibrils scattered densely throughout the cytoplasm. The nucleus stained with DAPI (Figure 3.14). However, using these cells, no change in intracellular Ca^{2+} could be detected in response to KCl-induced depolarization, thus the imaging experiments were done using freshly isolated coronary smooth muscle cells.

(A) SMC growth after 10 days



(B) SMC growth after 17 days

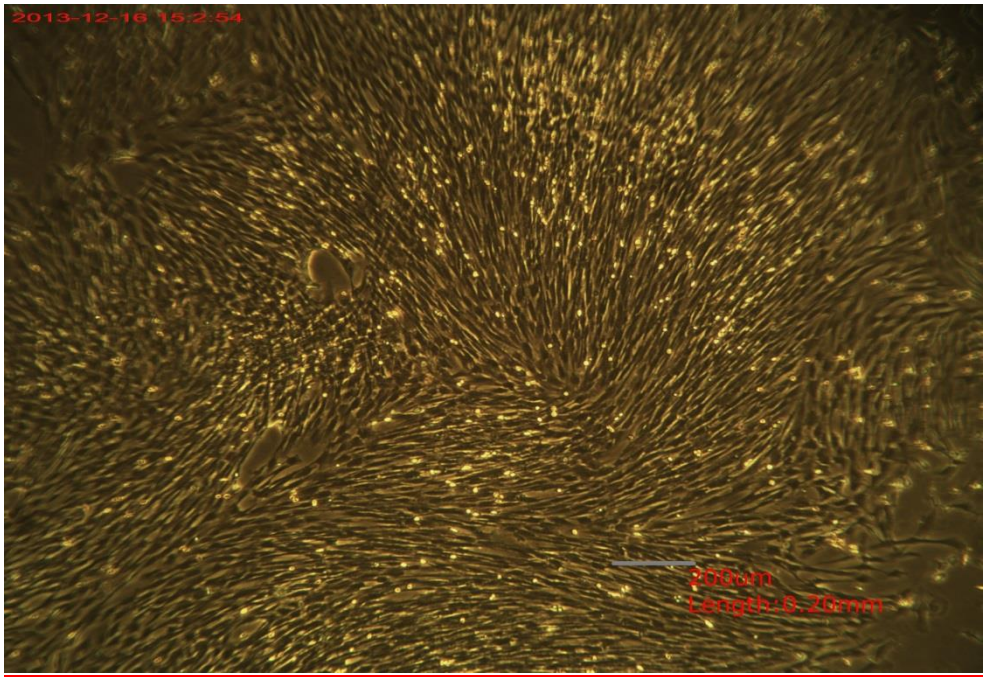


Figure 3.13. Coronary Smooth muscle cells in culture **A-** after 10 days **B-** after 17 days with a 'hill-and-valley'-like pattern which is characteristic of smooth muscle cell growth. Micrographs were captured with light microscope. The magnification is 10X.

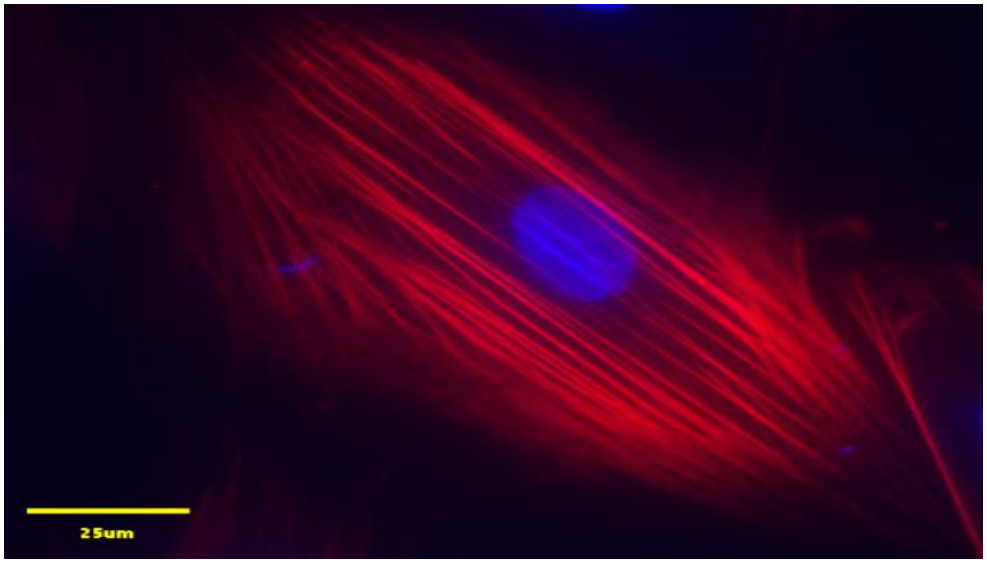


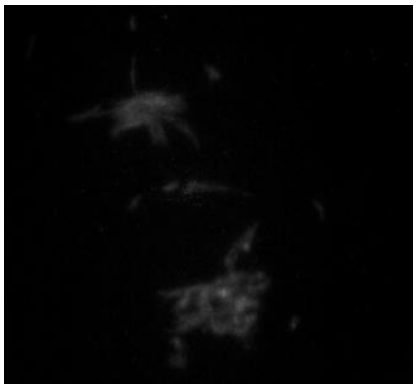
Figure 3.14. Immunofluorescent image for fixed α-smooth muscle cells actin grown from porcine coronary vessels, 3-weeks in culture. The cells were fixed in 4% PFA, and then immunohistochemistry performed. Micrographs were captured with DMRB Fluorescence Microscope Luca ^{EM}, magnification 63X, scale bar 25 μm.

3.5.8.1 Freshly isolated smooth muscle cells

Unlike cultured primary smooth muscle cells, freshly isolated smooth muscle cells produced an increase in intracellular calcium in response to KCl (figure 3.15 A). In the presence of 10 μM simvastatin, this response was significantly reduced (figure 3.15 B & C).

A)

1



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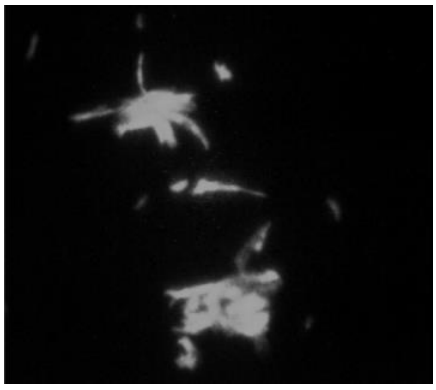

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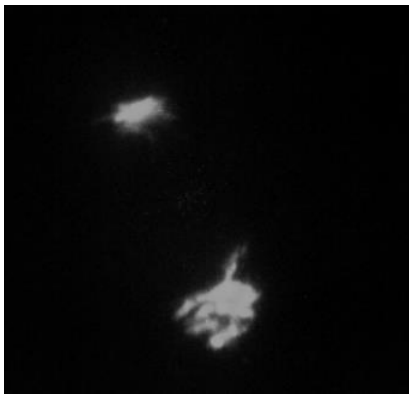
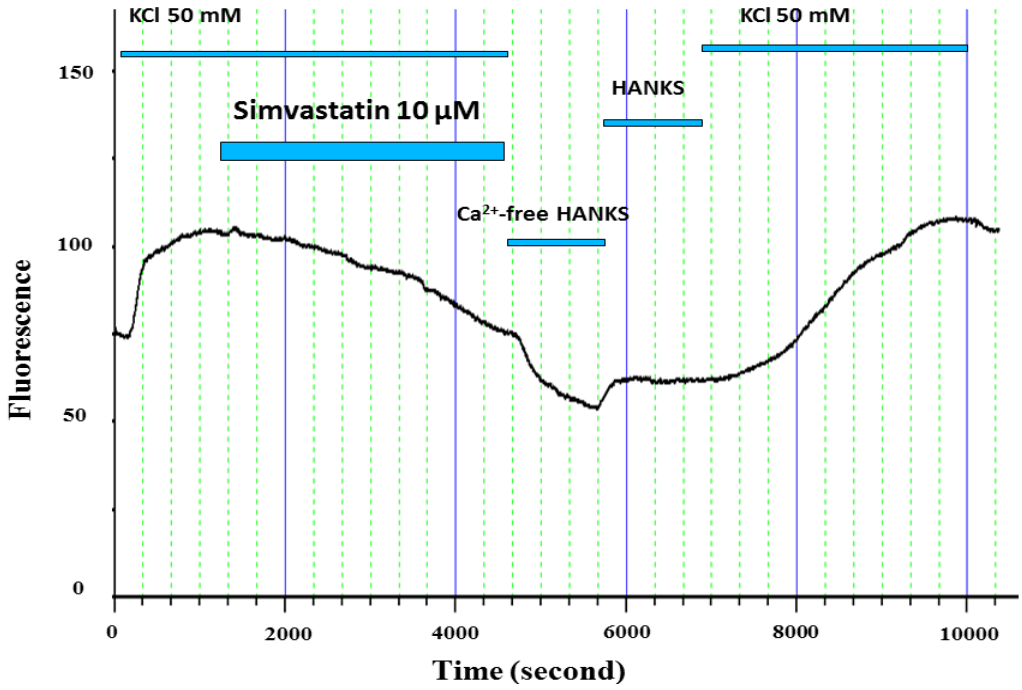


B)


KCl 50 mM
KCl 50 mM
HANKS
Simvastatin 10 µM
Ca2+-free HANKS
150
100
50
0
Fluorescence
0
2000
4000
6000
8000
10000
Time (second)

C)

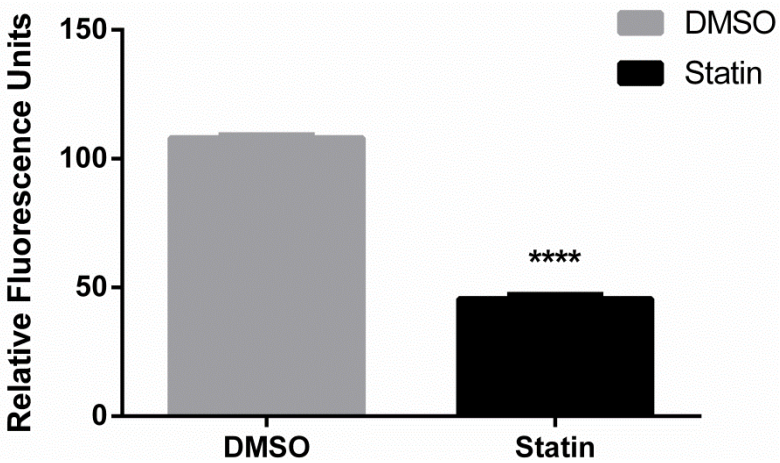


Figure 3.15. (A) Example images showing changes in fluo-4 fluorescence in freshly isolated coronary artery smooth muscle cells (cell clumps of 25–50 cells were often found). Before depolarization (basal fluorescence), after addition of 50 mM KCl (depolarized SMC), and after perfusion of 10 μM simvastatin. (**B)** time-response traces and **(C)** bar chart of mean data ± SEM from 239 (DMSO) and 291 (simvastatin) cells showing the changes in fluo-4 fluorescence in response to 50 mM KCl in the presence of 0.1 % v/v DMSO or 10μM simvastatin. **** indicates $p<0.0001$ v DMSO, 2-tailed, unpaired t-test.

3.6. Discussion

The present study provides evidence that simvastatin induces a concentration- dependent relaxation in the isolated proximal porcine coronary artery segments after pre-contraction with KCl depolarization. The relaxation is likely to arise from the ability of the simvastatin to inhibit Ca^{2+} influx. The effect of simvastatin on Ca^{2+} channels was supported by two observations. First, simvastatin inhibited coronary artery contraction in response to increasing concentrations of $CaCl_2$ in a Ca^{2+} free, high K^+ containing solution. Second, simvastatin hindered the increase in intracellular Ca^{2+} in vascular smooth muscle isolated from porcine coronary arteries, as indicated by fluo-4.

The relaxation induced by simvastatin after pre-contraction with KCl was similar to that seen after pre-contraction with U46619, and endothelin-1

(see chapter II), indicating that the relaxant effect of simvastatin was not due to an interaction with K^+ channels. Similarly, treatment with L-NAME did not affect simvastatin-induced responses, thus ruling out the involvement of NO. Agonist-induced calcium signal is provisioned by extracellular calcium influx through voltage operated calcium channel (VOCC), store-operated calcium channel (SOCC), or release from intracellular calcium store (Goto et al., 1989, Tosun et al., 1998b, Miwa et al., 2005, Pang et al., 2005). In this context, it is perhaps easier to hypothesize for KCl stimulation to narrow down the mechanism of calcium blockade, since there is a general consensus for KCl mechanism of activation of contraction (Ratz et al., 2005). Elevation of K^+ is known to activate L-type Ca^{2+} channels with an increase in Ca^{2+} via membrane depolarization in vascular smooth muscle (Fransen et al., 2012). Effects of Ca^{2+}-induced Ca^{2+} release by the sarcoplasmic reticulum might also be involved (Fernandez-Tenorio et al., 2011). However, incubation with CPA, a Ca^{2+} ATPase inhibitor (Seidler et al., 1989) had no effect on simvastatin relaxation, ruling out an effect of simvastatin on calcium uptake into intracellular stores as a possible mechanism for relaxation (Zheng et al., 1993, Moritoki et al., 1996). Consistent with our data, Bergdahl *et al* study provided evidence that lipophilic statins inhibit L-type Ca^{2+} current and attenuate contraction in cerebral vascular smooth muscle (Bergdahl et al., 2003). Similarly, simvastatin inhibited Ca^{2+} increase by KCl in rat islet pancreatic β-cells (Yada et al., 1999). Furthermore, simvastatin, cerivastatin, and fluvastatin blocked the hypoxia-induced Ca^{2+} rise in human umbilical cord endothelial cells (Schaefer et al., 2006). However, simvastatin (50 μM)-treated rat aortic smooth muscle cells showed a slight increase in basal Ca^{2+} compared with control (Tesfamariam et al., 1999), which could be related to the high concentration of simvastatin used.

On the other hand, incubation of human umbilical vein endothelial cells for 24 hours with cerivastatin or fluvastatin (1 μM) significantly increased the resting Ca^{2+} (Heinke et al., 2004). Similarly Lorkowska (2004) proved that incubation of bovine aortic endothelial cells with atorvastatin, cerivastatin, simvastatin, lovastatin, but not pravastatin, at a concentration of 10–30 μM, induce an immediate increase in Ca^{2+} and such effect may correlate with the immediate release of NO and PGI_2 by these drugs (Luckhoff et al., 1988, Lorkowska et al., 2004). Collectively, such findings suggest that vascular effects of simvastatin involve both mobilization of Ca^{2+} from intracellular stores and/or extracellular Ca^{2+} entry via membrane cation channels (Su et al., 2014), which may be induced as a consequence of intracellular Ca^{2+} store depletion (Alvarez de Sotomayor and Andriantsitohaina, 2001). On the other hand, blockade of extracellular Ca^{2+} entry into smooth muscle. The latter promotes relaxations independent of the presence of endothelium (Alvarez de Sotomayor et al., 2001). The data in isolated smooth muscle confirmed that simvastatin inhibits the increase in intracellular calcium as a result of exposure to KCl. It is worth mentioning that Ca^{2+} influx could not be elicited in cultured porcine vascular smooth muscle cells by KCl-induced depolarization, suggesting that the culturing conditions result in loss of calcium channel expression (Kuga et al., 1996, Gollasch et al., 1998). The same phenomenon has been reported previously (Clunn et al., 1997). Therefore, freshly isolated cells were used for measuring changes in intracellular calcium levels.

The mechanism by which statins may inhibit Ca^{2+} influx is not clear; the high K^+ depolarization was completely abolished by a selective L-type Ca^{2+} channel blocker, nifedipine (1 μM). However, the same concentration of nifedipine failed to inhibit the simvastatin relaxation in U-46619 pre-contracted arteries. Such effect could point to the indirect inhibition of Ca^{2+} influx by simvastatin.

NO may inhibit calcium efflux (Collins et al., 1986, Blatter and Wier, 1994). However, this study has shown that NO inhibition had no effect on simvastatin relaxation, thus ruling this out as a mechanism.

Rho-kinase can be seen as a major player for receptor-mediated, but not for KCl stimulation (Nobe and Paul, 2001). It is worth mentioning that voltage-gated calcium channels not only mediate the Ca^{2+} influx that triggers contraction, but also metabotropically regulate intracellular Ca^{2+} release and RhoA activation to sensitize the contractile machinery (Fernandez-Tenorio et al., 2011, Urena and Lopez-Barneo, 2012). Thus, the involvement of Rho in vasorelaxation mediated by simvastatin cannot be discounted, and it remains to be investigated.

Previous studies have shown that pre-incubation with mevalonate restored the $CaCl_2$ -induced contraction (Alvarez de Sotomayor et al., 2001) and reverse the changes induced by simvastatin on Ca^{2+} signal after stimulation with noradrenaline (Alvarez de Sotomayor et al., 2001) and vasopressin (Ng et al., 1994). This study has shown that mevalonic acid incubation for one hour had no effect on simvastatin relaxation, suggesting that the acute effects of simvastatin involves direct actions at Ca^{2+} pools unrelated to mevalonate products (Bergdahl et al., 2003). Longer incubation, up to 48 h with mevalonic acid might have different effects which in turn might explain the discrepancy with other studies (Clunn et al., 1997). However, Escobales *et al* have shown that the inhibitory effect of simvastatin was reduced 50% by 30 min incubation with mevalonic acid in rat aortic rings (Escobales et al., 1996b).

Membrane cholesterol plays a role in regulating L-type calcium current, both under basal conditions and in response to selected agonists (Bialecki et al., 1991). Several published observations seem to refute the possibility that the acute effects of simvastatin on vascular tone and Ca^{2+} influx are due to reduced cholesterol content. For examples, cyclodextrin reduced the

arterial contents of cholesterol by 20%, but was without any effect on contraction induced by depolarisation (Lohn et al., 2000). Enrichment of cholesterol, on the other hand, leads to increased L-type current (Sen et al., 1992), which may involve incorporation of cholesterol into membrane domains where it is not normally present (Sen et al., 1992). Thus, inhibition of Ca^{2+} influx by simvastatin is likely to be independent of cholesterol biosynthesis.

Acute application of simvastatin on human skeletal muscle fibres triggers a large release of Ca^{2+} from the SR as an early event for myotoxicity. The Ca^{2+} release results from alterations in mitochondrial function with subsequent mitochondrial membrane depolarization and Ca^{2+} efflux to the cytoplasm (Sirvent et al., 2005b). Mitochondria appeared as the cellular structure responsible for the earlier event leading to a subsequent large sarcoplasmic reticulum Ca^{2+} release (Liantonio et al., 2007, Pierno et al., 2009). Because of the structural proximity between the mitochondria and the sarcoplasmic reticulum, mitochondria ensure efficient sarcoplasmic reticulum refilling and minimize cytosolic Ca^{2+} elevations (Poburko et al., 2009, Restini et al., 2006). Furthermore, the principal internal Ca^{2+} stores within vascular smooth muscle cells are the sarcoplasmic reticulum (SR) and mitochondria (Drummond and Fay, 1996). Accordingly, mitochondrial inhibition produces a small, transient increase in Ca^{2+} as a result of mitochondrial Ca^{2+} efflux. On the other hand, mitochondrial inhibition reduces the amount of Ca^{2+} released by IP_3 (McCarron and Muir, 1999). Indeed, IP_3 receptors became inoperative during mitochondrial depolarization and were reversible upon repolarization of mitochondria, the latter is considered as a protective mechanism to prevent Ca^{2+} dependent apoptosis (Collins et al., 2000). A previous study by Escobales *et al* could not prove the precise mechanism by which simvastatin releases calcium from IP_3-sensitive pools (Escobales et al., 1996a). Our study suggests that

simvastatin effects on vascular smooth muscle involve Ca^{2+} release from intracellular stores by mitochondrial depolarization while on the same time blockade of extracellular Ca^{2+} entry. However, the involvement of mitochondria needs more studies to confirm the hypothesis.

Application of the complex I inhibitor rotenone (10 μM) completely inhibited Ca^{2+} waves with accompanied increase in basal Ca^{2+} in interstitial cells from rabbit urethra (Sergeant et al., 2008). Rotenone (10 μM) also reduced Ca^{2+} spark and wave frequency and elevated global Ca^{2+} in smooth muscle cells of intact arteries (Cheranov and Jaggar, 2004) and induced a rapid increase in Ca^{2+} in the presence of extracellular Ca^{2+} but not in the absence of extracellular Ca^{2+} (Wyatt and Buckler, 2004). This suggests that mitochondrial inhibition might influence Ca^{2+} wave activity, possibly due to a close spatial relationship of mitochondria and the sarcoplasmic reticulum. On the other hand, It has been found that rotenone inhibits dilation in human coronary vessels by suppression of $O^{-\bullet}$ and H_2O_2 formation (Liu et al., 2003). This mechanism has been discussed in chapter I.

In the present study, rotenone prevented the inhibitory effect of simvastatin on the calcium-induced contraction (figure 3.12). Rotenone also inhibited the relaxation to simvastatin after pre-contraction to KCl (figure 3.9) which is likely to be due to inhibition of calcium influx. Previous study has shown that rotenone inhibited acetylcholine-induced, endothelium-dependent relaxation of rat and rabbit aorta by inhibiting NO production (Weir et al., 1991). However, in our study, NO synthase inhibition with L-NAME had no effect on relaxation induced by simvastatin, ruling this out as a mechanism. Furthermore, uncoupling of the mitochondria with FCCP had no effect on simvastatin relaxation in KCl pre-contracted vessels (figure 3.10) indicating that the inhibitory effect of rotenone is unlikely to be due to a non-selective effect of inhibition of

mitochondrial function. It is also worth mentioning that the relaxation induced by nifedipine on KCl-contracted coronary rings was unaffected, unlike simvastatin, by rotenone incubation. This provides further support to the suggestion that the inhibitory effect of rotenone on the simvastatin relaxation is not due to a non-specific effect of rotenone.

A Previous study has shown that reactive oxygen species generated from complex III of the mitochondria result in Ca^{2+} channel inhibition in cardiac cells (Scragg et al., 2008), other studies have shown that oxidized LDL enhance Ca^{2+} current via mitochondrial reactive species(Fearon, 2006). These differential responses may relate to the concentration and type of ROS, species differences and experimental conditions. The locus of increased superoxide was suggested to be beyond complex I. To examine whether Complex III was the site of enhanced superoxide production pharmacological inhibition of the individual mitochondrial complexes was used, inhibition of the respiratory chain by rotenone or myxothiazol block electron flow at the Q_o superoxide site of complex III (Viola and Hool, 2010). Both compounds successfully attenuated the increase in superoxide, thus inhibit complex III ROS generation and prevent the effects of ROS on Ca^{2+} channels. Therefore inhibition of complex III by simvastatin would no longer have any effect.

The data presented here suggest that simvastatin acts as inhibitor of complex III. If the effects of simvastatin are mediated through inhibition of complex III, then the effects of simvastatin on vascular tone should be mimicked by a complex III inhibitor. Thus, the effects of the mitochondrial inhibitors on the precontracted porcine coronary segments, with special reference to antimycin A as complex III inhibitor, will be investigated in the next chapter.

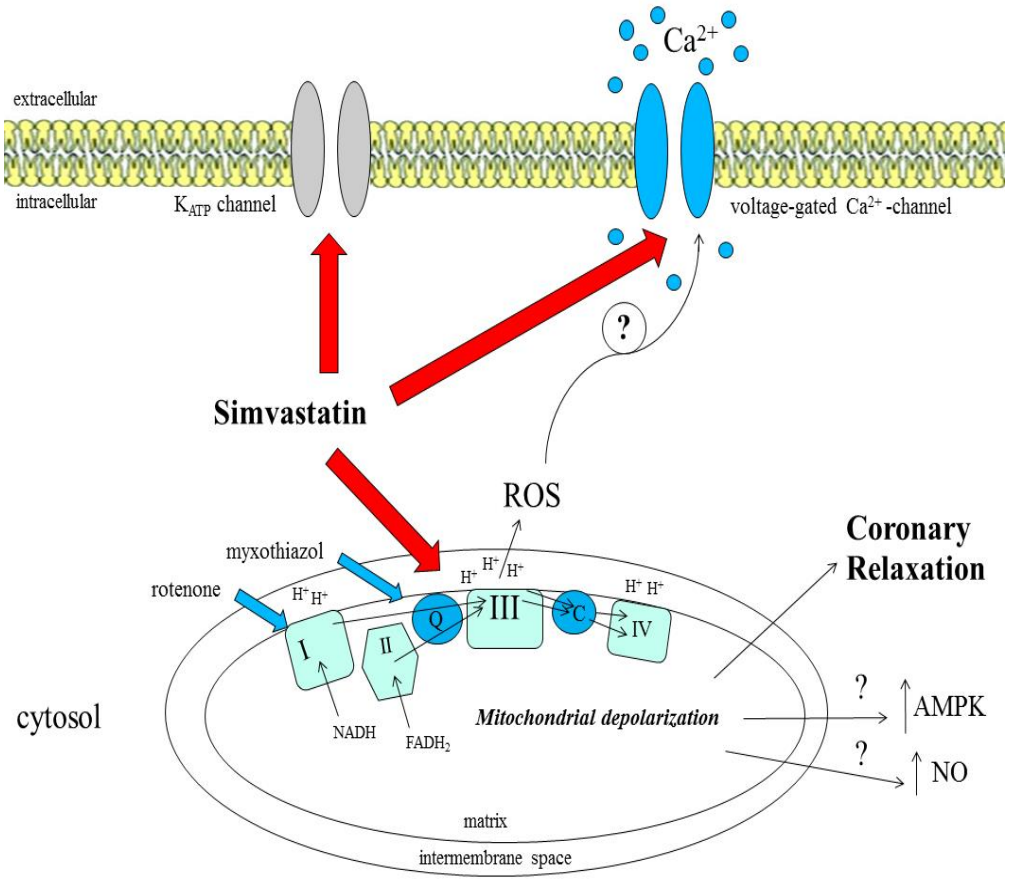


FIGURE 3.16. Role of mitochondria in simvastatin induced porcine coronary relaxation. The three complexes of the mitochondrial (inner) membrane that form the proton gradient (*I, III,* and *IV*) are indicated, and two established inhibitors of electron flow are shown, rotenone and myxothiazol. The sites of simvastatin action proposed by our study, as indicated by red arrow, are mitochondrial complex III thereby causing mitochondrial depolarization. Furthermore simvastatin inhibits Ca^{2+} influx with subsequent relaxation. Mitochondrial depolarization may be the upstream for AMPK stimulation and NO production proposed by other studies. Simvastatin blocks K_{ATP} channels thus they have no role in simvastatin relaxation. Our proposal includes also ROS generation as a result of complex III inhibition, in-turn ROS may inhibit Ca^{2+} channels (as indicated by *?*), however more studies are recommended to confirm the mechanism of simvastatin induced Ca^{2+} channels inhibition.

CHAPTER IV: Effect of complex III inhibition with antimycin A on porcine coronary tone: mechanism of action & comparison with other mitochondrial inhibitors

4.1 Introduction

Traditionally mitochondria are known as the energy generating centre of the cells, However, there is growing consensus demonstrating that mitochondria actively participate in intracellular signalling, such as production of ROS and regulation of the intracellular Ca^{2+} concentration (Michelakis et al., 2002). The process of ROS generation is tightly regulated (Sena and Chandel, 2012). In the mitochondrial matrix, superoxide ($O_2^{\cdot -}$) is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. H_2O_2 then diffuses across the mitochondrial membranes. In the cytosol H_2O_2 can be further be metabolized to water (H_2O) by glutathione peroxidases (Zhang and Gutterman, 2007, Xi et al., 2005). Mitochondrial superoxide is mainly produced by complexes I and III of the electron transport chain (Chen et al., 2003). The oxidant generation can be modulated by mitochondrial potential and intracellular Ca^{2+} (Camello-Almaraz et al., 2006a). Antimycin A (a complex IIIQi inhibitor) is known to increase $O_2^{\cdot -}$ generation, and myxothiazol (a complex IIIQo inhibitor) has the same effect (Starkov and Fiskum, 2001). With rotenone (a complex I inhibitor) this point is controversial. While inhibition of complex I with rotenone reduced mitochondrial ROS in intact human coronary arterioles (Liu et al., 2003), increased mitochondrial ROS was observed in isolated mitochondria (Andreyev et al., 2005). On the other hand, uncoupling oxidative phosphorylation with (FCCP) reduces $O_2^{\cdot -}$ production (Saitoh et al., 2006).

The increase in ROS production has been considered a pathological response since excess $O_2^{\cdot -}$ generation influences vasomotor tone by quenching NO. However, recent findings suggest that ROS can play an important supportive role in the control of a large variety of vascular cell signalling processes including activation of nitric oxide synthase (NOS),

modulation of intracellular Ca^{2+}, and AMP kinase activation (Cardaci et al., 2012). AMP kinase in turn can increase NO bioavailability (Rossoni et al., 2011) and modulates vascular tone (Mackenzie et al., 2013). In small coronary arterioles, complex I and III –derived H_2O_2 is found to be responsible for flow-mediated vasodilatation that is independent of NO (Liu et al., 2003, Cai, 2005). Rotenone and myxothiazol markedly attenuated flow-induced dilation and associated $O_2^{\cdot -}$ and H_2O_2 generation in the coronary circulation (Liu et al., 2003). On the other hand, mitochondrial depolarization using nanomolar concentrations of the protonophore CCCP induces ROS elevation with an increase in calcium spark frequency, which in turn stimulates calcium-activated K^+ channels, resulting in cerebral artery vasodilation (Xi et al., 2005). By contrast, marked depolarization using micromolar CCCP or rotenone reduces calcium spark activity thereby reducing opening of calcium-activated potassium channels (Cheranov and Jaggar, 2004, Xi et al., 2005).

Previous studies have demonstrated that inhibiting the mitochondrial electron transfer chain leads to vasodilatation (Qamirani et al., 2006, Hao et al., 2006, Sward et al., 2002, Weissmann et al., 2003). However, other studies have demonstrated opposing actions in different blood vessels e.g. inhibition of complex I constricted the pulmonary artery, but stimulated dilation of the renal artery (Michelakis et al., 2002). Such differences of the mitochondrial inhibitors on the vascular tone could be related to mitochondrial diversity between different vascular beds (Michelakis et al., 2002).

It is now well recognised that the large negative potential across the inner membrane (up to −180 mV) accumulates Ca^{2+} very efficiently and rapidly (McCarron et al., 2013, Szabadkai and Duchen, 2008). The uptake of Ca^{2+} into mitochondria is proposed to stimulate mitochondrial ATP production, thereby providing a means for matching increased energy demand. Despite

reports of their high-capacity and low affinity for Ca^{2+}, mitochondria are also accepted as being important regulators of intracellular Ca^{2+} concentration, thereby regulating smooth muscle tone. Mitochondria could be transiently exposed to local Ca^{2+} concentrations much higher than those measured in the bulk cytosol sufficient to activate their low-affinity Ca^{2+} uptake (McCarron et al., 2012). In arterial smooth muscle cells, the results suggested that mitochondrial Ca^{2+} uptake is important over low Ca^{2+} range (Kamishima and Quayle, 2002).

Chapters 2 and 3 have indicated that simvastatin produces a relaxation response, which we hypothesise is through an effect on complex III of the mitochondria. If this is the case, then inhibition of complex III should produce similar effects. The direct impact of the mitochondrial inhibitors on the vascular tone of the coronary vessels is unknown. Therefore the goals of the present chapter were to determine the effects of antimycin A as a complex III inhibitor, in comparison with other mitochondrial inhibitors, on the vascular tone of the isolated porcine coronary artery and to elucidate the underlying signalling pathways involved.

4.2. Materials and mehods

4.2.1. Materials

Rotenone, antimycin A, 3-nitropropionic acid (3-NP), 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TTFA), FCCP, oligomycin, TEA, 4-aminopyridine, ebselen, PEG-SOD, PEG-catalase, diethylthiocarbamate (DETCA), U46619, indomethacin, Rb^+, fluo-4 AM, bovine serum albumin (BSA) were obtained from Sigma-Aldrich, Poole, UK. Antibodies specific to AMPKα and total AMPK (New England Biolabs, Hitchin, UK).

4.2.2. Solutions

Krebs-Henseleit buffer (NaCl 118, KCl 4.8, $CaCl_2.H_2O$ 1.3, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4.7H_2O$, glucose 11.1 in (mM). Hanks buffer containing (in mM): 5.6 KCl, 138 NaCl, 4.2 $NaHCO_3$, 1.2 NaH_2PO_4, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose and 10 HEPES [pH 7.4]. Homogenisation solution: 80 mM sodium β-glycerophosphate, 20 mM imidazole, 1 mM dithiothreitol (DTT), 1 mM sodium fluoride (NaF) [pH 7.6]. Laemmli sample buffer (4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (v/v) bromophenol blue and 0.125 M Tris HCl at pH 6.8). Electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS in distilled H_2O at pH 8.3). Transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol in distilled H_20 at pH 8.3). Tris-buffered saline [25 mM Tris, 125 mM NaCl to pH 7.6 in distilled H_2O].

4.2.3. Data Analysis

The relaxation responses were expressed as a percentage change from the U46619- or KCL induced pre-contraction over time. Values for all figures refer to mean ± SEM. Relaxation responses over time were compared by using two-way analysis of the variance (ANOVA) followed by a Bonferroni

post-hoc test. Differences were considered to be significant when the P value was < 0.05. Contractile responses to calcium chloride were expressed as a percentage of the 60 mM KCl response. Changes in fluo-4 fluorescence were analysed by unpaired Student's t-test.

4.2.4. Method

Porcine hearts from pigs of both sexes were obtained from a local abattoir and transported back to the laboratory in ice-cold Krebs-Henseleit buffer (NaCl 118, KCl 4.8, $CaCl_2.H_2O$ 1.3, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4.7H_2O$, glucose 11.1 in (mM) gassed with a mixture of 95% O_2 and 5% CO_2). The anterior proximal descending branch of the coronary artery was dissected out and cleaned of fat and connective tissues and set up for isometric tension recording (see chapter 2). Tissues were pre-contracted with cumulative additions of U46619 (a thromboxane A_2-mimetic) to about 50-70% of the second KCl response before addition of a single concentration of antimycin A (3 μM to 10 μM), myxothiazol, rotenone, 3-NP, oligomycin, (all 10 μM) and FCCP (1 μM), and the tone measured for 2 hours. Control tissues contained vehicle only (0.1% v/v DMSO). In separate experiments, the effect of antimycin A after pre-contraction with KCl was determined.

In order to determine the role of endothelial factors on the effects of antimycin A, tissues were incubated with the nitric oxide synthase inhibitor, L-NAME (300 μM), or the cyclo-oxygenase inhibitor indometacin (10 μM) for 20–30 minutes prior to pre-contraction with U46619. The role of AMP kinase inhibition on the effects of antimycin A was also determined by pre-incubation with dorsomorphine (10 μM). In some experiments, to evaluate the role of K^+ channels, the segments were pre-incubated with the non-selective K^+ channel blocker tetraethylammonium (TEA; 10 mM),

glibenclamide 3 μM or the K_v channel blocker 4-aminopyridine (1 mM) for 60 minutes prior to addition of U46619.

4.2.4.1 Role of reactive oxygen species in antimycin A-induced relaxation

The role of reactive oxygen species (ROS) in the relaxation response to antimycin A was investigated by pre-incubation with ebselen, a scavenger of hydrogen peroxide (3 μM), and PEG-superoxide dismutase (100 Uml^{-1}) which metabolises superoxide free radicals. In a separate set of experiments, tissues were incubated with diethylthiocarbamate (DETCA), which inhibits superoxide dismutase (10 mM).

4.2.4.2. Effect of antimycin A on calcium-induced contractions

To assess the effect of antimycin A on calcium influx, a concentration–response curve for $CaCl_2$ was constructed. Arteries were incubated for 1 h in Ca^{2+}-free Krebs-Henseleit buffer with antimycin A 10 μM. Tissues were then exposed to 60 mM KCl and then increasing concentrations of $CaCl_2$ (1 μM to 3 mM) added to induce a contraction. In another set of experiments, tissues were incubated with rotenone, myxothiazol, oligomycin (10 μM) or FCCP (1 μM) in place of antimycin A.

4.2.4.3. Effect of antimycin A on calcium influx in freshly isolated smooth muscle cells.

Coronary smooth muscle cells were isolated from the proximal coronary artery segments by incubating with type II collagenase (2 mg/mL) for 30 min at 37 ºC and plated on coverslips. The cells were then loaded with 5 μmol/L fluo-4 AM for 1 hour at room temperature in HEPES-buffered Hanks solution (same as chapter 3). Coverslips were then superfused with Hanks buffer at 37°C for 5 min for basal recording. The Hanks buffer was then replaced with high K^+ (50mM) Hanks containing which elicited a sustained

increase in the fluorescence ratio. Subsequently, antimycin A 10 μM or DMSO (0.1% v/v) as a control were added to the perfusion media.

4.2.4.4. Atomic absorption spectrophotometric determination of $3Na^+/2K^+$ ATPase activity

Rubidium-uptake was measured with atomic absorption spectrophotometry (Perkin-Elmer) to study the sodium-potassium pump activity as previously described (Ferrer-Martinez et al., 1996). Porcine coronary segments (PCA) were cleaned of adherent connective and fatty tissues. The segments were cut open longitudinally, ~1.5 cm in length. They were placed into 6-well plates pre-filled with 3 ml Krebs-Henseleit solution previously gassed with 5% CO_2 and 95% O_2. Test agents 100 nM ouabain, 10 μM antimycin A or DMSO were added into the respective wells. Plates were incubated at 37°C in a shaker for 1 h. After complete incubation, Krebs solution was replaced with K^+-free Krebs'-Henseleit solution containing 4 mM RbCl with respective inhibitors and incubated at 37 °C in a shaker for an additional 30 minutes. Arteries were then rapidly washed three times with ice-cold 0.2 M $MgSO_4$ then stored at -20 °C until further assay. Frozen segments were fixed with 2 mL fixative (containing 50% ethanol, 49% distilled water and 1% acetic acid) and left to evaporate in a fume hood overnight. Intracellular Rb^+ was then extracted in 2 mL of distilled water and the Rb^+ content was determined with flame photometry on an atomic absorption spectrometer using wavelength of 780 nm. Rb^+-uptake levels were interpolated from a standard Rb^+ curve and individual PCA reading was divided by the wet weight of the respective artery segment.

4.2.4.5. Measurement of AMP Kinase activation

4.2.4.5.1. Tissue preparation

The phosphorylation state of AMP Kinase was measured using Western immunoblotting. Porcine coronary artery segments were set up in 5 ml organ baths connected to a force transducer for isometric tension recording. The baths contained Krebs-Henseleit solution and were connected to a thermostat to maintain the temperature at 37°C and constantly gassed with carbogen (95% O_2, 5% CO_2). After two consecutive KCl (60 mM) challenges, arteries received one of the following treatments: U46619 (20 – 30 nM), in order to achieve 60–85% of the maximal KCl contraction. After a steady contractile tone was achieved, antimycin A was added to a final concentration of (10 μM). DMSO as a solvent control was added to the second channel. Timing of drug treatment was 2 h which is consistent with other antimycin A experiments. Control arteries were removed at the same time. The segments were frozen rapidly by placing the arteries on dry ice. Finally, the arteries were stored at -80ºC. On the day of the experiments, the rings were chopped into smaller pieces using scissors and homogenised for approximately 30 seconds (FisherBrand 0.1 ml glass-glass homogeniser) in ice-cold buffer containing 80 mM sodium β-glycerophosphate, 20 mM imidazole, 1 mM dithiothreitol (DTT), 1 mM sodium fluoride (NaF) [pH 7.6] with a protease inhibitor cocktail (Calbiochem).

4.2.4.5.2. Bradford Protein Assay

A fraction of each homogenate was used to estimate protein concentration using the Bradford protein assay. Firstly, a standard concentration curve of bovine serum albumin (BSA) was prepared using the following

concentrations: 2 mg ml^{-1}, 1 mg ml^{-1}, 0.5 mg ml^{-1}, 0.25 mg ml^{-1} and 0.125 mg ml^{-1}. The diluent used contained homogenisation buffer at concentrations listed above. 10 µl of each sample was loaded in pairs onto a 96-well plate, along with each concentration of BSA and a pair of blanks (diluent only). Each well was topped up with 40 µl BioRad protein assay dye reagent concentrate and 150 µl distilled water. Absorbance readings, measured at 595 nm, were generated using a microplate reader and used to estimate the concentration of protein in each sample. These estimates in turn were used to determine the volume of each homogenate sample to be used in the next stage of the experiment.

4.2.4.5.3. Western immunoblotting

The remainder of the homogenised samples were diluted (1:1) in Laemmli sample buffer (4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (v/v) bromophenol blue and 0.125 M Tris HCl at pH 6.8) and heated at 95ºC for 5 min. Equal amounts of each sample (10 µg protein) were carefully loaded into wells of pre-cast 4-20% (w/v) acrylamide SDS-PAGE gels. Loaded gels were slotted into an electrode tank containing electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS in distilled H_2O at pH 8.3). A potential difference of 150 V was placed across the gel for 40 min, causing protein separation. Protein was transferred from gel to nitrocellulose membrane (GE Healthcare Life Sciences, Amersham, UK) using a mini Transblot (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) immersed in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol in distilled H20 at pH 8.3). A voltage of 100 V was applied for 60 min.

To prevent non-specific attachment of antibodies to the nitrocellulose membrane, the membrane was 'blocked' by incubating for 60 min in 5% (w/v) milk solution (Marvel skimmed milk powder dissolved in Tris-

buffered saline [25 mM Tris, 125 mM NaCl to pH 7.6 in distilled H_2O] containing 0.1% Tween-20 (TBS Tween). Membranes were incubated overnight at 4°C in 5% BSA (w/v) solution containing a 1 in 1000 dilution of "primary" antibodies specific to AMPKα and total AMPK (Cell Signaling Technology, UK).

The following morning, membrane blots were washed in TBS Tween for 1 hour and then incubated at room temperature for 60 min in 5% (w/v) milk solution containing IR dye taged "secondary" antibodies (1 in 10,000 dilution) raised in goat against mouse for AMPK and rabbit for phosphorylated AMPK (LI-COR Biotechnology Ltd., Cambridge, UK). Blots were washed for 1 hour in TBS Tween again, and then washed in distilled water. Finally, the blots were then scanned at 700 nm and 800 nm wavelengths using an Odyssey Infrared Imaging System (LI-COR Biotechnology Ltd., Cambridge, UK). Odyssey software (LI-COR Biotechnology Ltd., Cambridge, UK) was used to analyse the optical densities and molecular weights of the visualised bands. In order to determine if there was a difference in the level of phosphorylation of AMP kinase, the density of the bands to phospho-AMP kinase were expressed as a ratio of the bands to total AMP kinase. Statistical significance between control and antimycin A-treated vessels was determined using a 2-tailed, unpaired Student's t-test with $p<0.05$ determining significance.

4.3. Results

4.3.1. Acute effects of antimycin A and myxothiazol on porcine coronary artery tone

As shown in figure (4.1 & 4.2), antimyci*n* A (1 μM & 10 μM) caused essentially immediate relaxation of the porcine coronary artery pre-contracted with U46619 reaching a maximum response at around 100 minutes. Myxothiazol also produced relaxation of the porcine coronary artery, although the response was slower to develop (figure 4.4). DMSO as a vehicle control had no effect. Pre-constriction with KCl instead of U46619 attenuated the relaxation response to both antimycin A and myxothiazol (figure 4.3 and 4.4 respectively).

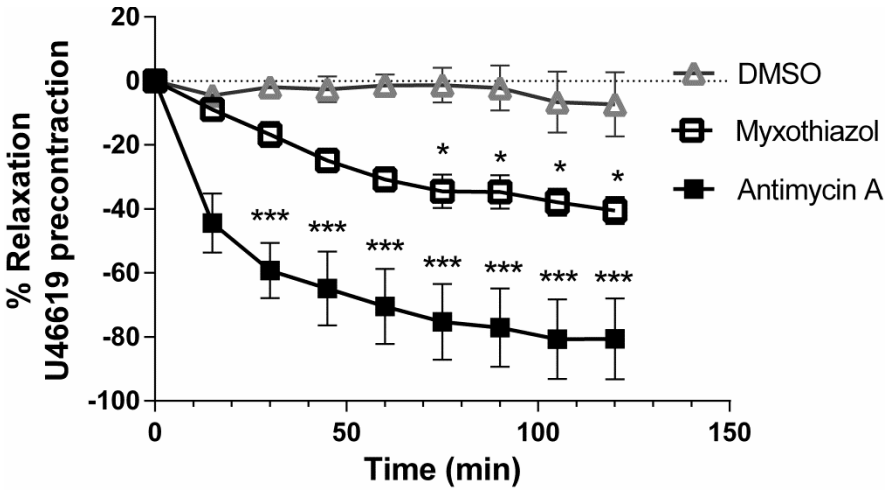


Figure 4.1. Relaxation responses to antimycin A and myxothiazol (both 10 μM) in segments of porcine coronary artery precontracted submaximally with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different experiments. * indicates P<0.05 and *** indicates P<0.001.

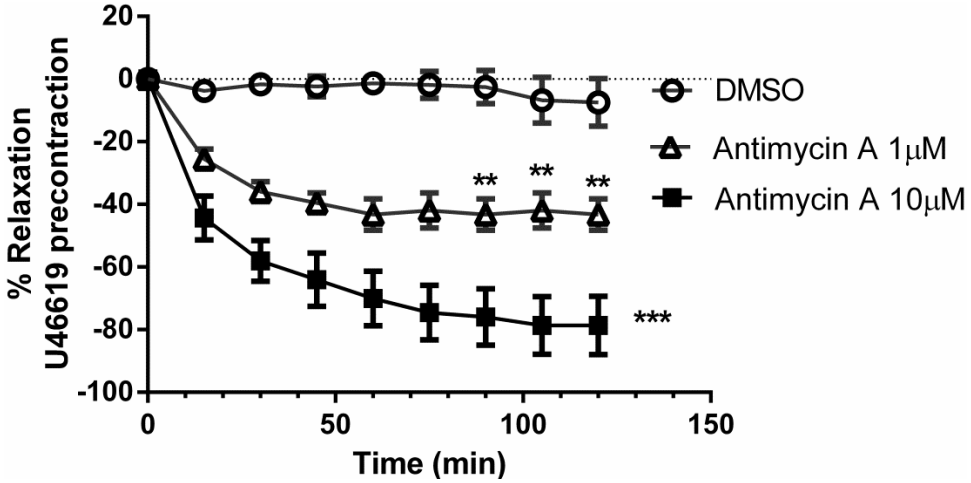


Figure 4.2. Relaxation responses to antimycin A (1 μM and 10 μM) in segments of porcine coronary artery submaximally precontracted with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 8 different experiments. ** indicates P<0.01 and *** indicates P<0.001.

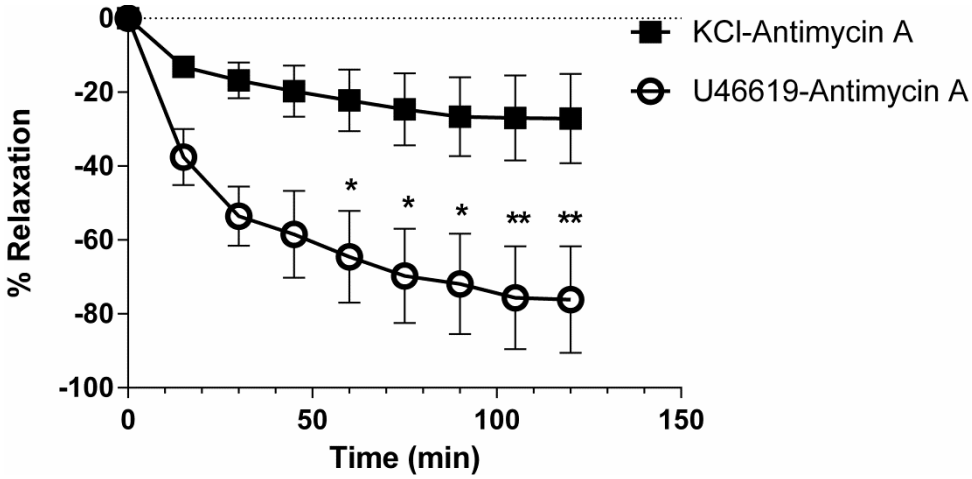


Figure 4.3. Effect of antimycin A (10 μM) in segments of porcine coronary artery precontracted submaximally with U46619 in comparison with KCl precontraction. Data are expressed as a percentage relaxation of the U46619 or KCl-induced contraction and are mean ± SEM from 6 different experiments. * indicates P<0.05 and ** indicates P<0.01.

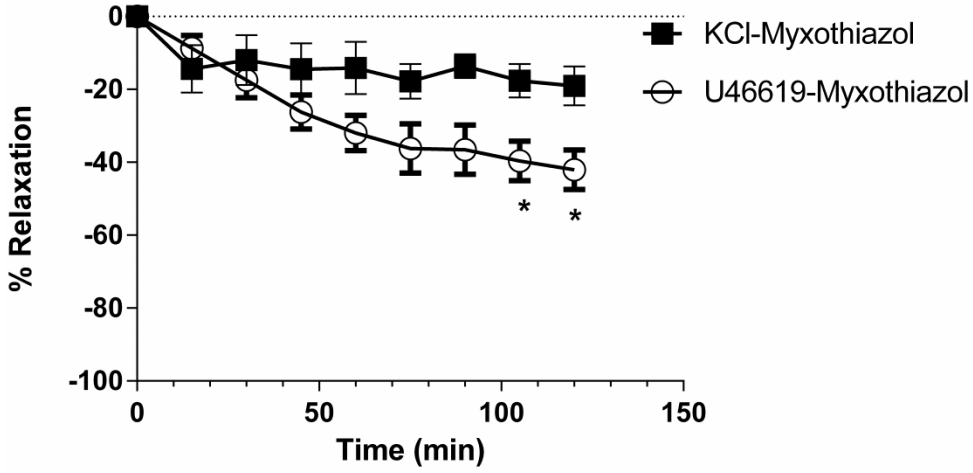


Figure 4.4. effect of myxothiazol (10 μM) in segments of porcine coronary artery precontracted submaximally with U46619 in comparison with KCl precontraction. Data are expressed as a percentage relaxation of the U46619 or KCl-induced contraction and are mean ± SEM from 5 different experiments. * indicates P<0.05.

4.3.2. Effects of complex I inhibitor rotenone, and complex II inhibitor on PCA tone

Neither rotenone (10 μM) nor 3-NP (10 μM), had any significant effect and the contraction was well maintained over the time course of the experiment (figure 4.5).

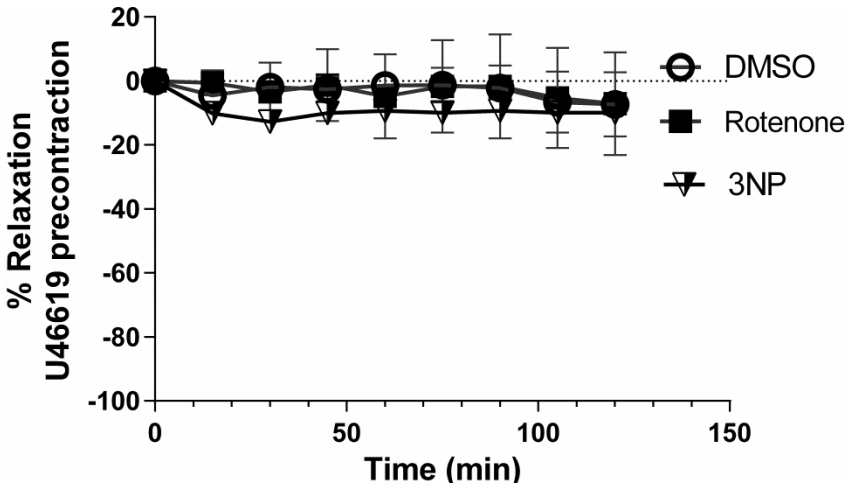


Figure 4.5. effect of rotenone and 3NP (both 10 μM) in segments of porcine coronary artery precontracted submaximally with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different experiments.

4.3.3. Effects of mitochondrial uncoupler FCCP on PCA tone

The mitochondrial uncoupler FCCP at (100 nM), but not at (1 μM), also produced a time-dependent relaxation, reaching a maximum at around 120 minutes (figure 4.6). At the higher concentration of 1 μM, FCCP caused an initial, transient contraction, before returning to the same level as the control (figure 4.6).

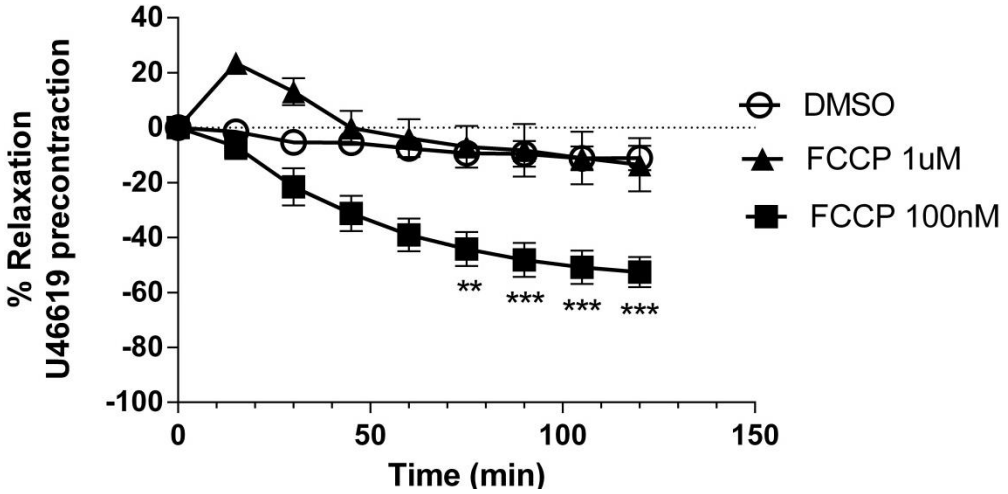


Figure 4.6. effect of FCCP (100 nM and 1 μM) in segments of porcine coronary artery precontracted submaximally with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different experiments. ** represents P<0.01 and *** indicates P<0.001.

Unlike antimycin A, pre-constriction with KCl instead of U46619 had no significant effect on the relaxation response induced by 100 nM FCCP (figure 4.7).

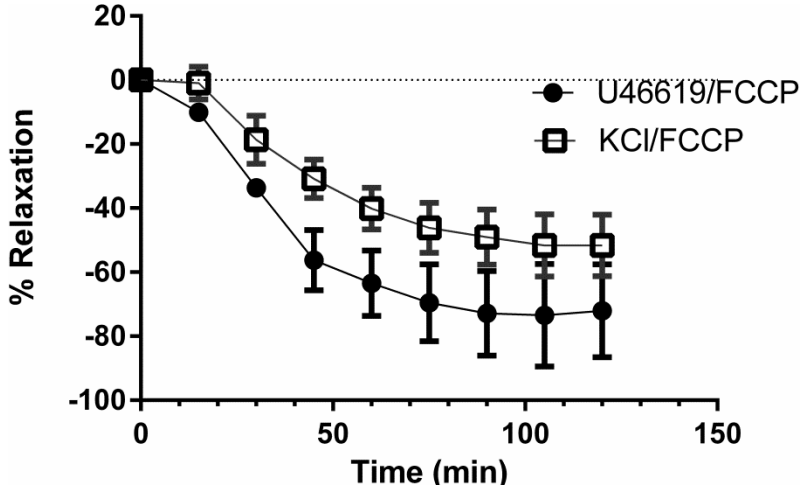


Figure 4.7. effect of FCCP (100 nM) in segments of porcine coronary artery precontracted submaximally with U46619 and KCl. Data are expressed as a percentage relaxation of U46619 or KCl-induced contraction and are mean ± SEM from 5 different experiments.

4.3.4. Effects of ATP synthase inhibitor oligomycin on PCA tone

The ATP synthase inhibitor oligomycin (10 μM) also produced a slow, time-dependent relaxation of the coronary artery, which was still relaxing at 120 minutes (figure 4.8).

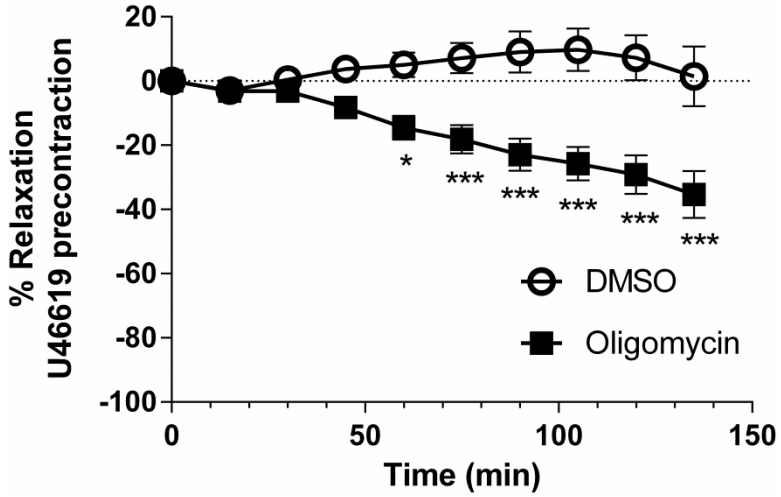


Figure 4.8. effect of oligomycin (10 μM) in segments of porcine coronary artery precontracted submaximally with U46619. Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 9 different experiments. * indicates $P<0.05$ and *** indicates $P<0.001$.

4.3.5. Role of NO, Prostacyclin in the relaxation response to antimycin A

A previous study by Rowlands *et al*. has shown that mitochondrial ROS play a role in endothelial NOS activation (Rowlands et al., 2011). Moreover, mitochondrial depolarization promotes eNOS activation without ROS generation (Katakam et al., 2013). Prostacyclin may be also produced by endothelial cells in the presence of mild oxidative stress (Toniolo et al., 2013). However, pre-treatment of the porcine coronary rings with a nitric oxide synthase inhibitor, L-NAME (300 μM), or a cyclooxygenase inhibitor, indomethacin (10 μM), had no effect on antimycin A-induced relaxation (figure 4.9 & 4.10 respectively).

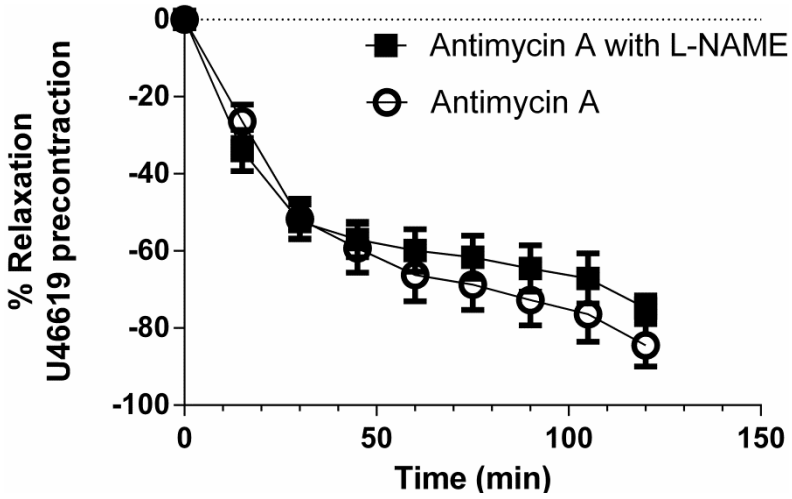


Figure 4.9. Relaxation responses to antimycin A in segments of porcine coronary artery precontracted submaximally with U46619 in the presence of L-NAME (300 μM). Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 7 different experiments.

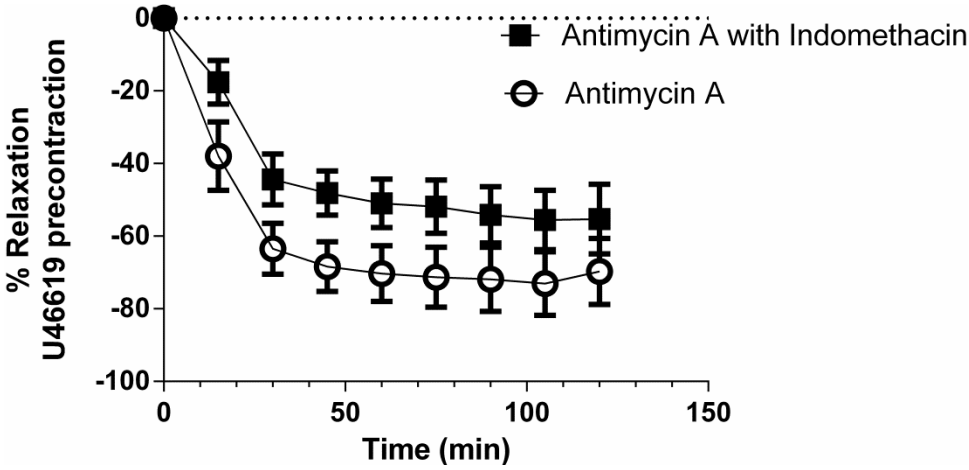


Figure 4.10. Relaxation responses to antimycin A in segments of porcine coronary artery precontracted submaximally with U46619 in the presence of indomethacin (3 μM). Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 9 different experiments.

4.3.6. Role of AMPK in the relaxation response to antimycin A

Inhibition of mitochondrial respiration at complex III with 10 μM antimycin A interrupts ATP production and hence elevates ADP concentration. This in turn would elevate the concentration of AMP with subsequent AMPK activation. A variant mechanism for AMPK activation is by RNS or ROS, those latter have been shown to activate AMPK when mitochondrial respiration is blocked (Quintero et al., 2006, Mackenzie et al., 2013). Pre-incubation with dorsomorphin, an AMP kinase inhibitor, significantly reduced antimycin A relaxation (figure 4.11), suggesting that antimycin A-induced vasodilation is mediated in part by AMP kinase activation.

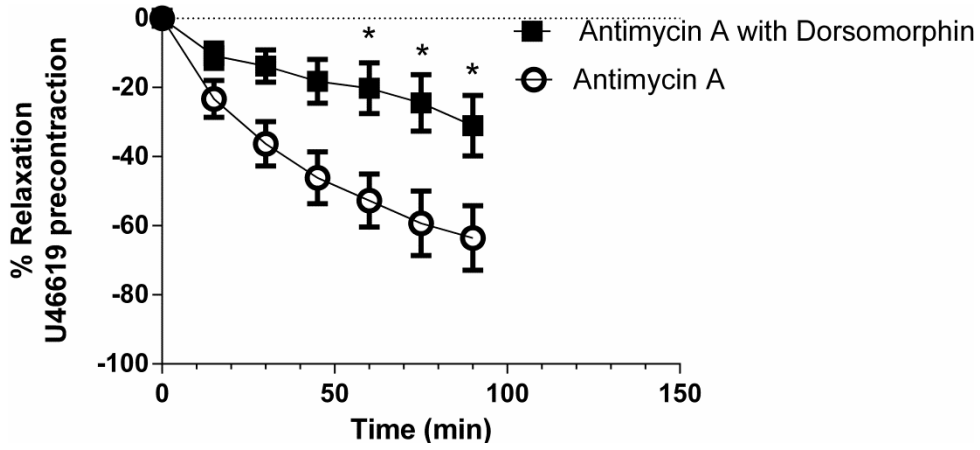


Figure 4.11. Relaxation responses to antimycin A in segments of porcine coronary artery precontracted submaximally with U46619 in the presence of dorsomorphin (10 μM). Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 9 different experiments. * indicates P<0.05.

4.3.7. Role of K^+ channels in the relaxation response to antimycin A

The incubation of coronary rings with the K_{Ca} channel blocker (TEA, 10 mM), K_{ATP} blocker (glibenclamide, 3 μM) or Kv blocker (4-aminopyridine, 1 mM) had no effect on the antimycin A-induced coronary relaxation (figure 4.12, 4.13 & 4.14 respectively).

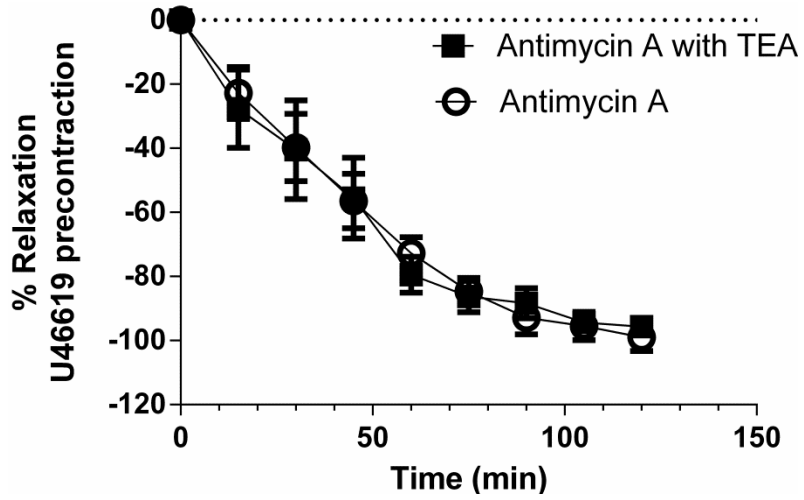


Figure 4.12. Relaxation responses to antimycin A in segments of porcine coronary artery precontracted submaximally with U46619 in the presence of TEA (10 mM). Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 5 different experiments.

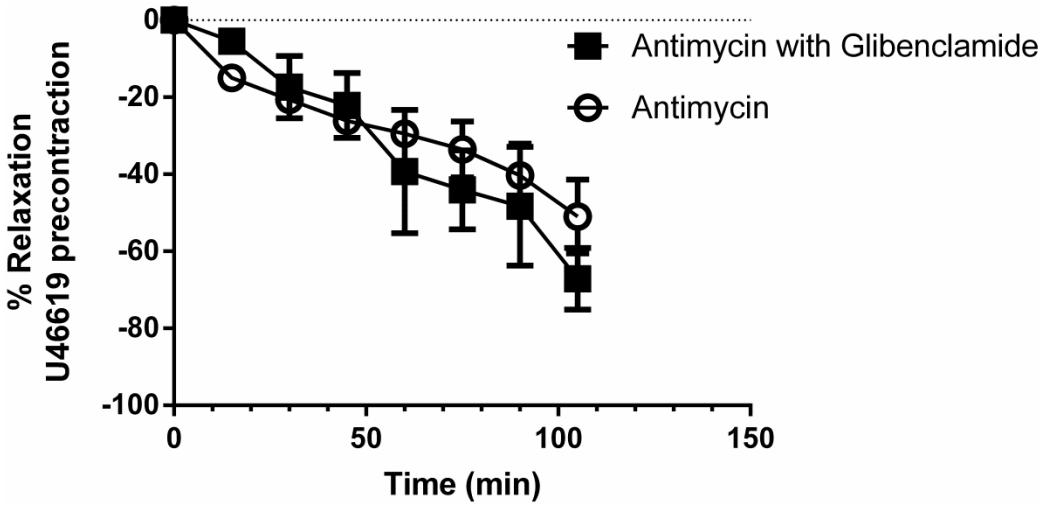


Figure 4.13. Relaxation responses to antimycin A (10 μM) in segments of porcine coronary artery precontracted submaximally with U46619 in the presence of glibenclamide (3 μM). Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 5 different experiments.

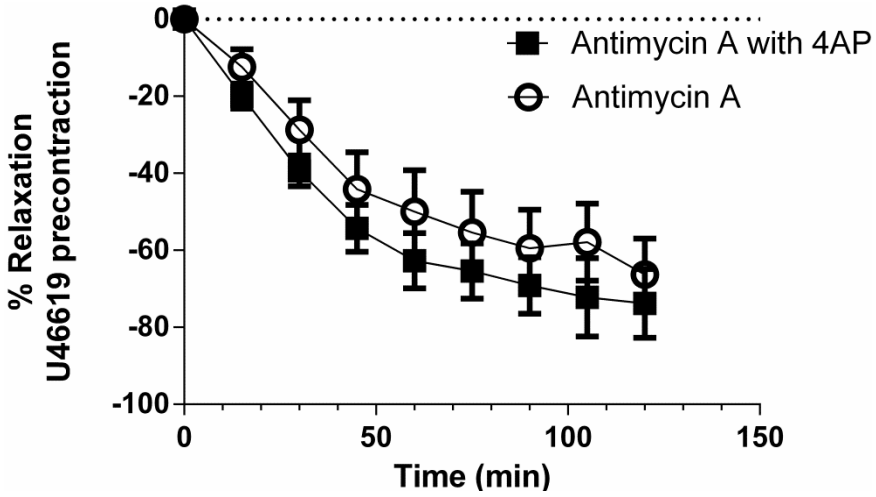


Figure 4.14. Relaxation responses to antimycin A (10 μM) in segments of porcine coronary artery precontracted submaximally with U46619 in the presence of 4-AP (1 mM). Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 5 different experiments.

4.3.8. Effect of pre-incubation with FCCP, or rotenone, on the relaxation response to antimycin A

Pre-incubation with the mitochondrial uncoupler FCCP (1 μM), significantly reduced the relaxation to antimycin A. On the other hand rotenone (10

μM), a complex I inhibitor, caused a partial inhibition of the antimycin A relaxation (figure 4.15). The relaxation response to antimycin A was reduced after pre-contraction with KCl as mentioned before (figure 4.3). Under these conditions, pre-incubation with 10 μM rotenone caused complete inhibition of the relaxation to antimycin A (figure 4.16).

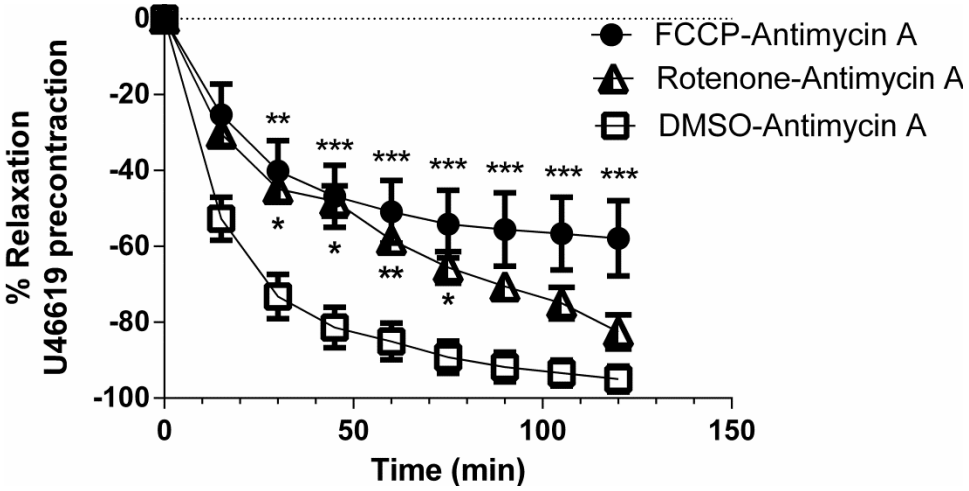


Figure 4.15. Relaxation responses to antimycin A (10 μM) in segments of porcine coronary artery precontracted submaximally with U46619 in the presence of FCCP (1 μM) or rotenone (10 μM). Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 10 different experiments. * indicates P<0.05, ** indicates p<0.01 and *** indicates P<0.001.

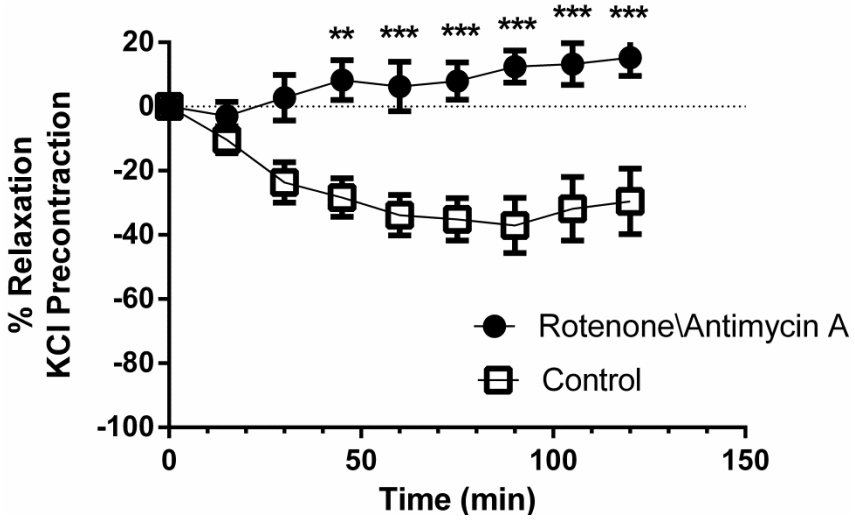


Figure 4.16. Relaxation responses to antimycin A in segments of porcine coronary artery precontracted submaximally with KCl in the presence of rotenone (10uM). Data are expressed as a percentage relaxation of the

U46619-induced contraction and are mean ± SEM from 5 different experiments. ** indicates P<0.01 and *** indicates P<0.001.

4.3.9. Role of Na^+/K^+ ATPase pump in the relaxation response to antimycin A

A study by Qamirani et al provided evidence that NaN_3, a mitochondrial complex IV inhibitor, directly dilates porcine coronary arterioles by the activation of Na^+/K^+ ATPase pump (Qamirani et al., 2006). In the presence of ouabain, a Na^+/K^+ ATPase inhibitor, at 100 nM concentration, the antimycin A-induced coronary relaxation was significantly attenuated (figure 4.17), suggesting that antimycin A affects the Na^+/K^+ ATPase pump. However, the results obtained from rubidium-uptake experiments showed that Na^+/K^+ ATPase pump was not altered by antimycin A (figure 4.18).

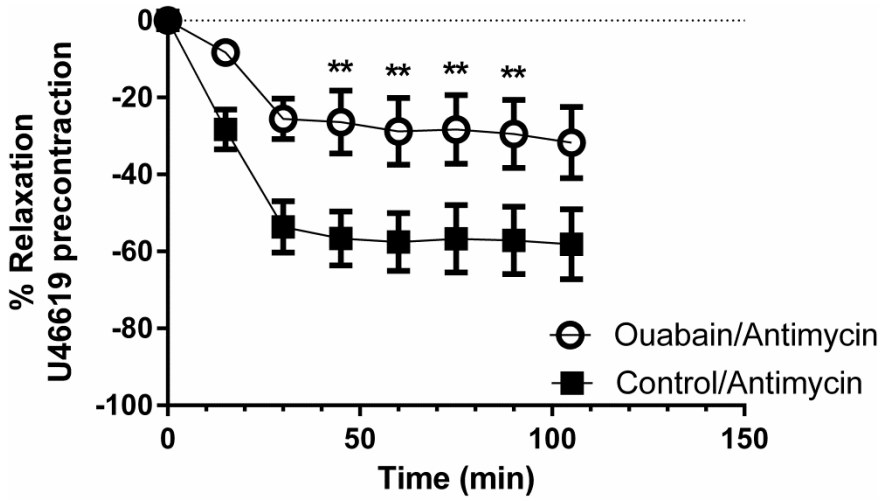


Figure 4.17. Relaxation responses to antimycin A (10 μM) in the absence or presence of ouabain (100 nM), Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different experiments. ** indicates P<0.01.

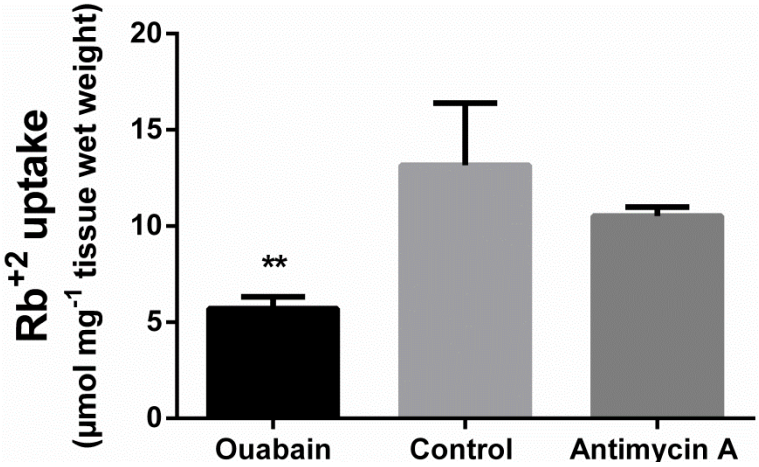


Figure 4.18. Measurement of rubidium-uptake (30 min incubation time) in porcine coronary arteries to determine the activity of Na^+/K^+ pump in the presence of 100 nM ouabain or 10 µM antimycin A using atomic absorption spectrophotometer used in a flame emission mode. Data are expressed as rubidium uptake in µmol mg^{-1} of tissue wet weight and are mean ± S.E.M. of 6 experiments. **P < 0.01, one-way ANOVA followed by Dunnett's multiple comparison test compared to the control.

4.3.10. Role of reactive oxygen species in the relaxation response to Antimycin A

PEG-superoxide dismutase (100 Uml^{-1}) had no effect on the relaxation response to antimycin A (figure 4.19). By contrast, ebselen (3 µM) or DETCA (10 mM) inhibited the antimycin A relaxation in the coronary rings pre-contracted with KCl (figure 4.20 & 4.21). Pre-contraction with KCl was used under these conditions as both ebselen and DETCA reduced the U46619-induced tone; the contraction is better maintained with KCl under these conditions (Takase et al., 1999).

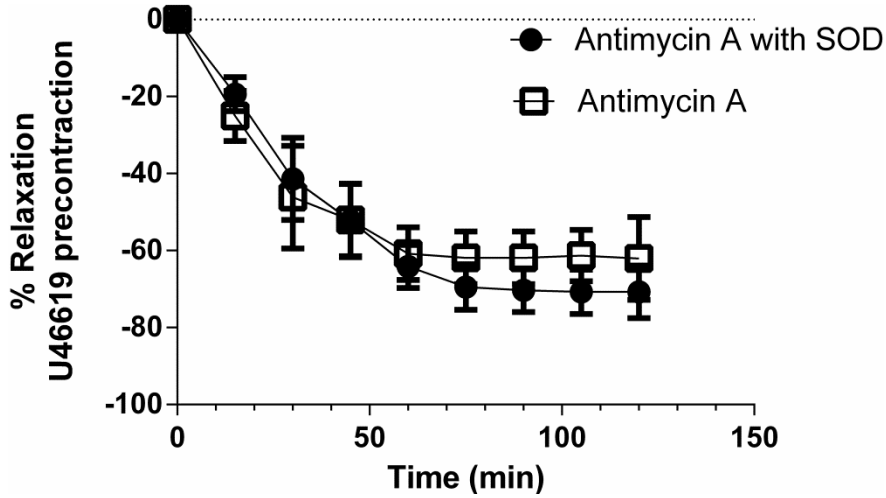


Figure 4.19. Relaxation responses to antimycin A (10 μM) in the absence or presence of SOD (100 Uml^{-1}), Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 6 different experiments.

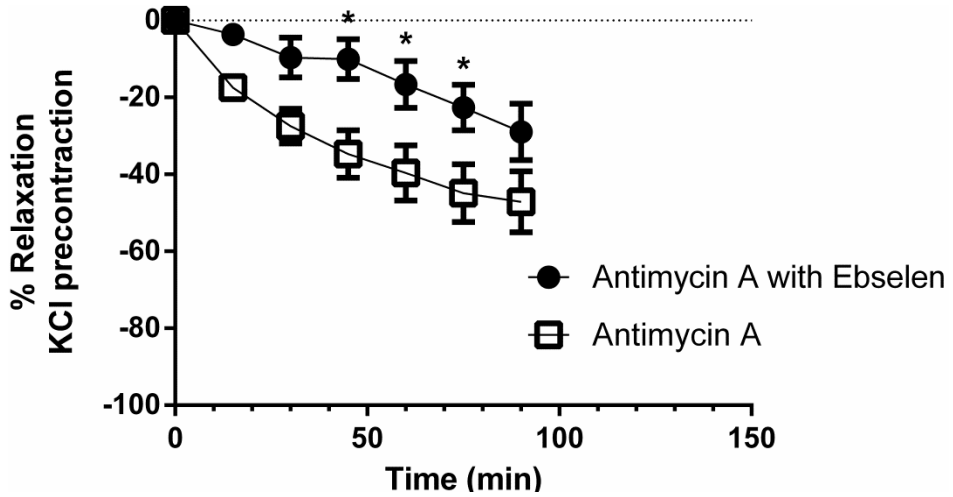


Figure 4.20. Relaxation responses to antimycin A (10 μM) in the absence or presence of ebselen (10 μM), Data are expressed as a percentage relaxation of the KCl-induced contraction and are mean ± SEM from 6 different experiments. * indicates $P<0.05$.

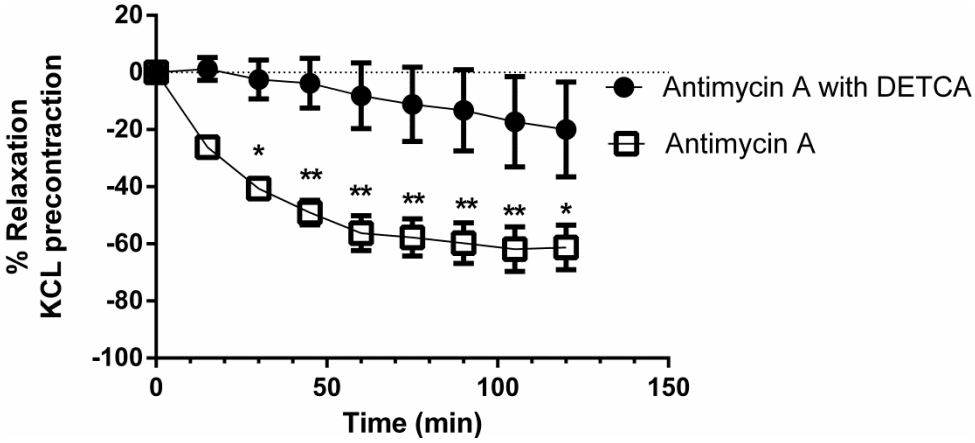


Figure 4.21. Relaxation responses to antimycin A (10 μM) in the absence or presence of DETCA (10 mM), Data are expressed as a percentage relaxation of the U46619-induced contraction and are mean ± SEM from 7 different experiments. * indicates P<0.05, ** indicates P<0.01.

4.3.11. Effect of antimycin A on Calcium-Induced contractions

In chapter 3, it was demonstrated that simvastatin inhibits calcium-induced contractions. Therefore, as a comparison, the effect of antimycin A on Ca^{2+} influx was also determined. Re-addition of calcium in the presence of 60 mM KCl produced a concentration-dependent contraction in the porcine coronary artery. Pre-incubation with antimycin (10 μM) produced a concentration-dependent inhibition of the contractile response (figure 4.22). Similarly, the complex III inhibitor myxothiazol (10 μM) produced significant inhibition of the contraction (figure 4.23). On the other hand, complex I inhibitor (rotenone, 10 μM) had no effect on the calcium-induced contraction (figure 4.22). ATP synthase inhibitor (oligomycin, 10 μM) and the respiratory uncoupler (FCCP, 1 μM) also inhibited the calcium-induced contraction (figure 4.22 & 4.23).

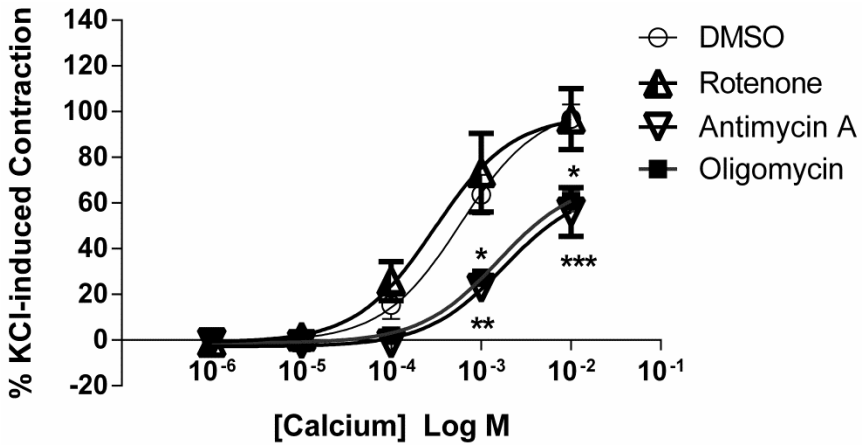


Figure 4.22. Effect complex I inhibitor rotenone (10 μM), complex III inhibitors antimycin A (10 μM) and ATPase inhibitor oligomycin (10 μM) on calcium-induced contractions in the porcine coronary artery.* indicates P<0.05 and *** indicates P<0.001.

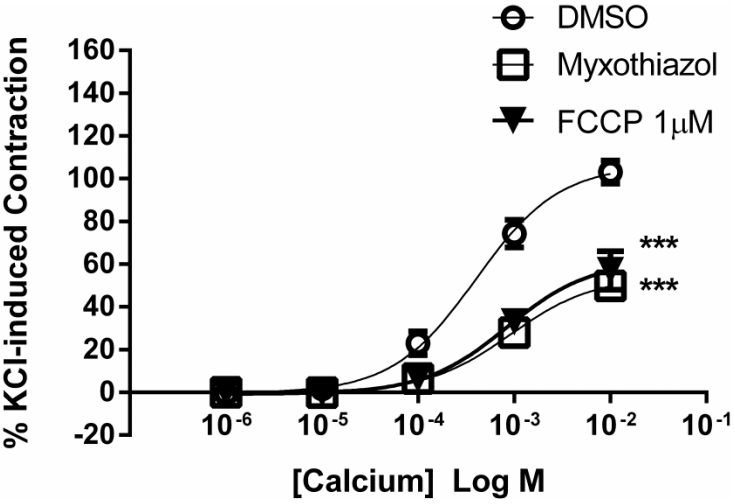


Figure 4.23. Effect complex III inhibitor myxothiazol (10 μM), and FCCP (1 μM) on calcium-induced contractions. Data are expressed as a percentage of the contraction to 60 mM KCl in the presence of calcium. *** indicates P<0.001.

4.3.12. Effect of antimycin A on calcium influx in freshly isolated smooth muscle cells.

Freshly isolated smooth muscle cells produced an increase in intracellular calcium in response to 50 mM KCl. In the presence of 10 μM antimycin A, this response was significantly reduced (figure 4.24).

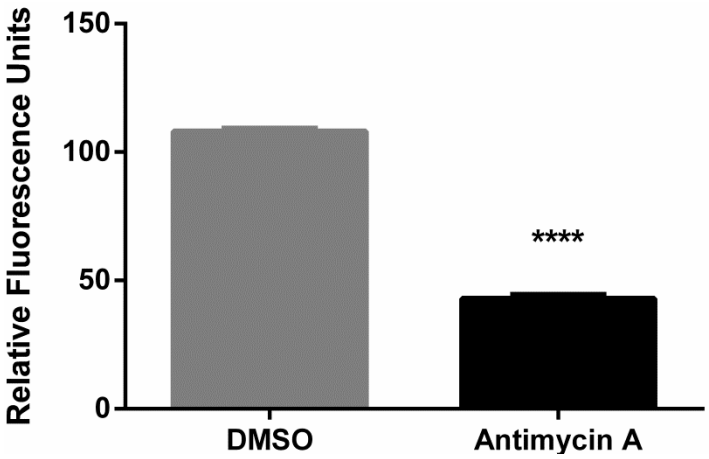
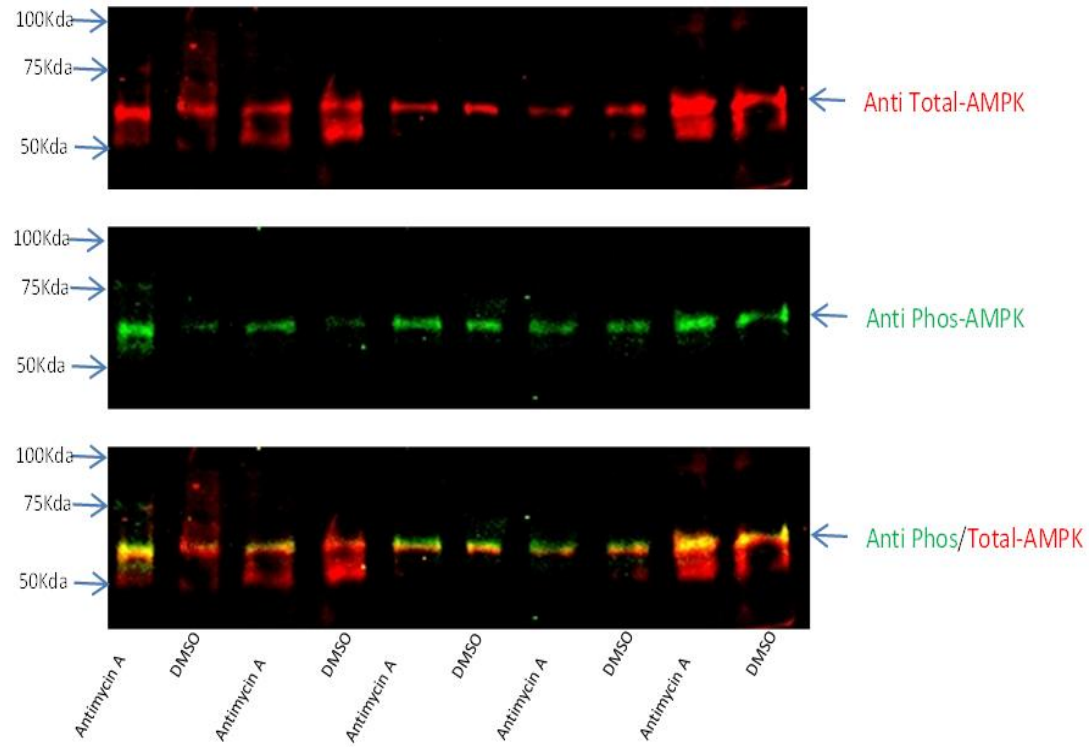


Figure 4.24. Bar chart of mean data ± SEM from DMSO and from 111 (antimycin A) cells showing the changes in fluo-4 fluorescence in response to 50 mM KCl in the presence of 0.1 % v/v DMSO or 10 μM antimycin A. **** indicates p<0.0001 v DMSO, 2-tailed, unpaired t-test.

4.3.13. Effect of antimycin A on AMPK activation in porcine coronary artery

As shown in figure (4.25-A). A representative blot of western immunoblotting experiment assessing the effect of antimycin A incubation on AMPK activation in isolated PCA rings incubated in tissue baths for 2 h in the presence or absence of antimycin. In PCA incubated with antimycin A for 2 h, the level of phospho-AMPK to total AMPK was significantly increased in comparison with PCA incubated with DMSO as a solvent control using 2-tailed, unpaired Student's t-test (figure 4.25-B).

A-



B-

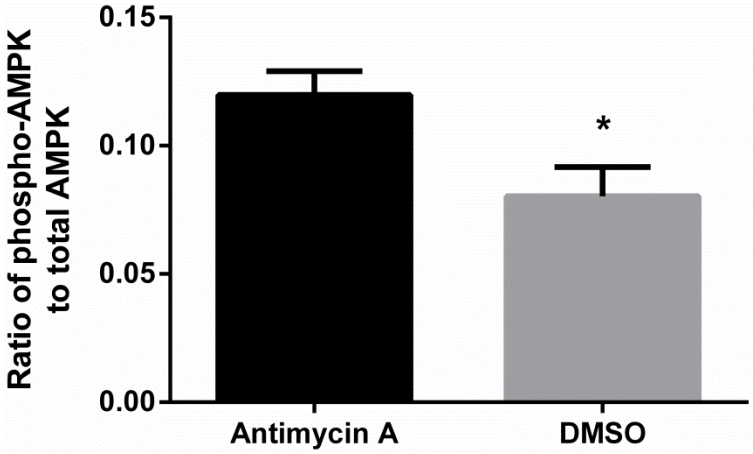


Figure 4.25. A- Representative blot of western immunoblotting experiments assessing the effect of antimycin A on AMPK activation in PCA. Prior to freezing and homogenisation, PCA rings were incubated in tissue baths for 2 h in the absence or presence of antimycin **B-** Bar chart shows the ratio of phospho-AMP kinase to total AMP kinase from antimycin A and DMSO treated segments of 5 different tissues. Data are presented as mean ± SEM, 2-tailed, * indicates $p<0.05$ unpaired Student's t-test.

4.4. Discussion

The data presented in chapters 2 & 3 demonstrate that simvastatin produces a relaxation of the porcine coronary artery, which is thought to be due, a least in part, to inhibition of the mitochondria, probably at complex III. The present study demonstrates that antimycin A, a complex IIIQi inhibitor, evokes a similar time-dependent relaxation of the isolated porcine coronary artery. Myxothiazol, a complex IIIQo inhibitor, also produced a relaxation of the porcine coronary artery; however, the response was smaller and slower to develop. On the other hand, inhibition of complex I with rotenone or complex II with 3-NP had no effect on coronary artery tone. These data suggest that mitochondria play a role in the regulation of vascular tone through complex III. The relaxation responses seen with antimycin A and myxothiazol are not due to general inhibition of mitochondrial function as inhibition of mitochondrial complexes I and II had no effect on tone. Moreover, uncoupling the mitochondria with a high concentration of FCCP (1 μM) did not induce coronary relaxation. The current study also provided evidence that the relaxation response to antimycin A was inhibited by AMPK inhibition. By contrast the relaxation was independent of K^+ channel, Na^+/K^+ ATPase pump, nitric oxide or cyclooxygenase activation.

In most cells, the mitochondrial electron transfer chain is one of the major sources of ROS (Turrens, 2003). While complex I can deposit O_2^- into the mitochondrial matrix to be dissipated by the mitochondrial antioxidant defence (Chen et al., 2003), complex III (site IIIQo) can release O_2^- to the cytosol where it is converted to hydrogen peroxide by superoxide dismutase 1 (Cai, 2005, Hool, 2006, Hawkins et al., 2007). Cytosolic hydrogen peroxide is believed to be the primary form of ROS signalling in the cell (Liu et al., 2003). Antimycin A is known to increase complex III O_2^-

generation. It prolongs the lifetime of ubisemiquinone at the Qo site with a resultant increase in O_2^- release (Mukhopadhyay et al., 2007, Wolin, 2009). This may explain why inhibition of complex III, but not complex I or II, results in relaxation of the coronary artery. This is supported by the evidence that ebselen, a glutathione peroxidase mimic (Liu and Gutterman, 2009) significantly inhibits antimycin A-induced coronary relaxation. Moreover, DETCA, a superoxide dismutase inhibitor, inhibited the antimycin A relaxation suggesting that H_2O_2 formation is important for the antimycin A-induced coronary relaxation.

Uncoupling the mitochondria with FCCP (1 μM) did not produce a relaxation in the porcine coronary artery, but inhibited the relaxation to antimycin A. Furthermore, incubation with rotenone (10 μM) abolishes antimycin A-induced relaxation. Both of these compounds might reduce the amount of ROS produced by complex III inhibition, and provide evidence that mitochondrial ROS may underlie the relaxation effects seen with antimycin A (Chen et al., 2003, Liu et al., 2003, Hsieh et al., 2001).

The U46619-induced contraction is associated with Ca^{2+} release from the sarcoplasmic reticulum and Rho-kinase–mediated Ca^{2+} sensitization (Dorn and Becker, 1993). On the other hand, exposure to high KCl (60 mM) activates smooth muscle contraction by changing the K^+ equilibrium potential and clamping the membrane potential above the resting level with subsequent Ca^{2+} channel activation. Studies have shown that IP_3 induced Ca^{2+} release becomes inoperative during mitochondrial depolarization. Therefore, antimycin A may inhibit U46199- induced Ca^{2+} release by mitochondrial depolarization (Collins et al., 2000, McCarron et al., 2012). The data presented also suggest that antimycin A inhibits Ca^{2+} influx as it inhibited the contraction to increasing concentrations of calcium, in the presence of KCl. This was confirmed in freshly isolated coronary artery smooth muscle cells in which the KCl-induced increase in

intracellular calcium was inhibited by antimycin A. Similar effects on Ca^{2+} influx have been seen in vascular and intestinal smooth muscle (Nakagawa et al., 1985) and rat tail artery (Sward et al., 2002). Rotenone produced complete inhibition of the relaxation to antimycin A after pre-contraction with KCl (figure 4.16). The latter suggested that complex I Inhibition with rotenone prevents the effect of antimycin A on calcium influx. However, rotenone has no effect on the Ca^{2+} depletion induced by antimycin A, therefore the inhibitory effect on the U46199-precontracted vessels is less pronounced (figure 4.15). Previously, rotenone has been shown to inhibit dilation in human coronary vessels by suppression of O_2^- and H_2O_2 formation (Liu et al., 2003). This ties-in with the data showing that ebselen and DETCA inhibit the antimycin A-induced relaxation and supports the notion that antimycin A increases ROS formation, leading to coronary relaxation. Previous results have shown that H_2O_2 inhibits Ca^{2+} entry and the force of contraction of the porcine coronary arteries via SOC inhibition (Pande et al., 2012); similarly, superoxide inhibits voltage-dependent Ca^{2+} channels of artery myocytes (Fusi et al., 2001). Some of antimycin A effects on calcium influx could be due to activation of plasma membrane K^+ channels; opening of these channels would lead to hyperpolarization of the membrane with closure of the voltage gated calcium channels. However, as mentioned above, inhibition of K^+ channels with glibenclamide, TEA and 4-aminopyridine, had no effect on the antimycin A-induced relaxation in the porcine coronary artery, indicating that this is unlikely to be the mechanism of action. Collectively, the effects of antimycin A suggest a role for mitochondria in regulating L-type Ca^{2+} channel activity (Kubota et al., 2003).

A study by Janssen *et al.* has suggested that free radicals can activate the Na^+-K^+ pump, hyperpolarize the cell membrane and relax tracheal smooth muscle (Janssen et al., 2000). On the other hand, oxygen radicals can

damage Na^+-pumps in coronary arteries (Elmoselhi et al., 1994). Our data showed that incubation with ouabain, the Na^+/K^+ ATPase inhibitor, partially reduced antimycin A-induced coronary segments relaxation (figure 4.17). However, the results of the rubidium-uptake experiments showed that Na^+/K^+ ATPase pump activity was not altered by antimycin A (figure 4.18). Therefore, ouabain might act non-specifically to inhibit antimycin A coronary relaxation. Previous studies have shown that inhibition of the sodium pump results in cell membrane depolarization (Aalkjaer and Mulvany, 1985) with a concomitant increase in calcium influx into vascular smooth muscle cells (Pulina et al., 2010). The latter may have opposing effect to the relaxation induced by antimycin A.

Mitochondrial ROS have been shown to activate AMP kinase (Quintero et al., 2006, Mackenzie et al., 2013). Pre- incubation with dorsomorphin, an AMP kinase inhibitor, reduced the antimycin A relaxation, suggesting that the antimycin A-induced vasodilation is mediated in part by activation of AMP kinase. Activation of AMP kinase inhibits agonist-induced contractions of the mouse aorta, which is independent of NO and cyclo-oxygenase inhibition. Such relaxation induced by AMPK activation, like antimycin A, was reduced in the presence of high K^+ (Davis et al., 2012). AMP kinase is thought to have a direct action on the smooth muscle via phosphorylation and inactivation of smooth muscle myosin light chain kinase and thus induce relaxation (Horman et al., 2008). Therefore, it is plausible that mitochondrial ROS generation by antimycin A could lead to AMP kinase activation with subsequent coronary vascular relaxation. Western blotting experiments have shown that acute incubation 2 h of isolated porcine coronary arteries with antimycin A 10 μM increased AMPK activation as compared to control segments. These findings support the contention that AMPK activation in vascular smooth muscle imparts a brake on contraction. Previous results have shown that metformin increases mitochondria-

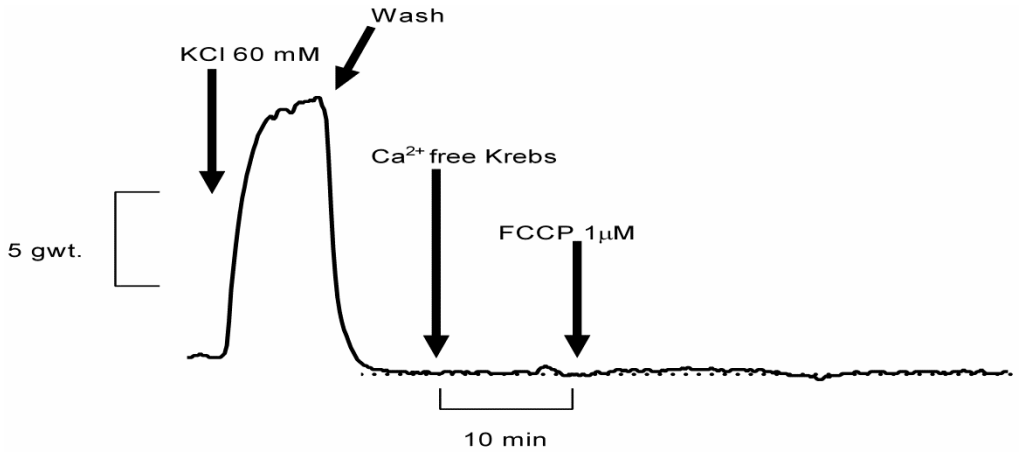
derived reactive nitrogen species to activate AMPK in vivo (Zou et al., 2004). It also inhibited phenylephrine-induced contraction in aortic rings due to AMPK activation (Sung and Choi, 2012a). Similarly, acetylcholine inhibited phenylephrine-mediated contraction in endothelium-denuded rat aorta via AMPK-dependent mechanism (Lee and Choi, 2013). On the other hand, metabolic challenge could also activate AMPK (Rubin et al., 2005). Thus, further studies are recommended for ROS estimation and ATP level measurement in order to determine the exact mechanism of antimycin A-induced AMPK activation. Data from the present study demonstrate that AMPK is activated over the time course of antimycin A relaxation; however, additional studies are necessary to determine the role of AMPK in coronary smooth muscle relaxation.

Additional findings of the current study is that FCCP (100 nM) and oligomycin (10 μM) induced coronary relaxation while FCCP (1 μM) has no significant effect on the coronary tone. A previous study has shown that FCCP (1 μM) increases Ca^{2+} by activating Ca^{2+} influx (Restini et al., 2006). However, FCCP is also capable of mobilizing Ca^{2+} from the intracellular Ca^{2+} pools. The prevention of Ca^{2+} uptake into the mitochondria, driven by the mitochondrial proton gradient, may contribute to the FCCP induced Ca^{2+} increase (Park et al., 2002). Similarly, the present study clearly shows that FCCP (1 μM) induced a transient increase in the contraction. This initial increase in contraction can be explained by Ca^{2+} release from the intracellular store as well as by activating Ca^{2+} influx since the contractile response is markedly reduced in Ca^{2+} free Krebs (figure 4.26). Increasing calcium influx could explain the mechanism by which FCCP inhibited the antimycin A relaxation. An interesting observation of the present study is that FCCP at a low concentration, 100 nM, induced coronary relaxation (figure 4.6). Previous studies have shown that mitochondrial uncoupling with low-dose FCCP improves post-ischaemic

functional recovery via a ROS-dependent pathway that is not mediated via cellular ATP depletion (Brennan et al., 2006). Therefore at low concentrations, FCCP may produce a relaxation through a ROS-dependent mechanism. However, at higher concentrations, transient increases in calcium leads to vascular contraction (Nicholls, 2005).

The effect of antimycin A on the coronary artery tone could be a toxic effect. However, the fact that the relaxation can be inhibited by further inhibition of the mitochondria (i.e. rotenone and FCCP) and by inhibition of AMP kinase would rule out a toxic effect. Furthermore, the response of the antimycin A-treated segments to U46619 and KCl depolarization was comparable to the control segments (figure 4.27). It is also possible that inhibition of mitochondrial complexes causes a reduction in ATP (Nakagawa et al., 1985). However, again, the fact that the relaxation can be inhibited by further inhibition of the mitochondria, would suggest that this is not the mechanism. In conclusion, the data presented in this chapter indicate that alteration of mitochondrial function at complex III with antimycin A inhibits Ca^{2+} influx and causes relaxation of porcine coronary artery.

A)



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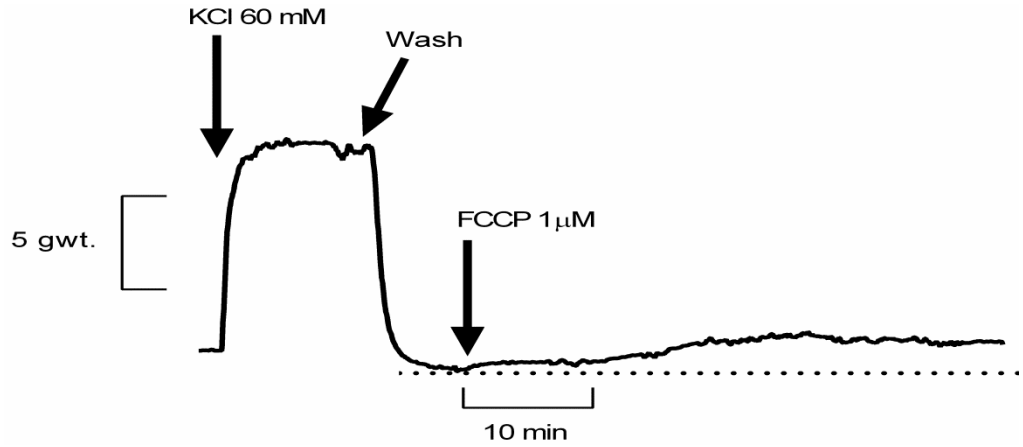

Figure 4.26. Effect of FCCP (1 μM), mitochondrial uncoupler, on basal porcine coronary artery tone. A- in normal Krebs **B-** in Ca^{2+} free Krebs, the contractile response is markedly reduced in Ca^{2+} free Krebs.

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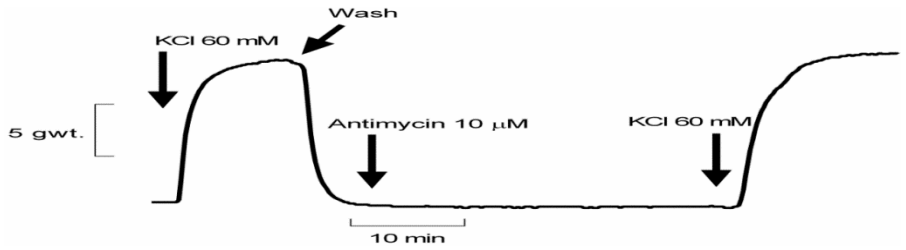

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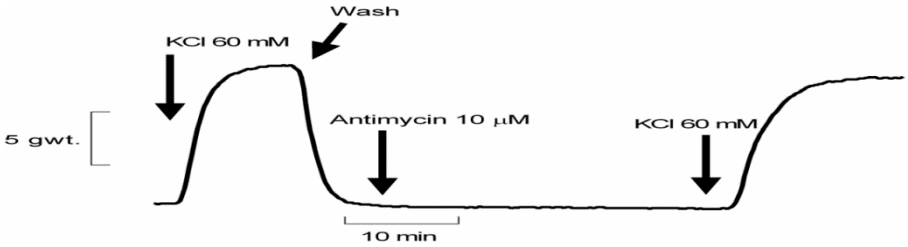

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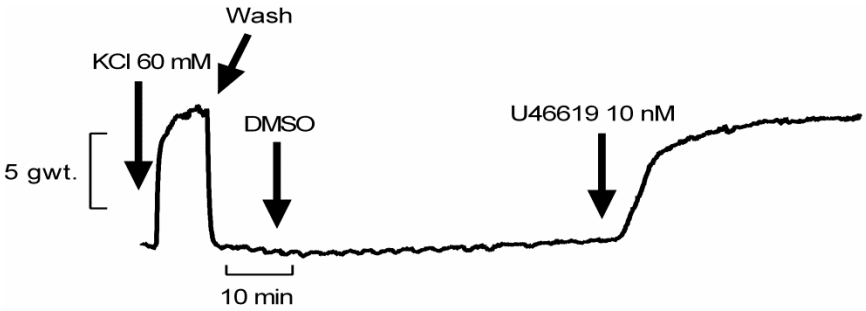

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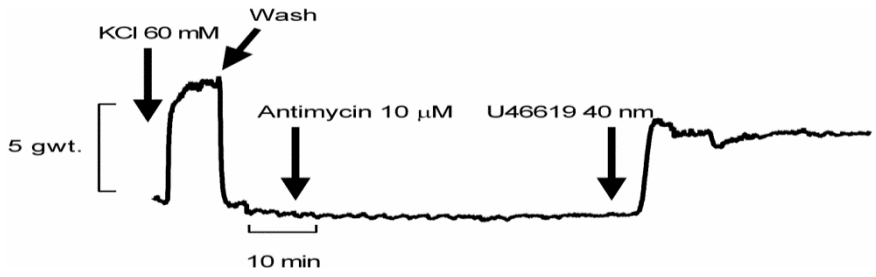


Figure 4.27. Effect of antimycin A (10 μM) incubation on porcine coronary artery tone. **1&2-** vessels contracted with KCl 60 mM **3&4-** vessels contracted with U46619.

CHAPTER V: Effect of statins on mitochondrial membrane potential and K_{ATP} ion channel activity in mouse primary β-cells.

5.1 Introduction

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, collectively referred to as statins, are key drugs to lower plasma cholesterol levels in the treatment of hypercholesterolaemia (Pedersen et al., 2004, Kostis et al., 2012). Patients with type 2 diabetes may also be candidates for statin therapy regardless of cholesterol levels (Betteridge, 2005, Rocco, 2012). However, in recent years, a number of intriguing and still unresolved questions have arisen as to whether the use of statins is associated with an increased risk of glucose intolerance and diabetes mellitus (Macedo et al., 2014). In fact, evidence from a number of clinical and experimental trials have highlighted an association between treatment with lipophilic statins and increased risk of glucose intolerance and diabetes (Sattar et al., 2010, Bellia et al., 2012, Jukema et al., 2012, Wang et al., 2012, Carter et al., 2013, Yoon and Lee, 2013, Bang and Okin, 2014).

A randomized double-blind placebo-controlled study investigating the use of rosuvastatin in the primary prevention of cardiovascular diseases revealed a link with accelerated diabetes in statin users (Ridker et al., 2008). A large study on 345,417 subjects over a two-year time period demonstrated that fasting plasma glucose (FPG) increased with statin use from 102 mg/dL to 141 mg/dL, by contrast FPG increased from 100 mg/dL to 129 mg/dL among non-statin users (Sukhija et al., 2009). In a further meta-analysis, there was a small (2,082) but significant increase in the incidence of diabetes among 57,593 patients on statins during 3.9 years (Rajpathak et al., 2009). In fact, in 2012, the Food and Drug Administration (FDA) issued a safety communication that statins can increase fasting plasma glucose concentrations (2012). In 2003, A population based study by Carter *et al* suggested that there is increased

incidence of diabetes, between 10-22%, with the lipophillic compounds atorvastatin, rosuvastatin, and simvastatin in comparison with the hydrophilic pravastatin (Carter et al., 2013). Similarly, clinical studies in patients with type 2 diabetes have demonstrated that simvastatin administration raises fasting glucose from 7.66 ± 0.55 mmol/l to 8.32 ± 0.28 mmol/l after six months along with deterioration of β-cell function (Bellia et al., 2012). By contrast, pravastatin, a hydrophilic statin, is reported to have no adverse influence on glycaemic control in type II diabetes patients (Freeman et al., 2001, Guclu et al., 2004). Therefore, despite causing comparable improvements in plasma cholesterol levels, different statins have differential risks in development of diabetes (Koh et al., 2009, Koh et al., 2011, Koh et al., 2013). The risk of new-onset diabetes is associated with intensive-dose (80 mg) daily versus moderate-dose (20 mg) simvastatin daily (Preiss et al., 2011, Rajpathak, 2012, Rocco, 2012). Moreover, this risk was more evident in postmenopausal women (Culver et al., 2012, Chen et al., 2013, Ma et al., 2013) and elderly patients (Sattar et al., 2010, Ma et al., 2012).

The diabetogenic effect of simvastatin and rosuvastatin is likely to be mediated by a deterioration of insulin secretion (Bellia et al., 2012). An *in-vitro* study by Zhou *et al* (2014) in the β-cells, MIN6, showed that after 48 h of treatment, simvastatin significantly inhibited glucose-stimulated insulin synthesis and secretion (Zhou et al., 2014). Similarly lovastatin inhibited MIN6 insulin secretion and content after 24 h incubation (Tsuchiya et al., 2010).

Glucose entry across the β-cell plasma membrane initiates the cascade of metabolism–secretion coupling (Figure 5.1). Glucose is phosphorylated by glucokinase initiating glycolysis and pyruvate production. Then, mitochondrial metabolism generates ATP, increased ATP concentration promotes the closure of ATP-sensitive K^+ channels (McTaggart et al., 2010)

which leads to plasma membrane depolarization. The latter results in Ca^{2+} influx through voltage-gated Ca^{2+} channels with subsequent triggering of insulin exocytosis (Wollheim, 2000, Mears, 2004).

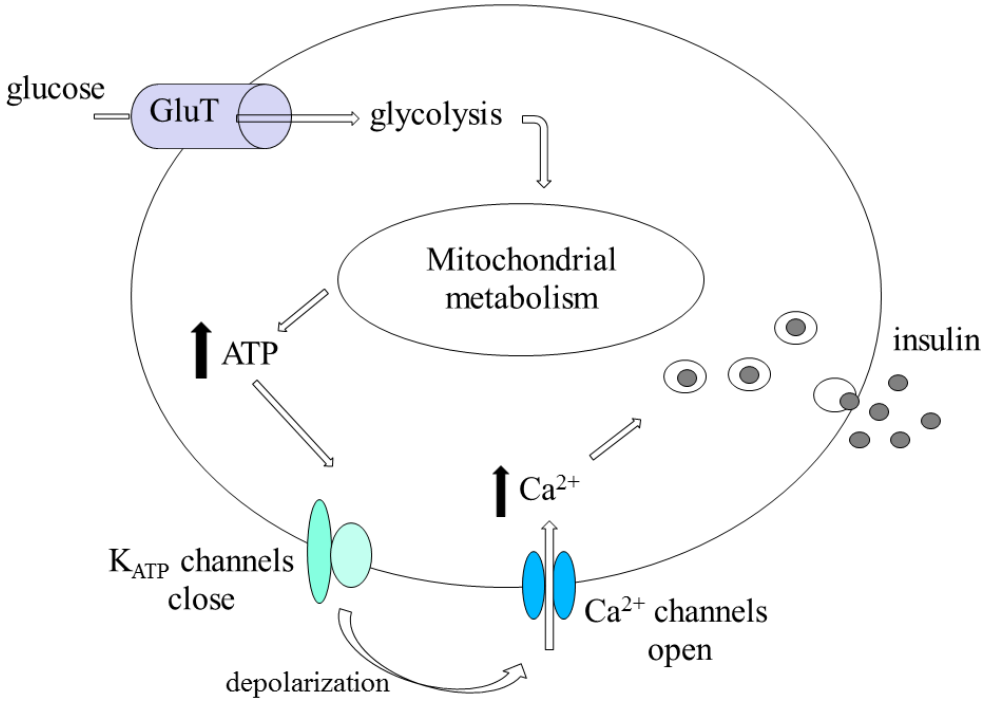


Figure 5.1. coupling of glucose metabolism to insulin secretion in β-cell and the role of K_{ATP} channels. Glucose enters the cell via the glucose transporter, GLUT. Glycolytic and mitochondrial metabolism of the sugar leads to an increase in ATP. This results in K_{ATP} channel closure, membrane depolarization, opening of voltage-gated Ca^{2+} channels, Ca^{2+} influx, and exocytosis of insulin granules.

The effects of statins on blood glucose levels in humans suggest that these compounds may alter pancreatic β-cell function. Simvastatin, but not pravastatin, can inhibit calcium signalling-dependent insulin secretion by the blockade of L-type Ca^{2+} channels in rat islet β-cells (Yada et al., 1999). Alternatively, the synthesis of ubiquinone, a central factor involved in the mitochondrial production of ATP, has been shown to be suppressed by statins with subsequent reduction in insulin secretion (Mabuchi et al., 2005, Sampson et al., 2011, Larsen et al., 2013). Statins also decreased

the expression of glucose transporters (GLUTs) (Nakata et al., 2006), they affect the structure and function of GLUTs by reducing plasma cholesterol content with subsequent decrease in glucose uptake in adipocytes, skeletal myocytes, and hepatic cells (Takaguri et al., 2008, Nowis et al., 2014).

On the other hand, MIN6 incubation with simvastatin or atorvastatin for 24-48 h resulted in an unexpected dose-dependent increase in basal insulin secretion at low glucose concentration (Ishikawa et al., 2006). Similarly, *in-vivo* randomized, single-blinded, placebo-controlled study showed that simvastatin, but not pravastatin, administration for two months significantly increased plasma insulin levels (Koh et al., 2009).

K_{ATP} ion channel activity and mitochondrial metabolism are crucial for the coupling of glucose recognition to the exocytosis of insulin granules (Rustenbeck et al., 1997, Ashcroft et al., 1984, Wollheim, 2000), thus K_{ATP} channels as well as mitochondria have to be considered as a potential off-target for statins.

The modulation of K_{ATP} ion channel activity by statins has been observed in different tissues. Simvastatin, but not pravastatin has been reported to inhibit K_{ATP} channel -induced relaxation of the porcine coronary arteries (Uhiara et al., 2012). This is consistent with recent data demonstrating that simvastatin inhibits K_{ATP} channel in porcine coronary artery myocytes (Seto et al., 2013). On the other hand, cerivastatin, but not pravastatin, is thought to induce relaxation of rat aortic rings through activation of K_{ATP} channels (Sonmez Uydes-Dogan et al., 2005). Such effects suggest that statins have differential effects on K_{ATP} channels of different cells.

On the other hand, other studies have demonstrated that lipophilic statins can alter mitochondrial function. In skeletal muscle fibres, simvastatin alters the mitochondrial respiratory chain leading to mitochondrial membrane potential depolarization (Sirvent et al., 2005b, Sirvent et al., 2012). Simvastatin has also been shown to cause a concentration-

dependent mitochondrial membrane potential depolarization in rat hepatocytes (Abdoli et al., 2013, Tolosa et al., 2014). The importance of mitochondrial metabolism for β-cell function is well established and oxidative mitochondrial metabolism is central to glucose stimulated insulin secretion (Lowell and Shulman, 2005, Maechler et al., 2010, Wiederkehr and Wollheim, 2012). Therefore, another potential way in which statins could alter β-cell function is through effects on the mitochondria.

The aim of this present work was to identify the effects of statins on mitochondrial membrane potential ($\Delta\psi_m$) and to determine whether the statin drugs, simvastatin and pravastatin, functionally interact with K_{ATP} ion channel activity in β-cells, and thereby contribute to the observed side-effects of these drugs.

5.2. Materials and Methods

5.2.1. Solutions:

For the measurement of K_{ATP} currents and $\Delta\psi_m$ the extracellular (bath) solution contained (in mM): 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 1.2 NaH_2PO_4, 4.2 $NaHCO_3$ 10 HEPES (pH 7.4 with NaOH). For patch clamp, the pipette solution contained (in mM): 140 KCl, $NaHCO_3$ 4.2, $MgCl_2$ 1.1, HEPES 10 (pH 7.4 with NaOH). For inside-out experiments, the intracellular face of the membrane was bathed in a solution which contained (in mM): KCl 140, $CaCl_2$ 4.6, EDTA 10, K-HEPES 10 (pH 7.2; free [Mg^{2+}] <6 nM; free [Ca^{2+}] < 30 nM). This solution does not support protein phosphorylation since it does not contain Mg^{2+} or ATP, it also reduces rundown of K_{ATP}-channels activity in excised patches (Ashcroft and Kakei, 1989, Smith et al., 1994). Simvastatin was dissolved in either DMSO or ETOH. carbonyl-cyanide-*p*-trifluoro- methoxy-phenylhydrazone (FCCP) was dissolved in DMSO, final concentration of the solvents kept less than

<0.1%. Pravastatin was dissolved in distilled water. Mitosox (Invitrogen) was dissolved in DMSO. All other drugs were obtained from Sigma.

5.2.2. Preparation of the β -cell

β-cells were isolated from mouse islets of Langerhans. The latter were acutely dissociated from pancreatic islets isolated from the pancreata of 30-35g CD1 mice as previously described (Daunt et al., 2006). For fluorescence measurements, β-cells were plated onto 22 mm glass coverslips. For electrophysiology, cells were plated onto 35 mm plastic Petri dishes (Nunc). Cells were maintained and grown in RPMI-1640 media supplemented with 11 mM glucose, 10 % FCS, 25 mM HEPES and were kept at 37°C in humidified air/5% CO_2. For all patching experiments, cells were preincubated prior to use in a sugar free Hanks solution at 37°C for 20-30 minutes, unless stated otherwise. Experiments were performed in a HEPES-buffered Hanks solution which contained (in mM):138 NaCl, 4.2 $NaHCO_3$, 1.2 NaH_2PO_4, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES (pH 7.4 with NaOH).

5.3. Method

5.3.1. Patch clamp recording

Patch pipettes were made from borosilicate glass capillaries (GC150TF-15; Harvard, UK). The pipettes were pulled using 2-stage vertical puller (Narishige PP83, Japan). The diameter of the pipette tip was assessed using the bubble number method. Those with bubble numbers of 7 were used for patch clamp. The pipettes were coated with Sylgard (Dow Corning Corp) below the pipette tips in order to reduce electrical noise and the pipette tips were fire-polished prior to use. Typically, the pipette resistances were between 2.5 and 5 MΩ. The pipettes were then filled with

high K^+ solution using a microsyringe, with care taken to remove bubbles from the tip.

The β-cells were washed with Hank's solution prior to patching. Only cells that appeared to be round and morphologically distinct were patched. Recordings were made, cell-attached or inside-out configuration, using an Axopatch patch clamp amplifier (Molecular Devices Inc., Sunnyvale, CA, USA). Single-channel currents were filtered at 2 kHz prior to digitisation at 10 kHz using Clampfit 10.3 (Molecular Devices Inc., Sunnyvale, CA, USA). Axopatch-1D patch clamp amplifier (Axon instruments). All experiments were carried out at a room temperature, 21-23 $^{\circ}$C.

In the cell-attached mode, single channel currents were recorded at pipette potential of 0 mV i.e. at the resting membrane potential. In the excised inside-out mode, single- channels recordings were made with pipette voltage of +60 mV.

NPo (open probability) was used as a measure of channel activity. All NPo values were calculated from three minutes of single-channel recording. Data from each patch membrane served as the control i.e. NPo after seal and HANKS perifusion as a basal record, was compared with NPo after 3 minutes and 10 minutes post drug treatment.

5.3.2. The use of rhodamine 123 for detection of mitochondria polarization.

Mitochondria are involved in a large variety of cellular functions (Duchen, 1999). A characteristic feature of mitochondria is the generation of a large transmembrane potential mediated by the H^+ transport through activity of the respiratory chain. Modulation of the mitochondrial potential has been implicated in many different pathological situations (Duchen, 2004, Lu et al., 2010). Therefore, the determination of the mitochondrial potential is

important for the understanding of cellular functions. In the current study, β-cells isolated from mice were plated on glass coverslips. Cells were then loaded with 10 μg ml^{-1} Rh 123 at 37°C for 10 min in Hanks solution (5 mM glucose). There was no apparent cytotoxicity in cells treated at 10 μg ml^{-1} (Johnson et al., 1980). Moreover, results of a previous experiment in our laboratory by Dr Paul Smith concluded that 10 μM of Rh123 has no effect on β-cell oxygen consumption. Rh123 is commonly used to follow changes in mitochondrial potential with time following acute cellular insults (Palmeira et al., 1996). It is well established that this lipophilic dye is predominantly localized to mitochondria (Johnson et al., 1980). The attraction of rhodamine 123 molecules by the relatively high negative electric potential across the mitochondrial membrane is the basis for the selective staining of isolated mitochondria as well as mitochondria in living cells (Scaduto and Grotyohann, 1999, Perry et al., 2011, Baracca et al., 2003). Addition of glucose to the mitochondria leads to the generation of a potential with subsequent uptake of Rh123 (figure 5.14). On the other hand, agents known to depolarize or de-energize mitochondria results in the redistribution of the dye across the inner membrane, relieving quench, with associated increase in fluorescence (Johnson et al., 1981). Using this property, the effects of simvastatin or pravastatin perifusion on β-cell mitochondrial potential was measured at 32 °C. Control cells were perfused with vehicle only (0.1% DMSO).

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential, $\Delta\Psi m$, was monitored with Rhodamine-123 (Rh-123) as described previously (Daunt et al 2006). After loading with Rh123 (10 μg ml^{-1} for 10 minutes at 37°C in 5 mM glucose), cells were continuously illuminated at an excitation wavelength of 485±10 nm at the lowest light intensity that gave a fluorescent signal. The emitted

light was long pass filtered at 515 nm and visualised with a Zeiss PlanNeofluar 40X/1.3 oil objective (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK) heated to 32°C with an Bioptechs objective heater (Intracel LTD, Royston, Herts, UK). Images were captured at a frequency of 1Hz with a Photonics ISIS CCD camera, DT3155 frame grabber (Data translation, UK) and Imaging workbench software (IW5.2 INDEC BioSystems, Santa Clara, CA, USA). Images were analysed using equation below as shown in the (figure 5.2).

$$(A-100)/ (B-100)*100$$

Where **A** value represents the increase in fluorescence induced by simvastatin, & **B** value represents the maximum fluorescence induced by FCCP perifusion

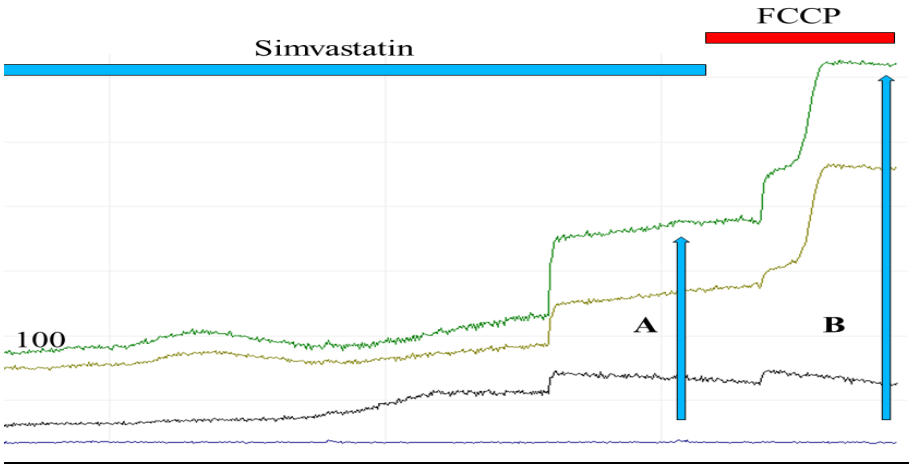


Figure 5.2. Representative fluorescence trace showing method of calculating the FL increase induced by statins. mitochondria are considered to be fully polarized =100. **A** value represents the increase in fluorescence induced by simvastatin, and **B** value represents the maximum fluorescence induced by FCCP perifusion.

5.3.3. Superoxide measurement using MitoSOX:

MitoSOX is a red mitochondrial superoxide indicator with excitation/emission of approximately 510/580 nm. It is a highly selective dye for detection of superoxide in the mitochondria of live cell (Robinson et al., 2006). In the mitochondria, the dye is oxidized by superoxide. Then the oxidation product exhibits red fluorescence upon binding to the nucleic acids (Robinson et al., 2008). The dye was prepared from 5 mM stock solution. MIN6 cells were incubated with 5 μM MitoSOX in HANKS with 5 mM glucose for 10 minutes at 37°C. The cells were then washed gently three times with HANKS followed by incubation with 1 μM simvastatin or 1 μM antimycin A for two hours duration. Lastly, the cells imaged with DMRB Fluorescence microscope Leica^{EM}.

5.4. Statistical analysis:

Single channel kinetics and NPo were estimated using the half-amplitude threshold technique as implemented in clampfit. Channel kinetics from only single events were then exported into a bespoke program that fitted a conditional probability density function as described in (Smith et al., 1994) to determine the effect of the drug we used the open time of highest probability and the closed time which represents closure between events. Data were ascertained to be normally distributed by the D'Agostino-Pearson omnibus Normality test. In this study statistical analysis was accomplished by Student's unpaired t-test or 1-way ANOVA, statistical significance was attributed when $P<0.05$. The analysis was accomplished using Prism 6. Data are given as mean ± S.E.M and n is the number of experiments in cell attached, inside out recordings, or mitochondrial potential measurement. Each experiment came from a different petri dish.

5.5. Results:

5.5.1. Patch clamp recordings

5.5.1.1. Effect of glucose on K_{ATP} channel activity

The aim of these experiments was to investigate the effects of simvastatin on K_{ATP} channels of pancreatic β-cells. The cell-attached configuration was chosen for such purpose, so that the β-cells retain an intact glucose metabolism (Ashcroft et al., 1984). As a point of reference, the NPo of K_{ATP} channels was calculated in zero glucose conditions followed by the NPo examination in 5 mM glucose. Significant inhibition of K_{ATP} channel activity by glucose addition was observed (figure 5.3). It is concluded that the β-cells used for the experiments were intact and responded to the glucose with increased ATP generation and subsequent K_{ATP} channel inhibition.

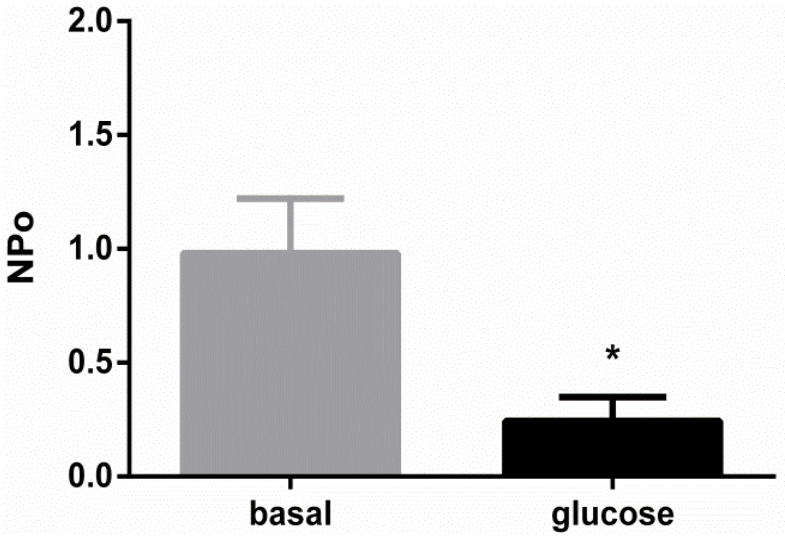
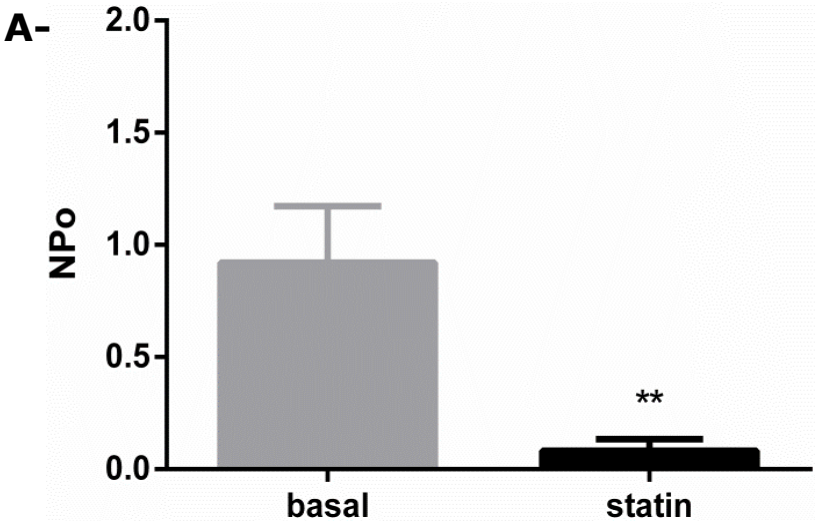
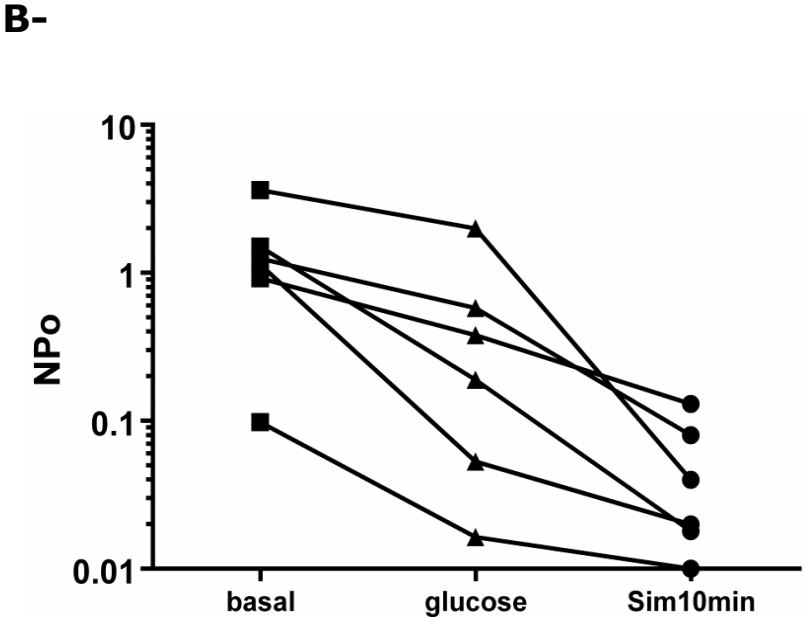


Figure 5.3. Effect of glucose 5 mM on K_{ATP} channel activity in pancreatic β-cells: changes of the NPo after the addition of 5 mM glucose**.** * indicates P<0.05, Student's unpaired *t*-test of 5 different patches.

5.5.1.2. Effect of statins on K_{ATP} channel activity

In cell-attached patch clamp experiments, the channel opening probability of the cells treated with 10 µM simvastatin was calculated alike. As shown in figure (5.4-A), simvastatin (10 µM) significantly reduced K_{ATP} channel activity by 71 ± 16% (n=6, $p<0.05$). Figure (5.4-B) illustrates the individual NPo values for the block of K_{ATP} channel activity. A maximal block was observed after perifusion of 10 µM simvastatin for 10 minutes in 5 mM glucose conditions. On the other hand, DMSO (0.1%) had no effect on NPo in the presence of 5mM glucose (Figure 5.4-C).





C-

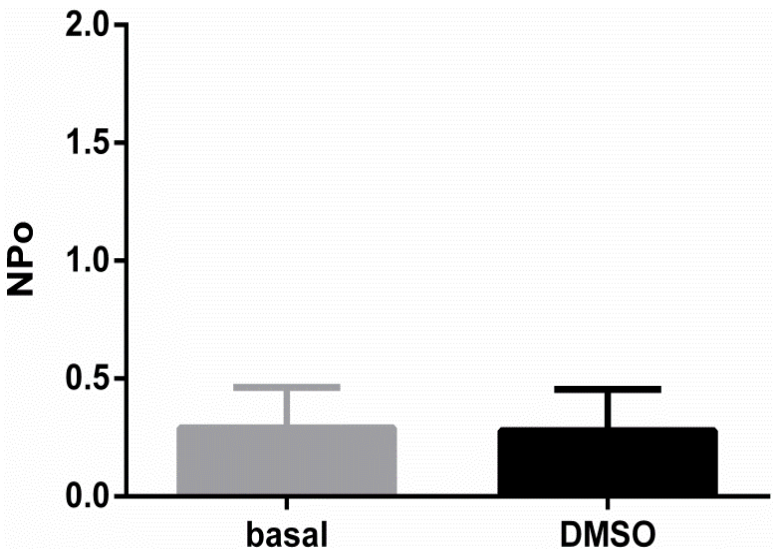
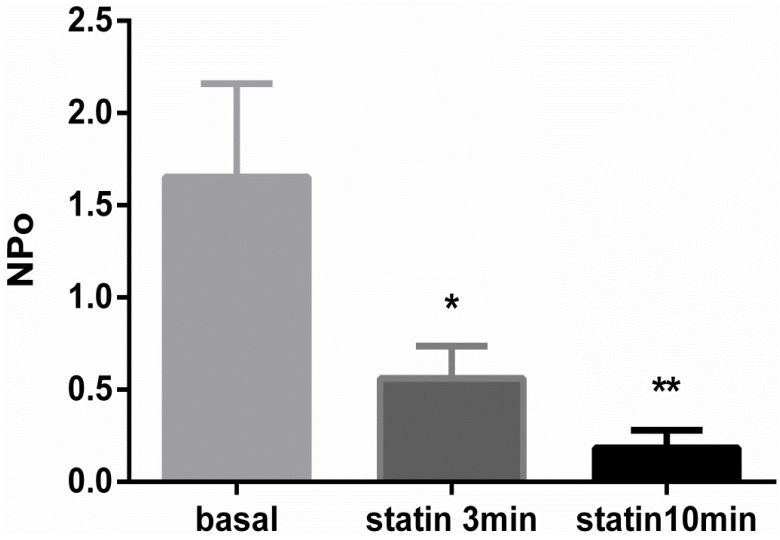


Figure 5.4. Effect of simvastatin on K_{ATP} channel activity in the presence of 5 mM glucose. A- changes of the NPo after the addition of 10 μM simvastatin (statin) in 5 mM glucose **B-** changes of the NPo in the progress of the experiments, the addition of 5 mM glucose significantly decreased NPo, subsequent addition of 10 μM simvastatin caused a time dependent block in comparison to equivalent experiment for the vehicle control. After 10 min statin had almost abolished channel activity unlike the vehicle control. **C-** changes of the NPo after DMSO perifusion in 5 mM glucose. ** indicate P<0.01, Student's unpaired *t*-test of 6 different patches.

5.5.1.3. Effect of simvastatin on K_{ATP} channel activity in the absence glucose:

In the absence of glucose, 10 μM simvastatin significantly reduced K_{ATP} channel activity (Figure 5.5-A). Figure (5.5-B) is a representative K_{ATP} channel recording trace showing the effect of simvastatin perifusion on the K_{ATP} channel activity NPo.

A-



B-

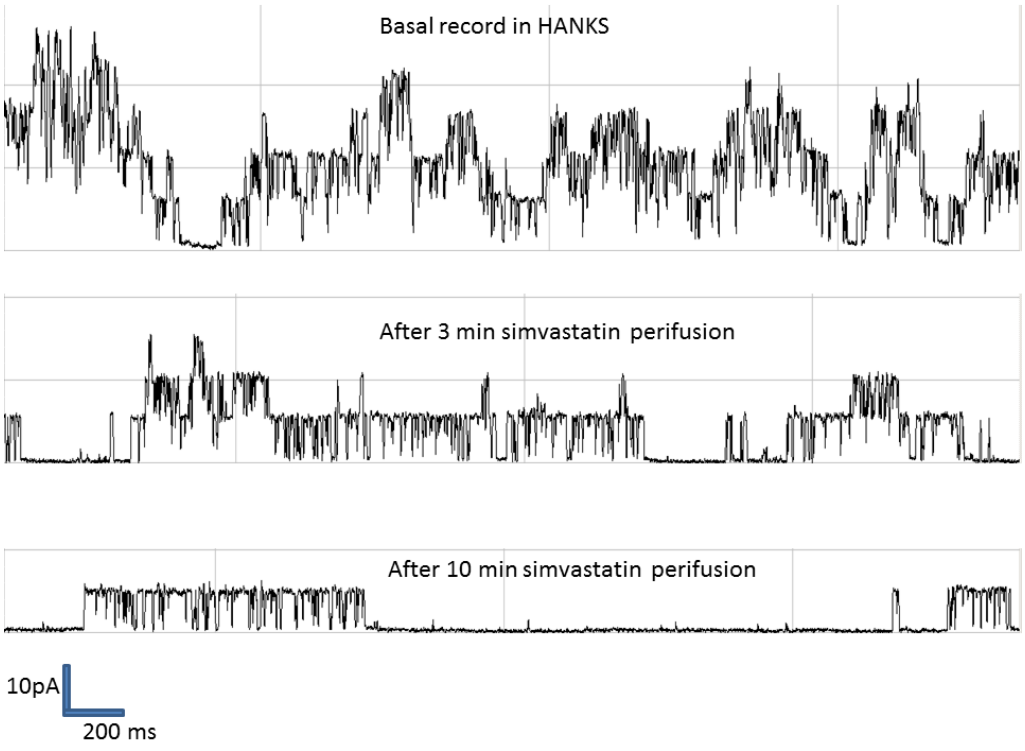
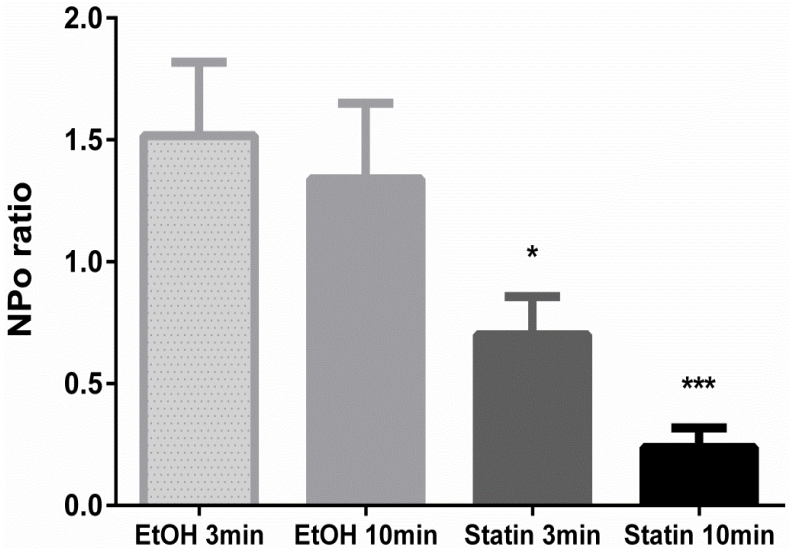


Figure 5.5. Effect of 10 μM simvastatin on K_{ATP} channel activity (NPo) in the absence of glucose: A- K_{ATP} channel activity after 3 min and 10 min of 10 μM simvastatin (statin) perifusion dissolved in DMSO compared to the K_{ATP} channel basal activity (NPo) prior to addition of simvastatin from a cell-attached patch on isolated β-cells in a glucose-free HANKS solution, simvastatin caused block ~67 % block after 3 min

perifusion and by ~90 % block after 10 min perifusion. * P< 0.05, ** P<0.01, 1 way ANOVA with Dunnett's multiple comparisons of 11 different patches. **B-** representative K_{ATP} channel recording trace showing the effect of simvastatin perifusion on the K_{ATP} channel activity NPo.

Changes of the NPo ratio after 3 min and 10 min of 10 μM simvastatin (dissolved in ethanol) were also calculated (figure 5.6-A). Figure (5.6-B) is a representative K_{ATP} channel recording trace showing the effect of ethanol perifusion on the K_{ATP} channel activity NPo.

A-



B-

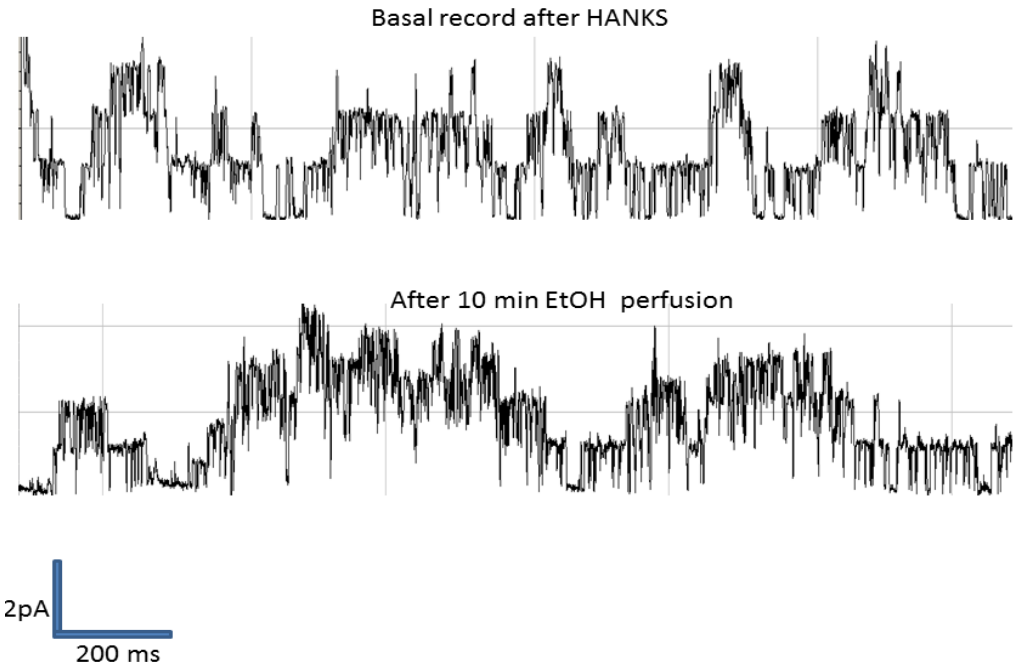
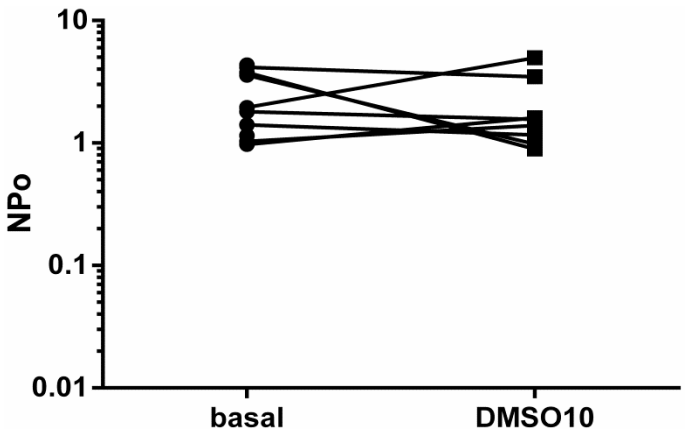


Figure 5.6. Effect of 10 µM simvastatin (statin) and its vehicle control (EtOH) on NPo ratio: A- effect of 10 µM simvastatin (statin) after 3 min and 10 min on K_{ATP} channel activity NPo normalized to basal channel activity in comparison with ethanol (EtOH) as a solvent control 3 min and 10 min perifusion using cell attached patch from a cell-attached patch on β-cells isolated in a glucose-free HANKS. * P< 0.05, ** P<0.01, 1 way ANOVA with Dunnett's multiple comparisons of 10 different patches. **B-** representative K_{ATP} channel recording trace showing the effect of (EtOH) perifusion on the K_{ATP} channel activity NPo.

5.5.1.4. Effect of the solvent control on K_{ATP} channel activity

In order to verify that results made with simvastatin were not due to the solvents used, the effects of DMSO and ethanol on K_{ATP} channels were evaluated separately. Neither of the two solvents affected NPo values (figure 5.7-A&B). In chapter II ethanol induced a transient contraction to the coronary vessels incubated with 300 µM L-NAME, thus DMSO was used as alternative solvent.

A-



B-

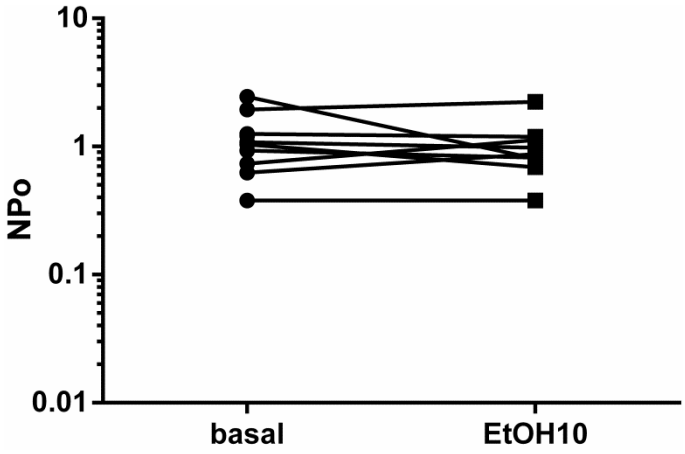
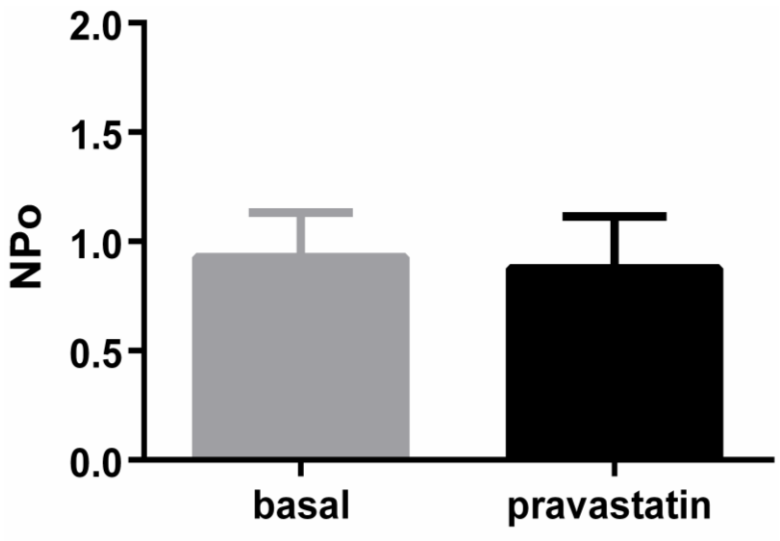


Figure 5.7. K_{ATP} channel activity (NPo) after 3 min and 10 min of **A-** DMSO & **B-** ethanol (EtOH) perifusion as a vehicle control compared to the K_{ATP} channel basal activity. 1 way ANOVA with Dunnett's multiple comparisons of 10 different patches for each.

5.5.1.5. Effect of the pravastatin on K_{ATP} channel activity

As part of the comparison, the effects of the hydrophilic statin, pravastatin, on the channel opening probability was also examined. As shown in (figure 5.8-A) 10 μM pravastatin had no effect on the parameter tested. Figure (5.8-B) is a representative K_{ATP} channel recording trace showing the effect of pravastatin perifusion on the K_{ATP} channel activity NPo.

A-



B-

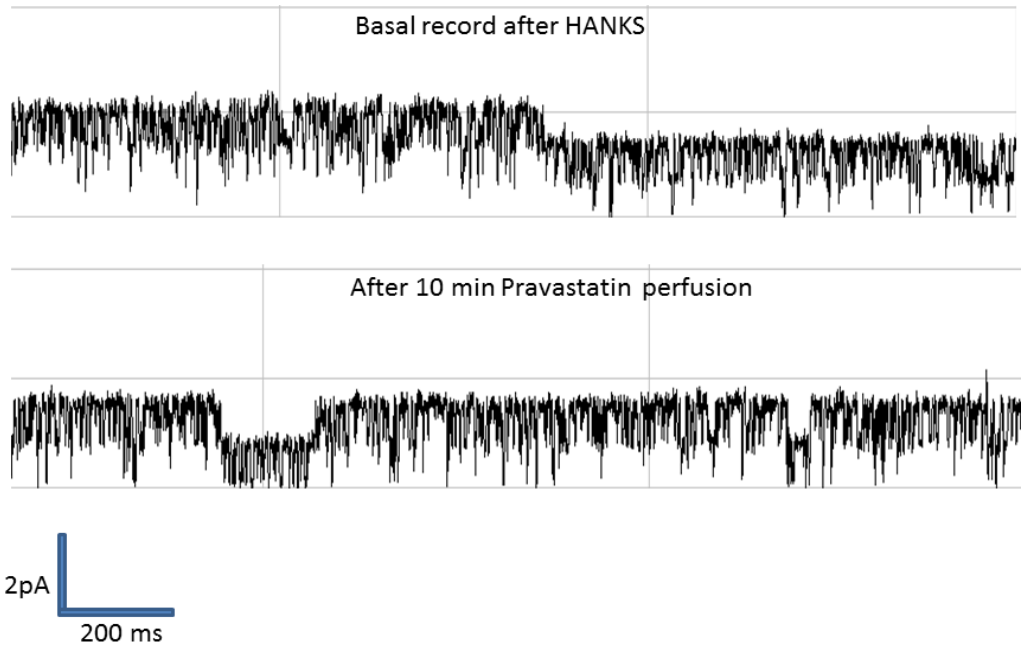
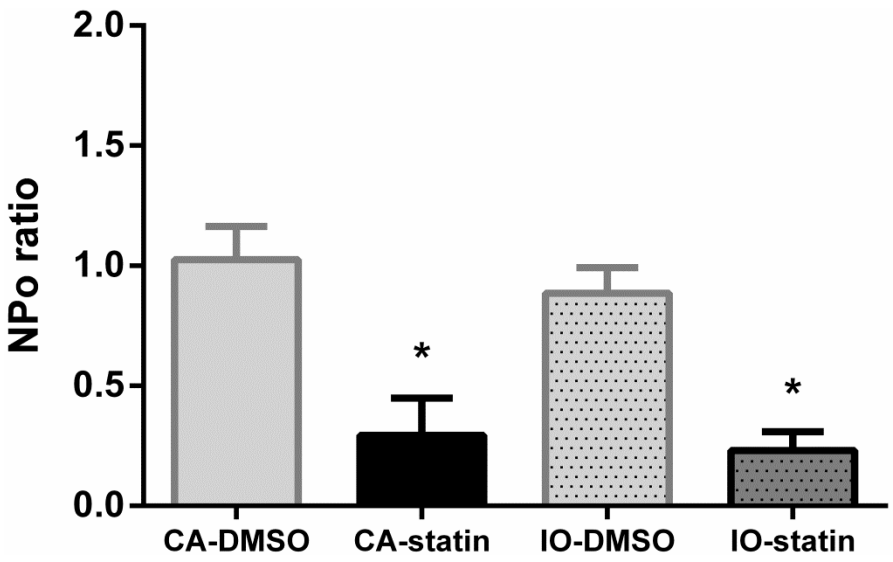


Figure 5.8. Effect of pravastatin on K_{ATP} channel activity with cell attached patch: A- Single channel current recorded from a cell-attached patch on β-cells after 10 min perifusion of 10 μM pravastatin in HANKS solution. Pipette potential was held at 0 mV throughout the experiment. Student's unpaired *t* test of 7 different patches. **B-** representative K_{ATP} channel recording trace showing the effect of pravastatin perifusion on the K_{ATP} channel activity NPo.

5.5.1.6. Effect of simvastatin on K_{ATP} channel activity using inside-out configuration:

Similar to the results obtained with cell attached patch, using inside-out configuration the addition of 10 μM simvastatin caused a significant reduction in K_{ATP} channel activity. The reduction in the NPo ratio with cell attached patch was 71.57% and with inside-out patch it was 73.86% (figure 5.9-A). Figure (5.9-B) is a representative K_{ATP} channel using inside-out configuration showing the effect of simvastatin perifusion on the K_{ATP} channel activity NPo.

A-



B-

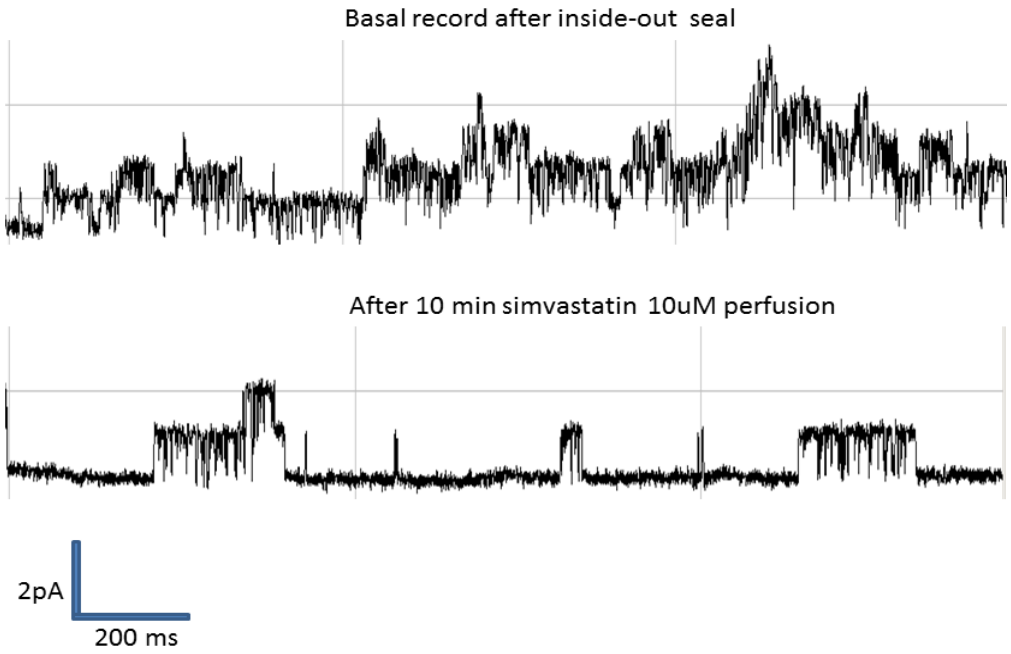


Figure 5.9. comparison of the effect of simvastatin (10 µM) on NPO in both cell attached and inside-out patch: A- effect of 10 µM simvastatin (statin) after 3 min on K_{ATP} channel activity NPo normalized to basal channel activity using cell attached patch in comparison with inside-out patch on β-cells in a glucose-free HANKS. Pipette potential was held at 0 mV with the cell attached patch and the Pipette potential was held at -60 mV with inside-out experiments. * indicates P<0.05, 1 way ANOVA with Dunnett's multiple comparisons of 6 different patches. **B-** representative K_{ATP} channel using inside-out configuration showing the effect of simvastatin perifusion on the K_{ATP} channel activity NPo.

5.5.1.7. Effects of simvastatin on K_{ATP} channel kinetics:

To determine further the molecular nature of the interaction between simvastatin and the K_{ATP} channel, an analysis of single channel kinetics was performed. In this study simvastatin 10 µM significantly inhibited the single channel amplitude by 18.17 % of the basal amplitude after 3 min of treatment, additional inhibition of the channel amplitude was observed after 10 min of drug treatment 44.8 6% (figure 5.10).

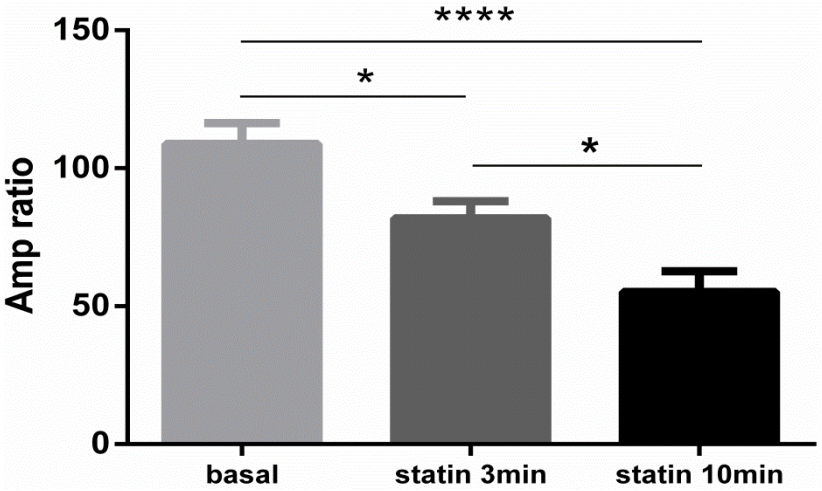


Figure 5.10. Effect of 10 μM simvastatin on K_{ATP} channel amplitude with cell- attached patch: effect of 10 μM simvastatin (statin) after 3 min and 10 min on K_{ATP} channel amplitude, data were normalized to basal channel amplitude (Amp ratio) using cell attached patch on β-cells in a glucose-free HANKS. * indicates P<0.05, **** indicates P<0.0001. One way ANOVA by Tukey's multiple comparisons test of 9 different patches.

A smaller but significant effect on the amplitude (14.55 %) was seen after 10 min of simvastatin perifusion in an inside-out patch configuration (figure 5.11). On the other hand, 10 μM simvastatin significantly increased the K_{ATP} channel opening time (figure 5.12).

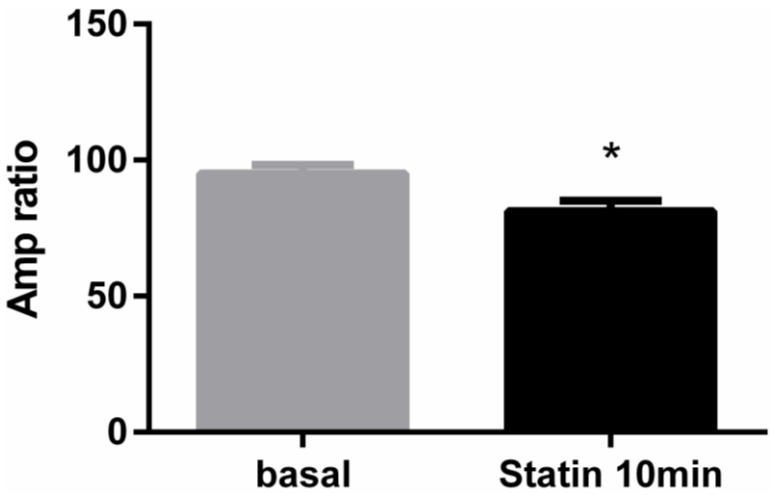


Figure 5.11. Effect of 10 μM simvastatin on K_{ATP} channel amplitude with inside-out patch: effect of 10 μM simvastatin (statin) after 10 min on K_{ATP} channel amplitude, data were normalized to basal channel amplitude (Amp ratio) using inside-out patch on β-cells. Pipette potential was held at -60 mV throughout the experiment. * indicates P<0.05, Student's unpaired *t*-test of 6 different patches.

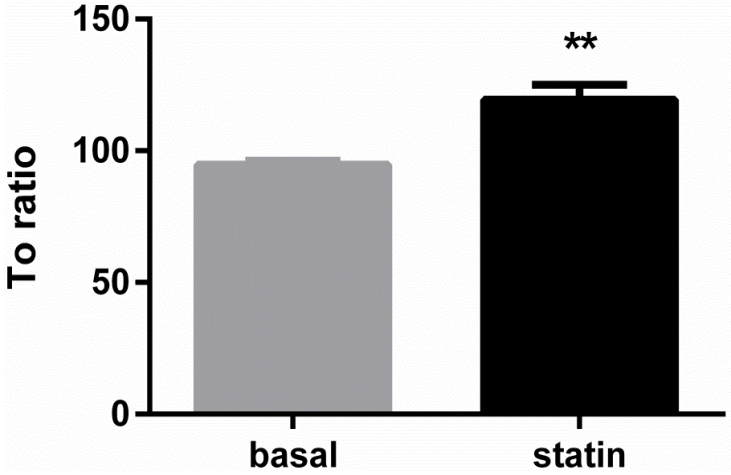


Figure 5.12. Effect of 10 μM simvastatin on K_{ATP} channel open time: effect of 10 μM simvastatin (statin) after 10 min on K_{ATP} channel open time (To), data were normalized to basal channel open time (To ratio) using cell-attached patch in a glucose-free HANKS. ** indicates P<0.01, Student's unpaired *t*-test of 5 different patches.

5.5.1.8. Effect of antimycin A and myxothiazol on K_{ATP} channel activity:

In our study simvastatin is proposed to act as a mitochondrial complex III inhibitor, thus as part of the comparison, the effects of the standard complex III inhibitors, antimycin A and myxothiazol, on β-cell NPo were examined. Results showed that myxothiazol inhibited the open probability of the K_{ATP} channel (figure 5.13), while antimycin A had no significant effect on the K_{ATP} channel activity.

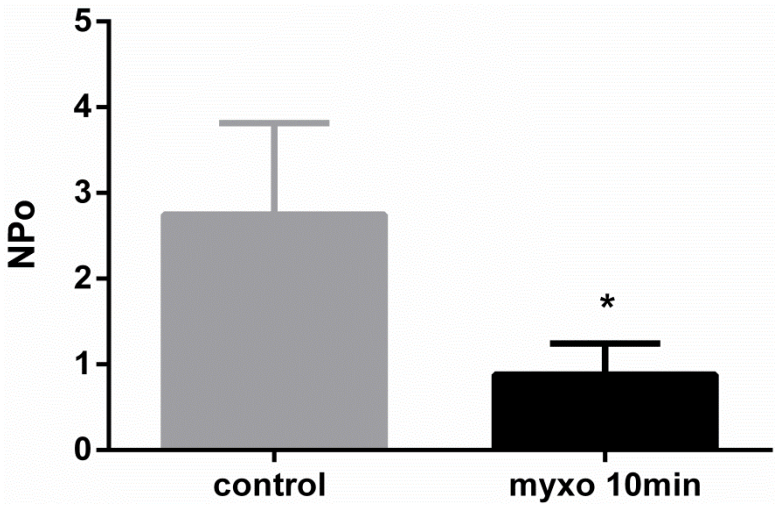


Figure 5.13. Figure 4: Effect of 10 μM complex III inhibitor, myxothiazol, on K_{ATP} channel activity (NPo): K_{ATP} channel activity after 10 min of 10 μM myxothiazol (myxo) perifusion dissolved in DMSO compared to the K_{ATP} channel basal activity (NPo) from a cell-attached patch on isolated β-cells. * indicates P<0.05, Student's unpaired *t*-test of 9 different patches.

5.5.2. Effects of simvastatin on mitochondrial potential in B-cell:

In the current study, the distribution and subsequent quenching of the fluorescent lipophilic cation Rh123 was used to monitor changes in mitochondrial potential ($\Delta\psi_m$). In the presence of 5 mM glucose, FCCP, a proton ionophore, dissipated $\Delta\psi_m$ and increases Rh123 fluorescence. On the other hand, FCCP had negligible effect on Rh123 fluorescence in the absence of glucose. This suggests that β-cell mitochondrial potential is depolarized in the absence of glucose. Furthermore, the mitochondrial potential is restored as the glucose concentration is increased (figure 5.14 at second 1000). Thus, the effect of simvastatin or pravastatin on β-cell mitochondrial potential was examined in 5 mM glucose (figure 5.15 & 5.16 respectively). Simvastatin (10 μM & 3 μM) caused depolarization of $\Delta\psi_m$ as evidenced by a 94% & 76% increase in Rh123 fluorescence respectively (figure 5.17 & 5.18). DMSO, as a solvent control, had no effect on mitochondrial potential (figure 5.14). By contrast, the water soluble statin (pravastatin, 10 μM) had no effect on mitochondrial potential (figure 5.16 & 5.17).

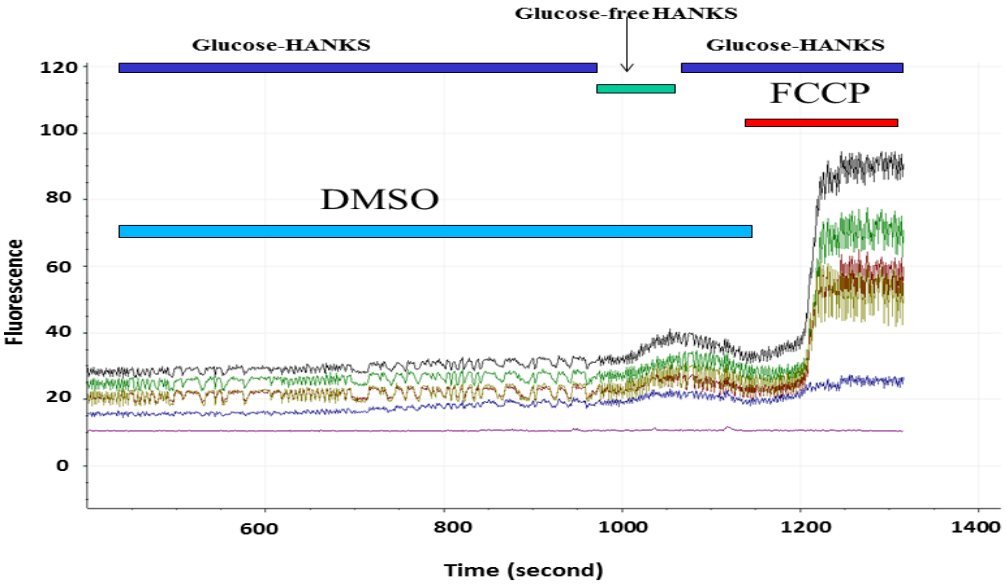


Figure 5.14. Representative effect of DMSO on Rh 123 fluorescence: Rh 123 fluorescence traces for 4 primary β-cells examined the effects of DMSO, applied at 200s, as a solvent control on the mitochondrial potential. DMSO clearly had no effect on the mitochondrial potential. At 1000s, superfusion of β- cells with glucose-free HANKS for one minute, caused increase in the fluorescence signal due to mitochondrial depolarization. After few seconds superfusion of HANKS with glucose resulted in reversal of the basal Rh123 signal due to mitochondrial repolarization with subsequent RH123 quenching. At the end of the recording, at 1150s, FCCP 1 μM is added as positive control to get the maximum fluorescence.

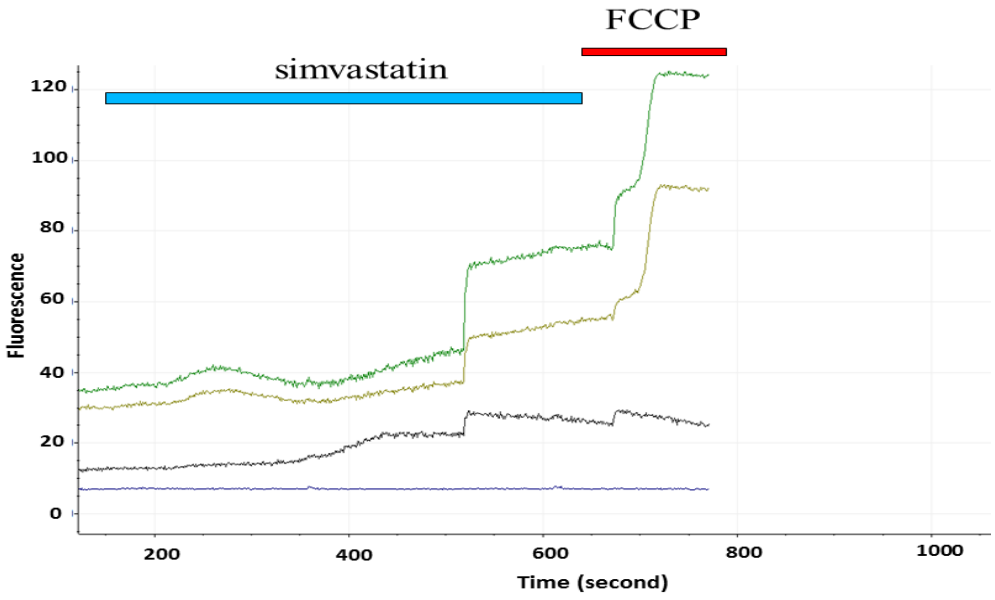


Figure 5.15. Representative effect of simvastatin on Rh 123 fluorescence: The trace for 3 single pancreatic β-cells isolated from mice, examined the effects of 10 μM simvastatin on the mitochondrial potential. Simvastatin was applied for the duration of the bars, at 620s, FCCP 1 μM was perfused as positive control to get the maximum fluorescence.

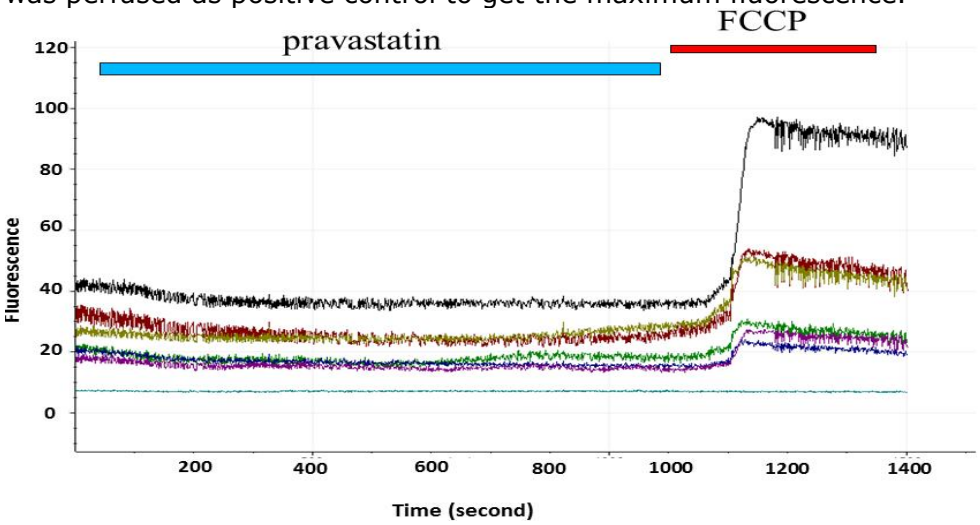


Figure 5.16. Representative effect of pravastatin on Rh 123 fluorescence: The trace for 5 single pancreatic β-cells isolated from mice, examined the effects of 10 μM pravastatin on the mitochondrial potential. Pravastatin was applied at the point indicated on the trace in the presence of 5 mM glucose. At 1150s, FCCP 1 μM was perfused as a positive control to get the maximum fluorescence.

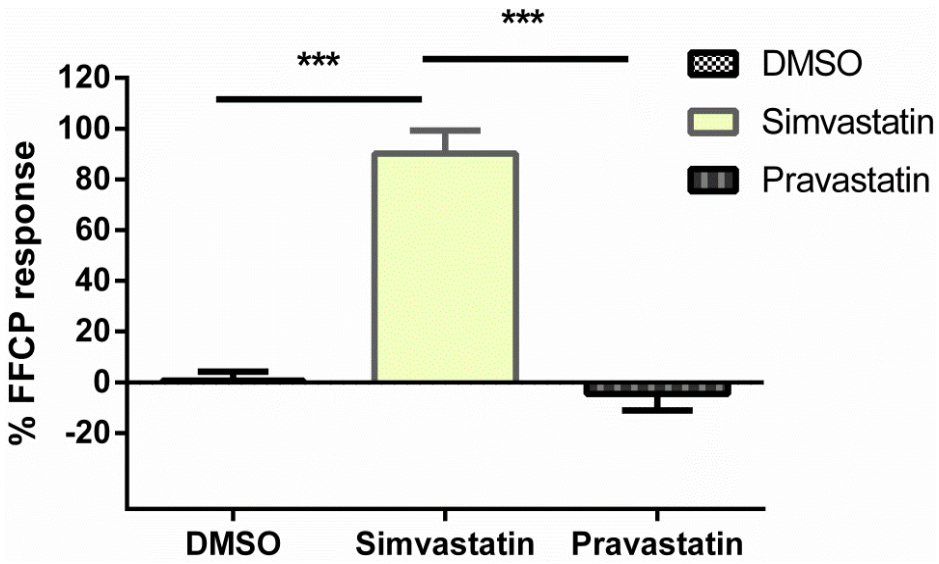


Figure 5.17. effect of simvastatin 10 μM on mitochondrial potential. Cumulative fluorescence data from pancreatic β-cells loaded with Rh123 in the presence of simvastatin (10 μM), pravastatin (10 μM), or vehicle control (0.1% v/v DMSO). Data are expressed as a percentage of the response to 1 μM FCCP in the same cells and are mean ± SEM from n=7 separate experiments. *** indicates $p<0.001$, one-way ANOVA, followed by Tukey's multiple comparisons test.

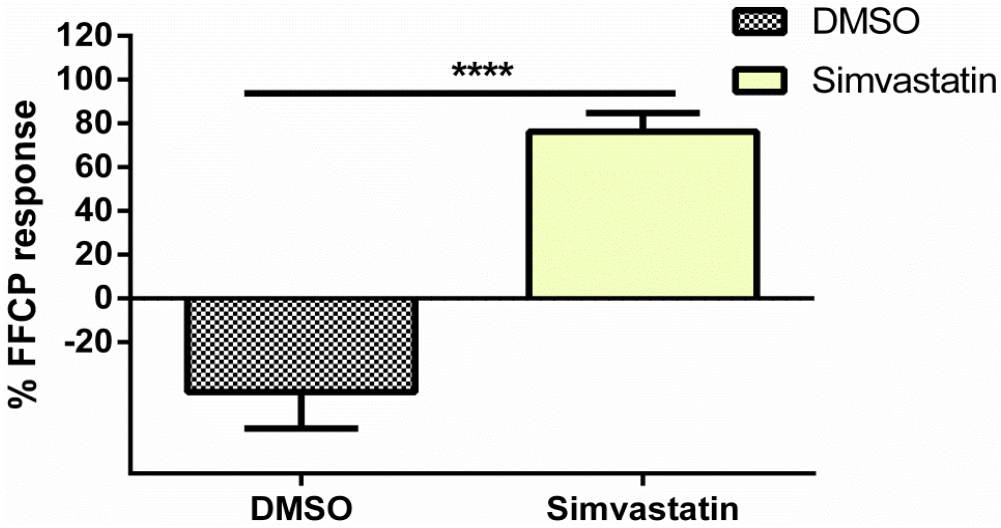


Figure 5.18. effect of simvastatin 3 μM on mitochondrial potential. Cumulative fluorescence data from pancreatic β-cells loaded with Rh123 in the presence of 3 μM simvastatin or vehicle control (0.1% v/v DMSO). Data are expressed as a percentage of the response to 1 μM FCCP and are mean ± SEM from n=14 separate experiments Student's unpaired *t*-test. **** indicates p<0.0001.

5.5.3. Effects of simvastatin on superoxide generation in MIN6:

Mitochondria are the most important cellular source of ROS in β-cells principally through complexes I and III of the respiratory chain (Chen et al., 2003, Muller et al., 2004). Results in the previous section have shown that simvastatin depolarizes the mitochondria in pancreatic β-cells. Thus, the effects of simvastatin on superoxide generation were examined. MIN6 cells were used as a model for pancreatic β-cells. Figure (5.19) are representative images showing the increase in superoxide production in the mitochondria of MIN6 in the presence of 10 μM simvastatin or 1 μM antimycin A. Quantitative data expressing changes in mean fluorescent intensity of basal MitoSOX fluorescence following 10 μM simvastatin exposure in comparison with 1 μM antimycin A is shown in (figure 5.20).

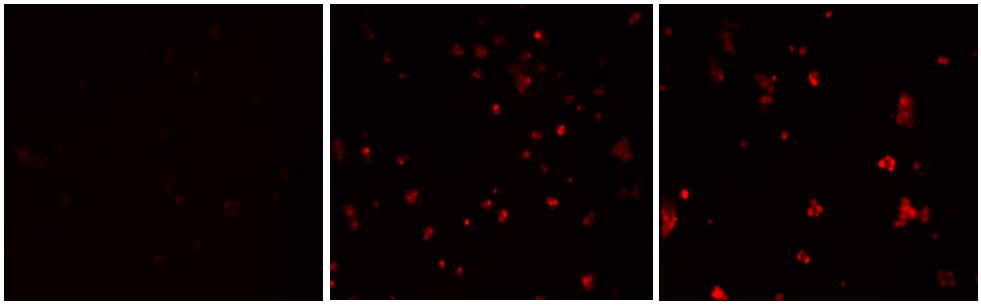


Figure 5.19. effect of simvastatin on superoxide production in MIN6: Representative images showing the increase in superoxide production in the mitochondria of MIN6 using Mitosox. A- basal fluorescence in the presence of vehicle (0.1% DMSO), B- in the presence of 10 μM simvastatin and C- in the presence of 1 μM antimycin A.

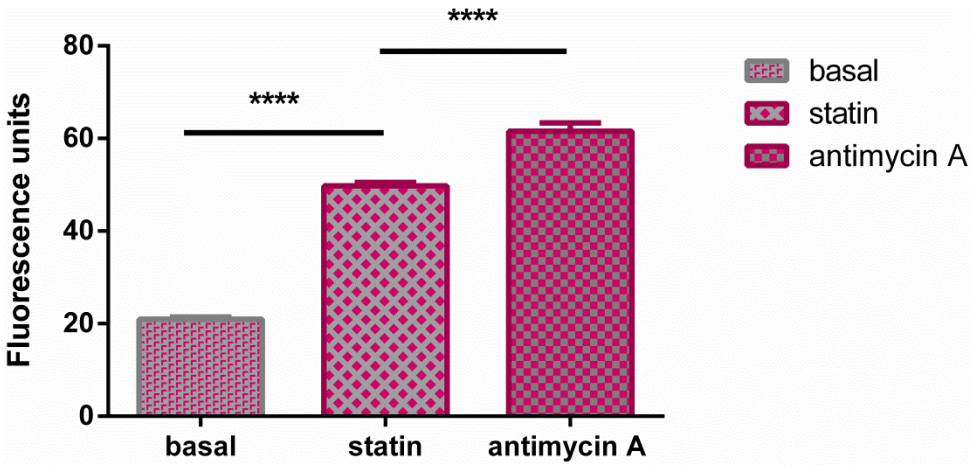


Figure 5.20. Effect of simvastatin and antimycin on mitochondrial superoxide formation in β-cells (**MIN6):** Quantitative data expressing changes in mean fluorescent intensity of basal MitoSOX fluorescence following 10 μM simvastatin (statin) exposure in comparison with 1 μM antimycin A measured by DMRB Fluorescence microscope Leica^{EM}. Data presented as average ± standard deviation of 124 cells from 4 different repeats. ****P < 0.0001, one-way ANOVA followed by Tukey's multiple comparisons test.

5.6. Discussion

The statins, simvastatin and pravastatin are currently important drugs for the treatment of hyperlipidaemia. The effects of these drugs on the early steps of the insulin-secretory pathway in the pancreatic β-cells were examined in the present study. The rationale for this investigation was a recent meta-analysis of clinical data which highlighted an increased risk of type II diabetes mellitus in hyperlipidaemic patients when administered certain statins (Baker et al., 2010, Carter et al., 2013, Bang and Okin, 2014, Macedo et al., 2014). Primary pancreatic β-cells isolated from mice were used for this investigation. The K_{ATP} channel open probability (NPo), open time and amplitude were investigated using cell-attached and inside-out patch configuration. The effects of simvastatin on single channel activity were analysed in the absence and the presence of 5 mM glucose.

K_{ATP} channels were blocked by glucose, consistent with the previously documented effects of glucose on K_{ATP} channel kinetics i.e. decrease NPo and amplitude (Ashcroft et al., 1984). This is due to the concomitant increase in cell metabolism with subsequent raising of the ATP/ADP ratio which inhibits K_{ATP} channels. Subsequently, simvastatin or pravastatin were perifused at 10 μM concentrations. The experiments revealed that simvastatin, but not pravastatin, inhibits channel activity (NPo). Further analyses of the channel kinetics showed that simvastatin significantly reduced channel amplitude and increased the open time of K_{ATP} channels (To).

In order to further investigate the action of simvastatin on β-cell function, our study examined the effects of simvastatin on β-cell mitochondrial membrane potential as possible target of statins. Simvastatin has been previously shown to depolarize mitochondria in skeletal muscle (Sirvent et

al., 2005b, Sirvent et al., 2012), liver cells (see appendix) (Abdoli et al., 2013, Tolosa et al., 2014) and smooth muscle (chapter II). The present study clearly shows that 10 μM simvastatin depolarizes the mitochondria membrane potential in β-cells within 10 min of perifusion. Similar results of mitochondrial depolarization were obtained with 3 μM simvastatin, however longer perifusion period, around 20 min, was usually required in order to depolarize the mitochondria at this concentration. The effect of 1 μM simvastatin on mitochondrial membrane potential was also tried. At this concentration the mitochondria depolarized after about 30 min of continuous simvastatin perifusion. However, because of the continuous bleaching of Rh123, the results failed to reach a significant effect. Thus, our study confirms that simvastatin at 3 μM & 10 μM depolarizes the mitochondria of the β-cell, which in turn may lower ATP concentration provided by the oxidative phosphorylation. However the exact mechanism of mitochondrial depolarization remains to be defined and further studies are recommended to shed light on this proposal.

The studies with excised patches demonstrate that the effect of simvastatin on K_{ATP} channels is likely to be direct and independent of changes in the mitochondria or other intracellular signalling. Accordingly, it is clear that simvastatin acts on different targets in β-cells which exert opposing effects, the first one is a direct inhibition of K_{ATP} channel, and the second effect is β-cell mitochondrial depolarization which gives the reason behind the increase in K_{ATP} channel opening time (figure 5.21). A previous study by Smith *et al* (1999) has shown that tolbutamide uncouples the β-cell mitochondria and has a blocking effect of K_{ATP} channel (Smith et al., 1999). The Seto *et al* study (2013) illustrated that simvastatin acutely inhibits vascular K_{ATP} channel openings in coronary artery myocytes (Seto et al., 2013), which was thought to be mediated via AMPK activation (Seto

et al., 2013). The study by Zou *et al* demonstrated that respiratory chain inhibition generate mitochondrial O_2^- which then leads to AMPK activation (Zou et al., 2004). In fact, the AMPK activator, AICAR, inhibited K_{ATP} opening in mouse pancreatic islets β-cells (Wang et al., 2005). However, it is implausible that K_{ATP} channel inhibition by simvastatin in pancreatic β-cells is mediated by AMPK phosphorylation since the inhibitory effect of simvastatin was observed in excised patches, i.e., in the absence of metabolic regulation. On the other hand, pravastatin, a hydrophilic statin had no effect on either measurement. The latter evidence suggests that statin action on β-cell function is related to the lipophilicity of these compounds (Joshi et al., 1999).

The reduction in the amplitude suggests a direct block of K_{ATP} channels by simvastatin. Results suggest also that simvastatin acts by interaction with the SUR subunit of the K_{ATP} channels like e.g. tolbutamide which would decrease NPo. In cell-attached patches the amplitude reduction appears larger than inside-out patches. The latter suggests membrane potential changes with cell depolarization due to block of K_{ATP} channels by simvastatin. However, K_{ATP} channel opening time appear to be increased with simvastatin perifusion, although NPo decreased, this may be due to inhibition of mitochondrial metabolism and reduced ATP generation.

The inhibition of K_{ATP} channels by simvastatin is likely to underlie the increase in insulin secretion observed by previous in-vitro study after 24 h incubation (Ishikawa et al., 2006) and *in-vivo* study after two months daily administration of 20 mg (Koh et al., 2009, Moutzouri et al., 2011). On the other hand, the block of K_{ATP} channels is unlikely to elicit exocytosis in β-cells in a situation of a decreased ATP supply induced by mitochondrial depolarization (Rustenbeck et al., 1997). The latter may explain the impaired glycaemic control and insulin secretion after 12 months of 20 mg

daily use (Bellia et al., 2012). In the pancreatic β-cell, mitochondrial metabolism plays a pivotal role in the generation of signals coupling glucose recognition to insulin secretion (Maechler, 2003, Maechler et al., 2010). Glucose equilibrates across the plasma membrane through specific transporters thereby initiating glycolysis, subsequently mitochondrial metabolism leads to an elevated intracellular [ATP]/[ADP] ratio which promotes the closure of ATP-sensitive K^+ channels (Ashcroft et al., 1984, Koster et al., 2005, Remedi et al., 2006, McTaggart et al., 2010). Plasma membrane depolarisation promotes Ca^{2+} influx, which raises cytosolic Ca^{2+} and triggers insulin exocytosis (Eliasson et al., 2008). Besides the importance for ATP generation, mitochondria promote the generation of metabolite coupling factors, such as succinate, amplifying the action of Ca^{2+} signal for sustained insulin secretion (Leibowitz et al., 2005). Therefore, interfering with mitochondrial metabolism by blocking the respiratory chain abolishes glucose-stimulated insulin secretion (MacDonald and Fahien, 1990, Wollheim, 2000, Martin and McGee, 2014, Green et al., 2004).

Inhibition of K_{ATP} channels by simvastatin causes depolarization of the β-cell membrane. In turn, this should trigger the opening of the voltage-gated Ca^{2+} channels, eliciting Ca^{2+} influx, which should stimulate the exocytosis of insulin-containing secretory granules (Bokvist et al., 1995, Satin, 2000). Moreover, the elevation of cytosolic Ca^{2+} is also a prerequisite for sustained insulin secretion (Mears, 2004, Maechler et al., 2006). Unfortunately, our study did not examine the effects of simvastatin on Ca^{2+} influx in pancreatic β-cells. However, a previous study by Yada *et al,* showed that lipophilic HMG-CoA reductase inhibitors inhibit glucose-induced Ca^{2+} signalling and insulin secretion by blocking Ca^{2+} currents in rat β-cells (Yada et al., 1999). Data have shown that mitochondria

maintain Ca^{2+} oscillations (Straub et al., 2000, Camello-Almaraz et al., 2002, Camello-Almaraz et al., 2006b). Furthermore, mitochondria could act as a buffer of the localized increase in Ca^{2+} just beneath the plasma membrane (Theler et al., 1992, Toescu et al., 1993, Tinel et al., 1999, Rizzuto et al., 2012). Due to its localization in the vicinity of Ca^{2+} release or influx channels (Brini, 2003), the mitochondria have the ability to rapidly accumulate and then release large quantities of Ca^{2+} (Park et al., 2001). A previous study by Li *et al,* in diabetic mice has demonstrated that defective mitochondrial function in pancreatic β-cells is a key cause of abnormal insulin secretion by altering Ca^{2+} influx (Li et al., 2012). Moreover, mitochondrial complex III block by antimycin A blocks the increase in Ca^{2+} induced by succinate. On the other hand rotenone, a mitochondrial complex I inhibitor, was without effect. Accordingly we may suppose that simvastatin by blocking mitochondrial respiratory chain downstream of complex II may lead to Ca^{2+} desensitization (Maechler et al., 1998). However, further studies are recommended to determine if this is the case.

Simvastatin and ROS generation:

Significant increase in superoxide production was observed in MIN6 cells after 2 h treatment with 10 μM simvastatin (figure 5.20). Similarly, simvastatin (5 μM, 1 h) incubation induced human liver carcinoma cell (HepG2) mitochondrial depolarisation with a significant increase in mitochondrial superoxide (Tolosa et al., 2014). The exact relationship between insulin secretion and reactive oxygen species generation in pancreatic β cells is still debated (Sakai et al., 2003, Pi et al., 2007). Superoxide O_2^- ions are normally converted by superoxide dismutase to H_2O_2 that is then reduced to water by catalase or glutathione peroxidase. ROS may have different actions according to cellular concentrations being

either below or above a specific threshold, i.e. signalling or toxic effects. Accumulated evidence suggests that small fluctuations in the steady state concentration of ROS play an essential role in insulin secretion (Leloup et al., 2009). However, exposure to oxidative stress is sufficient to impair glucose stimulated insulin secretion in pancreatic islets (Maechler et al., 1999, Sakai et al., 2003, Green et al., 2004, Li et al., 2009). In fact, β-cells are characterized by relatively weak expression of free radical-quenching enzymes SOD, CAT, and GPx (Grankvist et al., 1981, Lenzen et al., 1996). Thus, mitochondrial inner membrane components are at a high risk for oxidative injuries, resulting in depolarized mitochondrial membrane and impaired ATP production. Lastly, ROS alone cannot promote insulin release since glucose induced elevations in ATP are necessary for glucose-stimulated insulin secretion (Kajikawa et al., 2002).

In summary, although it is clear that statins have beneficial effects on cardiovascular disease prevention; our study suggests that long-term use of large doses of lipophilic simvastatin may have adverse effects on glucose metabolism and insulin secretion by acting on K_{ATP} channels and β-cell mitochondrial potential. Therefore, patient blood glucose levels should be monitored to reduce adverse events caused by simvastatin especially in elderly patients (Sattar et al., 2010, Ma et al., 2012) and those using intensive simvastatin dosing (Preiss et al., 2011, Rajpathak, 2012, Rocco, 2012). Aging *per se* is associated with a continuous decrease, 1% per year, in basal insulin release beginning early in life (Iozzo et al., 1999, Chiu et al., 2000). Thus, high-dose simvastatin may be detrimental in the elderly, particularly with chronic treatment. Future research should focus not only on determining the incidence of new-onset diabetes with various statins, but also on clarifying the potential pharmacologic mechanism for such a relationship.

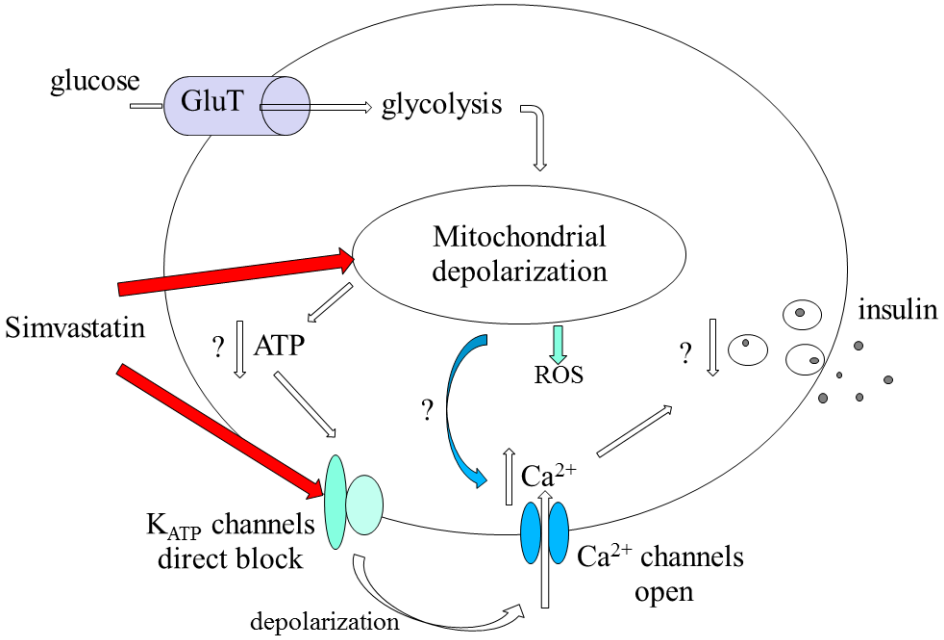


Figure 5.21. effect of simvastatin on pancreatic β-cell: The sites of simvastatin action proposed by our study, as indicated by red arrow, are mitochondrial depolarization. Furthermore simvastatin blocks K_{ATP} channels. Our proposal includes also ROS generation as a result of mitochondrial inhibition, mitochondrial depolarization may inhibit Ca^{2+} influx (as indicated by *?*). However, more studies are recommended to examine the effect of simvastatin on Ca^{2+} influx.

6. CHAPTER VI: DISCUSSION & CONCLUSION

6.1. GENERAL DISCUSSION

6.1.1. Short term effect of Simvastatin in comparison with Lovastatin and Pravastatin:

The benefits of long-term statin administration, i.e. lowering of serum cholesterol and treatment of hypercholesterolemia, are well established as the primary mechanism underlying therapeutic benefits in cardiovascular diseases (Bestehorn et al., 1997, Pedersen et al., 2004). In recent years, however, clinical and basic science research associated with statins showed that statins might also exert early cholesterol independent effects by acting on multiple targets (Wassmann et al., 2003, Takagi et al., 2006, Iida et al., 2007). These cholesterol independent effects were collectively known as pleiotropic effects (Bonetti et al., 2003). In fact, little has been addressed with regard to the matter of how statins affect the coronary circulation. Clinical studies have shown that patients with acute coronary syndrome may benefit from early statins use (Laufs et al., 2001, Stenestrand et al., 2001, Spin and Vagelos, 2003, de Lemos et al., 2004); however, it was not investigated whether these positive results were related to the lipid lowering effects of statins and/or specifically caused by the relatively early pleiotropic effects of statins (Kirmizis and Chatzidimitriou, 2009, Antoniades et al., 2011). Thus, the attention is now turning to the more immediate therapeutic effects of statins in the management of acute vascular conditions, which in turn would favour an immediate beginning of statin therapy (Spin and Vagelos, 2003).

In order to assess the vascular pleiotropic of statins our research was designed to study the very short-term effects of statins which appear over a range of 1-2 hours after *in vitro* incubation. The existence of non-lipid effects was further investigated by indirect evidence, such as studying

statins with different pharmacokinetics profile and/or treating animals, with different age and sex, obtained from local abattoir.

Currently simvastatin, pravastatin and lovastatin are approved for treatment of hypercholesterolemia in humans (Armitage, 2007). Despite differences in their pharmacokinetic profiles (Joshi et al., 1999), all have the characteristic in common of inhibition of enzyme HMG-CoA reductase (Sirtori, 1990). Thus, in order to expand our knowledge concerning the effects of statins on vascular contractility, we investigated the effects of simvastatin and lovastatin, both available in the lactone prodrug form (Schachter, 2005b), on the contraction of isolated porcine coronary rings by U46619. The action of these inactive, lactone prodrugs was then compared with that of pravastatin, available as the hydroxylated active form. The ability of the lipophilic simvastatin to interfere with vascular tone *in vitro* at bath concentrations nearly similar to the maximal therapeutic concentrations (0·3–1 μmol/L) may thus have potential clinical significance (Lilja et al., 1998). The hydrophilic pravastatin, with a lipid-lowering effect similar to simvastatin, but without an *in-vitro* effect on coronary tone, provides a suitable control for this non-lipid related effect of simvastatin. Lovastatin, another example of a lipophilic statin, similarly depressed the U46619-induced contraction of the coronary rings. The link between lipophilicity and relaxation is supported by the lack of effect of pravastatin on the coronary tone (Gryglewski et al., 2001, Mukai et al., 2003, Bergdahl et al., 2003) (Lorkowska and Chlopicki, 2005).

6.1.2. Simvastatin induced relaxation of the coronary vessels precontracted with U46619, ET-1 and KCl:

There is significant evidence that TXA_2 plays a role in the cardiovascular diseases such as sustained hypertension, cerebral vasospasm, unstable angina, acute myocardial infarction, heart failure, and various thrombotic

disorders (Tada et al., 1981, Houston et al., 1986, Mistry and Nasjletti, 1988, Sellers and Stallone, 2008). Thus pharmacological modulation of TXA_2 is of great clinical interest. Our results have clearly shown that incubation with simvastatin attenuated U-46619, a TXA_2 mimetic, mediated vasoconstriction, independent of NO and PGI_2 pathway inhibition. Previous data have shown that simvastatin possesses a significant component of vascular relaxation that is independent of endothelium NO (Alvarez De Sotomayor et al., 2000, Mraiche et al., 2005). Similarly, chronic statin treatment suppresses Rho-kinase-mediated rat aortic contraction via NO independent mechanism (Budzyn et al., 2004). However, others have shown that L-NAME incubation inhibits endothelium-dependent aortic relaxation induced by simvastatin (de Sotomayor et al., 2005). The reason could be related to different artery species (Christie et al., 1989).

The involvement of ET-1 in the development of hypertension, coronary atherosclerosis and endothelial dysfunction has been suggested by previous studies (Lerman et al., 1995, Ikeda et al., 1997, Schiffrin, 2001, Cediel et al., 2002, Iglarz and Schiffrin, 2003). Moreover, endothelin antagonism prevents the development of endothelial dysfunction in experimental hypercholesterolemia and hypertensive humans (Hopfner and Gopalakrishnan, 1999, Schiffrin, 2001). This study has also demonstrated that simvastatin inhibits ET-1 mediated coronary vasoconstriction. Both ET-I and TXA_2 induced contraction utilises extracellular Ca^{2+} entry through the L-type Ca^{2+} channels in addition to Rho dependent Ca^{2+} sensitisation (Egashira et al., 1990, Tosun et al., 1998b, Nobe and Paul, 2001). Simvastatin, by inhibiting Ca^{2+} entry through the L-type Ca^{2+} channels, shown by our results, is likely to be the main mechanism of inhibition of the U-46619 and ET-1 contractions by simvastatin (Tesfamariam et al., 1999, Kang et al., 2014).

On the other hand, the depolarization of vascular smooth muscle cells, regardless of the mechanism, would result in enhanced Ca^{2+} permeability which is a fundamental response of the vasculature to high blood pressure (Van de Voorde et al., 1992). Moreover, enhanced vascular depolarization is a stimulus for Ca^{2+} channel upregulation that may contribute to the development of abnormal vascular tone (Harder et al., 1985, Van de Voorde et al., 1992, Pesic et al., 2004). Simvastatin according to our study inhibits Ca^{2+} influx in the depolarized vascular smooth muscle cells and significantly relaxed the depolarized coronary vessels which in turn may explain the clinically meaningful effect of simvastatin on blood pressure (Strazzullo et al., 2007).

Our data also demonstrated that simvastatin can attenuate vasoconstriction independently of endothelial function, which is clinically relevant given the prevalence of endothelial dysfunction amongst patients with vascular diseases (Yasue et al., 2008). Collectively, these lipid-independent effects of simvastatin, which have been found *in vitro* using animal tissue, could easily explain the substantial cardiovascular benefits observed with simvastatin in clinical practice both in normo- and hypercholesterolemic individuals (Bonetti et al., 2003, Koh et al., 2008, Juncos et al., 2012).

6.1.3. Clinical significance of mitochondria and K_{ATP} channel inhibition induced by simvastatin:

The aggregate of our results and of other experimental trials in vascular smooth muscle and coronary segments support the notion that simvastatin induce coronary relaxation by a mechanism/s other than K_{ATP} activation furthermore, previous evidence suggests that simvastatin inhibits K_{ATP} channel activation (Seto et al., 2013). Indeed, K_{ATP} channel activation is an important mechanism in the hypotension characteristic of vasodilatory

shock e.g. in hypoxia, septic shock due to lipopolysaccharide administration, hemorrhagic shock, and cardiogenic shock. It is for this reason simvastatin may be expected to reduce vasodilatation and peripheral vascular resistance reduction in such cases (Landry and Oliver, 1992, Landry and Oliver, 2001). However, the inhibition of K_{ATP} channel activation by simvastatin may interfere with β-adrenoceptor agonist relaxation (Uhiara et al., 2009). Such issue requires more investigation to determine the clinical significance of simvastatin interaction with β-adrenoceptor agonist mediated relaxation *in-vivo* and other K_{ATP} channel openers.

K_{ATP} channels may also be an effector of myocardial ischemic preconditioning, i.e. protection conferred by brief periods of ischaemia against infarction produced by a subsequent long ischaemia (Hanley and Daut, 2005, Zhao et al., 2006). This idea was reinforced by the findings that K_{ATP} channel openers like cromakalin, bimakalim, or pinacidil could also mimic myocardial protection (Critz et al., 1997). Several studies have suggested that simvastatin may exert cardioprotective effects in animal models via enhancement of endothelial NO release or K_{ATP} channel activation (Di Napoli et al., 2001, Zhao et al., 2006, Yang et al., 2007). In fact, during ischaemia, mitochondria generate cytotoxic reactive oxygen species that contribute to cardiomyocyte damage (Becker et al., 1999). Two different studies by Bacher *et al*, and Lesnefsky *et al*, have shown that inhibition of the mitochondrial electron transport chain with rotenone, complex I inhibitor, or myxothiazol, complex III inhibitor, lessens damage to mitochondria (Becker et al., 1999, Lesnefsky et al., 2004). Thus simvastatin cardioprotective effects may be mediated at least in part through mitochondrial inhibition. On the other hand, mitochondrial membrane potential has a critical role in Ca^{2+} accumulation. Thus, depolarization reduces the driving force for mitochondrial Ca^{2+} uptake and

thereby prevents mitochondrial Ca^{2+} overload as a mechanism of cellular protection induced by simvastatin (Brennan et al., 2006). Further studies are needed to determine the effect of simvastatin on K_{ATP} in myocardial ischaemic conditions and the role of ischaemic oxidant generation as vital treatment strategies for ischaemia and reperfusion.

Mitochondrial complex I has a vital role in organic nitrate biotransformation (Sydow et al., 2004). Findings presented by Garcia-Bou *et al.* suggest that mitochondrial dysfunction results in the appearance of tolerance to nitrates (Garcia-Bou et al., 2012). Thus our results regarding mitochondrial inhibition lead us to propose a possible interaction of simvastatin with Glyceryl trinitrate (GTN). Such a possibility needs more investigation, especially in patients receiving chronic treatment with GTN and in comparison with hydrophilic statins. The latter have protective effects against nitrate tolerance by counteracting NAD(P)H oxidase superoxide production (Otto et al., 2006). Similarly, simvastatin-induced vascular relaxation with subsequent blood pressure reduction in patients with hypertension may be induced as a downstream effect of mitochondrial depolarization. Previous studies have demonstrated that mitochondrial depolarisation causes cerebral artery vasodilatation via activation of nitric oxide synthase (Katakam et al., 2013, Katakam et al., 2014). This effect may account for a part of the beneficial effects of simvastatin in the prevention of cardiovascular disease (Jiang and Roman, 1997, Correa et al., 2014, Juncos et al., 2012). In comparison to simvastatin, the administration of pravastatin did not induce additional blood pressure lowering effect in hypertensive patients (Mancia et al., 2010), which is supported by the data presented here showing that pravastatin does not produce relaxation of the coronary artery.

6.1.4. Effect of Statins on Pancreatic β-cells.

It seems likely that the simvastatin effect on K_{ATP} channels is not selective for cardiovascular tissues. Our results clearly have shown that simvastatin inhibits pancreatic β-cell K_{ATP} channels directly. ATP-sensitive K^+ channels play a major role in controlling β-cell membrane potential and thereby insulin release (Cook and Hales, 1984, Miki et al., 1999), such effect would explain the results of the increase in insulin secretion obtained by some *in-vivo* and *in-vitro* experiments (Ishikawa et al., 2006, Koh et al., 2009).

On the other hand, the present study clearly provides evidence for mitochondrial depolarization by simvastatin in mice pancreatic β-cells. Mitochondrial capacity is central to the key function of the pancreatic β-cell-regulated insulin secretion (Wollheim, 2000). If these findings translate to humans, then this could explain the glucose intolerance induced by the lipophilic simvastatin (Szendroedi et al., 2012). In contrast, pravastatin had no effect on the mitochondrial potential. Therefore, the current results may explain the differential metabolic effects between simvastatin and pravastatin in hypercholesterolemic patients, which in turn may be clinically relevant in patients prone to metabolic diseases (Koh et al., 2009, Koh et al., 2013). Studies have shown that thiazide diuretics and β blockers exhibit undesirable glycaemic effects, thus simvastatin combination with such agents may be associated with an increased risk of incident diabetes especially in elderly patients (Betteridge, 2005) and those on prolonged intensive dose therapy (Preiss et al., 2011). The use of pravastatin or alternate-day dosing may be helpful with fewer side effects (Koh et al., 2009, Marcus et al., 2013). However we need to make greater efforts to understand the relationship between statin use and diabetes in clinical trials and as part of mechanistic exploration.

6.1.5. Additional Benefits of Statins in Cardiovascular Disease

Proliferation and migration of vascular smooth muscle cells are pivotal events in atherogenesis. In *in vitro* studies, the lipophilic simvastatin and lovastatin, but not the hydrophilic pravastatin, may induce vascular smooth muscle cell apoptosis in a dose-dependent manner (Guijarro et al., 1999, Indolfi et al., 2000, Corpataux et al., 2005a, Corpataux et al., 2005b). The difference might be attributed to differences in the ability of the statins to penetrate the cell. Our study demonstrated that the lipophilic simvastatin, but not the hydrophilic pravastatin, induced mitochondrial depolarization in vascular smooth muscle cells, which may represent the mechanism of simvastatin-induced smooth muscle cell apoptosis. Similar data of smooth muscle cell apoptosis with lipophilic statins have been provided by previous studies (Corsini et al., 1991, Guijarro et al., 1999, Corpataux et al., 2005a). Furthermore simvastatin causes inhibition of Ca^{2+} influx. In fact, in experimental atherosclerosis, there is increase in calcium transport and subsequent treatment with Ca^{2+} channel blockers was able to suppress such experimental atherosclerosis (Bangalore et al., 2007, Martin-Ventura et al., 2008). Thus, simvastatin administration by the inhibition of Ca^{2+} influx could have beneficial additive effects. In view of the association of deranged Ca^{2+} homeostasis and atherosclerosis, there may be additional benefits of simvastatin that is not related to the lipid lowering effects. However, such effects may eventually lead to functional abnormality and/or apoptosis after prolonged exposure (Dirks and Jones, 2006, Sung and Choi, 2012b, Salabei and Hill, 2013). Vascular smooth muscle dysfunction may parallel the statin induced-myotoxicity observed in skeletal muscle, which is manifested as muscle weakness and rhabdomyolysis (Kang et al., 2014). In this context, potential beneficial effects of statin in pathological settings like inhibition of smooth muscle

proliferation in atherosclerosis could be detrimental to the normal vasculature.

As shown from our imaging and organ bath experiments, simvastatin inhibits Ca^{2+} influx, thus simvastatin co-administered with other Ca^{2+} channel blockers like nifedipine may result in additive effects on the haemodynamic parameters like heart rate, blood pressure and total peripheral resistance, as suggested by other studies (Jasinska et al., 2006, Clunn et al., 2010). In fact, the usefulness of the conventional L-type Ca^{2+} channel blockers are sometimes limited by their inability to dilate the venous circulation with resultant ankle oedema as a side effect (Thakali et al., 2010). Simvastatin may represent a useful combination therapy. Further studies are aimed to ascertain whether these findings are translatable to the clinical setting. On the other hand, the β-blocker propranolol, like simvastatin, causes mitochondrial inhibition (Wagner et al., 2008). Thus simvastatin and propranolol combination might increase the risk for developing muscle myopathy.

6.1.6. Simvastatin concentrations used in the study

The effect of simvastatin observed *in vitro* might be of potential clinical importance since the concentrations that induced coronary relaxation in our experiments are within the maximum range encountered in plasma of treated patients 0.03-1 μM (Lilja et al., 1998). However, for the majority of studies investigating the mechanism of relaxation, higher concentrations of simvastatin (3-10 μM) were used. These concentrations that we have used are higher than would be expected in the plasma of patients. Since the study aim was to determine the role of mitochondria and K_{ATP} channels in the acute coronary artery relaxation induced by simvastatin, accordingly the exposure to simvastatin must be for short periods of time. For isolated tissue bath experiments the exposure time was around 2-hours and for the

imaging experiments the exposure time was usually less than 30- min to avoid bleaching of the dye. In comparison, the long term therapeutic *in-vivo* use of simvastatin is sometimes maintained over several years. Therefore, it can be argued that higher simvastatin concentrations were needed to detect cellular responses over this short period. Indeed, the higher concentration of simvastatin required to induce mitochondrial depolarization of the smooth muscle cells in comparison to pancreatic B-cell may be related to the unusual rhodamine 123 retention of the smooth muscle (Summerhayes et al., 1982).

Long-term exposure to statins may lead to accumulation within cells, such that the intracellular concentration is greater than that measured in the plasma. Previous studies have shown that statin-induced skeletal muscle cytotoxicity is associated with intracellular accumulation (Kobayashi et al., 2008), since the lipophilic statins enter the skeletal muscle by passive diffusion, while on the other hand the transporters play less significant role (Ohtawa et al., 1999). Sidaway *et al.* have demonstrated that the ratio of simvastatin accumulation in gastrocnemius muscle relative to plasma was higher than cerivastatin. They also proved that the hydrophilic rosuvastatin had the lowest ratio of accumulation (Sidaway et al., 2009). In addition, lipophilicity plays a major role in mitochondrial uptake i.e. lipophilic molecules had higher uptake compared to hydrophilic molecules (Durazo et al., 2011). Lipophilic compounds can easily pass through the mitochondrial membrane and as the pH in the mitochondrial matrix is higher than the pH in the cytosol, these lipophilic compounds can become charged. As a consequence mitochondria can trap lipophilic compounds (Rashid and Horobin, 1991). Our hypothesis is that lipophilic statins accumulate within the mitochondria. Therefore, the concentration of statin within the mitochondria may be higher than that measured in the plasma. The time taken for uptake into the mitochondria would explain why the relaxation to

lower concentrations of simvastatin (300 nM) was slow and reached significance after around 145 minutes after addition. Further studies are required to measure accumulation of statins within mitochondria.

Concomitant administration of simvastatin with agents that inhibit cytochrome P-450 isoenzyme CYP3A4, responsible for the metabolism of simvastatin, for e.g. macrolide antibiotics like erythromycin, Ca^{2+} channel blockers like verapamil and diltiazem (Kantola et al., 1998), antifungal agents like ketoconazole and itraconazole (Neuvonen et al., 1998), and even grapefruit juice, taken daily, considerably increase simvastatin plasma levels (Lilja et al., 1998, Lilja et al., 2004). It is noteworthy to mention that human smooth muscle cells are more sensitive to statins than experimental animals, thus lower concentrations are required in human in order to inhibit smooth muscle cell proliferation (Corsini et al., 1993). Similarly, we might expect that lower concentrations might be required to induce human coronary relaxation.

Further studies to investigate the outcomes of the current body of work in isolated human vessels are needed to estimate the clinical significance of the experimental results and whether these findings are translatable to the clinical setting.

6.2. General Conclusion

In summary, we have demonstrated that simvastatin alters mitochondrial membrane potential in vascular smooth muscle cells and pancreatic β-cells. The relaxation to simvastatin in the porcine coronary artery is dependent, in part, upon mitochondrial activity. Alteration of mitochondrial membrane potential by simvastatin may lead to inhibition of calcium influx, hence stimulation of relaxation. How the mitochondria might regulate influx of extracellular calcium through calcium channels is unknown. ROS from the mitochondria into the cytosol can inhibit L-type calcium channels. However, as mentioned, antioxidant treatment had no effect on the relaxation to simvastatin. Mitochondria are able to regulate intracellular calcium homeostasis through accumulation (McCarron et al., 2013). For example, the reduction in intracellular calcium after depolarization in colonic smooth muscle is thought to be due to, in part, uptake into mitochondria (McCarron and Muir, 1999). Uptake of calcium into the mitochondria is also thought to prevent calcium-induced inhibition of voltage-gated calcium channels (Demaurex et al., 2009). On the other hand, the effects on mitochondrial membrane potential in pancreatic β-cell may be detrimental, particularly with chronic treatment due to the increased risk of diabetes observed with lipophilic statins.

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Appendix:

1. Effect of simvastatin on K_{ATP} channel activity in human aortic smooth muscle cells

As described earlier with β-cells, patch clamp experiment performed on smooth muscle cells isolated from PCA. The enzyme mixture consists containing DTT (6.0 mg ml^{-1}), papain (2.0 mg ml^{-1}) collagense type II (7.5 mg ml^{-1}) and bovine serum albumin (fatty-acid free; 2.0 mg ml^{-1}) for 45 – 60 mins at 37ºC. In fact, it proved difficult to isolate healthy smooth muscle cells and only a few cells stuck to the glass bottom. Furthermore, attempts to generate seals using suction were frequently unsuccessful, presumably due to a loss of cell membrane integrity during enzymatic digestion. The decision was finally taken to abandon the isolation procedure. As alternative, human aortic smooth muscle cells (Lonza; passages 7 to 9) were grown on sterilized glass cover slips were used to examine the effect of simvastatin on K_{ATP} channels in smooth muscle cells. Results of 5 repeats show that simvastatin 10 μM significantly inhibits K_{ATP} channels (figure 1). However more experiments are recommended to examine the effects in inside out patch and to compare the results with DMSO as a solvent control.

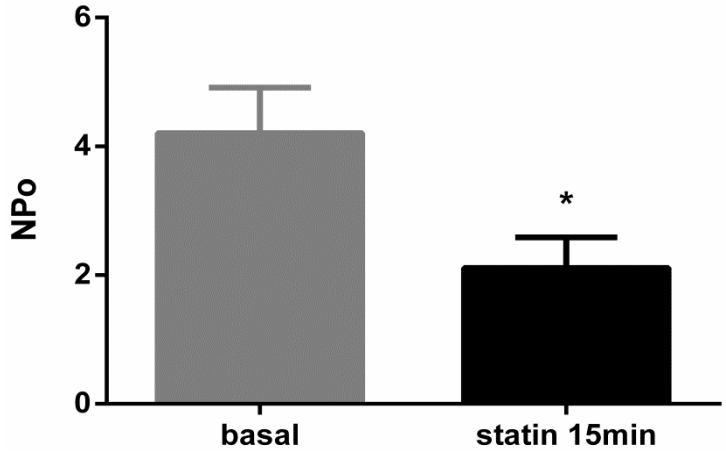

Figure 1. Effect of 10 μM simvastatin on K_{ATP} channel activity (NPo) in human aortic smooth muscle cells: A- K_{ATP} channel activity after 15 min of 10 μM simvastatin (statin) perifusion dissolved in DMSO compared to the K_{ATP} channel basal activity (NPo) prior to addition of simvastatin from a cell-attached patch on human aortic smooth muscle in a glucose-free HANKS solution. * P< 0.05, Student's unpaired *t* test of 5 different experiments.

2. Macro scripts for Ca^{2+} image analysis

```
//calcium 27Feb2014//
draw -n ds -d 1 -w 1.5 -l -v 2;
draw -n de -d 1 -w 1.5 -l -v 3;
draw -n ks -d 1 -w 1.5 -l -v 4;
ds.hmove=1;
de.hmove=1;
ks.hmove=1;
layer -d;
worksheet -d B%[%h,>'A'];
%n=C%h;
%A=%h;
d=0;K=0;
ncs=wks.ncols;
nrs=xindex(wks.nrows,wcol(1));
xen=nrs-1;
//type %h %n $(i) $(ncs);//
//normalize//
// for(i=ncs;i>1;i--){
//   %A=%h!wks.col$(i).label$;
//   wcol(i)=100*wcol(i)/%a;
//check cells//
 for(i=ncs;i>1;i--){
  window -t plot calibrate1 %n;
  layer -w %A i 1 i nrs 200;
  getyesno (Use record) Y;
```

```
 layer -d;
 if (y==0){
  d=d+1;
  del wcol(i);
 }
 else{
  k=k+1;
 };
};
//now form average//
ncs=wks.ncols;
nct=ncs+1;
tot=ncs-1;
worksheet -c average;
col(average)[nrs]=0;
wcol(nct)=0;
for(i=2;i<=ncs;i++){
wcol(nct)=wcol(nct)+wcol(i);
};
//calculate average and plot//
wcol(nct)=wcol(nct)/(ncs-2);
wks.col$(nct).label$=$(ncs-1);

%n=%h;
window -t plot calibrate2 G%nAverage;
layer -w %n nct 1 nct nrs 200;
de=xen-60;
//get timings//
getnumber
(50K start) Ks
(Drug start) Ds
```

```
(Drugend) De


(Measurement times);
layer -d;
kk=ds-180;
//find max and normalize//;
  loop (n,2,ncs){
   ym=0;
   yn=ds-kk+1;
    loop (m,kk,ds){
     ym=ym+%(%n,n,m);
    };
   wcol(n)=100*yn*wcol(n)/ym;
  };



 ncc=wks.ncols-1;
 nrs=wks.nrows;
 d=0; k=0;
 for(i=ncc;i>1;i--){
  worksheet -s i 1 i xen;
  worksheet -p 200 calcium1;
  getyesno KEEP Y;
  layer -d;
   if (y==0){
    del wcol(i);
    d=d+1;
   else{
    k=k+1;

//now form average//
```

```
ncs=wks.ncols-1;
nct=ncs+1;
col(average)[nrs]=0;
wcol(nct)=0;
for(i=2;i<=ncs;i++){
wcol(nct)=wcol(nct)+wcol(i);
};
//calculate average and plot//
wcol(nct)=wcol(nct)/(ncs-2);
wks.col$(nct).label$=$(ncs-2);

%n=%h;
worksheet -s 2 1 ncs xen;
worksheet -p 200 calcium1;
window -r %h G%n;

//draw limits and reset if necessary//
draw -n ds -d 1 -w 1.5 -l -v ds;
draw -n ks -d 1 -w 1.5 -l -v ks;
draw -n de -d 1 -w 1.5 -l -v de;
ds.hmove=1;
ks.hmove=1;
de.hmove=1;
//get timings//
getnumber
(50K start) Ks
(Drug start) Ds
(Drug end) De
(Measurement times);
draw -n ds -d 1 -w 1.5 -l -v ds;
draw -n ks -d 1 -w 1.5 -l -v ks;
```

```
draw -n de -d 1 -w 1.5 -l -v de;
//output//
window -n n results;
type.notes$=results;
type.redirection=2;
type \n;
type Worksheet %n;
type Basal 50K drug;
type $(KS) $(DE);
type Cell\tarea\tbasal\t50K\tdrug;

loop (n,2,ncs){
 Cbasal=0;c50k=0;cdrug=0;
  loop (m,1,180){
  Cbasal=Cbasal+%(%n,n,ks-m);
  c50k=c50k+%(%n,n,ds-m);
  cdrug=cdrug+%(%n,n,de-m);
  Cbasal=Cbasal/180;
  c50k=c50k/180;
  cdrug=cdrug/180;
 type $(n-1)\t%(n,@L)\t$(int(cbasal))\t$(int(c50k))\t$(int(cdrug));
```