Pharmacological cardioprotection of the human myocardium in diseased states

Thesis submitted by

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Declaration

I, Paul Stuart Chadwick Rees, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

BACKGROUND

Coronary artery disease is the leading worldwide cause of death. Even despite restoration of blood flow following acute myocardial infarction, further myocardial damage is seen during the reperfusion phase. Pharmacological strategies to induce resistance to ischaemia reperfusion injury have been shown to share common pathways. The most important common signalling pathway involved is the Reperfusion Injury Salvage Kinase pathway, and in animal models this can be pharmacologically activated, resulting in inhibition of the opening of the mitochondrial permeability transition pore, with a resultant cardioprotective effect. Atorvastatin has been shown to act in this way in animal models of ischaemia-reperfusion injury, although this has not been demonstrated in humans. This thesis examines (a) the role of atorvastatin in acute protection from ischaemia-reperfusion injury in human myocardium, (b) the ability of high-dose atorvastatin to recapture cardioprotection in human myocardium in the setting of chronic statin therapy, and (c) the functionality of the mitochondrial permeability transition pore in human hypertrophic cardiomyopathy, and the role of atorvastatin in inhibiting pore opening following oxidative stress.

METHODS AND RESULTS

Using human models to simulate ischaemia-reperfusion injury we have demonstrated: (1) Human atrial myocardium can be protected from simulated ischaemia-reperfusion injury by treatment with atorvastatin at the time of reperfusion; (2) Cardioprotection against simulated ischaemia-reperfusion injury can be recaptured with high-dose atorvastatin; (3) The mitochondrial permeability transition pore is a critical determinant in cell death in hypertrophic cardiomyopathy, and its inhibition can be achieved using atorvastatin.

CONCLUSION

The key aim of emergency reperfusion therapy in the setting of myocardial infarction is salvage of myocardium and preservation of cardiac function. These studies contribute to the field by exploring the cardioprotective effects of atorvastatin in human myocardium and delineating protective cascades involved. They offer a key translational step in our understanding of statin cardioprotection, and an insight into cardioprotection in hypertrophic cardiomyopathy.

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LIST OF ABBREVIATIONS

ACS	acute coronary syndrome
Akt	non-specific serine/threonine protein kinase, Protein Kinase B
ATP	adenosine triphosphate
Ca^{++}	calcium ion
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CK	creatine kinase
CK-MB	creating kinase – myocardial-bound
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
Erk-1/2	extracellular-signal-regulated, kinase
eNOS	endothelial-derived nitric oxide synthase
HCM	hypertrophic cardiomyopathy
ICD	implantable cardioverter-defibrillator
IL	interleukin – secreted cytokine signalling molecule
iNOS	inducible nitric oxide synthase
IPC	ischaemic preconditioning
K^{+}	potassium ion
K-ATP	ATP-sensitive potassium channel

LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride
LAD	left anterior descending coronary artery
LDL	low-density lipoprotein
L-NAME	L-Nitro-Arginine Methyl Ester
LV	left ventricle
µM	micromolar
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
mPTP	mitochondrial permeability transition pore
MRI	magnetic resonance imaging
Na^+	sodium ion
NO	nitric oxide
OPCABG	off-pump coronary artery bypass grafting
PCI	primary percutaneous coronary intervention
PI3K	phosphatidlyinositol-3-kinase
PTEN	phosphatase and homolog deletion on chromosome ten
RIPC	remote ischaemic preconditioning
RISK	reperfusion injury salvage kinase
ROS	reactive oxygen species
SPECT	single positron emission computed tomography
Statin	3-hydroxy-3-methylglutaryl co-enzyme A inhibitor
UO126	1,2-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene

LIST OF PUBLICATIONS

Published Abstracts

1. **P S C Rees**, SM Davidson, SE Harding *et al*. The mitochondrial permeability transition pore is a functional target for cardioprotection in human hypertrophic cardiomyopathy. European Heart Journal, Sep 2010;31(Suppl 1).

2. **P S C Rees**, S M Davidson, S E Harding *et al.* The mitochondrial permeability transition pore as a target for cardioprotection in ventricular cardiomyocytes harvested from patients with obstructive hypertrophic cardiomyopathy. Heart Sep 2010;96**:**e26

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4. **P S C Rees**, SM Davidson, SE Harding *et al.* The mitochondrial permeability transition pore is functional and serves as a target for cardioprotection in the setting of human hypertrophic cardiomyopathy. Journal of Molecular and Cellular Cardiology, May 2010;48(5)S21

5. **P S C Rees**, DJ Hausenloy, DM Yellon. Atorvastatin, administered at the onset of reperfusion, protects human atrial muscle against lethal reperfusion injury through the activation of the RISK pathway. Journal of Molecular and Cellular Cardiology, May 2010;48(5)S102

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Chapter One: INTRODUCTION

Coronary Artery Disease – The Leading Killer

Despite major advances in its prevention and treatment, coronary artery disease (CAD) remains the leading worldwide cause of death, killing around 8 million people annually. In the UK, 1 in 5 men, and 1 in 7 women will die from this disease (1). In low and middle income countries in 2001, CAD accounted for 11.8% of all deaths, rising to 17.3% in more affluent states (2). World Health Organisation predictions suggest that this trend will continue to accelerate well into this century, with CAD becoming the leading cause of both death and disability by 2020 (3), owing to a rapid increase in prevalence in developing countries, eastern Europe and the rising incidence of obesity and diabetes.

Pathophysiology

Following the development of an accumulation of lipid-laden cells beneath the vascular endothelium – a fatty streak – atherosclerotic lesions develop (4). These are asymmetric focal thickenings of the intima, the innermost arterial layer. With progression, these lesions may become obstructive to coronary blood flow, resulting in myocardial ischaemia under conditions of demand. This leads to the clinical presentation of exertional angina. The condition is treated by aggressive medical therapy with anti-anginal and secondary prevention medications (5), and revascularisation by means of

percutaneous coronary intervention (PCI) or coronary artery bypass grafting (6).

The most dramatic presentation of CAD is that of myocardial infarction. In this process, activation of an atheromatous plaque leads to thrombus formation on its surface, leading to vessel occlusion. The actions of a cascade of inflammatory cytokines, proteases, coagulation factors, free radicals and vasoactive substances combine at the culprit lesion in the coronary tree (7). Their combined actions destabilise the coronary lesion by attacking collagen within, leading to rupture of its fibrous cap. This may occur even in the absence of angiographically severe coronary stenosis (8). Plaque rupture, detectable in 70% of cases, exposes pro-thrombotic material from the plaque core to the blood, and this triggers acute thrombus formation (9). Thrombus propagation leads to total coronary occlusion, and downstream ischaemia then follows. Untreated this will lead to myocardial infarction, with the development of myocardial scar, and impairment of left ventricular function. The most important determinant of future prognosis is infarct size (10-12), and experimental animal studies in the 1970s sought to address this.

Emergency Reperfusion Therapy

The most effective strategy for salvaging myocardium in the setting of acute myocardial infarction due to occlusive thrombus is prompt reperfusion therapy (13-15). This life-saving therapy is now best delivered through an

organised primary PCI network (16) combining direct transport to a PCI centre, optimal antiplatelet (17) and antithrombin treatments (18), followed by emergency PCI with thrombus aspiration (19) to re-open the vessel. This will result in reperfusion of the vessel in over 95% of cases. If unavailable, thrombolytic therapy to dissolve the occlusive thrombus remains an option, though with less efficacy and more side effects (20-23). Emergency reperfusion therapy has been shown to save life, reduce recurrent myocardial events and preserve left ventricular function and should be offered even when cardiac arrest ensues (24;25)

Myocardial Reperfusion Leads to Further Cell Death – Reperfusion Injury

Despite prompt reperfusion, some cardiomyocytes in the culprit territory will go on to die. In 1960, Jennings *et al* first described a host of adverse histological findings found in myocardium which had been reperfused following temporary coronary artery occlusion in a canine model (26). This phenomenon of myocardial reperfusion injury may account for up to 50% of the final infarct size (27). A number of different contributory factors have been postulated, and form the basis for attempts to develop therapies with which to combat myocardial reperfusion injury (28;29) .

Oxidative Stress and Reactive Oxygen Species Generation

A large burst of oxidative stress is generated following reperfusion of ischaemic myocardium. In 1988, Zweier demonstrated a direct relationship between free radical generation and subsequent impaired myocardial contractile function. The study confirmed that reactive oxygen species (ROS) are generated in the reperfused myocardium, and that these radicals appear to be key mediators of myocardial reperfusion injury (30;31). The increase in ROS is thought to be due to damage to electron transport chain components, leading to inefficient transfer of electrons and generation of superoxides (32). Additionally, oxidative stress during reperfusion leads to depletion of nitric oxide (NO), an agent known to possess cardioprotective effects through its ability to regulate coronary flow, inactivate superoxide forms and inhibit neutrophil chemotaxis to the injured site (33). Thus, the at-risk myocardium in the jeopardised territory is further deprived of the potential benefits of an in-house cardioprotective agent.

pH flux and calcium accumulation

Following reperfusion, the pH is rapidly restored to within normal physiological limits. Paradoxically, this rapid correction in pH following reperfusion has been shown to contribute to lethal reperfusion injury (34). pH changes are also implicated in inducing adverse changes in calcium handling. During reperfusion, following the restoration of normal extracellular pH, intracellular calcium levels transiently rise as hydrogen and

sodium ions are extruded from the cell by ion exchange pumps. A sodium/calcium exchange pump, triggered by elevated intracellular sodium, actively imports calcium into the cell. Subsequent oscillations in intracellular calcium can lead to hypercontracture of the myocytes, depleting myocyte ATP levels and causing them to deteriorate. In some cells intracellular calcium levels remain high even after immediate reperfusion, and these cells are typically irreversibly damaged (32). Direct damage to the sarcoplasmic reticulum and sarcolemnal membrane also contribute, overwhelming the cell's ability to regulate calcium, leading to cell and mitochondrial calcium overload (35).

Cell Death - Necrosis, Apoptosis and Autophagy

The myocardial cellular injury sustained during ischaemia-reperfusion injury is believed to be due to a combination of necrosis, apoptosis and autophagy (32;36;37). Necrosis is an irreversible and non-regulated process of cell death associated with prolonged periods of ischaemia, whereas apoptosis and autophagy are programmed events which involve the activation of complex signalling pathways (38).

Apoptosis is an energy-requiring process that follows a defined time-sensitive signalling pathway. It leads to cell shrinkage, plasma membrane changes, intracellular proteolysis, loss of mitochondrial function and DNA fragmentation (39). Its immediate objective is the safe dismantling of intracellular components and two central pathways are involved in triggering

the process. An “extrinsic” pathway involves activation of cell surface death receptors by binding of ligands to tumour necrosis factor family receptors (40). A second “intrinsic” pathway is activated by apoptotic signals inside the cell which involve mitochondria as either initiators or signal magnifiers (41). Loss of ventricular myocytes by apoptosis has been heavily implicated in ischaemia-reperfusion injury (42), with apoptotic cascades being enhanced during the reperfusion phase as substrate levels and subsequent metabolic processes are restored (43;44), and post-ischaemic salvage strategies are aimed at reducing this myocyte loss with its concomitant potential for deterioration in ventricular contractile function (45).

Autophagy is a highly orchestrated cellular catabolic process by which proteins and organelles are degraded to generate free amino acids and sugars during periods of metabolic stress (46). Autophagy in mammalian cells occurs via three main pathways, the commonest being macroautophagy which will be discussed further (47). In cardiomyocytes, autophagy is seen under normal physiological conditions and represents a housekeeping function, removing damaged proteins and macro-molecular structures. During periods of cellular stress, autophagy is rapidly induced, possibly as a survival mechanism initially, eventually leading to cell death when repair of the injured cardiomyocyte is impossible (48). Studies have shown that autophagy is upregulated during myocardial ischaemia-reperfusion injury (49), and in these conditions many of the autophagosomes contain (presumably damaged) mitochondria (50). The exact role of

autophagy within the heart is not fully understood, and its regulation is not fully delineated. Work in this field remains ongoing. However it seems likely that cross-talk exists between autophagy and apoptosis as means of cell death, and this probably involves the mitochondrion as a common point in both pathways (46).

The Mitochondrial Permeability Transition Pore

The onset of reperfusion has been shown to activate a nonselective channel of the internal mitochondrial membrane, termed the mitochondrial permeability transition pore (mPTP) (27;51-53). This is a large-conductance megachannel comprising a voltage-dependent anion channel in the outer mitochondrial membrane, the adenine nucleotide transporter on the inner membrane, and cyclophilin D in the matrix (54). Induced in the first few minutes following reperfusion, triggered by mitochondrial calcium excess, oxidative stress, electron transport chain abnormalities and ATP depletion (31;55), the pore is a critical determinant of cell death (56). Studies in 1988 first confirmed the involvement of the mPTP in reperfusion injury by demonstrating its sensitivity to intracellular calcium levels and oxidative stress, all conditions present during reperfusion (57).

mPTP opening triggers apoptosis

Formation and opening of the mPTP results in depolarisation of the inner mitochondrial membrane potential and matrix swelling. Mitochondrial

swelling, in turn, leads to rupture of the outer mitochondrial membrane with subsequent release of pro-apoptotic factors such as cytochrome c and apoptosis-inducing-factor into the cytosol (58). These go on to activate caspase cascades involving caspase-9 (59). Caspase activation is detectable within 30 minutes of the onset of ischaemia, with DNA fragmentation seen after 60 minutes. Both features accelerate during the reperfusion phase, and inhibition of this process has been shown to reduce apoptosis in experimental models (60;61). Unchecked, the downstream effect is cellular fragmentiation and eventual cell death. mPTP opening is also believed to have a role in triggering autophagy, which could possibly act as a myocyte-rescue mechanism, saving myocytes from apoptotic death (46).

Inhibition of the mPTP is cardioprotective

Crompton's group demonstrated that pharmacological inhibition of the pore could be induced by the administration of cyclosporin A, which inhibits the enzymatic action of cyclophilin D within the pore (62), . Later this agent was used to successfully improve cell survival following anoxia in adult rat cardiomyocytes (63). Studies in human atrial myocytes by our research group have confirmed that the mPTP is a target for cardioprotection in humans, and that its direct pharmacological inhibition can be used to lengthen individual cell survival when exposed to oxidative stress, as well as improve contractile function after hypoxia-reoxygenation injury (64) .

Inhibition of the pore can be also achieved by activation of prosurvival signalling cascades which will be discussed in more depth later in this chapter (65). Targeting this pore has become a key focus of experimental and clinical studies attempting designed to optimise outcome from myocardial infarction by limiting lethal myocardial reperfusion injury.

Limiting ischaemia-reperfusion injury by preconditioning

Having observed that the rate of myocardial ATP depletion was reduced in hearts exposed to brief periods of ischaemia prior to experimental infarction, Murry's group used a canine model to further test this concept. Hypothesizing that brief periods of ischaemia applied up-stream to an experimental myocardial infarction might reduce infarct size, they exposed a control group to a 40 minute ischaemia-reperfusion injury protocol by occluding the circumflex artery, then reperfusing the territory at the end of the experiment. A second group of dogs was exposed to a "preconditioning" stimulus comprising four 5 minute occlusions of the circumflex, followed by reperfusion, immediately prior to the main 40 minute occlusion. They noted that electrocardiographic changes of ischaemia and infarction were slower to develop and less marked in the preconditioned group. Subsequent histological analysis confirmed a 25% reduction in final infarct size, despite a similar amount of myocardium being at risk (66). The concept of ischaemic preconditioning was thus proven, and the phenomenon has subsequently been demonstrated in a range of mammalian hearts, including

in humans. Interestingly, protection of the heart is not limited to the coronary territory exposed to the preconditioning stimulus, but has a pan-myocardial protective effect, with protection seen in areas remote from the preconditioned territory (67). These are indeed key findings, but of limited clinical applicability in the setting of acute coronary syndromes, which are effectively random unpredictable clinical events. To be of any clinical benefit, a cardioprotective strategy would need to be applied either immediately prior to, alongside or after reperfusion.

However, delineating the mechanisms by which these techniques might protect the myocardium remains important, as it may offer trigger mechanisms that could be exploited in clinical practice. In 2002, Yellon's group first published the importance of the reperfusion injury salvage kinase (RISK) pathway. They demonstrated the importance of this group of pro-survival kinases which, under specific pharmacological conditions, could be activated at reperfusion to the benefit of the myocardium at risk (68). In 2005, using an isolated perfused rat heart model of ischaemic preconditioning, they went on to assay the phosphorylation states of the protein kinase Akt and extracellular signalling kinasesErk1/2. They found that preconditioning also resulted in a transient increase in enzyme phosphorylation following the trigger stimulus, but also a four-fold increase at the point of reperfusion, associated with an expected decrease in final infarct size. Inhibiting these enzymes abrogated protection, demonstrating their key role in cardioprotection. They confirmed that preconditioning targets the

reperfusion phase, and activates the prosurvival kinase RISK cascade (69). Being able to activate this pathway acutely during the perfusion phase might allow cardioprotection to be applied during acute coronary syndromes. Pharmacological activation of the RISK pathway has been shown to induce cardioprotection by phosphorylation of a variety of downstream effectors including p70S6K (70), Bcl-2-associated death protein promoter (BAD) (70), GSK3β (71) and endothelial nitric oxide synthase (eNOS) (72;73). Also downstream of RISK activation is the mammalian target of rapamycin (mTOR), a serine-threonine kinase that regulates protein synthesis and cell growth. Interestingly, mTOR activation is linked to regulation of authophagy (46).

Postconditioning

In 2003, Zhao tested the hypothesis that repetitive ischemia applied during early reperfusion is cardioprotective by attenuating reperfusion injury. Using an anaesthetised, open-chest canine model of left anterior descending (LAD) artery occlusion for 3 hours, they applied a preconditioning protocol to one group, another group acted as a control, and the study group underwent 3 cycles of 30 seconds reperfusion followed by 30 seconds re-occlusion of the LAD at the onset of reperfusion. They showed that infarct size was significantly less in the preconditioning (15±2%, $p<0.05$) and postconditioning (14±2%, $p<0.05$) groups compared with controls (25±3%). Tissue oedema, leucocyte accumulation, and endothelial function were also

more favourable in the postconditioned groups. These data suggested that postconditioning could be as effective as preconditioning in reducing infarct size and preserving endothelial function. The authors then went on to suggest that postconditioning could potentially be applied in the setting of coronary interventions, coronary artery bypass surgery, organ transplantation, and peripheral revascularization where reperfusion injury is expressed (74). In subsequent studies they went on to confirm that part of the benefit may be derived from an anti-apoptotic effect (75)

In the light of this growing experimental evidence, retrospective data were published by Darling *et al*, assessing biomarker release in cases where multiple balloon inflation had been performed during primary PCI. In cases where ≥4 balloon inflations were performed, peak CK levels were lower (1655 versus 2272 IU/L; $p < 0.05$), consistent with the concept that relief of sustained ischemia in a stuttered manner (analogous to postconditioning) may evoke cardioprotection in the clinical setting (76)

Postconditioning activates the RISK pathway

To better delineate the signalling mechanisms involved, in 2004 Yellon's group performed a postconditioning study using isolated perfused rat hearts. They confirmed that postconditioning reduced infarct size from 51.2±3.4% to 31.5±4.1% ($p < 0.01$), and that inhibiting phosphatidylinositol 3-kinase (PI3K) at reperfusion using the agents LY294002 or Wortmannin during the

first 15 minutes of reperfusion completely abolished postconditioning-induced protection. Western blot immunochemical analysis demonstrated that postconditioning induced a significant increase in phosphorylation of Akt and endothelial nitric oxide synthase (eNOS). These findings strongly support the suggestion that the effects of postconditioning are mediated by activation of the prosurvival kinases PI3K, Akt and eNOS, key components of the RISK pathway (77).

In 2005, Staat and colleagues performed a prospective, randomized, controlled, multicenter study, to establish whether postconditioning could protect the human heart in the setting of primary PCI for acute myocardial infarction. Randomising 30 patients with acutely occluded major coronary arteries presenting within 6 hours after the onset of chest pain, the control group underwent standard PCI with direct stenting. The postconditioning group underwent 4 episodes of re-inflation of the angioplasty balloon for 1 minute, followed by restoration of flow for a further minute. First of all, no adverse events were noted as a result of applying the postconditioning protocol suggesting the technique can be safely applied. Analysis of the area of myocardium at risk was comparable between the groups. Total creatine kinase release assessed by serial measurements over the next 72 hours, used as a marker of infarct size, was 36% less in the postconditioned group. Myocardial blush grade - a marker of myocardial reperfusion – was also improved in the postconditioned group. This is interesting as current

PCI practice is to avoid repeated balloon inflation during primary PCI due to concerns that it might induce the “no-reflow” phenomenon and inhibit downstream perfusion of the myocardial capillary bed. These results suggest that might be far from the case. Overall, this small pilot study suggested that postconditioning during primary PCI is safe, feasible and protects the human heart during acute myocardial infarction (78). Longer term follow-up data on 38 patients subjected to postconditioning have been published, combining nuclear imaging and echocardiography techniques. The data confirm improvements in infarct size using single positron emission computed tomography (SPECT) at 6 months, and improved overall left ventricular function using echocardiography at 1 year, with a statistically (and clinically) significant 7% improvement in ejection fraction, confirming that postconditioning confers long-term benefits (79).

In a study of 41 patients undergoing primary PCI within 90 minutes of admission to hospital, Zhao’s group published further data in 2007. Using three 30 second balloon re-inflations/deflation cycles during the reperfusion phase, infarct size represented by creatine-kinase activity during the first 72 hours of reperfusion was reduced by 27%. At 7 days post-PCI, SPECT revealed that this translated into a reduced final infarct size (31.3±8.6% vs. 22.8±6.7% of left ventricle, $p<0.05$) (80).

A recent systematic review and meta-analysis of the six relevant published studies in this field confirms a highly-significant (p=0.005) benefit in terms of reduction of CK release associated with postconditioning. Perhaps more clinically important still, analysis of the echocardiographic left ventricular function ejection fraction shows a highly significant improvement of 4.2% where postconditioning is applied, though long-term outcome data have yet to be evaluated (81). Technically, postconditioning with repeated balloon inflation is counterintuitive to most interventional cardiologists. The overwhelming drive during a primary PCI is to reopen the occluded vessel with as little balloon inflation as possible, for fear of dislodging thrombus downstream and injuring the microvasculature. Despite having evidence to support it (albeit from small studies), postconditioning has had, as yet, little clinical impact.

Activation of the RISK pathway by postconditioning protects human myocardium

Recent studies by our group, using an isolated human atrial trabecular model of hypoxia-reoxygenation injury recently delineated the underlying cardioprotective cascades in the setting of ischaemic postconditioning. Right atrial samples were taken from 30 consenting patients undergoing elective CABG, during the establishment of cardiopulmonary bypass. Sivaraman *et al* exposed isolated atrial trabeculae to a standard hypoxia re-oxygenation protocol, and compared a control group with groups exposed to an ischaemic preconditioning protocol and a postconditioned group. In this

group, following a 90 minute hypoxia episode, either four 30 or 60 second episodes of alternating hypoxia-reoxygenation were applied at the onset of reoxygenation. Recovery of baseline contractile function was 26.7±2.1% in the control group, but 45.2±2.2% in the group given the 60 second cycles of postconditioning. This compared favourably with the improvement in function seen in the preconditioned group (45.4±3.2%). These studies demonstrated, for the first time, that postconditioning protects the human myocardium ex-vivo (82). Importantly, administration of LY294002 (a PI3-kinase inhibitor) and UO126 (an Erk-1/2 inhibitor) abolished the cardioprotective effect afforded by postconditioning. This confirmed that the effect was related to activation of prosurvival RISK signalling pathway, already well-established as a key mediator of cardioprotection in human tissue (83) and animal models (84).

Clinical cardioprotection with statins

3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors (statins) have a well-proven and established role in the management of cardiovascular disease (85;86). As primary prevention agents, they can be used to reduce serum levels of low density lipoprotein cholesterol, and prevent the development of coronary heart disease (87), and can reduce event rates even where cholesterol levels are considered normal (88;89). Used as secondary prevention in patients with established coronary disease, they significantly reduce further rates of death and recurrent myocardial

infarction (90) . In addition, they have been shown to be able to reduce the progression of atherosclerosis, and intravascular ultrasound studies have shown evidence of regression of markers of coronary disease burden with very intensive statin treatment (91;92).

More recently, attention has turned to the beneficial effects of statins in the setting of acute coronary syndromes (ACS). In the Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study, the effect of early administration of atorvastatin 80mg following ACS was examined against placebo. With over 3000 patients randomised, the results showed reduced readmission for ischaemia at 4 months (93). Further positive data were yielded by a report from the first Euro Heart Study, which showed a 2.2% absolute mortality reduction at 7 days where statin therapy was initiated early in the setting of ST-elevation myocardial infarction. The same didn't hold for the non-ST elevation myocardial group, but the concept that very early statin therapy might modulate the infarction process is supported by these findings (94). Intensive lipid lowering using atorvastatin 80mg, compared alongside pravastatin 40mg was also shown to be beneficial following acute ACS, in the Pravastatin or Atorvastatin Evaluation and Infection Therapy – Thrombolysis in Myocardial Infarction 22 (PROVE-IT-TIMI 22) trial (95). 4162 patients were randomized within 10 days of presentation with ACS. At the 2 year point, the atorvastatin group had demonstrated a reduction in the composite endpoint (of death, myocardial

infarction, ACS, coronary revascularisation and stroke) of 16% (95% CI 5-26%, p=0.005). Rather than being lipid-dependent, it was suggested that some of these differences may be due to other “pleiotropic” effects of statins. Alternative mechanisms whereby statin therapy alters the course of ACS are thought to include anti-inflammatory effects (88), immune modulation, improvement of endothelial function, inhibition of coagulation ,stabilisation of unstable coronary atherosclerotic plaques and reduced adhesion molecule expression. (96-98).

Outside the setting of ACS, the process of PCI itself can sometimes lead to myocyte necrosis, especially where the procedure is technically difficult, where coronary anatomy is challenging or where the patients has not been exposed to extensive medical pre-treatment, especially where antiplatelet loading has been suboptimal (99). As well as biomarker release, this damage has been documented using magnetic resonance imaging (MRI) techniques, and where present, is linked to worsened clinical outcomes. In an attempt to investigate whether it was possible to pharmacologically influence this, Pasceri et al conducted the Atorvastatin for the Reduction of MYocardial Damage During Angioplasty (ARMYDA) study. This was a prospective, randomized study in statin-naïve patients undergoing elective PCI. Randomising patients to either atorvastatin 40mg/day or placebo, they demonstrated a striking reduction in periprocedural MI from 18% to 5% in the atorvastatin arm (p=0.025) (100). Further work by the same group in the setting of ACS was undertaken in the ARMYDA-ACS study, randomizing 171

patients with ACS to atorvastatin 80 mg 12 h before PCI, with a further 40-mg pre-procedure dose (n = 86) or placebo (n = 85) followed by long-term atorvastatin treatment thereafter (40 mg/day) (101). The primary end point (death, MI, emergency revascularization) occurred in 5% of patients in the atorvastatin arm and in 17% of those in the placebo arm (p=0.01), mostly driven by reduction of myocardial infarction incidence (5% vs. 15%, p=0.04). Post procedural elevation of creatine kinase-MB and troponin-I was also significantly lower in the atorvastatin group (7% vs. 27%, p=0.001 and 41% vs. 58%, p=0.039, respectively). Following multivariable analysis, pretreatment with atorvastatin conferred an 88% risk reduction of 30 day major adverse cardiac events (odds ratio 0.12, 95% confidence interval 0.05 to 0.50; p=0.004). The short pre-treatment time (12 hours prior to PCI) clearly indicates that these effects are independent of lipid-lowering, as this takes weeks to achieve, and points more strongly towards one of the pleiotropic effects outlined above, or a direct effect on ischaemia-reperfusion injury associated with either the index ACS event or the PCI. A later meta-analysis of studies of statin use in the setting of PCI confirmed a significant reduction in myocardial infarction from 5.2% to 3.0% (odds ratio 0.57, 95% confidence interval 0.42 to 0.78, p<0.0001) (102).

Evidence that statins reduce acute ischaemia-reperfusion injury

As far back as 1998, Laufs et al confirmed one of the pleiotropic effects which probably plays a key role in the ability of stains to confer

cardioprotection. Using human endothelial cells they demonstrated that simvastatin and lovastatin unregulated eNOS expression almost four-fold, and confirmed that it was likely that the beneficial effects of statins seen in atherosclerosis were more than a product of lipid-lowering.

An early example of statin cardioprotection was a provided by Lefer et al in 1999. They demonstrated that simvastatin preserved contractile function and coronary flow in isolated perfused rat hearts exposed to ischaemia-reperfusion injury. They attributed this to inhibition of neutrophil mediated cardiac dysfunction, and possibly enhanced eNOS release, rather than the more direct role in inhibiting ischaemia-reperfusion injury that now seems more likely.

Bell, working at our laboratory in 2003, confirmed that atorvastatin has a cardioprotective effect when administered at reperfusion. Using isolated perfused mouse hearts subjected to 35 minutes of ischaemia followed by 30 minutes of reperfusion he demonstrated that atorvastatin caused a marked dose-dependent reduction in infarct size. Optimal cardioprotection was achieved at a dose of 50 μM/L, resulting in a final infarct size of 16±2% in the atorvastatin group versus 33±2% in the control arm, $p<0.01$. This protection was lost with inhibition of PI3 kinase, and was absent in eNOS knockout mice. Western blot analysis revealed that atorvastatin resulted in activation of the PI3-K/Akt signaling cascade in the first 5 minutes of reperfusion and that both Akt and eNOS phosphorylation were significantly increased.

Phosphorylation was also lost in the presence of the PI3-K inhibitor. The key finding was that atorvastatin not only attenuates lethal reperfusion-induced injury, but also that the protection afforded is reliant on PI3K and Akt activity and the presence and activity of eNOS. Further work by Birnbaum's group in Texas has confirmed this finding (103;104), and has sought ways in which to pharmacologically enhance the protection by combining atorvastatin with agents which further activate eNOS and or inducible NOS (iNOS) such as phosphodiesterase inhibitors sildenafil (105) and cilostazol (106;107). Synergistic effects were also found with co-administration of atorvastatin with dipyridamole, an adenosine reuptake inhibitor, as adenosine also has a role in activating eNOS, and triggering cardioprotective mechanisms (108). Activation of the RISK pathway by statins has also been confirmed in a porcine model of myocardial infarction by Vilahur and colleagues, using pretreatment for 7 days with high-dose rosuvastatin. They confirmed a cardioprotective effect, with a significant 7% absolute decrease in infarct size, and a 12% improvement in ventricular function, independent of plasma lipid levels. There was elevated protein expression of the RISK kinases Akt and Erk-1/2 in the myocardial border zone (109).

The Role of PI3-Kinase in Pharmacological Cardioprotection

Central to pharmacological activation of the prosurvival, antiapoptotic RISK pathway lies activation of effectors by phopshatidylinositol 3-kinase (PI3-K) (71). Rather than being a single entity, PI3-K is a family of conserved lipid

and protein kinase enzymes which are widely expressed in cells throughout the body, including in the myocardium. As a group, they modulate cell survival, hypertrophy, contractility and metabolism. Importantly, in cardiac tissue, PI3-K is responsible for the phosphorylation of phosphatidylinositol 4,5-bisphosphonate to form phosphatidylinositol 3,4,5-triphosphate ($PtdIns(3,4,5)P_3$), an action reversed by PTEN. PI3-Ks are divided into three main classes according to their substrate specificity, though a huge range of catalytic and accessory subunit variation is seen (110). Regarding the heart, the Class IA variants PI3K α, β and δ, and the PI3K Class IB γ predominate (111). PI3-K activation enhances cardiomyocyte survival and antagonises apoptosis (112). A recent murine study confirms that PI3-Kγ plays a key role in both myocardial ischaemic preconditioning and pharmacological-mediated cardioprotection (113). One central role of PI3-K in cardioprotection is the activation of Akt isoforms, as part of the RISK signalling cascade (114). Other downstream targets include glycogen synthase kinase-3 and mTOR (112).

Human data supporting a direct cardioprotective effect of statins

Human experimental studies which adequately explain the findings of the large clinical studies of statin use have been scant. Using a cellular model of hypoxia-reoxygenation injury, Verma provided positive evidence of these cardioprotective effects extending to human myocytes. Using cells harvested from the right ventricular outflow tracts of patients undergoing

surgery to correct congenital heart disease, his group used 90 minute hypoxia/ 30 minute reoxygenation cycles. Pravastatin treatment directly prevented cardiomyocyte death, independent of endothelial or other cell types. In keeping with all the animal data outlined above, this effect was mediated by both an increase in NO release and an increase in protein kinase Akt activation.

Atorvastatin in clinical context

Atorvastatin is the most widely used HMG-CoA reductase inhibitor, and is safe and well tolerated, even at the intensive therapy dose of 80mg daily (115). Developed during the mid 1990s, atorvastatin is more potent than other similar agents with the exception of rosuvastatin. In terms of lipid modulation, 14 days therapy with 80mg daily will result in a 58% reduction in LDL-cholesterol. Based on work in healthy volunteers, at the 80mg dose it achieves its maximum observed plasma concentration (252 ng/ml) 2 hours after ingestion (116). This rapid bioavailability lends itself well to use in the emergency setting, and if given during the prehospital phase, it is likely that a clinically active serum level would be present by the time primary PCI is performed, given that current targets aim for a balloon inflation time of less than 90 minutes from first medical contact (20).

Summary and basis for experimental studies

Whilst shown to be protective in most animal and cellular experimental models, the ability of atorvastatin to directly protect the human myocardium

against ischaemia-reperfusion injury has not been established, and this will form the basis for the experimental studies outlined below. Additionally, it is planned to examine whether, when administered at the onset of reperfusion, prosurvival signalling pathways including the RISK enzymes PI3K, Erk-1/2, and NOS isoforms can be activated with this agent.

Cardioprotection during revascularisation procedures

Surgical revascularisation

Cardiac surgery in the United Kingdom is performed for two main indications. The most common requirement is as part of a strategy of revascularisation for patients suffering from coronary artery disease, termed coronary artery bypass grafting (CABG). First performed in the USA in 1967, this rapidly became established as the standard of care for patients suffering from symptomatic coronary artery disease (117). During this procedure, vascular conduits are used to bypass blocked or severely stenosed epicardial blood vessels, with the aims of improving patient survival, reducing the symptoms of angina, and preserving function of the left ventricle. The conduits used are most commonly the left internal mammary artery onto the left anterior descending artery, and saphenous vein grafts harvested from the leg, onto the circumflex and right coronary artery territories Even with marked advances in minimally invasive percutaneous coronary techniques, CABG holds its place as the treatment of choice for some patient groups (6). Around 23,000 CABG procedures are performed annually in the UK (118).

Treatment of cardiac valvular disease is the next largest group undergoing cardiac surgery. Degenerative disease affecting the aortic valve is the most common form requiring surgical treatment, followed by mitral valve regurgitation, totalling around 6000 and 3900 cases per annum respectively

in the UK (118). In both aortic and mitral valve surgery, intervention is performed to prolong life by preventing left ventricular injury, and to reduce distressing symptoms such as angina and dyspnoea. Valve disease and coronary artery disease frequently co-exist, mandating a joint procedure to repair or replace the affected valve alongside CABG surgery. Percutaneous treatment for aortic and mitral valve lesions is now developing in some selected UK centres. This obviates the need for a full thoracotomy and cardiopulmonary bypass, but the technique is currently only available in a handful of specialist centres. Additionally, the current evidence base restricts this approach to include only patients at very high risk of death or serious disability following a conventional surgical approach (119). It is likely to take some years to fully evaluate these minimally-invasive procedures, and there will be a further delay in availability of the service caused by the requirement to train interventional cardiologists in this technique. In the meantime a conventional surgical treatment is the best option for the majority patients.

Circulatory support during surgery

The majority of cardiac surgery in the UK is performed using cardiopulmonary bypass to oxygenate and circulate the blood during the operation. Anticoagulated blood is diverted from the venous circulation using large bore tubes inserted into the right atrium or proximal vena cava. Having been routed through a filter, blood is cooled, oxygenated and

pumped back into the proximal aorta. This allows cardioplegia to be used, a technique which results in an intentional cardiac arrest. This gives the surgeon a motionless heart during the delicate phases of the surgery and thus facilitates the creation of anastomoses between conduit and native vessels. It also reduces the risk of air entering the circulation and causing embolic damage such as stroke. CABG comprises four main manoeuvres – i) surgical access to the thoracic cavity and connection to the perfusion circuit, ii) the establishment of cardiopulmonary bypass, iii) cross-clamping of the aorta and induction of cardiac arrest using a cardioplegia solution alongside cooling and iv) attaching the vascular grafts. As well as an inflammatory response due to connection to the bypass circuit, and a risk of stroke from the procedures which involve instrumenting the aorta, direct myocardial injury is also seen in CABG surgery and is discussed below. The procedures which require aortic surgery (e.g. graft attachment) all predispose the patient to the risk of stroke. At the end of the operation, normal heart rhythm is restored, and the patient is rewarmed. As extracorporeal support is no longer required, it is “weaned” off and the access pipes are removed.

Cardiac surgery is associated with significant morbidity and mortality

The physiological and biochemical insults faced by cardiac surgical patients in the perioperative phase can translate into adverse clinical events. A recent international prospective observational study suggests that composite

morbidity and mortality in CABG patients ranges from 12-24% (120). As well as general systemic insults including exposure to the bypass circuit, direct cardiac manifestations include coronary atheroembolic events with downstream myocardial necrosis following instrumentation of the coronary arteries, and direct myocardial injury from handling or over-distension. Some perioperative degree of myocardial necrosis is ubiquitous following cardiac surgery.

Direct myocardial ischaemia-reperfusion injury occurs during CABG

Intermittent cross-clamping of the aorta, performed to allow the attachment of vascular conduits during CABG results in repeated pan-regional episodes of myocardial ischaemia-reperfusion injury, in addition to damage induced by direct surgical contact with the myocardium. The extent of myocardial necrosis experienced during surgery can be quantified by measuring cardiac-specific markers such as troponins and myocardial-bound creatine kinase (CK-MB). Recent clinical research has shown that perioperative release of troponin-T is associated with poorer short and long-term outcomes. In a recent single centre prospective study of over 800 patients undergoing CABG, an elevated level of troponin-T when measured at 24 hours was found to be an independent predictor of death, heart failure or need for inotropic support. Conversely, a postoperative troponin-T below 1.6ng/ml had a high negative predictive value for excluding post-CABG

complications, suggesting that interventions aimed at reducing perioperative myonecrosis may be clinically relevant (121). Additionally, MRI techniques confirm that detectable myocardial scar is seen following 32% of myocardial revascularisation procedures, with a mean infarct mass of 5g. This is associated with a three-fold increase in adverse events in this group (122) . Periprocedural myocardial injury is not limited to revascularisation by CABG, but also occurs in the setting of PCI, hence targeting treatments at periprocedural cardioprotection could have applicability in both clinical settings (123).

Attempts to reduce perioperative myocardial injury:

Optimal surgical revascularisation

Following a paper published by the Cleveland Clinic in 1986, the use of an internal mammary graft in preference of a vein graft became standard practice. Prior to this, it was standard practice to graft the left anterior descending artery with a saphenous vein graft. This new approach lead to a significant reduction in death, late myocardial infarction, recurrent angina and the need for repeat surgery. The benefits extend out to 10 years postoperatively (124). Even fuller revascularisation can be performed using bilateral internal mammary artery grafts, and an early systematic review suggested better survival outcomes in these cases (125). The prospective randomised multicentre international Arterial Revascularisation Trial recently published 1-year follow up data, comparing single versus bilateral arterial

grafts. In this study, CABG patients were enrolled in 28 hospitals in seven countries. 3102 patients were randomly assigned to single (n = 1554) or bilateral IMA grafts (n = 1548). The mean number of grafts was 3 for both groups. Mortality was identical at 30 days and 1 year The rates of stroke, myocardial infarction, and repeat revascularization were also the same in both (126). The data confirm that this approach to maximal revascularisation is feasible though the authors accept it is technically more challenging. However, uptake of the technique amongst cardiac surgeons is slow, with less than 10% of patients receiving the technique in Europe (127).

Off-pump surgery

In the mid-1990s, CABG without the use of cardiopulmonary bypass circuit started to become established – “off-pump” CABG (OPCABG) or “beating heart” surgery. OPCABG was believed to reduce the perioperative systemic inflammatory response syndrome, and some studies showed reduced levels of circulating inflammatory markers in this setting (128-130). The cerebral insult accrued during CABG, which can lead to long-term cognitive abnormalities, was also perceived to be related to exposure to the bypass circuit, and could thus potentially be diminished by OPCABG (131-133). Intraoperative haemodynamic parameters and some technical aspects of cardiac handling, especially in regard to accessing the posterior aspect of the heart for grafting the circumflex vessel and its branches, may also be improved by using OPCABG (134;135). In some settings, OPCABG was

shown to confer a shorter in-hospital stay, better renal parameters, reduced operative blood loss and reduced perioperative myocardial injury in comparison with conventional “on-pump” surgery (136) and a reduction in operative mortality amongst OPCABG cohorts was noted (137). Despite these potential advantages, OPBACG remains a “niche” tool, performed by a minority of UK surgeons in a subset of highly selected cases (138). However, the acute (139) and longer-term (140) patency of grafts performed during OPCABG has recently been questioned, Data from a recent prospective, randomised, controlled, single-blind study of 2203 patients undergoing CABG suggest a poorer outcome with OPCABG, even in experienced hands (141). The investigators showed worse composite adverse outcome and poorer graft patency in the OPCABG group. Incomplete revascularisation at the time of surgery, more common with OPCABG, has also been shown to be associated with a worsening in mid-term mortality (142). It remains to be seen whether OPCABG will increase in popularity, or wither on the vine. With the exception of the fledgling percutaneous interventional valve techniques outlined above, valve surgery always mandates full cardiopulmonary bypass. Whatever the case, it seems likely that, even with a fully optimised surgical technique, patients will still be exposed to some degree of cardiac and systemic insult if we are to perform these major procedures (130). Surgical “fine-tuning” alone is unlikely to provide the answers, particularly as cardiac surgeons face an ageing cohort

of patients with multiple comorbidities, leading to a higher incidence of high-risk cases (143).

Cardioprotective strategies during cardiac surgery:

Perioperative ischaemic preconditioning

First characterised in humans by Yellon *et al* in 1993, IPC was applied in the context of applying intermittent cross-clamping of the aorta following the establishment of cardiopulmonary bypass. Two, three minute cycles of aortic cross-clamping were performed as the preconditioning stimulus, applied at two minute intervals to allow intermittent reperfusion. They demonstrated higher levels of ATP in the IPC cohort (144). Following this, a subsequent study confirmed lower levels of perioperative cardiac biomarker release at 72 hours post-surgery, specifically troponin-T (145). However, cross-clamp fibrillation remains unpopular as repeated aortic handling may induce stroke, especially in the increasingly older cohort of patients who may exhibit extensive calcification of the vascular tree.

Remote ischaemic preconditioning during cardiac surgery

Remote ischaemic preconditioning (RIPC) refers to the cardioprotective effect seen where the preconditioning stimulus is applied to an organ or tissue remote from the heart itself (146). First described in canine hearts, the investigators reported that, by intentionally applying prior circumflex

territory ischaemia, the effects of a subsequent left anterior descending occlusion were ameliorated, evidenced by a reduced final infarct size (67). Cardioprotective effects are also seen where a preconditioning (ischaemic) insult is applied remote from the heart itself. Pell *et al*, using a rabbit model, demonstrated that intermittent renal ischaemia could be used to induce cardioprotection, and linked this with activation of adenosine receptors and K-ATP channels (147). Subsequently this was also demonstrated with surgically-induced mesenteric (148) and gastrocnemius muscle ischaemia (149), again in animal models. In the setting of human patients undergoing cardiac surgery, Kharbanda and colleagues demonstrated a more useful and clinically applicable approach, avoiding the need for surgically-induced remote ischaemia. Using transient limb ischaemia of the forearm, they were able to demonstrate a reduction in ischaemia-reperfusion injury-induced endothelial dysfunction of the contralateral limb in human volunteers. Using a pig model they confirmed that this could translate into a reduced myocardial infarction size (150). In 2007, Hausenloy and colleagues performed a randomised controlled trial of the effects of RIPC on myocardial injury in patients undergoing CABG. The results confirmed a 43% reduction in absolute troponin-T release (151). The mechanism through which this protective effect is gained, however, remains unclear, and it is likely that a neural or hormonal pathway, or a combination if the two, exists to transmit the cardioprotective signal to the myocardium (146). Despite good evidence for RIPC, and little likelihood of harm, it remains little used clinically and data

from outcome studies are awaited. Such a study is being undertaken by Yellon's group at University College London and University College London Hospitals. This is entitled the Effect of Remote Ischaemic Conditioning in Coronary Artery Bypass Graft (ERICCA) study, and it is a randomised, double-blind placebo controlled trial (152).

Pharmacological preconditioning in CABG

The administration of a drug which might reduce perioperative myocardial injury is an attractive prospect, and has been intensively studied to date. Heavily implicated in cardioprotective cascades, adenosine has been shown to act as a preconditioning mimetic, reducing perioperative biomarker release (153;154). A recent small prospective randomised study using adenosine alongside cold cardioplegia suggests that it may reduce troponin-I release, reduce the release of inflammatory mediators IL-6 and IL8, and reduce myocardial cellular damage. There was also evidence of a reduction in mitochondrial damage in the adenosine cohort (155). However, at the doses required to produce a meaningful effect, adenosine causes significant side effects including bradycardia and hypotension. Indeed, it is used to provoke ischaemia by inducing coronary vascular hyperaemia in testing for coronary insufficiency, so thus may actually be inducing perioperative ischaemia. Perhaps, in this setting, we might be seeing pharmacological induction of ischaemia similar to pre-infarct angina, and it is this which confers protection, rather than the effect of the adenosine itself. In an

attempt to avoid administration of exogenous adenosine, Mangano used acadesine, a modulator of endogenous adenosine, in a large randomised controlled multicentre study of 2698 patients undergoing elective CABG. In those patients suffering from postoperative myocardial infarction (3.7% of the cohort), 2-year survival was shown to be improved, but there was no difference in the non-MI group (156). This is in keeping with the modification of myocardial infarction by adenosine seen in studies of its acute cardioprotective effects in humans undergoing reperfusion by thrombolysis (157) and PCI (158;159).

In the GUARDIAN clinical study, the sodium-hydrogen exchange inhibitor, cariporide initially looked promising in terms of a small improvement in early mortality and myocardial infarction (160). However, the subsequent larger EXPEDITION trail confirmed that these findings did not persist beyond the 6 month point, and confirmed the unwanted complication of an increase in cerebrovascular mortality associated with the drug (161).

Statin therapy and perioperative cardioprotection - the data so far

Fully established in the primary (87) and secondary prevention roles (90;162), investigators have started to explore the potential value of statins in clinical cardioprotection (163;164).

In 2000, Dotani et al reported the findings of a West Virginia University Hospitals retrospective analysis of the effects of perioperative statin therapy and cardiac outcomes following CABG. Out of 323 cases analysed, 104

patients were taking statins, of which 80% were using atorvastatin. There was a striking reduction in composite adverse events in the statin group (18% versus 57%, p<0.0001), and a significant reduction in both early (0% vs. 3.7%, p=0.05%) and late death. However the cohorts here were relatively small, and the authors concluded that further prospective randomised data should be sought (165).

Lazar performed a study of pigs undergoing an acute ischaemic coronary insult followed by cardiopulmonary bypass and surgical revascularisation. 21 days of pretreatment with atorvastatin 40mg (in 35 kg animals) was shown to reduce perioperative haemodynamic instability, reduce arrhythmias, improve ventricular wall motion scoring and resulted in a smaller final infarct size than placebo (21±2% vs. 41±2%, p=0.0001), despite the area of myocardium at risk being the same across the groups. However, the model used is not a true simulation of elective CABG, with some features of the study being more a model of acute ischaemia-reperfusion injury rather than a pure perioperative cardioprotection model. CABG is simply not used for acute revascularisation in the context of acute total coronary occlusion. However, the study usefully confirms that oral treatment with atorvastatin can induce a cardioprotected state (166).

A predefined subset analysis of 5436 patients in the prospective McSPI Epidemiology II multicentre study confirmed that preoperative statin therapy was associated with a significant reduction in the risk of early cardiac death

(0.3% vs. 1.4%, p<0.03) after elective CABG (Adjusted odds ratio 0.25; 95% confidence intervals 0.07-0.87). Additionally, discontinuation of statin therapy after surgery was also noted as to be independently associated with an increased risk of late postoperative mortality compared with those receiving uninterrupted statin therapy (1.91 versus 0.45, p<0.01). These data further outline the importance of perioperative statin therapy (167).

Two randomised controlled trials of atorvastatin followed, one of which demonstrated that preoperative atorvastatin was able to reduce the incidence myocardial dysrhythmias, specifically atrial fibrillation (168) . The second demonstrated that atorvastatin therapy was able to reduce serum inflammatory cascade markers (neutrophil CD11b expression, IL-6, & IL-8), although interestingly, not the incidence of systemic inflammatory response syndrome (169).

In 2008, a single-centre retrospective study of 1389 consecutive elective cardiac valve operations at Brigham and Womens' Hospital, Boston, demonstrated that 26% were taking statins at the time of their surgery. (54% atorvastatin, 34% simvastatin). The operative mortality rate was 0.8% in the statin cohort and 2.3% in the non-statin group. However, a trend towards lower stroke rates emerged in the statin group. There was no evidence to point towards any particular underlying mechanism of the improved outcomes seen (170).

More robust clinical evidence in support of reduced perioperative myocardial necrosis was afforded by Mannacio's study, also in 2008. Performed in an Italian centre, 200 consecutive patients undergoing elective CABG were enrolled in a randomised, double-blind fashion. Study inclusion criteria were highly selective in order to preserve study group homogeneity. In the intervention arm, rosuvastatin 20mg daily was prescribed 7 days prior to surgery, irrespective of lipid status. All aspects of the anaesthesia, surgery and subsequent management on the surgical critical care unit were standardised. The results demonstrated that patients receiving a statin required less inotropic support, suffered less postoperative atrial fibrillation and required a mean of 1 day less hospitalisation, than did the control group. There was also a reduction in high-sensitivity C-reactive protein following statin therapy. Importantly, myocardial damage was reduced in the statin group, with reductions in peak levels of troponin-I (0.16±0.015 versus 0.32±0.26 ng/ml, p=0.0008), CK-MB (3.9±3.3 versus 9.3±8.1 ng/ml). These data further suggest that the role of statins in this cohort is due to a direct cardioprotective effect, rather than a more general anti-inflammatory pleiotropic benefit (171).

A Parisian study of consecutive elective high-risk cardiac valve surgery patients assessed preoperative statin therapy and outcome variables. Following multivariate and propensity-adjusted regression analysis

regression of 422 cases, the three mortality risk factors identified were lack of statin use, poor ventricular function and pulmonary hypertension. Statins were associated with a significant reduction of postoperative mortality (odds ratio of 0.41, p=0.04), further adding weight to their value in the setting of cardiac surgery (172;173).

A meta-analysis of over 30,000 patients from 19 studies by Liakopoulos confirms that statin therapy exerts a significant clinical benefit on early postoperative outcomes in cardiac surgical patients (174). A further meta-analysis suggests a 25% relative reduction in mortality may be achieved by the use of preoperative statin therapy. Surprisingly, the authors stopped short of advocating routine preoperative statin therapy (175).

Subsequent to these, further data confirming that atorvastatin can reduce perioperative myocardial injury comes from a small prospective controlled study of 40 patients undergoing CABG. The investigators demonstrated a reduction in postoperative biomarker release following the preoperative administration of atorvastatin (20mg/day). 24 hours after the surgery, troponin I and CK-MB levels were significantly lower in the atorvastatin group: for CK-MB levels, 12.9±4.3 versus 18.7±7.4 ng/ml, (p=0.004); for troponin I levels, 1.7±0.3 versus 2.7±0.7 ng/ml ($p < 0.001$). In addition, atorvastatin use was associated with a decrease in the duration of critical care unit admission (176). The same research group have previously shown

reduced high-sensitivity C-reactive protein and IL-6 levels following preoperative atorvastatin (20mg/day) in a similar setting (177).

Underlying Mechanisms

Possible underlying mechanisms for the perioperative benefits of statins include a range of pleiotropic actions. These include anti-inflammatory effects, antithrombotic and antiplatelet effects, and atheromatous plaque stabilisation (163;164;178;179). However, whilst these could be seen to contribute to improved haemodynamic variables and perhaps reduce critical care admission duration, they are unlikely, *per se*, to contribute to any reduction seen in perioperative myocardial ischaemia-reperfusion injury (96).

Chronic statin administration and loss of cardioprotection

Whilst the lipid-lowering effects of statins increase in the weeks following administration, it is understood that the cardioprotection offered against ischaemia-reperfusion injury wanes over time. Atorvastatin has been shown to be cardioprotective and reduce ischaemia-reperfusion injury when given acutely during the reperfusion phase of an experimental myocardial infarction (72) In a rat model, Mensah demonstrated that, after 1-2 weeks therapy with 20mg/kg atorvastatin, the ability to cardioprotect against myocardial infarction was lost. An additional 40mg/kg high-dose repeat exposure to atorvastatin, however, was able to “recapture” the lost

cardioprotective effect, when given 3-4 hours prior to myocardial ischaemia-reperfusion injury. Analysis of levels of phosphatase and tensin homolog deleted on chromosome ten (PTEN) – a ubiquitously present "house-keeping" dual phosphatase - using a Western blotting immunochemistry technique were performed. These confirmed that chronic administration of atorvastatin leads to elevated levels of PTEN (180). Atorvastatin is known to induce cardioprotection acutely through activation of the PI3K/Akt pathway (72). This is a short term pro-survival pathway, with an anti-apoptotic effect (181-183). However, unopposed long term up-regulation of this enzyme cascade would induce hypertrophy, and in some tissue lines may be oncogenic (184-188). PTEN acts as an inbuilt down regulator of the PI3K/Akt system, which prevents the activated enzymes remaining in a phoshorylated state long-term. In terms of inhibiting long-term cardioprotection conferred by statin therapy, PTEN has been termed the "Achilles heel" of myocardial ischaemia-reperfusion injury (189). The occurrence of this phenomenon in human myocardium has not been established. Similarly, the ability to "recapture" cardioprotection by using a high dose of statin prior to an ischaemia-reperfusion insult remains a concept unproven in humans, although clinical studies of periprocedural biomarker release following urgent PCI hint that this might be the case (178;190).

Summary and basis for experimental studies

The beneficial effects of statin therapy during the perioperative phase could be due to a variety of pleiotropic actions. However, data exist which support the likelihood that some of the benefit is derived from a reduction in perioperative myocardial ischaemia-reperfusion injury.

Any protection against myocardial ischaemia-reperfusion injury afforded by atorvastatin should reduce following chronic administration, and it is unknown whether high dose oral administration to human subjects can recapture cardioprotection in myocardial tissue. These issues will form the basis for experimental studies outlined below.

Hypertrophic Cardiomyopathy and Cardioprotection

Background

First formally characterised as a clinical entity in the late 1950s (191), hypertrophic cardiomyopathy (HCM) is a genetically inherited disorder characterised by unexplained hypertrophy of the left ventricle. Affecting up to 1 in 500 of the general population (192-194), HCM is the most common inherited cardiovascular disorder. Although it can manifest at any age, it is one of the most common causes of sudden death in young people and athletes (195). The clinical syndrome is caused by mutations in genes which encode cardiac sarcomeric proteins. Key features include myocardial hypertrophy, myocyte disarray and fibrosis, and small vessel coronary disease. HCM occasionally may present in association with other diseases such as Noonan's syndrome, mitochondrial disorders, and metabolic disorders such as Anderson-Fabry disease.

Genetic Basis

The first step in the pathogenesis of HCM involves mutation-induced dysfunction of the sarcomere – the functional subunit responsible for myocardial contraction (194;196). HCM is inherited as an autosomal dominant condition, but with significant and expanding heterogeneity (197). Over 1000 mutations in 13 or more genes have been identified to date,

encoding for a variety of proteins associated with myocyte structure and function (196;198;199). The most common HCM-causing genes encode ß-myosin heavy chain, cardiac troponin T and myosin-binding protein C, and these genes account for the bulk of cases. A smaller number of cases are due to mutations in genes encoding cardiac troponin I, myosin light chains, titin and α-myosin heavy chain. Many other genes and mutations are yet to be identified. Genetic testing of subjects using DNA analysis for mutant genes is possible, and provides a definitive diagnosis. However, due to its complexity and costs it is not current clinical practice, and the diagnosis is usually made based on clinical criteria in the majority of cases.

Clinical Diagnosis

Clinical examination may reveal features of left ventricular outflow tract obstruction, evidenced by a bifid arterial pulse and a systolic murmur audible on auscultating the chest with a stethoscope. However, true resting left ventricular outflow tract obstruction is only present in around 25% of cases of HCM (200-202), so these are unreliable markers in isolation. Baseline electrocardiography is abnormal in 75% to 95% of HCM patients, commonly demonstrating high voltages indicative of left ventricular hypertrophy, interventricular conduction defects (such as right or left bundle branch block), PR interval shortening and ST segment and T wave abnormalities (203-205). Formal diagnosis is achieved using 2-dimesional echocardiography, which confirms a hypertrophied, non-dilated ventricle, at

the same time excluding other structural causes of hypertrophy such as intercurrent aortic valve disease (206). Additionally, echocardiography is used to confirm and measure the presence of any left ventricular outflow tract obstruction, and assess for systolic anterior motion of the mitral valve – both hallmark features of HCM.

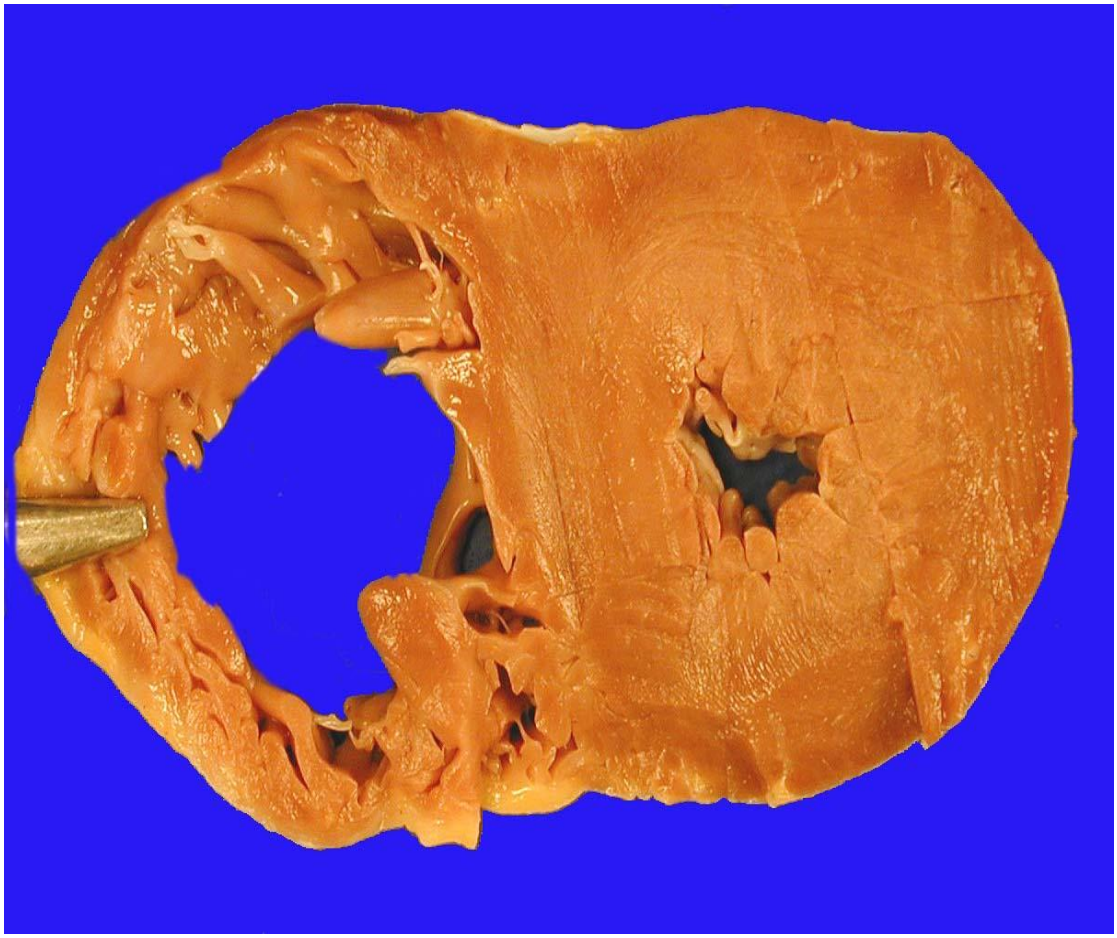

Figure 1.1 – post-mortem specimen showing massive hypertrophy of the interventricular septum and left ventricle, resulting in near obliteration of the left ventricular cavity when compared to the right ventricle (identified by forceps on left of image). Reproduced courtesy of Pinpoint Scotland Ltd.

Histological abnormalities

Microscopically, left ventricular tissue is hypertrophied, and disarrayed with chaotic cellular alignment and multiple intercellular connections which are not found in normal myocardium (207). This cellular disarray may be focal or more generalised throughout the left ventricle, and frequently also involving the right ventricle

Enhanced oxidative stress

Experimental animal models of left ventricular hypertrophy induced by pressure overload, for example using aortic banding to increase ventricular afterload, have demonstrated elevated markers of myocardial oxidative stress suggesting this is an important component in the pathogenesis (208-210) . One clinical study of HCM patients with LV dysfunction confirmed increased expression of 4-hydroxyl-2-nonenal-modified protein, a marker of myocardial oxidative stress (211). A recent study in patients with HCM and preserved LV function assessed serum levels of 8-isoprostglandin $F_{2\alpha}$ as a marker of oxidative stress and found HCM patients had significantly elevated levels compared with matched, non-HCM control subjects. Interestingly, the level of oxidative stress positively correlated with anatomical and haemodynamic indices of left ventricular outflow tract obstruction (212). Pressure overload triggers nitric oxide synthase-3 uncoupling, generating a

significant burden of reactive oxygen species (213). In experimental settings, antioxidant therapies such as tetrahydrobiopterin and N-actetylcysteine have been shown to reduce nitric oxide synthase-3 uncoupling, inhibit reactive oxygen species generation and block or reverse maladaptive changes in hypertrophic hearts(214;215) . Oxidative stress has also been shown to induce perivascular inflammation and cardiac fibrosis – both present in the setting of HCM (216).

HCM generates a state of chronic regional myocardial ischaemia

The increased myocardial mass leads, in turn, to an increase in basal myocardial oxygen requirements . Extravascular compressive forces, applied to the perfusing blood vessels by the abnormally bulky myocardium, reduce myocardial perfusion and can compromise flow in large septal perforator branches and even major epicardial vessels. In addition, the intramural coronary vessels are of relatively small calibre due to medial hypertrophy (217;218). At myocyte level, disarray leads to inefficient contraction, leading to further circulatory demands. These multiple factors combine to result in reduced coronary vasodilator flow reserve (219-224) and a myocardium which is frequently exposed to ischaemia, resulting in myocyte necrosis and scarring (225). The degree of circulatory impairment has been shown to be clinically significant, and correlates with long term adverse event rates (226).

HCM renders the myocardium more sensitive to ischaemia

Multiple co-existing factors render the HMC-affected heart more susceptible to the effects of ischaemia than normal heart tissue (227-229). Abnormalities of myocardial metabolism, calcium handling, a chronic inflammatory response, sympathetic nervous system and renin-angiotensin system activation co-exist in cardiac hypertrophy, and contribute towards and increased sensitivity of hypertrophied myocardium to ischaemia-reperfusion injury (230-232). Uncoupling of energy substrate use, with low glucose oxidation, and higher rates of glycolysis have been observed in hypertrophied rat hearts, leading to reduced functional recovery after an ischaemic insult (233;234). Reduced sarcosplasmic reticulum calcium ATPase activity leads to cellular calcium overload, and this subsequently further impairs post-ischaemic recovery (230;235). Additionally, the effects of powerful means of cardioprotection, such as pharmacological inhibition of the mitochondrial permeability transition pore, may not operate in the setting of some pathologies (229;231). Specifically it is not known whether the opportunity to manipulate this protective mechanism exists in HCM.

HCM frequently co-exists with coronary artery disease

First reported in an angiographic case series by Gulotta over 30 years ago, HCM and coronary artery disease can frequently co-exist, especially in older cohorts (236). In those over 45 years of age, significant angiographic epicardial lesions can be found in up to 24% of HCM cases (237-240). The

burden of stenotic lesions required to cause ischaemia is likely to be much lower in the setting of HCM, due to the pre-existing presence of reduced coronary flow reserve, as outlined previously (241). The paucity of long term outcome data in this important group of patients was recently addressed by Sorajja et al, who performed a retrospective case analysis of adult HCM patients undergoing coronary angiography over a 28 year period (242). Of a final study population of 433 patients, 26% has severe coronary artery disease, 27% mild-to-moderate disease, and 47% had normal angiograms. Patients with coronary artery disease were more likely to have co-existing risk factors such as hypertension, diabetes and stroke than those without coronary artery disease. The 10-year survival rate in those with HCM and severe coronary disease was startlingly low at 46%, despite these patients having good ventricular systolic function. The sudden cardiac death rate was 2.1%, which represents a doubling of that expecting in the background HCM population. All-cause annual mortality was markedly elevated at 6.65 which represents a doubling of the rate of death even when compared against 'complicated' HCM tertiary unit reported annual data (243-245)

Available supportive medical and surgical therapies for HCM

The overall annual mortality rate in patients with HCM is similar to the general population, and is around 1% (246). Many patients experience a normal life expectancy despite having the condition. However, a high-risk subgroup of younger patients exists, in which mortality rates of up to 6% per

year could be expected (206). To avoid sudden cardiac death from arrhythmia, risk stratification is performed to identify these high-risk individuals as they would benefit from an implantable cardioverter-defibrillator (ICD). Clinical markers of high-risk include a family history of sudden death, syncope, nonsustained ventricular tachycardia and a poor haemodynamic response to exercise (247). Risk assessment can be further refined by the use of cardiac magnetic resonance imaging to detect scar, which can suggest a 5 year probability of sudden cardiac death of 11%, versus 0.7% where scar is absent (248). Subcutaneously implanted ICDs allow for advanced pacing and cardioversion therapies to be delivered immediately should potentially fatal arrhythmias such as ventricular tachycardia or fibrillation ensue, with the aim of avoiding cardiovascular collapse, hypoxic brain injury and subsequent death.

Drug therapy to improve symptoms is typically aimed at reducing the left ventricular outflow tract gradient. A treatment regimen might typically include the use of negatively inotropic agents such as ß-blockers, verapamil or disopyramide. These agents are thought to enhance ventricular filling, decrease myocardial oxygen demand and increase myocardial perfusion. ß-blockers are considered first-line therapy, with the calcium channel blocker verapamil used where ß-blockers are either not tolerated or contraindicated, or where diastolic dysfunction is the predominant feature. Disopyramide, a class Ia sodium channel blocking antiarrhythmic, is usually reserved for

cases with severe functional limitation unresponsive to other agents (206). None of these agents has any proven prognostic benefit.

For severely symptomatic patients with prominent septal hypertrophy, interventional cardiology techniques can be employed to improve left ventricular outflow. Alcohol septal ablation can be performed percutaneously and will aid with breathlessness or syncope in carefully selected cases. The technique involves intubation of a large septal artery under echocardiographic and angiographic guidance, followed by injection of alcohol to effectively induce a limited infarction. The resulting loss of myocardial mass within the target area improves flow and relieves symptoms, with procedural success in over 80% of cases (206;247;249).

In refractory cases, surgical excision of excess bulky septal tissue can be performed with good results, though this technique is highly invasive and requires full cardiopulmonary bypass and a thoracotomy. It is most suited to cases where surgery is required concomitantly to treat valve lesions or co-existing coronary artery disease (206;247).

Disease Modification – the current state of play

Despite recent advances in cardiovascular medicine, no pharmacological therapies currently exist which can inhibit or actually reverse disease progression. Gene therapy has been suggested as the most likely source of

a meaningful disease-modifying therapy, but this is currently a long way from being a useful clinical tool. The condition is incompletely characterised in genetic terms, the clinical course is not fully documented, and there is a paucity of evidence-based therapy available (247;250).

The potential roles for statin therapy in HCM

Statins and cardiac hypertrophy

Cardiac hypertrophy and interstitial fibrosis are major determinants of morbidity and mortality from disease processes other than HCM, and in particular are found in hypertensive heart disease, and coronary artery disease (251;252). This has led to investigation of the pleiotropic effects of statin therapy in this context, in addition to their well-documented mortality and morbidity effects due to improved lipid profile.

In a study of rats with angiotensin-induced hypertension, treatment with simvastatin attenuated the development of hypertension, inhibited left ventricular mass increase and reduced circulating markers of oxidative stress (253). Laufs et al treated spontaneously hypertensive rats with atorvastatin, and their findings suggested that inhibition of pro-hypertrophic small GTP binding proteins Rac1 and RhoA may be responsible for statin-induced reduction of left ventricular mass (254). In a magnetic resonance study of left ventricular hypertrophy in rats exposed to surgically-induced myocardial infarction, Nahrendorf et al demonstrated that cerivastatin

attenuated post-infarction left ventricular hypertrophy and improved cardiac output. Interestingly, the protective statin effect was abolished by nitric oxide synthase inhibition with L-NAME (255). These results are further supported by the work of Luo et al, who showed that simvastatin can inhibit hypertrophy in a rat model of aortic stenosis (256). Indolfi et al implicated Ras activation as an important factor in the prevention of cardiac hypertrophy by simvastatin, using a pressure overload model of ventricular stress (257), and Chen et al confirmed that statins prevent hypertrophy in a rat model of hypertrophy (258).

Clinical trials of statins in the anti-hypertrophy role

Su et al examined the effects of pravastatin 10mg daily on left ventricular hypertrophy in drug-naive patients with an adverse lipid profile and essential hypertension in a small, prospective clinical study. There was a significant excess reduction of left ventricular mass index in patients treated with statin therapy, 11% over and above that achieved by maximal antihypertensive therapy. The authors suggested inhibition of angiotensin II mediated transcription factors as a possible mechanism for the anti-hypertrophy effect (259;260). In a clinical study of 300 angina patients with angiographically confirmed coronary stenosis, Nishikawa et al reported that therapy with pravastatin or simvastatin significantly reduced left ventricular mass evidenced by follow-up echocardiography by 14.7%, compared to controls (261).

Studies of statins in animal models of HCM

In an animal study investigating the antioxidant effects of statin therapy on cardiac myocyte hypertrophy, Takemoto et al treated neonatal rat cardiomyocytes with angiotensin II to increase reactive oxygen species production and subsequently induce hypertrophy, with and without simvastatin 5 μM. In the simvastatin treated cells, cardiac myocyte hypertrophy was inhibited, due in part to inhibition of isoprenoid synthesis, Rho gerenylgeranylation and reactive oxygen species production (262). Patel et al studied a ß-myosin heavy chain transgenic rabbit model of human HCM which expresses cardiac hypertrophy, interstitial fibrosis, myocyte disarray and cardiac dysfunction. Administering simvastatin 5mg/kg per day for 12 weeks induced regression of hypertrophy and fibrosis and improved cardiac function (263). Using the same transgenic rabbit model, Senthil et al later showed that that treatment with atorvastatin 2.5 mg/kg lead to beneficial effects over and above the 49% reduction of serum total cholesterol levels achieved. Multiple markers of cardiac hypertrophy were used to assess the effects of therapy, including echocardiographic assessments of left ventricular mass, histological studies of myocyte size, expression of molecular markers of hypertrophy and fibrosis and expression of oxidative stress response genes. At organ, cell and molecular levels, atorvastatin therapy was shown to reduce cardiac hypertrophy after a 1-year observation period (264). Importantly, there was a reduction in left ventricular mass index of ~30%. However, it is important to note that, unlike the clinical cohort

of HCM patients exposed to experimental studies, the intervention arms in these two studies consist of a genetically homogenous group with a predefined defect. The possibility exists that this effect may be less significant where another gene defect is present.

Clinical trials of statins on HCM

Clinical trials of statin therapy in HCM have, however, been inconclusive to date. In a single-centre randomised placebo-controlled double-blind pilot study, Bauersachs et al treated 11 HCM patients with atorvastatin 80mg daily for 9 months alongside placebo-treated controls. Using electrocardiography, echocardiography and cardiac magnetic resonance imaging they were unable to demonstrate a significant improvement in left ventricular mass or other associated indices following statin therapy. Only intended as a pilot study, the study design was underpowered to demonstrate anything other than a major difference in the two cohorts. Additionally, the number of patients taking calcium antagonists was almost doubled in the placebo arm, and this may have masked a possible treatment effect from statin therapy. Therapy was also relatively short in terms of the natural history of HCM, which evolves over several years, with final follow-up after only 9 months of therapy.

More recently, Nagueh et al performed a feasibility study in 21 HCM patients. After enrolment, atorvastatin was administered at 20mg per day for

3 months, increasing to 40mg per day for a further 3 months then continuing at 80mg daily for 18 further months. At the 2 year point, the dropout rate in the atorvastatin arm was exceptionally high compared to previous statin studies, at over 50%. However, the authors explain this was mainly due to patients' lack of perceived clinical benefit, and no major adverse effects were seen. Although no significant benefits were seen in terms of cardiac hypertrophy, it is interesting to note that reported exertional chest pain was markedly reduced, from 33% at baseline to 0% at 24 months.

Difficulties inherent in clinical studies of human HCM

The absolute numbers of patients investigated in these clinical trials are small. It may be that they simply suffer from inadequate power with which to detect a difference. Also, some of the difficulties lie in the genetic and phenotypic diversity of causal mutations and pathways involved in the pathogenesis of HCM in humans. This contrasts with the animal models where the subjects have a homogenous background, caused by a pre-specified genetic lesion.

Measurement of left ventricular hypertrophy, especially by echocardiography, is not an exact science, and the tolerances allowed, combined with scan reporting inter-operator variances could have hidden a difference. With cardiac magnetic resonance imaging this should become less of an issue (265), but technical scan reporting differences will still remain, even if all scans are done in one lab by one operator.

Rho and Rac signalling pathway activation, inhibited by statins, whilst important in some genetic forms, may not be activated in all forms of HCM caused by some genetic variants, and thus beneficial statin effects may not be present in all forms of the disease. Also unknown is whether the pleiotropic beneficial effects of statins are truly a group-effect, or merely limited to a few agents.

Even in the setting of prospective randomised clinical trials, the low overall event rate in the HCM population means that, to demonstrate a positive treatment effect, multi-centre studies would be needed, and follow-up needs to encompass many patients for many years, in order to accrue enough objective outcomes and clinical events. A recent working group highlighted the urgent need to identify putative targets for intervention in HCM (250)

Statins role may be more than anti-hypertrophy in HCM

Are the beneficial effects of statins limited to an anti-hypertrophy role, or could they be due to inherent cardioprotective effects, protecting the sub-optimally perfused myocardium from the effects of ischaemia-reperfusion injury? If the anti-anginal effect seen in Nagueh's study is real, this would support this possibility. Modification and mitigation of ischaemia-induced injury in this setting, and protection from ongoing, chronic injury, with subsequent replacement of damaged areas by scar, termed replacement fibrosis, could be a potentially useful effect of statin therapy, and may modify

its clinical course (225). However, the ability of statins to cardioprotect in HCM in this manner has not been investigated.

Summary and basis for experimental studies

As outlined above, coronary artery disease and HCM frequently coexist and protection of this highly vulnerable myocardium from ischaemia-reperfusion injury during periods of cardiopulmonary bypass and handling during surgery (e.g. during myectomy for HCM, valve surgery or concomitant bypass grafting) or during percutaneous coronary intervention has not been addressed or studied to date. Potential therapeutic avenues leading to the ability to pharmacologically cardioprotect in the setting of HCM will be examined in the experimental studies described below.

Introductory Summary

Preconditioning and postconditioning are cardioprotective techniques which reduce myocardial damage due to ischaemia-reperfusion injury. Both share activation of the RISK pathway as an effector mechanism (82), and their effects are focussed in the moments surrounding reperfusion (266;267). Activation of the RISK pathway mediates inhibition of the mPTP (65) and promotes myocyte survival. In terms of using these modalities in the clinical setting of ACS, the requirement to apply a stimulus upstream makes preconditioning clinically irrelevant. Postconditioning, despite positive

clinical data in its favour (81), has potential technical drawbacks which make it unattractive for the PCI operator.

Atorvastatin, a potent statin in widespread clinical use (115;116), has been shown to activate the RISK pathway and to reduce infarct size in experimental settings (72;103). The aim of any search for a cardioprotective strategy for use during ACS should focus on myocardial salvage, as final infarct size remains the most important determinant of future prognosis, (10-12), and infarct reduction is key to improving survival, preserving left ventricular function and avoiding heart failure.

The occurrence of myocardial damage during CABG surgery and PCI is well established and associated with deterioration in clinical outcomes (122;268). Atorvastatin has been shown to be clinically effective in reducing adverse events rates in both settings (102;163). However, the mechanism by which this is achieved remains to be delineated. Additionally, it is known that the anti-ischaemia-reperfusion injury effects of statins wane with time (180). The ability to recapture cardioprotection by an additional top-up dose of statin, a proven concept in animal models, has not been confirmed in humans.

In the sphere of HCM, the presence of chronic ongoing myocardial ischaemia is now well-established (225). Myocyte death leads to fibrosis, adverse ventricular remodelling and worsens clinical outcomes. The ability

of cardioprotective techniques (such as mPTP inhibition) to operate in HCM-affected human cells has not been investigated. Any protection possible could be exploited either to modify the disease process itself, or at least offer protection in the setting of ACS, CABG or other surgical intervention in these high-risk patients.

This thesis contributes by exploring the role of atorvastatin in acute cardioprotection in human myocardium, assessing whether it is possible to recapture statin-cardioprotection in human tissue, examining the mechanisms of statin-cardioprotection in humans and delineating the role of the mPTP in the setting of HCM.

Chapter Two: OBJECTIVES AND HYPOTHESES

Overall objective

The main objectives of these experiments were to elucidate whether it is possible to pharmacologically protect human myocardium from ischaemia-reperfusion injury and oxidative stress using atorvastatin, in two distinct pathological settings. Three separate hypotheses were formulated as a basis for these experimental studies :

Hypothesis one

Atorvastatin protects the human myocardium when administered at the onset of reperfusion by the activation of prosurvival signalling pathways including the RISK enzymes PI3K, Erk-1/2, and inducible nitric oxide synthase.

This hypothesis was examined by:

1. Investigating whether atorvastatin, given at the onset of reperfusion, protects the human atrial myocardium from simulated ischaemia-reperfusion injury.
2. Investigating whether inhibition of PI3K and Erk-1/2 pathways could abolish any cardioprotective effect elicited by atorvastatin

3. Investigating whether inhibition of nitric oxide synthase isoforms could abolish any protective effect elicited by atorvastatin.

Hypothesis two

High treatment with atorvastatin, administered orally, recaptures additional cardioprotection when compared to background chronic statin therapy, acting by the activation of prosurvival signalling pathways including the RISK enzymes PI3K and Erk-1/2, and inducible nitric oxide synthase.

This hypothesis was examined by:

1. Investigating whether high-dose atorvastatin pretreatment in patients undergoing cardiac surgery protects the human atrial myocardium from simulated ischaemia-reperfusion injury compared with standard dose background chronic statin therapy.
2. Investigating whether inhibition of PI3K and Erk-1/2 pathways at reperfusion could abolish any cardioprotective effects elicited by high-dose atorvastatin pretreatment.
3. Investigating whether inhibition of nitric oxide synthase isoforms could abolish any cardioprotective effect elicited by high-dose atorvastatin pretreatment.

Hypothesis three

Human ventricular myocardium in the setting of obstructive hypertrophic cardiomyopathy can be pharmacologically cardioprotected.

This hypothesis was examined by:

1. Investigating whether treatment with cyclosporin-A can delay mitochondrial permeability transition pore opening following oxidative stress in isolated human ventricular cardiomyocytes.
2. Investigating whether treatment with atorvastatin can delay mitochondrial permeability transition pore opening following oxidative stress in isolated human ventricular cardiomyocytes.

Chapter Three: GENERAL METHODS

Introduction

This chapter describes the human atrial superfusion model of simulated myocardial ischaemia-reperfusion injury. Also described is the human isolated ventricular myocyte model of oxidative stress, including assay of the mitochondrial permeability transition pore

The human atrial superfusion model of ischaemia-reperfusion injury

Study subjects

Patients undergoing elective coronary artery bypass grafting (CABG) and/or cardiac valve repair at the Heart Hospital, University College London Hospitals NHS Foundation Trust, London, were screened for eligibility for these studies.

Prior ethical approval for the study was obtained from the University College London/University College London Hospital Committee on the Ethics of Human Research, sub-committee alpha. The ethical approval reference was REC 00/00275. Fully informed consent was obtained at the time of admission to hospital, on the afternoon prior to surgery. Patients were not approached for consent discussions or possible recruitment if they had

received sedatives or opiate medication, to ensure consent was fully informed.

Exclusion criteria

The following patients were excluded:

1. Patients over the age of 79 years.

2. Patients presenting with unstable angina – cardiac chest pain at rest or dynamic ST-segment ECG changes.

3. Patients presenting with non ST-segment elevation myocardial infarction, i.e. an acute coronary syndrome presentation accompanied by a detectable troponin release.

4. Patients with arrhythmias including any form of atrial fibrillation, atrial flutter or any ventricular arrhythmia.

5. Patients taking primary antiarrhythmic drugs such as digoxin or amiodarone.

6. Patients with impaired left ventricular function (EF<50% on recent transthoracic echocardiogram) or assessed as impaired on perioperative transoesophageal echocardiogram), or presenting with cardiogenic pulmonary oedema.

7. Patients with renal impairment (estimated glomerular filtration rate <60ml/min).

Patient profiles

A total of 68 patients were recruited into these studies. Their basic profile is outlined in the table below:

Total patients in study	68
Male	54
Female	14
Age range	33-79
Mean age	63
CABG alone	51
Aortic valve replacement alone	9
Aortic valve replacement plus CABG	6
Mitral valve repair/replacement	2

Table 3.1 – profile of patients recruited into atrial appendage studies

Explantation and transport of right atrial appendages

During right-heart cannulation for the establishment of cardiopulmonary bypass, right atrial appendages were surgically excised. This was performed by the application of a haemostatic purse-string suture around the base of the atrial appendage, after which either scissors or a scalpel blade was used to remove the tissue. Using forceps it was gently placed into a

plastic tube containing 50ml modified Tyrode's buffer at 4°C. This was sealed, labeled and transported on ice to the laboratory. The buffer had been preoxygenated with 95% O_2/5% CO_2, achieving a PO_2 between 50 and 60 kPa and a PCO_2 of between 4 and 6 kPa. The pH of the transport buffer was checked to ensure it was between 7.35 and 7.45. The temperature remained below 4°C during transport. Transport time was <10 minutes in all cases.

Dissection, explantation and stabilisation of human atrial trabeculae

In the laboratory, the entire atrial appendage was secured in a dissection dish using pins, whilst still immersed in cold buffer. The appendage was gently opened to expose the endocardial surface, and individual atrial trabeculae were gently dissected and excised. They were then explanted into individual 25 ml organ baths equipped with pacemaker electrodes, on a custom-built superfusion rig (Radnoti Glass Technologies, USA). 5/0 silk sutures were used to secure one end of the trabeculae to a fixed glass hook, and the other to a force transducer. Five separate superfusion baths were available per experiment. Once mounted, the trabeculae were superfused with modified Tyrode's buffer comprising (in mmol/L) 118.5 NaCl, 4.8 KCl, 24.8 NaHCO3, 1.2 KH_2PO_4, 1.44 $MgSO_4$.7H20, 1.8 $CaCl_2.2H_2O$, 10.0 glucose and 10.0 sodium pyruvate. The solution was oxygenated with a 95%O_2/5%CO_2 mixture. pH was maintained between 7.35 and 7.45 with a

partial pressure of O_2 between 50 and 65 kPa and a partial pressure of CO_2 between 4.0 and 6.0 kPa. Buffer which had passed through the superfusion baths was recirculated using a peristaltic pump (5056, Watson-Marlow, UK). A constant temperature of 37° was maintained using a circulating water jacket and heat exchanger (Thermo Electron Corporation, USA), and this was regularly checked using a calibrated electronic thermometer throughout the experiment. Buffer pH, ppO_2, $ppCO_2$ and glucose levels were measured at predetermined points during the experiment by sampling fluid direct from the superfusion baths, and using an automated blood-gas analyser (ABL 700 series, Radiometer Limited, UK).

The atrial trabeculae were paced at 1 Hz using a dual pulse stimulator via the platinum electrodes in the superfusion baths (Harvard 6012 stimulator, Harvard Apparatus, UK). The stimulators were all set to deliver a fixed, continuous signal with a width of 2ms and an output of 30V. Once set up in this fashion, the trabeculae were allowed to stabilise for 75 minutes. Trabecular tension and force of contraction was amplified, monitored and recorded with a Powerlab/8sp instrument (AD Instruments, Australia). Peak contractile function at the end of the stabilisation phase was recorded and provided a baseline for comparison later.

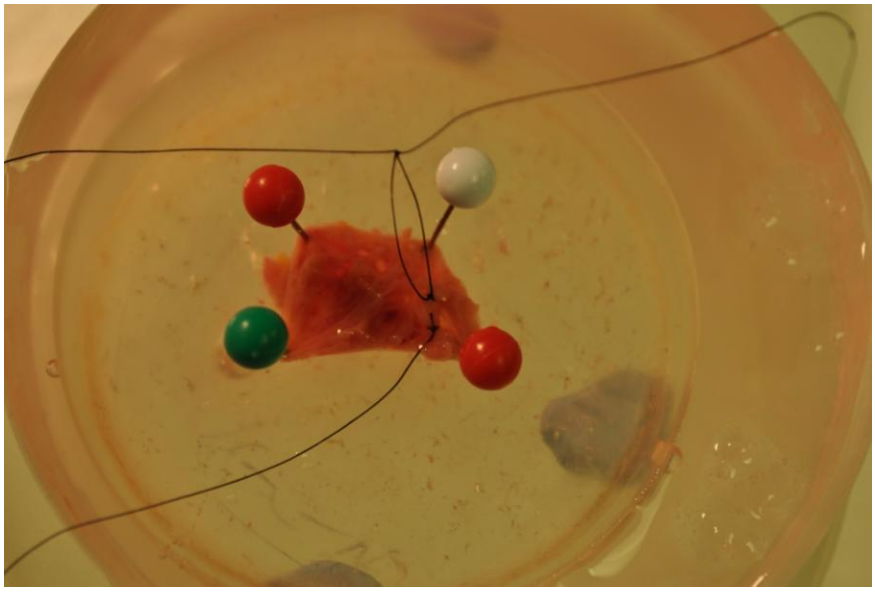

Fig 3.1 – human right atrial appendage during dissection, with trabecular sutures positioned immediately prior to explantation.

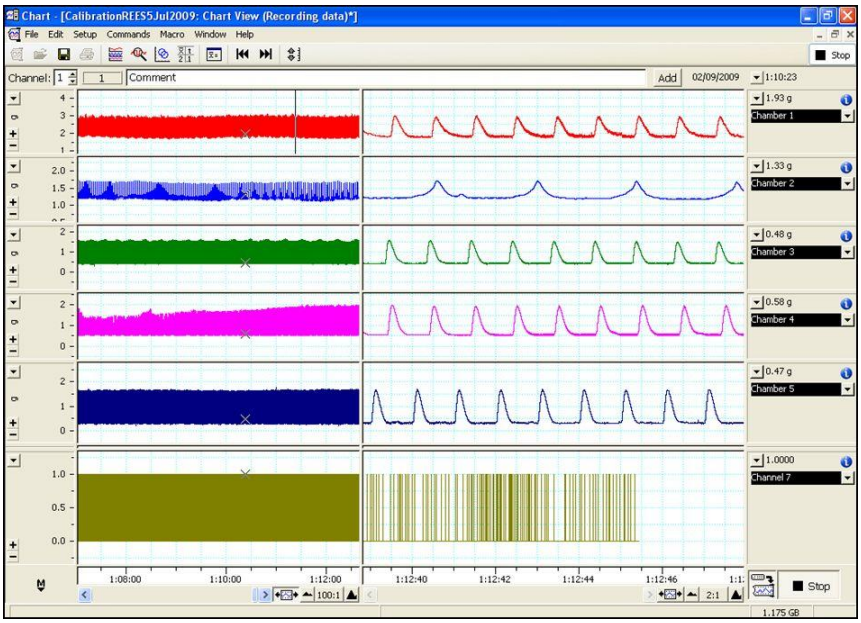

Fig 3.2 - computer screen showing transduced contractile activity from 5 human atrial trabeculae receiving 1Hz pacing stimulus during stabilisation phase.

Exclusion criteria

Trabeculae were excluded as follows:

1. If, on measurement, they were >1.2mm in diameter or <2mm in length.

2. If they were mechanically damaged during dissection or mounting (as assessed visually).

3. If time to explant took >20 minutes.

4. If the contractile force failed to reach 0.5g after 75 minutes of stabilisation, or;

5. If any evidence of electro-mechanical instability during stabilisation

Simulated ischaemia-reperfusion injury

Following stabilisation, atrial trabeculae were then subjected to a period of hypoxia which comprised 90 minutes of superfusion with glucose-free hypoxic modified Tyrode's buffer composed of (in mmol/L) 118.5 NaCl, 4.8 KCl, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, 1.44 $MgSO_4$.7H20, 1.8 $CaCl_2.2H_2O$, 7.0 choline chloride. Pacing was increased to 3Hz. The buffer had been previously bubbled with 95%N_2-5%CO_2 to achieve a partial pressure of O_2 to < 8kPa with a physiological pH. After 120 minutes, simulated reperfusion followed. To achieve this, trabeculae were superfused with the oxygenated, glucose-containing buffer used during the stabilisation phase, and pacing frequency was returned to 1 Hz. At the end of this phase, residual contractile function was measured and recorded as a percentage of baseline function. After the conclusion of the experimental protocol the trabeculae

were removed from the apparatus and the sutures were carefully removed. The length, width and weight of the trabeculae were measured and recorded.

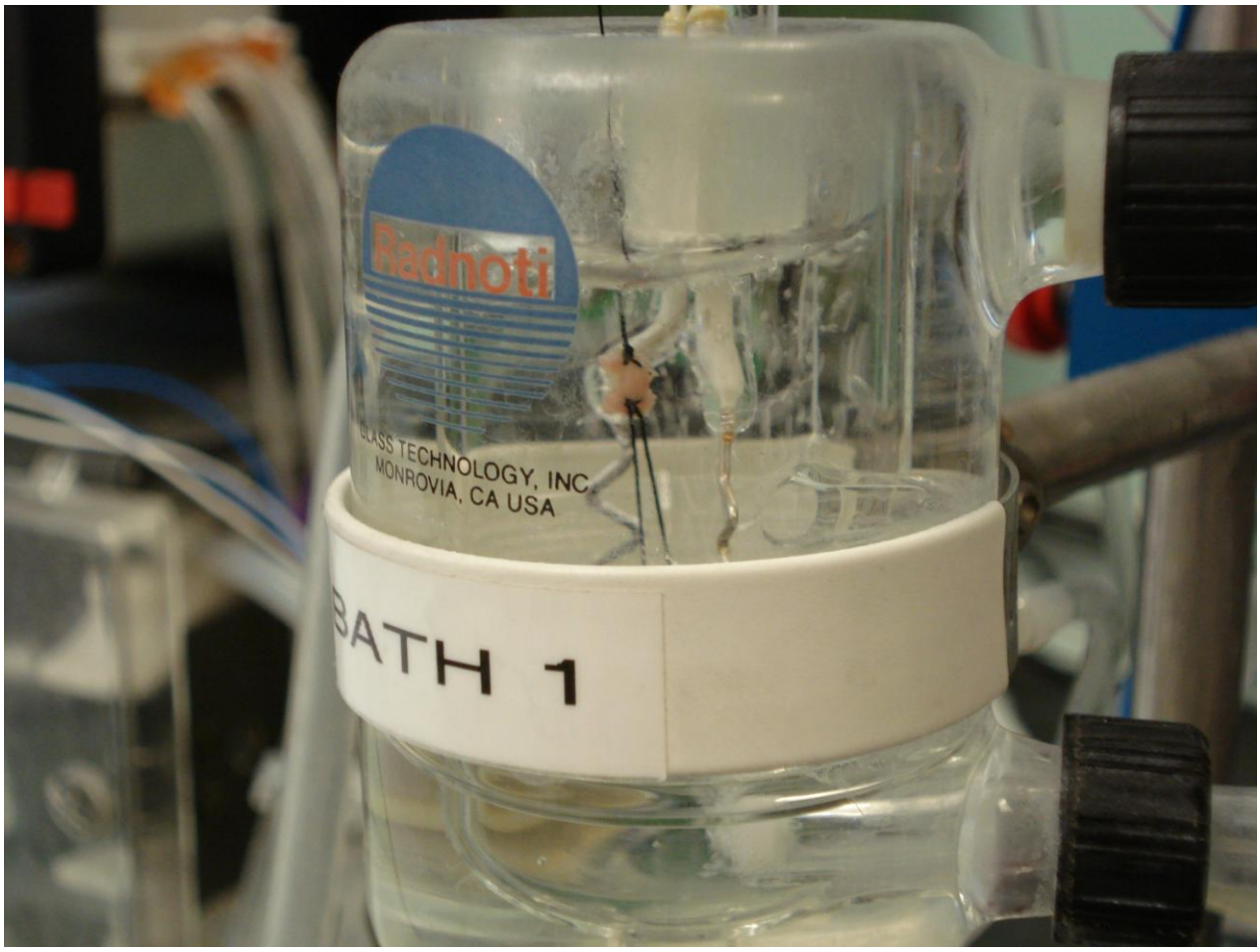

Fig 3.4 – human atrial trabecula in organ bath

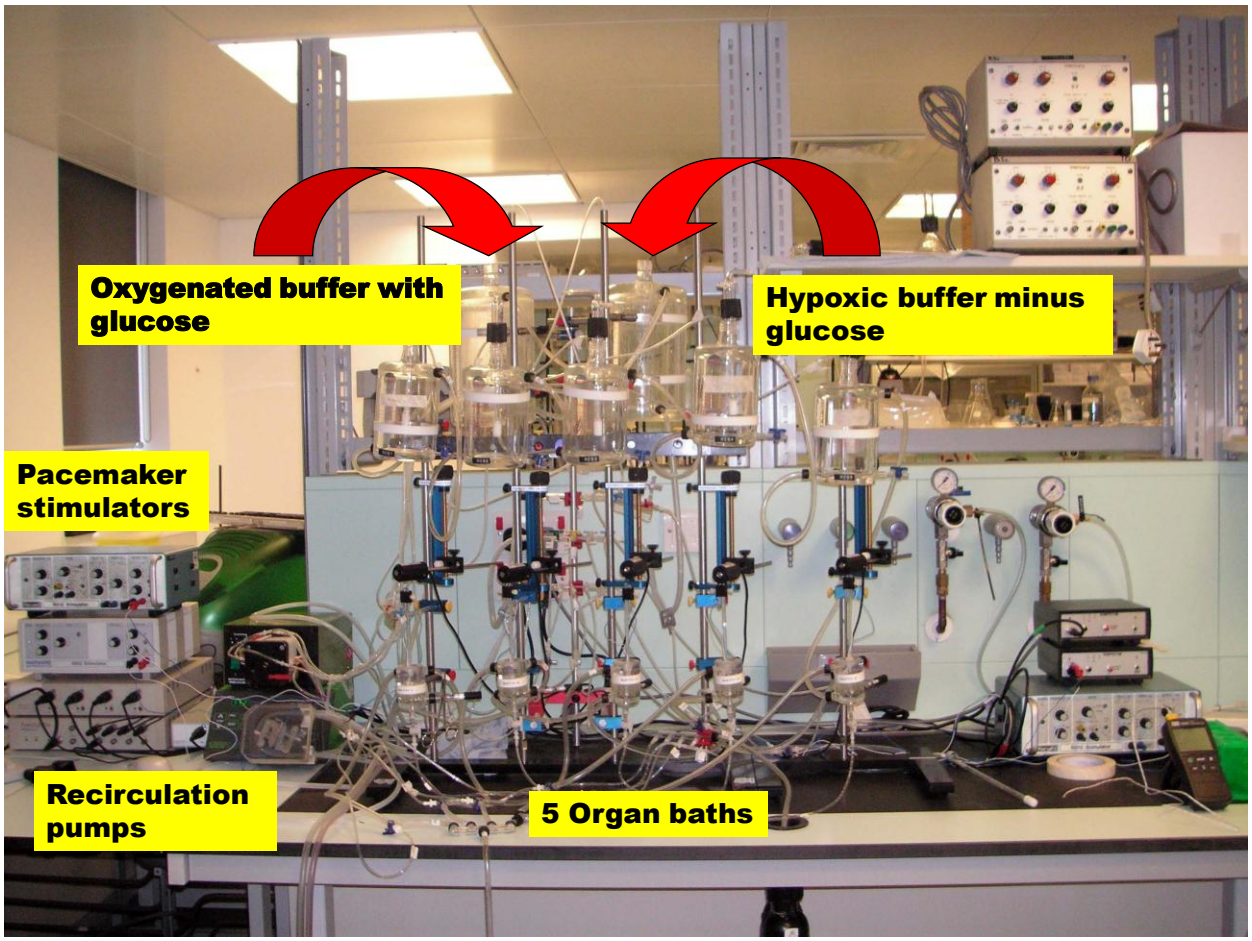

Fig 3.5 – human atrial superfusion equipment showing arrangement of reservoirs, organ baths and pacemaker stimulators

Application of experimental protocols and infusion of pharmacological agents

Using the apparatus outlined above, it was possible to apply experimental protocols incorporating ischaemic preconditioning or pharmacological cardioprotection. To simulate IPC hypoxic buffer was selectively diverted to

the appropriate tissue bath alone for a 3 minute interval, followed by a 7 minute restoration of stabilisation buffer immediately prior to the main 90 minute hypoxic insult.

To allow selective exposure to a range of pharmacological agents, individual 1 litre reservoirs were housed above each tissue bath. These could be operated as wholly isolated circuits via an array of separate tubing, a three-way tap and a recirculation pump. The effluents from the baths were carefully managed to ensure no cross-contamination between isolated circuits. Circuits were thoroughly decontaminated after each use, an received acid cleaning after each main experimental phase.

Trabeculae were randomly assigned to experimental protocols at the end of the stabilisation phase, using a computer-generated numbering system.

Recovery of baseline function as an indicator of cardioprotective efficacy

The force of trabecular contraction was carefully measured at the end of the experimental protocol, and was recorded. This was divided by the force of contraction at baseline and recorded as a percentage value.

Critical analysis of the model

This human atrial superfusion protocol examines and records functional recovery as a surrogate marker of the degree of myocardial injury sustained following a sustained period of glucose-free hypoxia. It is a simulation, ex-vivo, of the biochemical milieu encountered during an ST-elevation myocardial infarction, using explanted human myocardial tissue. The model has been shown to provide reproducible data when using human tissue to assess simulated ischaemia-reperfusion injury, simulated ischaemic preconditioning, and using pharmacological agents to provide cardioprotection (64;83;269-274). Recently it has also been used to examine the effects of ischaemic postconditioning (82).

One potential criticism of the models surrounds the use of atrial, rather than ventricular tissue. The only human tissue available to researchers in this field will be of atrial origin. Ventricular tissue is rarely resected during routine cardiac surgery. Ventricular wall tissue is removed only as part of a (now rarely-performed) aneurysectomy, in which case it is mostly non-contractile scar tissue. Septal myectomy for patients with hypertrophic cardiomyopathy is performed (discussed below) but this results in fragments of tissue being excised which are not presented as functional subunits which could be compared. Additionally, the underlying genotyic and phenotypic abnormality is present throughout the tissue removed.

Oxygen and substrate delivery to the cells during the experimental phase relies on diffusion from the surrounding buffer. It is not possible to perfuse the trabeculae independently as they are too small and lack an individual arteriolar entry point. To ensure that diffusion to the inner core of the tissue was not compromised, an exclusion cut-off diameter of 1.20mm was applied. This was based on the work of previous researchers using this model, and previous work using ventricular papillary muscles, showing a reproducible functional recovery using similar diameter tissue samples (64;82;83;269-274).

At the outset, it is acknowledged that all trabeculae were excised from patients undergoing cardiac surgery for either coronary artery disease, cardiac valve disease, or both. Thus the tissue samples, at baseline, were not truly normal. However the underlying diseased state was identical for all trabeculae isolated from an individual patient, and was similar overall for both intervention and control groups. Moreover, a control arm was maintained within each individual experiment where multiple protocols were being applied, to ensure overall function and reproducibility of the model. The ability to have an inbuilt control arm during each experiment is a particular strength of this model.

Human myocyte isolation

Study Subjects

Patients undergoing elective ventricular myectomy for obstructive hypertrophic cardiomyopathy at the Heart Hospital, University College London Hospitals NHS Foundation Trust, London, were screened for eligibility for these studies.

Prior ethical approval for the study was obtained from the University College London/University College London Hospital Committee on the Ethics of Human Research, sub-committee alpha. Fully informed consent was obtained at the pre-admission visit some weeks prior to planned surgery. Patients were not approached for consent discussions or possible recruitment if they had received sedatives or opiate medication, to ensure consent was fully informed.

Exclusion Criteria

The following patients were excluded:

1. Patients over the age of 79 years.
2. Patient with concurrent coronary artery disease or any history of myocardial infarction

3. Patients who had undergone previous alcohol septal ablation therapy for HCM

4. Patients with renal impairment (eGFR <60ml/min)

Patient profiles

Total patients in study	8
Male	5
Female	3
Age range	28-55
Mean age	47

Table 3.2 – Profile of patients undergoing myectomy for HCM

Tissue excision, handling and transport

A transport buffer comprising 10ml Harefield hospital formulation high-strength cardioplegia solution (comprising, in mmol/L, 147 Na, 84 K, 80 Mg, 2 Ca, 5 procaine , 400 Cl) made up to 50 mls with compound sodium lactate intravenous solution (comprising, in mmol/L 131 Na, 5 K, 2 Ca , 111 Cl, 29 lactate) was prepared in advance and stored at 4˚C. Pre-operatively, there was close liaison with theatre staff in order to optimise handling of the excised tissue. Usual practice was to request that the excised tissue be placed in a pot of saline prior to being handed to the researcher. If this was impractical, moistened gauze was used to cover the tissue to prevent exposure to the air and subsequent dessication and possible cell loss.

Following induction of general anaesthesia, perioperative transoesophageal echocardiography, and after establishing cardiopulmonary bypass, the surgeon performed an aortotomy. Having retracted the aortic valve, under direct vision and using echocardiography as a guide, the obstructive ventricular septal tissue was resected. The mass of tissue removed ranged from 2-11 grammes.

Following excision, approximately 0.5 to 1.0 grammes of septal tissue were placed into the transport buffer. The sample was handled gently using DeBakey forceps. This tube was then sealed, labelled, and placed into a flask of ice prior to rapid transport to a laboratory facility.

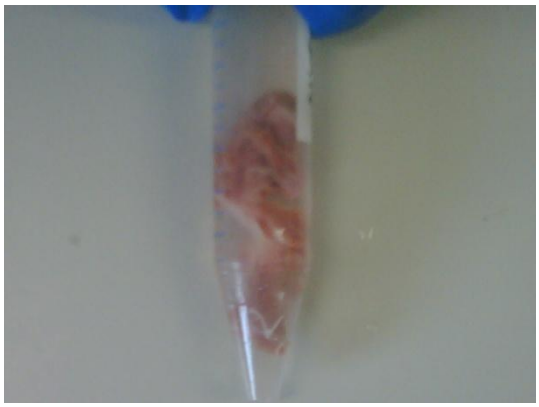

Fig 3.5 - Myectomy sample in transport buffer

Tissue dissection

The sample was placed on an inverted plastic dish and reduced to much smaller pieces (approximately $1mm^3$) by careful razor cleavage, using clean straight cuts over 5 minutes. Care was taken to avoid applying shearing

forces to the tissue where possible, which results in a reduced myocyte yield, and the tissue was kept moist by liberally applying aliquots of the transport buffer throughout. All containers used in the processing of the sample were plastic, and these were carefully rinsed. No glassware was used to avoid the possibility of introducing calcium contamination. Similarly, all reagents used for buffer composition were Aristar™ certified, i.e. low calcium content.

Once reduced in size, the tissue fragments were washed to remove debris and traces of the cardioplegia solution. 4 washes each of 3 minutes using 25ml low-calcium buffer (comprising, in mmol/L 120 NaCl, 5.4 KCl, 5 $MgSO_4$, 5 pyruvate , 20 glucose, 20 taurine, 10 HEPES, pH 6.96) were performed. During this phase the solution containing the tissue was stirred manually using a handheld device, with 100% oxygen bubbling through the solution. The container was immersed in a water bath to maintain a stable temperature, pre-heated to 35˚C. Between washes, the wash solution was poured through a 300 µm nylon gauze filter and discarded, retaining only the tissue. The wash cycle was continued until a total of 12 minutes incubation time was reached

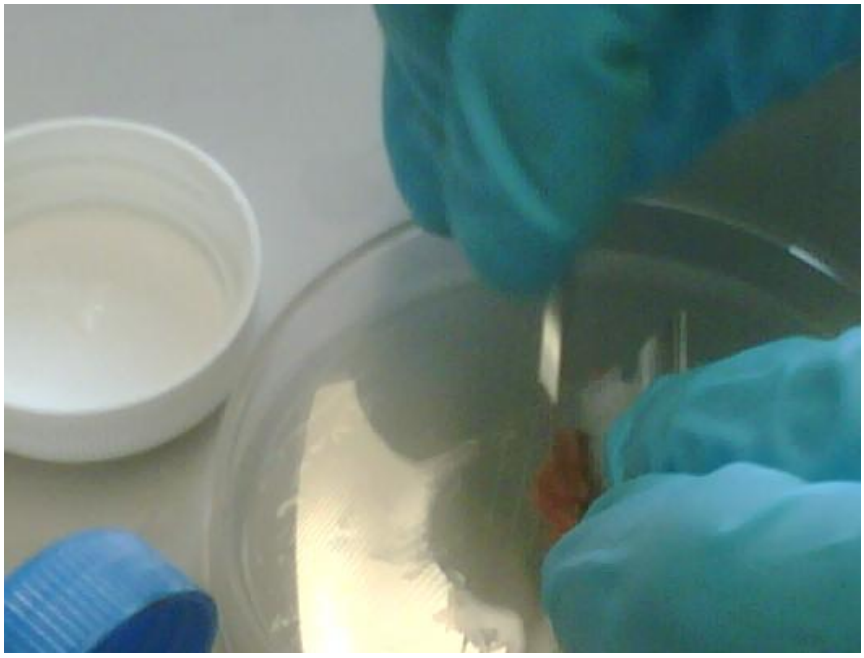

Fig 3.6 - Tissue cleavage using razor blade

Proteinase cycle

Following discarding the final 25ml of wash solution, the tissue fragments were placed in a 50ml plastic tube. 15 ml of 4U/ml bacterial type XXIV protease solution were added (8038-1G, Sigma Aldrich, UK). The tube was then immersed in the water bath, and a tubing array delivered oxygen across the surface of the solution. A flask-shaker (Stuart SF-1, Stuart Scientific) was used to hold the tube and agitate the solution for the next 45 minutes.

Collagenase cycle

The tissue suspension was poured through a fresh nylon gauze filter and funnel assembly, and the tissue fragments carefully placed into another 50 ml plastic tube. 15 ml of 400IU/ml collagenase solution were added (C9263-5G, Sigma Aldrich, UK) and the tube returned to water bath. Again, oxygen

flow across the surface of the solution was maintained, and the tube was agitated for a further 45 minute cycle.

Fig 3.7 - Oxygenated tissue fragments undergoing digestion & oxygenation in water bath

Centrifugation

Following the digestion cycles, the resulting suspension was filtered through a fresh 300µm nylon gauze filter, and the resulting filtrate collected in a plastic test tube. This was then centrifuged gently at 400 revolutions per minute for 1 minute. This resulted in the formation of a pellet of myocytes at the bottom of the tube. The supernatant was gently removed using a plastic pipette, and a further 5-10 ml of enzyme carrier solution (minus enzyme) was added to resuspend the myocytes. The centrifugation cycle was repeated, and the resulting supernatant discarded. This cycle served to further clean the filtrate. Finally, the pellet was resuspended and 2 ml of the enzyme solution. A drop of this suspension was examined using light microscopy to confirm the presence of myocytes. The myocyte suspension was transferred

into a fresh plastic tube, and topped up to avoid bubble formation, to avoid causing mechanical damage to cells during transit. The tube was also transported lying horizontally to prevent the myocytes from clumping, and potentially forming a hypoxic nidus in the middle of the clump.

Myocyte preparation

Krebs-Henseleit solution had been previously prepared (comprising, in mmol/L NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.22, KH_2PO_4 1.21, Ca^{2+} 1.84, glucose 11) to which HEPES 1M was added at a ratio of 1% per unit volume of buffer.

The tube containing the myocyte suspension was kept at room temperature and placed on a gyratory rocking platform (SSM3, Stuart) to gently prevent cell clumping without causing mechanical injury. 250 µL of the solution was now removed by micropipette, using a gentle action to further reduce damage, and carefully placed onto a 25mm diameter round coverslip. 250 µL of the Krebs/HEPES buffer was then gently added over 30 seconds. This served to gradually raise the ambient calcium concentration in the resulting solution. The slow addition was aimed at avoid sudden calcium flux which results in cell swelling and death.

This process has been extensively used for human myocyte isolation, and represents a robust and reproducible process for extracting viable human cells (64;275).

Fluorescent cationic dye loading

5 µL of tetramethyl rhodamine methyl ester (TMRM) 3 µM was added to the solution, and incubated at room temperature for 15 minutes. TMRM is a monovalent cationic fluorescent dye, which selectively accumulates within the mitochondrial membrane. The loaded coverslip wells were shielded from light during the incubation phase.

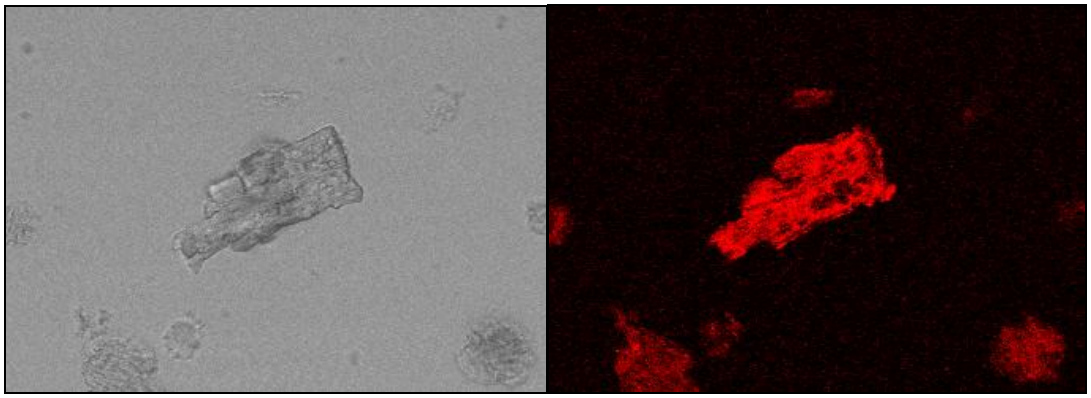

Fig 3.8 - Left-hand panel - isolated human cardiomyocte under light microscopy. Right-hand panel – the same cell viewed with confocal microscopy following loading with the cationic dye TMRM.

Drug treatment

In the groups randomised to receive drugs, the agents were added via micropipette 5 minutes after addition of TMRM, and allowed to incubate for a further 10 minutes. The total incubation time with TMRM present did not exceed 15 minutes. Control groups of cells received DMSO alone at this

point. In the group randomised to receive atorvastatin, 2.5 µL of 5 mM stock was added by micropipette to give a final concentration of 25 µM. In the ciclosporin-A group, 0.5 µL of 0.2 mM stock was added, resulting in a final concentration of 0.2 µM. These drug concentrations have previously been shown to be cardioprotective in animal models, ex-vivo isolated myocardial models, and isolated cellular models.

Confocal microscopy

The chamber containing TMRM-loaded myocytes and drug/carrier was mounted on the viewing stage of a confocal microscope (TCS SP5, Leica Microsystems GmbH, Germany), and cells were examined using a ×20 quartz objective lens. A 543-nm emission line from a HeNe laser at 20% power was used to illuminate the cells, using a sector sweep speed of 5.2 seconds. Comparability between different experiments was ensured by maintaining identical conditions with respect to laser power, confocal pinhole, and optical slice and detector settings. TMRM fluorescence was acquired via a 585nm filter, and the images were analysed using Leica software (Leica Application Suite, Advanced Fluorescence, Leica Microsystems GmbH, Germany).

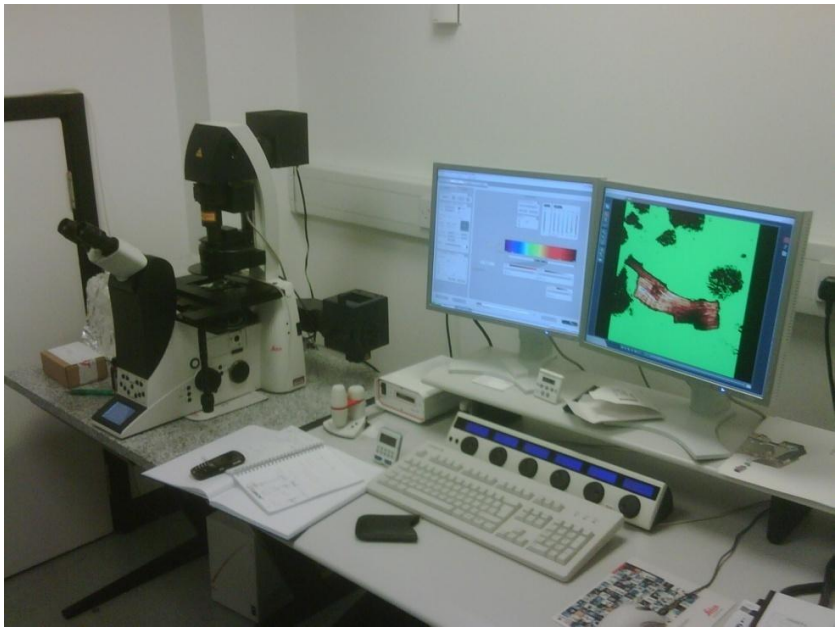

Fig 3.9 - Confocal microscope system

Exclusion criteria

Myocytes were selected that were as morphologically normal as possible.

Specifically, cells were excluded in the following circumstances:

1. Cells with >30% fibrillar disarray or where architecture unclear
2. Cells with multiple promontories
3. Cells which show evidence of partial depolarisation at start of imaging
4. Cells <60nm in length or <30nm wide
5. Cell batches >2 hours from completion of isolation

Laser Oxidative Stress

Once a suitable cell was identified, laser photosensitization was commenced. The high concentration of TMRM within the mitochondria effectively focuses the oxidative stress within the mitochondria. This eventually causes global loss of mitochondrial membrane potential, signifying opening of the mitochondrial permeability transition pore. As this occurs, TMRM is released into the cytosol, and an increase in signal can be detected. The opening of the mPTP has been previously demonstrated to be a critical determinant of cell death in a variety of animal models of ischaemia-reperfusion injury (27;51;52;56;276-280), and in isolated human atrial myocytes (64).

End Points

The time taken to the onset of mitochondrial depolarisation was recorded. The two preselected experiment end-points were either the establishment of a clear forward wave of depolarisation, or regional, progressive loss of subcellular structure.

It was possible to perform experiments on up to three groups of cells per isolation. After three groups (and especially after two hours post-isolation), live cell yield started to decline significantly, and further experiments were not pursued after this time.

The mean depolarisation time for each group was then normalised against the control group.

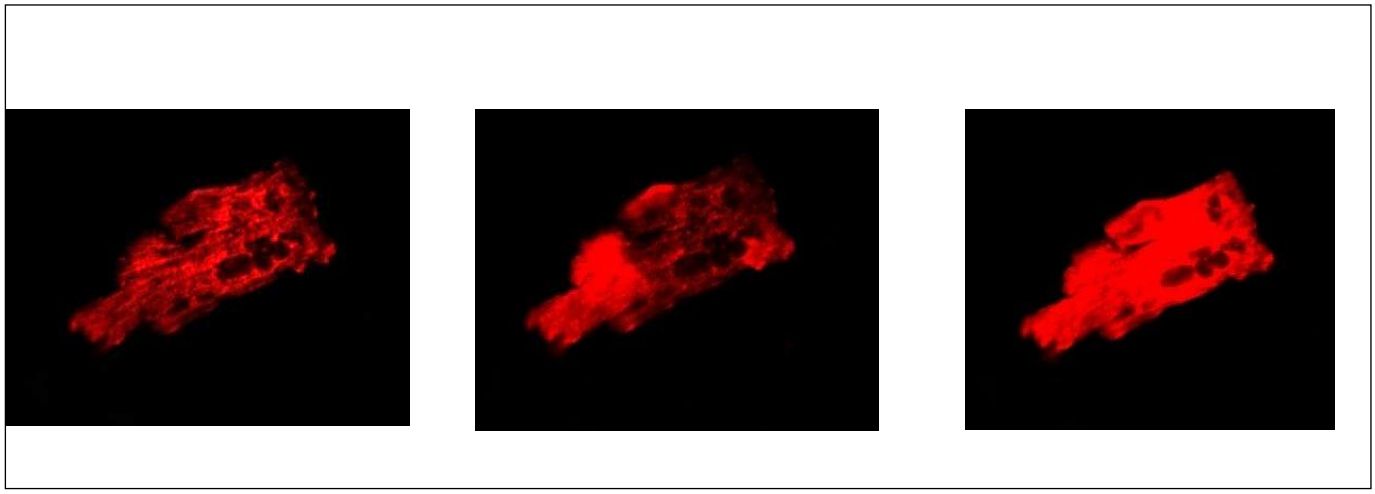

Fig 3.10 - Human ventricular myocyte under confocal microscopy, showing progressive wave of depolarisation signifying mPTP opening.

Critical Analysis of the Model

The laser oxidative stress model described uses a standardised laser insult to induce loss of mitochondrial membrane potential in human ventricular myocytes. The end point used is a precursor to cell death, rather than cell death itself. However, this model has produced robust and highly reproducible data in published studies using both animal and human atrial myocardial cells.

A particular strength of the model is that it allows analysis of freshly excised human ventricular cardiomyocytes. Studies of cellular mechanisms of

cardioprotection in human tissue are frequently limited to investigation of atrial tissue, which is more readily available.

Clearly, these investigations are limited to single-cell experiments, and do not necessarily reflect the behaviour of a whole, perfused piece of tissue or organ. Additionally, our studies were necessarily confined to the investigation of patients with hypertrophic cardiomyopathy. Ventricular tissue excision is effectively limited to surgery for this condition, in which tissue debulking is the surgical therapeutic aim. However this allows for specific investigation of cardioprotective mechanisms in the setting of this condition.

The cell isolation process is time consuming, and during this period cells are lost. Additionally, during the transport phase between isolation and confocal laser laboratories, further cell loss was noted. A finite time period of two hours from the end of isolation phase was imposed, limiting the numbers of experiments that could be performed

Chapter Four: ATORVASTATIN ADMINSITERED AT REPERFUSION PROTECTS HUMAN ATRIAL MYOCARDIUM AGAINST SIMULATED ISCHAEMIA-REPERFUSION INJURY THROUGH ACTIVATION OF THE RISK PATHWAY AND NITRIC OXIDE SYNTHASE

Introduction

Emergency reperfusion of an occluded coronary artery by means of percutaneous coronary intervention (PCI) limits infarct size, and reduces mortality and recurrent cardiac event rates (21;22) . Where PCI is unavailable, thrombolytic therapy remains an option for achieving reperfusion, albeit with a lower success rate (23;281;282).

Even after rapid reperfusion, further cardiomyocyte loss in the infarct territory occurs, believed to be caused by a combination of both necrosis and apoptosis (36;37;283). This represents lethal reperfusion injury, and may contribute to the burden of morbidity or mortality seen even after successful reperfusion therapy.

HMG Co-A reductase inhibitors (statins) have a proven and established role in the primary and secondary prevention of cardiovascular disease in humans. They are effective in reducing serum levels of low density

lipoprotein cholesterol, and have been shown to reduce rates of myocardial infarction and cardiovascular death in both these settings, and lead to regression of coronary atherosclerotic lesions. (87;89-91)

Experimental animal models have demonstrated that acute therapy with statins reduces infarct size independently of its effect on serum lipids (72). This effect is present whether initiation of statin therapy precedes infarction by a few days (104), is given at the point of reperfusion (72) following a severe ischaemic insult, or is administered enterally within 3 hours of myocardial infarction. The cardioprotective effect has been demonstrated most recently with simvastatin, atorvastatin and rosuvastatin (109). Additionally, in an animal model of ischaemia-reperfusion injury, upstream treatment with atorvastatin (as a preconditioning mimetic) has been shown to be cardioprotective and lead to a reduction in final infarct size (284) .

Acute statin therapy modulates ischaemia-reperfusion injury in both cellular and animal models, by pharmacological activation of the pro-survival PI3K-Akt and Erk-1/2 signalling cascades during the reperfusion phase (72;285). This cascade has been named the Reperfusion Injury Salvage Kinase (RISK) pathway (286;287). Pharmacological inhibition of PI3-K, abrogates this cardioprotective effect (72).

The proven beneficial cardiovascular effects of statins in humans have recently been extended to include administration to patients suffering from acute coronary syndromes undergoing urgent PCI (190), those undergoing cardiac surgery (170;288), and prior to major vascular non-cardiac surgery (289). Whether these benefits are derived primarily from a reduction in myocardial ischaemia-reperfusion injury or are related to a range of other pleiotropic effects is unclear. These actions include the stabilisation of unstable coronary atherosclerotic plaques, improved endothelial function, and reduced adhesion molecule expression. (96-98). There is currently no evidence regarding the ability of stain therapy to ameliorate the effects of IRI in human myocardial tissue, and less is known about the signaling pathways involved in human patients.

Hypothesis

Atorvastatin is cardioprotective in human myocardium, acting by the activation of prosurvival signalling pathways including the RISK enzymes and nitric oxide synthase isoforms.

Objective

The objective was to determine the role of atorvastatin with respect to potential acute cardioprotection of human myocardium following ischaemia-reperfusion injury. In experimental settings, cardioprotection derived from statins following ischaemia-reperfusion injury has been shown to be linked to

the activation of enzymes in the prosurvival RISK pathway (72;285;290), but the applicability of this mechanism in human myocardium has not been established. Additionally, statin-induced cardioprotection seen in animal models is also closely linked with up-regulation of nitric oxide synthase isoforms (103;107;291;292). It was intended to additionally examine the role of nitric oxide synthase in statin-induced cardioprotection in humans.

Materials & methods

Atorvastatin was supplied by Pfizer Incorporated, USA. It was dissolved in dimethyl sulphoxide (DMSO) and added to the Tyrode's reperfusion buffer at a concentration of 25 µM. The PI3-kinase inhibitor LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride, Sigma-Aldrich, Poole, UK) was dissolved in DMSO and added to the buffer at a concentration of 15µM. The p42/44 MAP kinase inhibitor, UO126 (Sigma-Aldrich), was dissolved in DMSO and added to buffer to give a concentration of 10 µM. The nitric oxide synthase inhibitor, L-NAME (Nitro-L-arginine methyl ester hydrochloride, Sigma-Aldrich)) was added directly to the reperfusion buffer at a concentration of 100µM. 1400W (N-([3-(Aminomethyl)phenyl]methyl)ethanimidamide dihydrochloride, Sigma-Aldrich), a specific inhibitor of inducible nitric oxide synthase, was dissolved directly into Tyrode's buffer giving a final concentration of 5 µM. The final concentration of DMSO used, where applicable, was <0.01%. The experimental methods have been outlined in chapter 3.

Pilot studies

To determine a suitable concentration of atorvastatin to use in this experimental setting, a series of experiments was performed to resolve technical issues. Since atorvastatin is highly insoluble and will not dissolve directly into aqueous buffer at the temperatures used in this experimental protocol, a suitable carrier solvent was required to allow the drug to be added to the superfusate buffer (see below).

A total of 10 trabeculae, isolated from 4 patients, were exposed to atorvastatin dissolved in varying concentrations, initially using methanol as solvent. Recovery of function in this group was below that of contemporaneous controls, suggesting either no effect from the atorvastatin, or possibly even a directly toxic effect attributable to either drug or solvent (see Fig 4.1 below). In an attempt to rectify this, the carrier agent was then changed from methanol to DMSO. Atorvastatin 25 µM dissolved in DMSO as a carrier solvent established enhanced recovery of function suggesting cardioprotection was occurring , and this concentration was used for subsequent experiments. The presence of DMSO alone in the superfusion buffer was investigated and had no protective effect on functional recovery, in keeping with previous data using this experimental model (64;82;83;269-274). Methanol was not subsequently used as a carrier solvent.

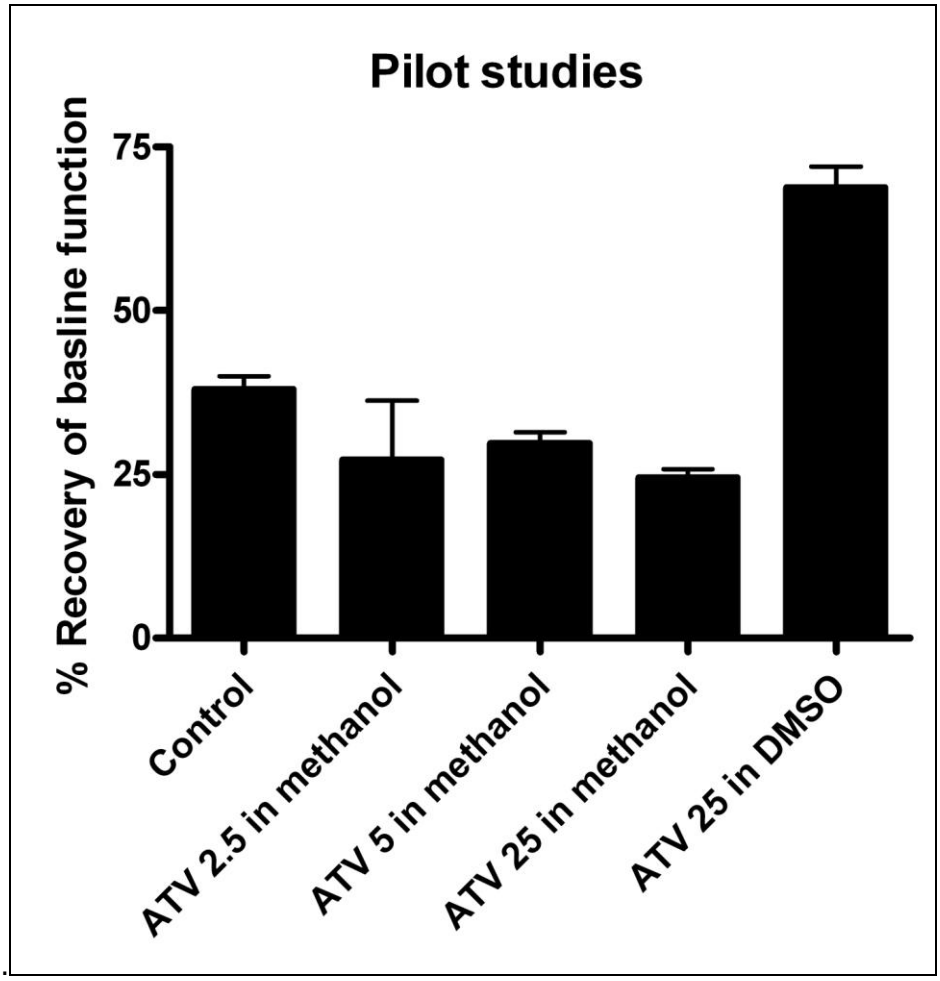

Fig 4.1 - Functional recovery of trabeculae during pilot phase using methanol then DMSO as carrier for atorvastatin

Study subjects

31 patients were consented for these experiments and had their results analysed and included. A further 9 patients consented, but the tissue samples were either too small for atrial trabeculae to be isolated, or they were damaged during the explantation process. The mean number of

trabeculae per patients was 2.3. The following table outlines the profile of surgical patients included in this study

Total patients in study	31
Male	25
Female	6
Age range	50-79
Mean age	64
CABG alone	23
Aortic valve replacement alone	4
Aortic valve replacement plus CABG	3
Mitral valve repair/replacement	1

Table 4.1 – Subjects recruited for atorvastatin studies

Experimental protocol

Explanted human atrial trabeculae were randomly assigned to one of the following treatment groups:

(1) Control group (n=17) – no additional agent during reoxygenation phase.

(2) Hypoxic preconditioning (n=4) – a standard hypoxic preconditioning (HPC) protocol was used as a positive control group. This allowed comparison with a preconditioning protocol consisting of 3 minutes superfusion with hypoxic glucose-free buffer and pacing at 3 Hz followed by 7 minutes of superfusion with oxygenated glucose-containing buffer and pacing at 1 Hz, immediately prior to the 90 minute hypoxic phase. HPC is well established in inducing resistance to HRI in this experimental model. (64;82;83;269-274)

(3) ATV (25μM) (n=9). A dose in the mid therapeutic range was selected based on preliminary investigations and preclinical animal cardioprotection studies (72). ATV was administered at the point of reoxygenation and throughout the 120 minute reoxygenation period.

(4) ATV with the P42/44 MAPK-kinase inhibitor LY294002 (15μM), both drugs administered at and throughout reoxygenation (n=4).

(5) ATV with the PI3-kinase inhibitor UO126 (10μM), both drugs administered at and throughout reoxygenation (n=4).

(6) ATV with the nitric oxide synthase inhibitor L-NAME (100μM), both drugs administered at and throughout reoxygenation (n=7).

(7) ATV with specific inducible nitric oxide synthase inhibitor 1400W (5μM)), both drugs administered at and throughout reoxygenation (n=5).

(8) ATV with DMSO vehicle control (n=4).

(9) Inhibitor-only control with LY294002 (15μM), control tissue with drug administered at and throughout reoxygenation (n=5).

(10) Inhibitor-only control with UO126 (10μM), control tissue with drug administered at and throughout reoxygenation (n=4).

(11) Inhibitor-only control with L-NAME (100μM), control tissue with drug administered at and throughout reoxygenation (n=5).

(12) Inhibitor-only control with 1400W (5μM), control tissue with drug administered at and throughout reoxygenation (n=5).

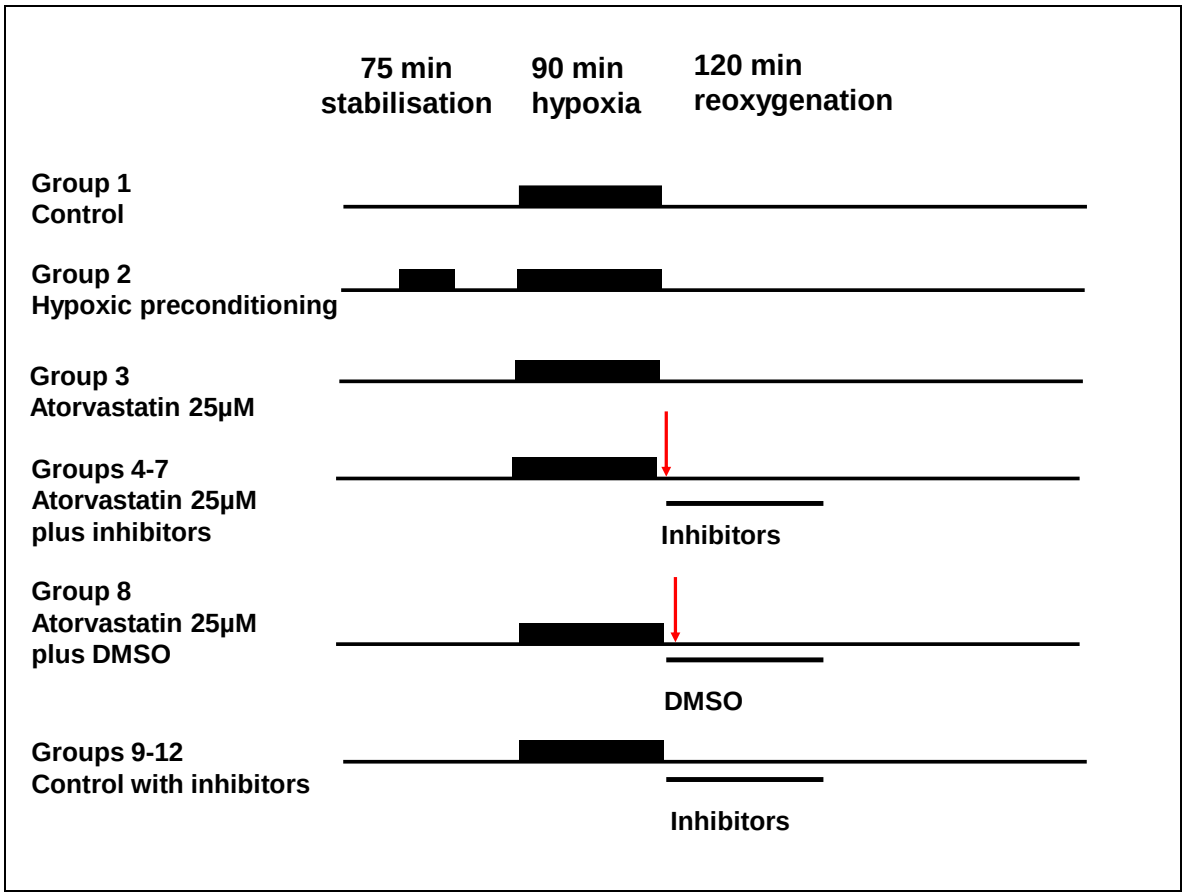

Fig 4.2 – Experimental protocol for atorvastatin studies

Results

Right atrial appendages were obtained from 31 patients with stable CAD/valve disease. A total of 71 trabeculae met inclusion criteria and were randomised into the treatment groups outlined above. Mean baseline contractility in the control group was 0.93+/-0.08g, which compares favourably with 0.92+/-0.1g in the arm randomised to receive atorvastatin at reperfusion.

Atrial trabeculae in the control group recovered 37.05±1.33 % of baseline function contractile function. This is in keeping with previous studies using the same model (64;82;83;269-274). Atorvastatin 25 μM markedly improved the recovery of contractile function to 61.0±2.3 %, an improvement which was highly statistically significant ($p<0.001$) when compared against the control group. This improvement in recovery of contractile function was of a similar magnitude to that achieved in the group of trabeculae exposed to hypoxic preconditioning (60.4 ± 3.8%).

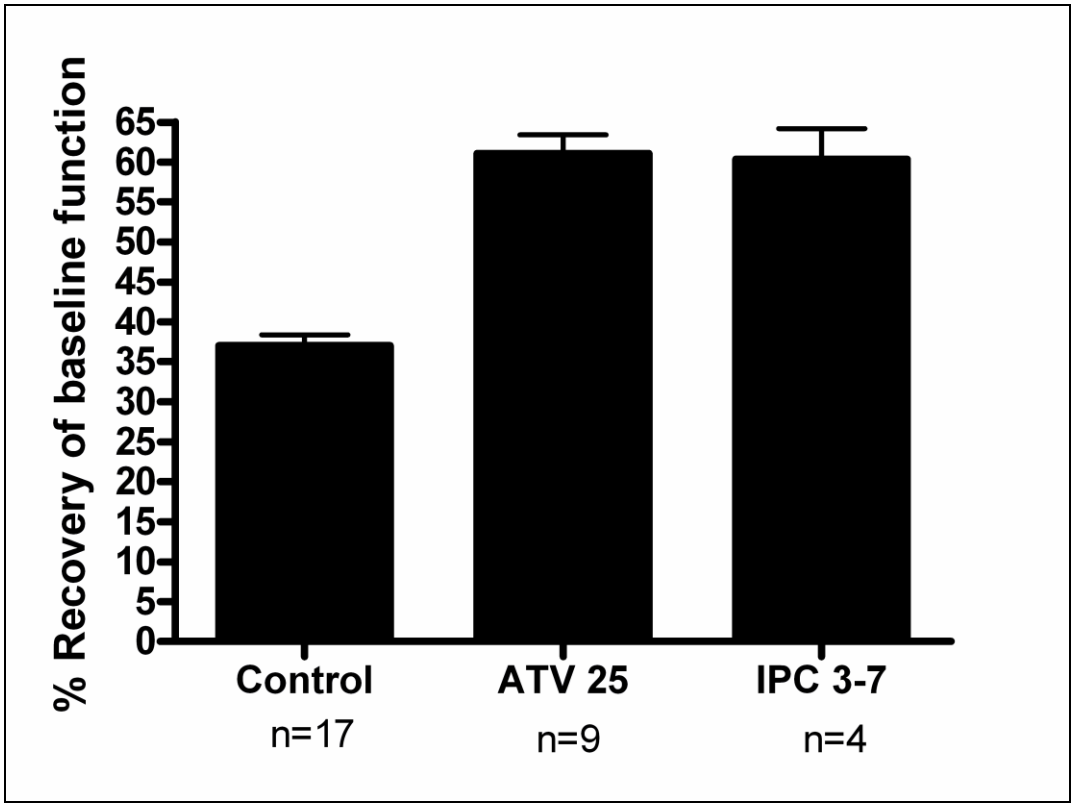

Fig 4.3 – Functional recovery of atrial trabeculae treated with atorvastatin compared to positive control with hypoxic preconditioning stimulus

The improvement seen with ATV 25 was abolished by the co-administration of LY294002 (the PI3-kinase inhibitor), UO126 (the p42/44 MAP kinase inhibitor), L-NAME (a non specific nitric oxide synthase (NOS) inhibitor) and 1400W (a specific inducible NOS inhibitor).

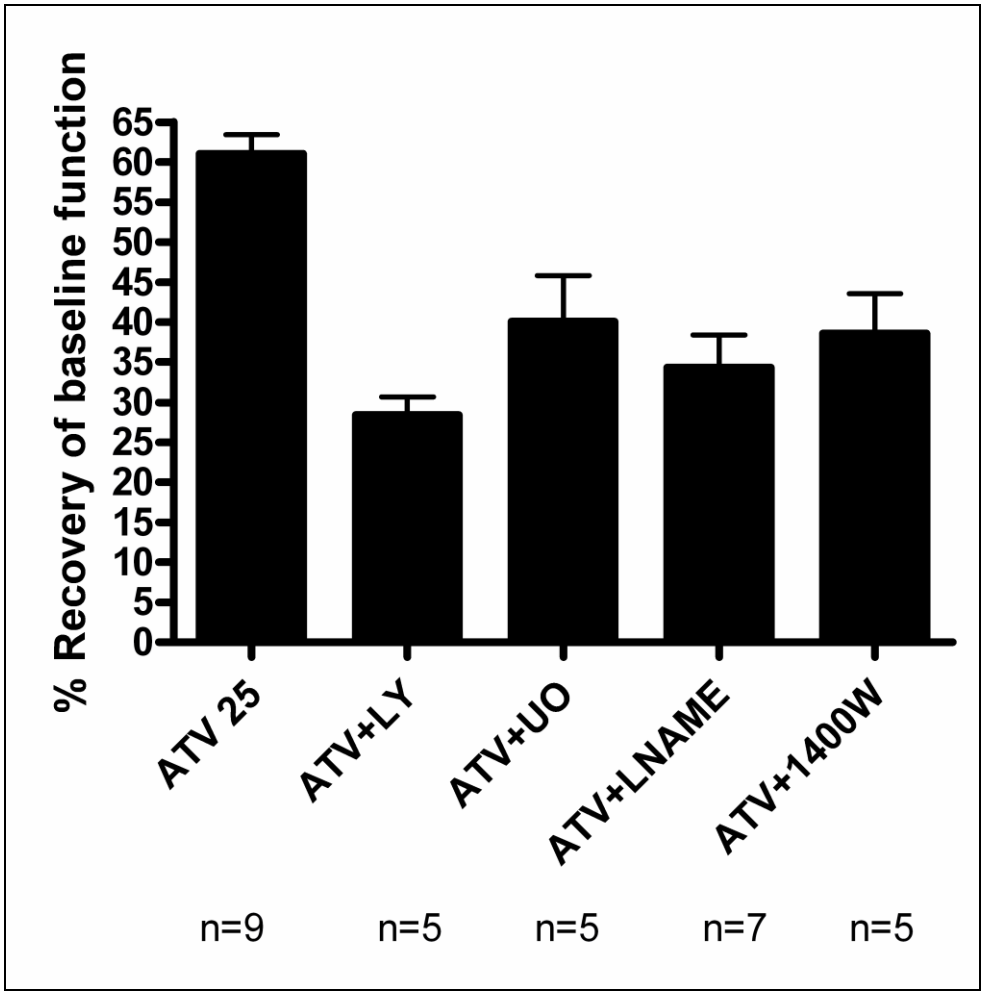

Fig 4.4 – Functional recovery data with atorvastatin plus inhibitor agents

The inhibitor agents alone, or DMSO carrier alone, had no effect on functional recovery, as shown below. Trabeculae in these groups recovered function as follows: LY 34.01±2.72%, UO126 35.12±4.90 %, L-NAME 30.59±3.33%, 1400W 29.62±2.05, and 35.20±2.98% in the DMSO carrier-control group. These values compare favourably with the control group, in which recovery was 37.05±1.33 % of baseline function contractile function, and are shown below.

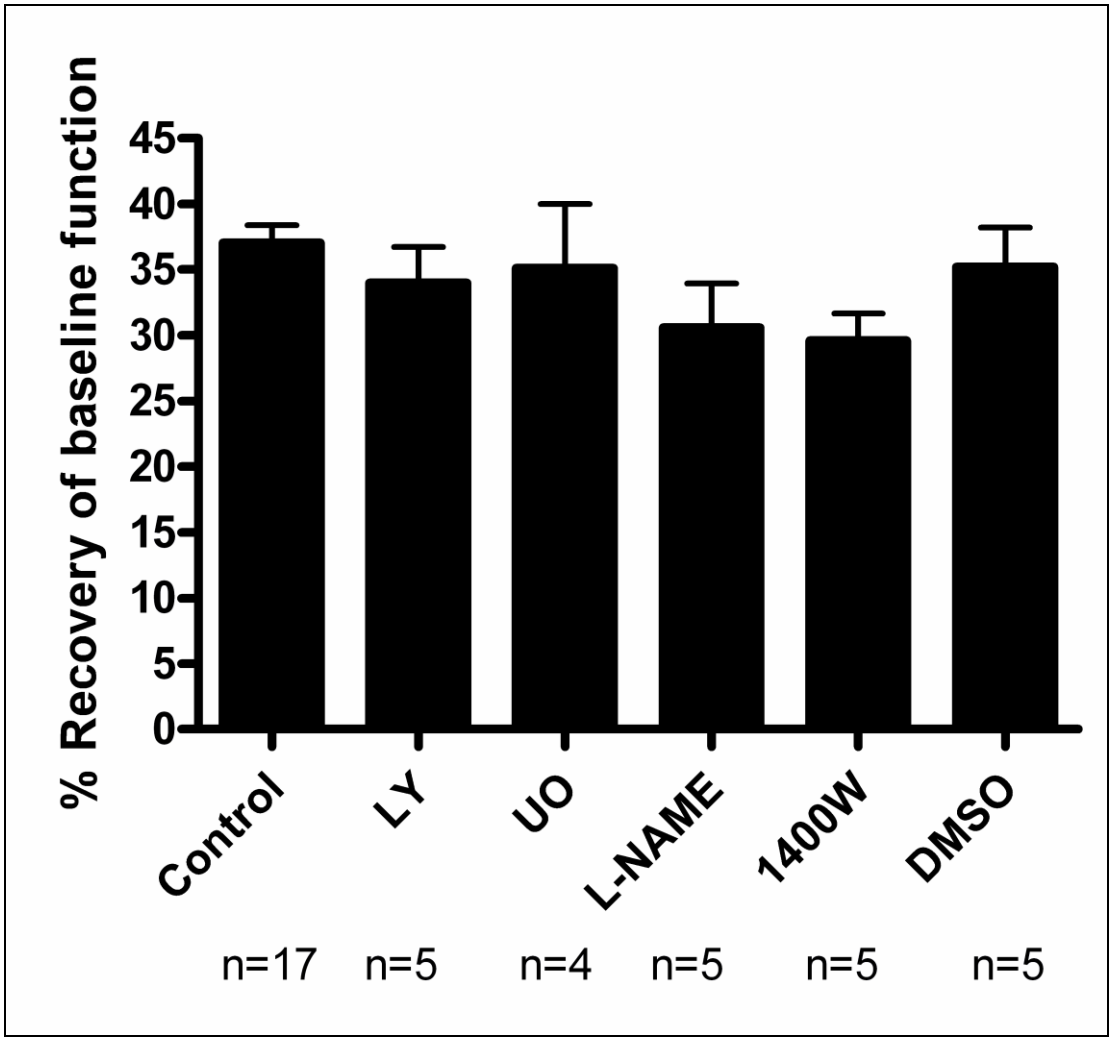

Figure 4.5 – functional recovery data using carrier or inhibitor-only controls

Diabetic subgroup analysis

Diabetic patients are at higher risk of developing cardiovascular disease than nondiabetic patients (293), with more than double the background population risk of myocardial infarction (294;295) . Once cardiovascular disease has become established, patients with diabetes have higher morbidity and mortality (296). Protection of the diabetic myocardium has received considerable experimental attention, but has been shown to be more difficult to achieve than in non-diabetic states. Some studies have suggested that traditional preconditioning techniques may fail to work altogether when applied in the setting of diabetes (229;297-299), possibly due to mitochondrial K-ATP channel dysfunction (300). Animal studies have confirmed that, if preconditioning is to be achieved, a higher intensity preconditioning stimulus must be applied when compared to non-diabetics (301), possibly due to impaired PI3K/Akt signalling (302;303) . Studies by our group using human atrial trabeculae support the concept that diabetic tissue can be successfully preconditioned, but a stronger stimulus was required (304;305). It appears that in the setting of diabetes, the threshold for cardioprotection is raised.

To investigate these findings, and to establish whether pharmacological cardioprotection is possible in the setting of diabetes, a direct comparison between diabetic tissue receiving atorvastatin treatment versus non-diabetic tissue was also performed.

Due to the randomisation process, the number of trabeculae in this analysis is small. Mean recovery of function in tissue from diabetics was 35.31±0.83% (n=4), compared with 37.59±1.71% in nondiabetics (n=13). A functional recovery value of 61.14±3.0% was obtained in non-diabetic trabeculae treated with atorvastatin (n=7), compared with 60.74±2.97% in treated diabetic tissue (n=2). It is accepted that these values are small, but the initial suggestion is that the cardioprotective effect is present irrespective of underlying diabetes mellitus.

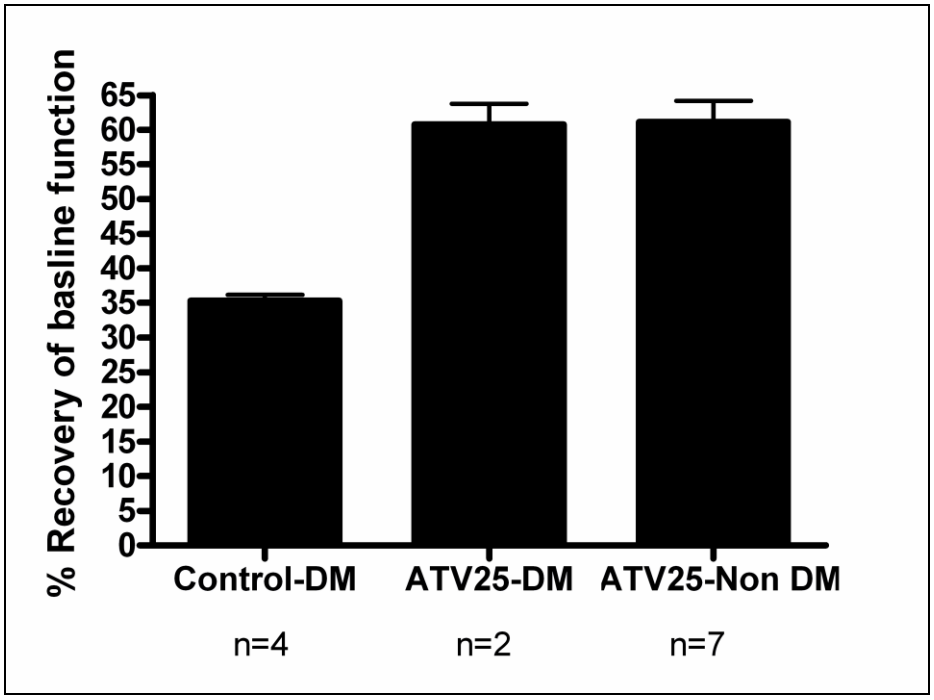

Fig 4.6 – Functional recovery in diabetic subgroup

Discussion

The major findings in this study are: (a) human atrial myocardium can be protected from simulated ischaemia reperfusion injury by treatment with atorvastatin 25 μM administered concurrent with reperfusion and (b) the protection offered by atorvastatin administration is abolished by the presence of PI3K inhibitors, Erk-1/2 inhibitors, and nonspecific NOS and specific inducible NOS inhibitors. This implicates a role for the RISK pathway and NOS in the mediation of atorvastatin-induced cardioprotection in humans.

Statin-induced cardioprotection has been described and characterised in isolated perfused murine (72;306) and rat hearts (180;284;307), and in-vivo in the mouse and rat (103-106;291;292). Perhaps the most consistent finding from the animal experimental data is the role of activation of the RISK pathway and NOS signalling systems (72;106;291). Activation of the PI3K and Erk-1/2 protein kinases at the time of myocardial reperfusion has been shown to confer powerful cardioprotection(286). The mechanism through which statin therapy may directly activate the RISK pathway is not clearly defined, but canine work suggests it involves the potentiation of myocardial adenosine by ecto-5'-nucleotidase (308).

Statin-mediated cardioprotection is also thought to be mediated by a direct increase in eNOS protein expression as a result of stabilising eNOS mRNA (309-311). Furthermore, statin cardioprotection cannot be elicited in the absence of eNOS, as evidenced by the lack of statin effect in eNOS-deficient

animal models (72;312) Studies have also suggested that eNOS phosphorylation by the PI3K and Akt signalling cascades may be responsible for an increase in protective eNOS activity(313). The importance of these systems in human myocardial tissue is supported by the findings above, in that inhibition of components of the RISK pathway and NOS signalling pathways were shown to abolish cardioprotection.
For the first time this data confirms that atorvastatin elicits its cardioprotection in humans through a direct myocardial effect involving prosurvival signalling pathways.

In this study, atorvastatin has been shown to protect the myocardium when administered at the time of reoxygenation, and this could be of clinical benefit in the setting of acute coronary syndromes especially ST-elevation myocardial infarction. Additionally, in situations where myocardial ischaemia can be predicted, pretreatment with atorvastatin could be a clinically useful tool with which to reduce myocardial injury. These settings could include elective cardiac surgery or percutaneous coronary interventions. Both procedures have been shown to be associated with myocardial injury as an unwanted side effect of the therapeutic intervention, as evidenced by biomarker release and magnetic resonance imaging data (122;268), and in both settings are associated with adverse clinical outcomes. Following PCI, unwanted biomarker release (indicating myocardial necrosis) occurs in 5-30% of cases (314), influenced by technical aspects of the procedure

difficulty, and the degree of pharmacological optimisation used upstream of the procedure (99). Clinical studies have demonstrated that statins (102;315;316), and in particular high-dose atorvastatin, can ameliorate this myocardial damage and drive down periprocedural event rates (100;101;190), and the experimental findings outlined above could form part of the basis for these clinical effects. The potential for this effect to extend to the high-risk diabetic population is an additional benefit, but further experimental data are required due to the low numbers in our study.

Conclusion

This study demonstrates, for the first time, that atorvastatin can protect human atrial myocardium ex vivo against simulated ischaemia-reperfusion injury, as evidenced by a marked improvement in recovery of contractile function. This cardioprotective effect has been shown to be mediated by pharmacological activation of the RISK pathway and NOS. The ability of atorvastatin to acutely activate this prosurvival mechanism provides a potential means to enhance myocardial salvage in the clinical setting of acute coronary syndrome patients undergoing emergency reperfusion therapy.

Chapter Five: HIGH-DOSE PREOPERATIVE ATORVASTATIN TREATMENT OF PATIENTS UNDERGOING CARDIAC SURGERY RECAPTURES PROTECTION OF ATRIAL MYOCARDIUM AGAINST SIMULATED ISCHAEMIA-REPERFUSION INJURY

Introduction

Since its introduction in 1968, coronary artery bypass grafting (CABG) became established as the standard of care for patients suffering from multi-vessel coronary artery disease (117). Vascular conduits are used to bypass diseased major epicardial blood vessels to improve survival, reduce angina, and preserve function of the left ventricle. The conduits used are most commonly the left internal mammary artery onto the left anterior descending artery, and saphenous vein grafts onto the circumflex and right coronary artery territories. More complete revascularisation using bilateral internal mammary artery grafts may improve survival outcomes (125) and is technically feasible (126), but uptake of the technique amongst cardiac surgeons is slow (127).

Even with recent advances in minimally invasive percutaneous coronary techniques, CABG holds its place as the treatment of choice for some

patient groups (6). Around 23,000 CABG procedures are performed annually in the UK (118)

Degenerative disease affecting the aortic and mitral valves is also treated by cardiac surgery (118). Surgical intervention in this setting prolongs life and reduces distressing symptoms. Valve disease and coronary artery disease frequently co-exist, mandating a combined procedure to repair or replace the affected valve alongside CABG surgery. Percutaneous treatments for aortic and mitral valve lesions are only currently available in a small number of centres.

The combined physical, physiological and biochemical insults faced by cardiac surgical patients in the perioperative phase translate into adverse clinical events. As well as general systemic insults including exposure to cardiopulmonary bypass, direct cardiac manifestations include coronary atheroembolic events and direct myocardial injury from handling or over-distension. A recent international prospective observational study confirms that composite morbidity and mortality in CABG patients ranges from 12-24% (120). A degree of myocardial necrosis is ubiquitous following cardiac surgery. The extent of this can be quantified by measuring cardiac-specific markers such as troponins and myocardial-bound creatine kinase (CK-MB). Recent clinical research has shown that perioperative release of cardiac biomarkers is associated with poorer short and long-term outcomes (121).

Additionally, MRI studies confirm that detectable myocardial scar is seen following 32% of myocardial revascularisation procedures, with a mean infarct mass of 5g. This is associated with a marked increase in adverse events in this cohort (122) . Even with relatively non-invasive PCI, periprocedural myocardial necrosis occurs in 5-30% of cases (314), influenced by technical aspects of the procedure, and the degree of pharmacological pre-optimisation employed (99).

A number of techniques have been developed which could potentially combat perioperative myocardial injury caused by ischaemia-reperfusion injury. Work in this field has included direct comparisons of varying cardioplegia regimens, use of off-pump cardiac surgery (136;137;141;317;318), the use of external preconditioning stimuli, such as remote ischaemic preconditioning to activate prosurvival signalling, and the use of pharmacological agents such as statins (138;151;319-322). It is this last approach which is investigated here.

The use of statin medications as an adjunct to perioperative care has been examined in the settings of elective cardiac surgery (164-166;168;170;171;321), and has been shown to exert substantial benefit on early postoperative adverse outcomes (174). Statins have also been demonstrated to reduce periprocedural injury in patients undergoing elective

PCI (98;100;316) and PCI for acute coronary syndromes (101;190), and in high-risk cohorts undergoing elective major vascular surgery (289)

Acutely, as discussed previously (see Chapter 1), statins have been shown to offer cardioprotection when administered prior to an experimental myocardial ischaemia-reperfusion insult (104;284) , and when given during the reperfusion phase (72), an effect independent of their lipid-lowering effects . These cardioprotective effects are mediated by activation of the pro-survival PI3K-Akt and Erk-1/2 signalling cascades during the reperfusion phase – termed the Reperfusion Injury Salvage Kinase (RISK) pathway (72;109;285-287)

Most patients undergoing cardiac surgery are taking chronic statin therapy, in accordance with European guidelines for secondary prevention (162). With chronic statin therapy, intrinsic cellular mechanisms down-regulate PI3K-Akt activation and lead to loss of protection against ischaemia-reperfusion injury – thought to be due to the effect of phosphatase and homolog deletion on chromosome ten (PTEN) (189) – a regulator of PI3K activity. In an animal model, following a demonstrable loss of protecting after 1-2 weeks of statin therapy, cardioprotection was recaptured by giving a high supplementary dose of atorvastatin prior to an ischaemia-reperfusion insult (180).

The ability or otherwise of an acute additional administration of statin to recapture resistance to myocardial ischaemia-reperfusion injury in humans is unproven. If present, it could suggest re-activation of the RISK pathway as a potential mechanism underpinning the ability of atorvastatin therapy to reduce periprocedural myocardial injury, and could contribute to the development of pharmacological strategies to reduce ischaemia-reperfusion injury associated with cardiac surgery or PCI.

Hypothesis

High-dose oral atorvastatin pre-treatment of patients undergoing cardiac surgery protects the human myocardium ex-vivo from simulated ischaemia-reperfusion injury by activation of the RISK pathway and NOS isoforms.

Study subjects

37 patients were consented for these experiments and had their results analysed. A further 9 patients consented, but the tissue samples were either too small for atrial trabeculae to be isolated, or they were damaged during the explantation process. 85 trabeculae were isolated and met the inclusion criteria outlined in Chapter 3. The mean number of trabeculae included per patient was 2.30. The following table outlines a profile of surgical patients included:

Total patients in study	37
Male	29
Female	7
Age range	33-77
Mean age	61
CABG alone	28
Aortic valve replacement alone	5
Aortic valve replacement plus CABG	3
Mitral valve repair/replacement	1

Table 5.1 – Outline of subjects consenting to studies of atorvastatin re-loading

Materials & methods

Atorvastatin was supplied by Pfizer Incorporated, USA. It was prescribed and administered at a dose of 160mg taken 10-12 hours prior to planned cardiac surgery. Enzyme inhibitors LY294002, UO126, L-NAME and 1400W were supplied and prepared as outlined above. The experimental methods have been outlined in chapter 3.

Objective

To investigate whether a high-dose preoperative treatment with atorvastatin in human subjects taking chronic background statin therapy confers cardioprotection on atrial trabeculae, and to establish whether pharmacologically inhibiting the PI3-K-Akt, Erk-1/2 pathways abolishes any cardioprotective effect. Additionally, it was intended to pharmacologically inhibit eNOS and iNOS, as likely downstream effectors of statin-induced cardioprotection.

Experimental protocol

Patients were randomly assigned, using sealed envelopes, into a control group or a group receiving high-dose pre-treatment with atorvastatin. Once they had been prepared and isolated, human atrial trabeculae were assigned to one of the following treatment groups. Control group trabeculae were randomly assigned to groups 1 or 3. Trabeculae exposed to atorvastatin pre-treatment were randomly assigned to experiments with or without pharmacological inhibitors, into groups 2, 4 or 5.

(1) Control group (n=27): Patients taking background statin therapy only (>4 weeks prior to surgery).

(2) High-dose atorvastatin pre-treatment (n=22): Patients received 160mg atorvastatin 10-12 hours prior to surgery on the background of chronic statin therapy (>4 weeks prior to surgery).

(3) Hypoxic preconditioning (n=12): Atrial trabeculae from patients in a control group (background chronic statin therapy only) which received a standard hypoxic preconditioning stimulus. This comprised 3 minutes of increased pacemaker frequency (3Hz) plus superfusion with glucose-free hypoxic buffer, followed by 7 minutes baseline pacemaker frequency (1Hz) plus superfusion with glucose-containing, oxygenated stabilisation buffer immediately prior to the main hypoxic episode. This preconditioning group was included as a positive cardioprotection control. This hypoxic preconditioning protocol has been previously documented to improve the recovery of baseline contractile function in this experimental model (82;83;270-274;305)

(4) High-dose atorvastatin plus LY, UO, L-NAME or 1400W (n=19): pre-treated atrial trabeculae exposed to the following agents during the reperfusion phase; LY294002 , a PI3-kinase inhibitor,15 µM (n=4); UO126, an Erk-1/2 inhibitor, 10µM (n=5); L-NAME, a non specific NOS inhibitor, 100µM, (n=5); and 1400W , a specific inducible NOS inhibitor, 5 µM, (n=5).

(5) High-dose atorvastatin plus DMSO (n=5): pre-treated atrial trabeculae exposed only to the carrier agent DMSO (<0.01% per unit buffer volume) during the reoxygenation phase.

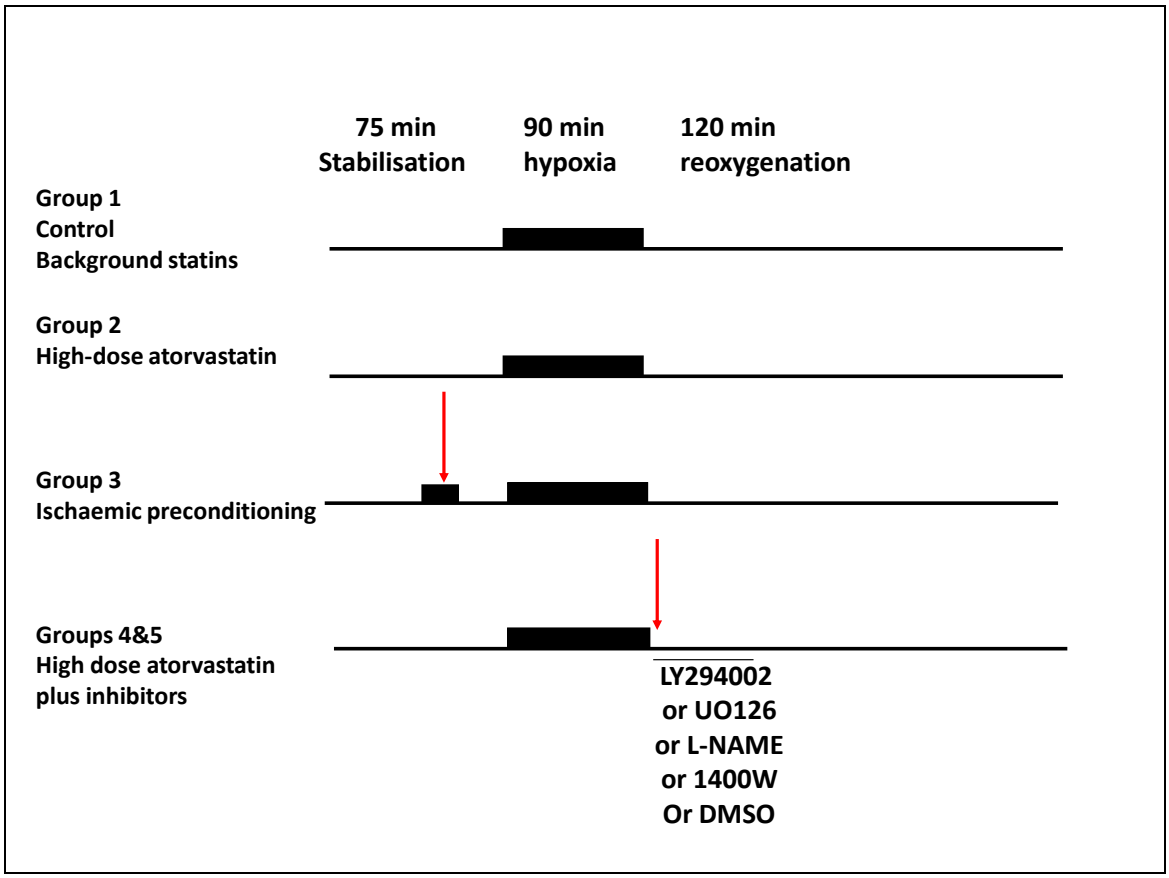

Fig 5.1 – Experimental protocol for trabeculae

Results

Baseline functional data

At baseline, the contractile function of atrial myocardium was comparable between groups, with a mean contractile force of 0.85±0.08g in the control group, and 0.88±0.04g in the high dose atorvastatin intervention arms.

Recovery of Function

Following 90 minutes of hypoxia, followed by 120 minutes of reoxygenation, the control-group atrial trabeculae recovered 35.54±1.1% of their baseline contractile function. Hypoxic preconditioning (using 3 minutes hypoxia and 7 minutes reoxygenation prior to the main hypoxic insult) included as a positive control, improved recovery of contractile function to 55.66±2.7%. In patients treated with high-dose preoperative atorvastatin, a similar improvement was seen, with trabeculae recovering 54.44±% of baseline function. This was highly statistically significant ($p<0.001$) when compared with the control group.

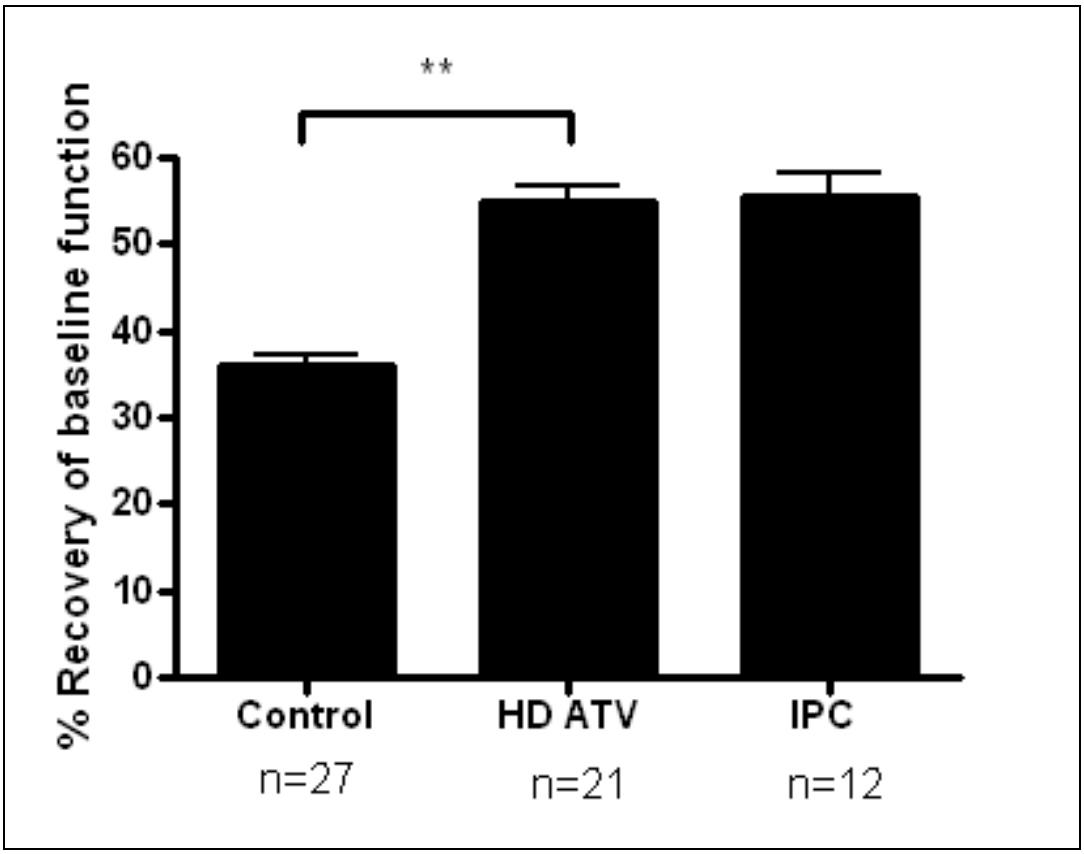

Fig 5.2 – Recovery of contractile function in trabeculae exposed to high-dose atorvastatin compared alongside control group and positive control.

This marked improvement in recovery of contractile function was not abolished by either the PI3-K inhibitor LY 294002, or by the Erk-1/2 inhibitor UO126. However, the nonspecific NOS inhibitor, L-NAME, and the specific iNOS inhibitor 1400W did significantly abolish the improvement seen with high-dose preoperative atorvastatin.

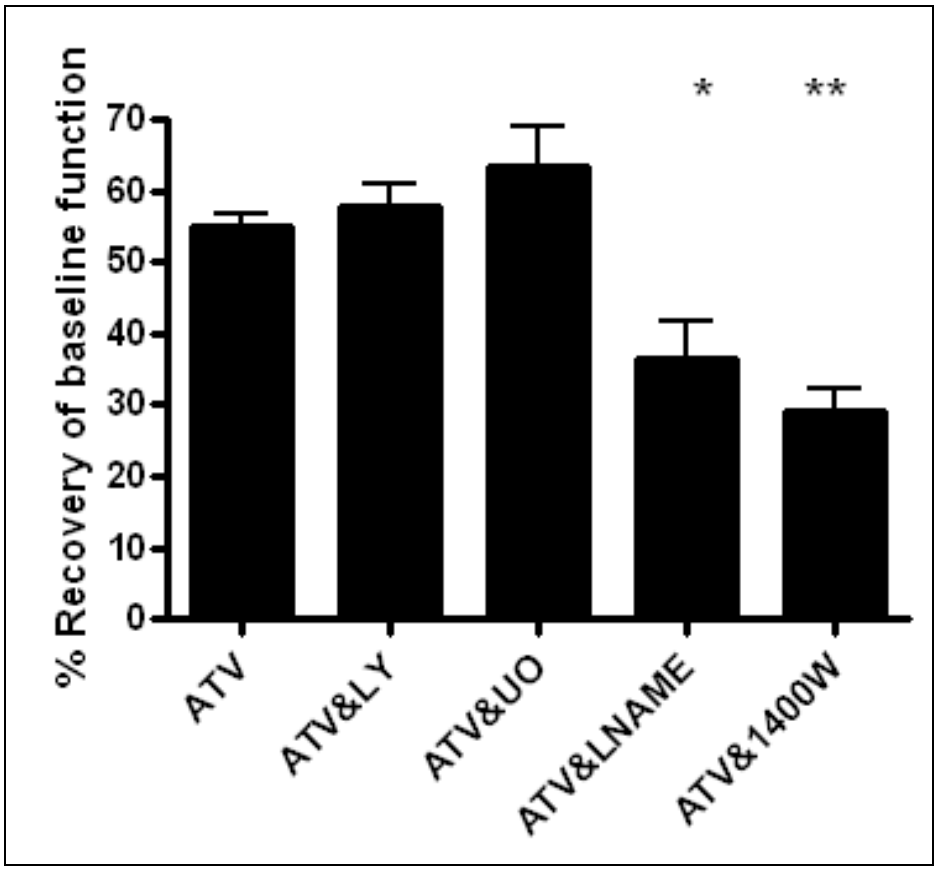

Fig 5.3 – Recovery of contractile function with RISK pathway and NOS inhibitors

Diabetic subgroup

A direct comparison between diabetic patients receiving high-dose atorvastatin pre-treatment against non-diabetic patients in the same control was performed. Mean recovery of function in diabetic patients receiving high-dose atorvastatin (n=9 trabeculae) was 55.07±2.99%. In the non-diabetic group receiving high-dose atorvastatin (n=14) trabeculae recovered 54.36%±1.89 of baseline function. By comparison, recovery in the diabetic control group was 35.14±2.1%. Interestingly, recovery of function was

effectively the same in both diabetic and no-diabetic control patients undergoing hypoxic preconditioning – 55.34±5.8% vs. 55.79±3.2% respectively.

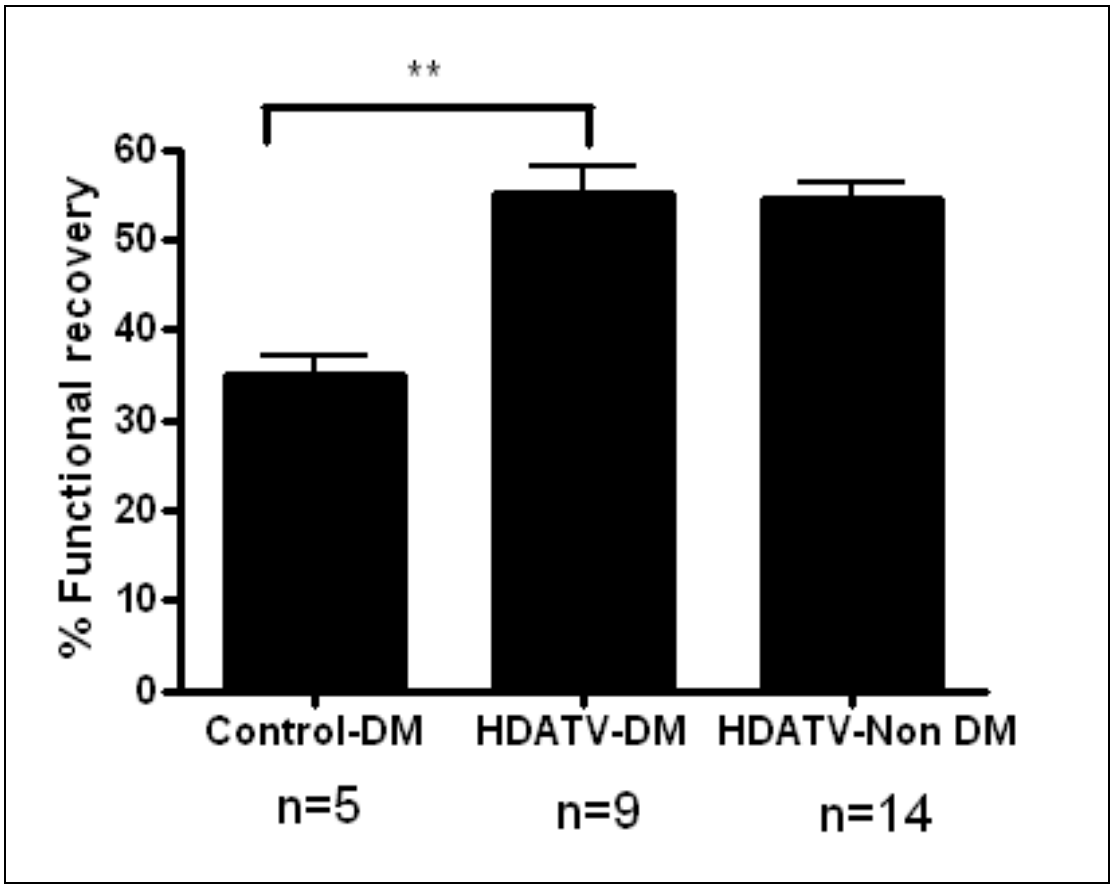

Figure 5.4 - Functional recovery of diabetic control trabeculae exposed only to chronic statins (Control-DM), high-dose preoperative atorvastatin trabeculae from diabetic patients (HDATV-DM) and high-dose preoperative atorvastatin trabeculae from non-diabetic patients (HDATV-Non DM)

Discussion

The major findings in this study are: (a) cardioprotection against simulated ischaemia-reperfusion injury of human atrial myocardium exposed to chronic statin therapy can be recaptured by treatment with high-dose atorvastatin and (b) the protection offered by this approach is abolished by inhibitors of NOS, but not by inhibitors of PI3K or Erk-1/2. This confirms the pivotal role of NOS in the mediation of atorvastatin-induced cardioprotection in humans in this setting.

Statin-induced cardioprotection

The ability of statins to protect against ischaemia-reperfusion injury has been established in isolated perfused hearts (72;180;284;306;307), in-vivo animal models (103;104;109;291;292) and in isolated human ventricular cardiomyocytes (323), with protection deriving from activation of the RISK pathway and NOS signalling systems (72;106;291).

NOS is a key component of the cardioprotective cascade

NO, produced by NOS isoforms, is a key regulator of vascular and myocardial function (324;325). eNOS derived NO has been shown to be markedly reduced after experimental myocardial infarction and in the setting of heart failure (326;327).

Experimental infarct size reduction is seen with the both the administration of NO precursors, and with direct eNOS enhancers, confirming its cardioprotective effect (328;329).

Statins enhance eNOS activity and expression

Enhanced eNOS expression and activity is central to statin-induced cardioprotection. Furthermore, statin cardioprotection cannot be elicited in the absence of eNOS, as evidenced by a lack of protective effect in eNOS-deficient animal models (72;312) Studies suggest that statin-induced cardioprotection is mediated by a direct increase in eNOS protein expression, as a result of a stabilising effect upon eNOS mRNA (309-311). Also, eNOS expression is increased with statin therapy, due to blocking of geranylgeranylation of the eNOS down-regulator, small GTPase Rho (254;311). Additionally, the protective effect of statins may be mediated by increased eNOS phosphorylation at the serine-1177 site, rather than an absolute increase in its absolute concentration. This phosphorylation is thought to be induced by the PI3K-Akt cascade (313). Specifically, atorvastatin has been demonstrated to act in this way in isolated mouse hearts exposed to ischaemia (72). If this is the upstream trigger, it is possible that the RISK cascade has already had an effect in this experimental setting, following oral administration of the drug, and the subsequent inhibition of RISK enzymes alone was too late to abrogate cardioprotection. In this experimental model, it seems likely that the RISK

cascade had already stabilised and enhanced eNOS activity in the hours following ingestion of the drug.

Why did RISK pathway inhibition fail to abrogate protection?

Activation of the RISK pathway is a key determinant of the acute cardioprotective effect of statins. However, in this experimental setting, the inhibition of PI3K and Erk-1/2 did not abolish cardioprotection, which would be expected if the RISK pathway was the sole or main mechanism in operation.

Whilst the RISK cascade is the main process involved in acute cardioprotection, the results here could be due to delayed form of preconditioning, or pharmacological preconditioning. In these forms of cardioprotection, RISK activation may be a key trigger, but with a diminished role later on. This will be discussed further below. The RISK pathway is certainly implicated in statin-induced cardioprotection (see Chapter 4), but in a way which relies on NOS isoforms as downstream effectors. It is possible that the RISK pathway had already exerted any effect it may have, and that its inhibition at the point of reoxygenation was simply too late to reverse the function of activated protective cascades downstream.

Is this a form of delayed preconditioning?

Delayed, or second-window preconditioning results in cardioprotection 24 hours after the conditioning stimulus, with a duration of 2-3 days (330). Atorvastatin has been shown to induce both acute and delayed myocardial preconditioning effects in vivo, with delayed preconditioning particularly mediated by iNOS (331). Both the presence and activity of eNOS and iNOS have been shown to be crucial in animal studies, with inhibition of either or both abrogating cardioprotection (72;103;105;107;291). Several studies confirm the key role of iNOS in delayed type (332-334) and pharmacological preconditioning (335-339). iNOS, when expressed in cardiomyocytes, is a profoundly protective protein (340). This is supported in this model by the finding that 1400w, a specific iNOS inhibitor, abolished cardioprotection. If the atorvastatin is inducing a delayed type of preconditioning, it could be argued that there exists a biphasic role of NOS isoforms, with eNOS acting as a trigger for initiating the protective cascade, and iNOS functioning as an essential mediator (339). In the delayed type cardioprotection, eNOS derived NO initiates a cascade of molecular events that results in the delayed activation of iNOS, which then confers protection (341). However, the ability of L-NAME, a nonspecific (and by inference eNOS) NOS inhibitor to abolish protection suggests that eNOS still has a role in this setting. It is likely that both eNOS and iNOS are both required to elicit delayed pharmacological cardioprotection (342)

Atorvastatin-induced NO interacts with mitochondria

As described previously, opening of the mitochondrial permeability transition pore during early reperfusion is a critical determinant of ischaemia-reperfusion injury (56;278;343). Enhanced bioavailability of NO may inhibit pore opening, and studies using NO donor agents confirm this link, as they offer cardioprotection due to their ability to protect with inhibition of the pore (344;345). Putative mechanisms suggested include interaction with the mitochondrial electron transport chain, and attenuation of mitochondrial depolarisation (346). Binding with the oxygen binding centre of cytochrome oxidase, NO effectively limits mitochondrial activity during an ischaemic insult. In turn, this could prevent generation of reactive oxygen species, reduce calcium uptake and inhibit formation of the pore (347;348). This interaction with components of the electron transport chain limits post-ischaemic damage, and provides a fundamental molecular explanation for the mechanism of NO-mediated cardioprotection (340).

Diabetic subgroup

Diabetic patients are at particular risk of cardiovascular disease (294;295) . Studies suggest that conventional preconditioning techniques fail in the setting of diabetes (229;297-299). Animal studies, and studies using human myocardium have confirmed that a higher intensity preconditioning stimulus must be applied when compared to non-diabetics (301), possibly due to impaired PI3K/Akt signalling (302-305). It appears that in the setting of diabetes, the threshold for cardioprotection is raised. These experimental

data confirm that the cardioprotective effect seen with atorvastatin extends to include the high-risk diabetic population.

Clinical applicability

Both CABG surgery and PCI have been shown to be associated with myocardial injury as an unwanted side effect of the therapeutic intervention, evidenced by biomarker release and magnetic resonance imaging data (122;268), and in both settings are associated with adverse clinical outcomes. Clinical studies have demonstrated that statins (102;315;316), and in particular high-dose atorvastatin, can reduce periprocedural event rates (100;101;190), and these experimental findings outlined above could provide an explanation of the underpinning mechanism of these clinical effects.

Conclusion

This study demonstrates for the first time that high-dose oral atorvastatin pre-treatment of patients undergoing cardiac surgery, taking chronic statin therapy, protects the human atrial myocardium ex vivo against simulated ischaemia-reperfusion injury. This protection is mediated by NOS. The ability of oral atorvastatin to induce a pharmacologically preconditioned state provides a potential explanation for clinical studies demonstrating reduced periprocedural events, and provides a basis for strategies to reduce

periprocedural myocardial injury in the clinical setting of cardiac surgery or PCI.

Chapter Six: THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE IS THE THERAPEUTIC TARGET FOR ATORVASTATIN AND CICLOSPORIN IN THE PROTECTION OF HUMAN VENTRICULAR CARDIOMYOCYTES ISOLATED FROM PATIENTS WITH HYPERTROPHIC CARDIOMYOPATHY

Introduction

In addition to the histological picture of ventricular hypertrophy and myocyte abnormalities outlined in the introductory chapter above, the pathophysiological features of hypertrophic cardiomyopathy lead to a milieu of ongoing oxidative stress (212) and chronic regional myocardial ischaemia (225;349). Increased myocardial mass leads, in turn, to an increase in basal myocardial oxygen requirements. In addition, the intramural coronary vessels are of relatively small calibre due to medial hypertrophy (217;218). The extravascular compressive forces applied by the bulky myocardium can further exacerbate myocardial perfusion, and can compromise flow in large septal and epicardial vessels. At the myocyte level, disarray leads to inefficient contraction, leading to further circulatory demands. These multiple

factors combine to result in reduced coronary flow reserve (219-224), and a myocardium which is frequently exposed to ischaemia, resulting in myocyte necrosis and replacement of the normal architecture with fibrotic tissue. HCM affected hearts are highly sensitive to the adverse effects of ischaemia (227;228). The combined effect of myocardial metabolic defects, an ongoing chronic inflammatory response and activation of adverse neuro-humoral pathways render the HCM myocardium more prone to injury than in unaffected subjects (230-232). Energy substrate use and calcium handling is impaired, and these factors further compromise post-ischaemic myocyte recovery (230;232;233;233;235).

To avoid sudden cardiac death from arrhythmia, risk stratification is performed to identify high-risk individuals who would benefit from an implantable cardioverter-defibrillator. Drug therapy using negatively inotropic antiarrhythmic drugs is employed to control symptoms, but has no disease modifying effect (206). Additionally, alcohol septal ablation can be performed percutaneously and will aid with breathlessness or syncope in carefully selected cases. In refractory cases, surgical excision of excess bulky septal tissue can be performed with good results (206). However, despite recent advances in cardiovascular medicine, no pharmacological therapies currently exist which modify or inhibit disease progression, and all hopes are currently pinned on gene therapy becoming available with which to combat this common condition (247). Similarly, protection of this highly

susceptible myocardium from ischaemia-reperfusion injury during cardiac surgery (e.g. during myectomy, valve surgery or concomitant bypass grafting) or during percutaneous coronary intervention has not been addressed or studied to date.

Coronary artery disease often co-exists with HCM from mid-life onwards (236-240). Long term follow-up data shows that the co-existence of these two disease processes carries a very adverse prognosis, with a very poor 10-year survival rate (242). The ability of conventional cardioprotective measures targeting prosurvival signalling pathways to help save myocardium at risk in this very high risk cohort of patients has not been investigated, but these therapies could have the potential to protect this cohort of very high risk patients. As an example, in a study of non-HCM human right ventricular cardiomyocytes, statin therapy was shown to induce resistance against hypoxia-reoxygenation injury, possibly acting via enhanced levels of nitric oxide synthase (323).

The ability of pharmacological agents to activate prosurvival signalling pathways in HCM, which might then act to inhibit adverse mitochondrial changes, would potentially confer powerful cardioprotective effects (65;280;350).

Hypothesis

Human ventricular myocardium from clinical subjects with hypertrophic cardiomyopathy can be pharmacologically cardioprotected.

Objectives

The objectives of this study were;

1. To establish whether inhibition of mPTP opening in response to oxidative stress in HCM could be achieved in isolated human ventricular cardiomyocytes using ciclosporin A, an agent known to inhibit pore opening in non-HCM human cardiac cells (64), and;

2. To establish if inhibition of mPTP opening in response to oxidative stress in HCM could be achieved in isolated human ventricular cardiomyocytes using atorvastatin, an HMG Co-A reductase inhibitor known to protect from ischaemia-reperfusion injury by activation of the prosurvival signalling pathways (72).

Materials and methods

Atorvastatin was supplied by Pfizer Incorporated, USA. It was dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO was <0.01%. Ciclosporin A was supplied by Merck Chemicals, UK. It was also dissolved in DMSO, with a final concentration of <0.01%. The experimental methods have been detailed in chapter 3.

Experimental protocol

Loaded coverslip wells containing freshly isolated human ventricular cardiomyocytes isolated from HCM patients undergoing surgical myectomy were randomised into control and drug-intervention groups, using a standardised isolation protocol (275). All wells were loaded with TMRM 3µM and allowed to incubate for 5 minutes at room temperature prior to confocal microscopy. In the drug-intervention arms, the agents were added via micropipette 5 minutes after the addition of the TMRM, and incubated for a further 10 minutes. In control groups, DMSO alone was instilled at this point and further 10 minutes incubation permitted. Total TMRM incubation time was limited to 15 minutes in all groups, to avoid preferential loading of any cell groups which could have resulted in potentially enhanced susceptibility to laser stress. In groups randomised to receive ciclosporin A, a final concentration of 0.2 µM in the buffer was administered. In the atorvastatin group, the concentration investigated was 25 µM. The concentrations were selected based on previously published data confirming cardioprotective efficacy at these doses in a variety of models including isolated cells (64;72;278;279).

Study Subjects

8 patients undergoing surgical myectomy for obstructive HCM consented to this study and underwent tissue harvest, myocyte isolation and experimental studies:

Total patients in study	8
Male	5
Female	3
Age range	28-55
Mean age	47

Table 6.1 – Study subjects undergoing septal myectomy consenting to myocyte isolation

Results

From 8 patients, a total of 20 groups of cells underwent analysis in a randomised fashion as described above. A control group was included in each experiment. In the control group, mPTP opening was induced after 188.7 ±22.7 seconds of oxidative stress, providing evidence for a functional mPTP in the setting of HCM. Pre-treatment with the known mPTP inhibitor, CsA, delayed the onset of mPTP opening by 51±10% ($P<0.001$) to 284.94±18.81 seconds. Treatment with atorvastatin delayed the onset of mPTP opening by 35±7% ($P<0.05$), to 252.48±11.32 seconds.

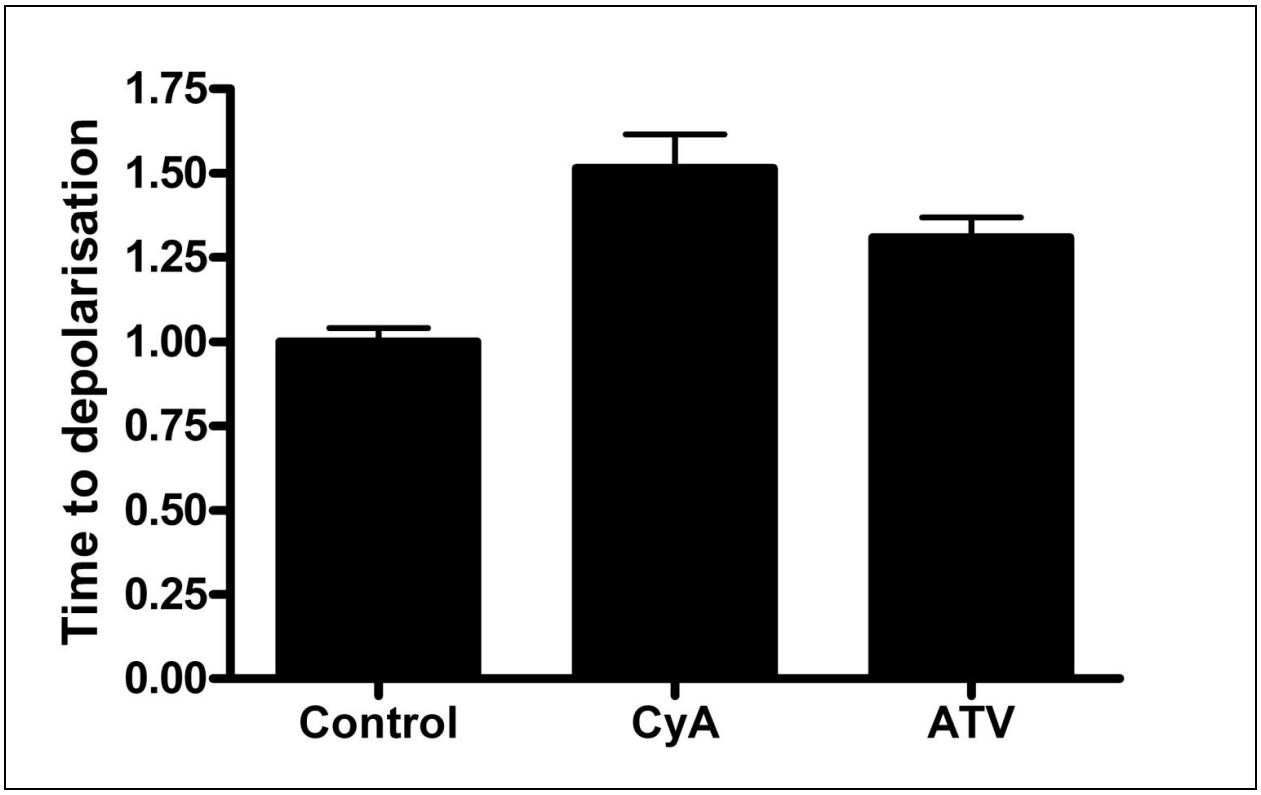

Fig 6.1 – Comparison of time to mPTP opening in groups of cells

Discussion

The main findings from this study are (a) that mPTP opening in isolated human ventricular cardiomyocytes with HCM is a critical determinant of cell death. This was evidenced here by the application of a standardised laser insult to induce mitochondrial oxidative stress, leading to pore opening with subsequent loss of mitochondrial membrane potential. This was detected by observing for an increase in cytosol TMRM fluorescence intensity, following loss of the dye from the mitochondria once the membrane had been breached; (b) inhibition of mPTP opening in response to oxidative stress in HCM can be achieved in isolated human ventricular cardiomyocytes using ciclosporin A; and (c) inhibition of mPTP opening in response to oxidative

stress in HCM could be achieved in isolated human ventricular cardiomyocytes using atorvastatin, an HMG Co-A reductase inhibitor known to protect from ischaemia-reperfusion injury by activation of prosurvival signalling pathways (72).

This is the first time in humans that the ability of HCM cardiomyocytes to derive benefit from cardioprotective mechanisms active in non-HCM settings has been established.

How could these findings be of potential clinical benefit?

The particular importance of ongoing ischaemia within the myocardium in the setting of HCM was first confirmed from an Italian post-mortem study which identified areas of myocardial damage within the hearts of patients who had suffered sudden death (351). Autopsy studies then went on to demonstrate structural abnormalities of intramural coronary vasculature, including thickening of the intima and medial layers of the blood vessels (218;221). These features can lead to an impaired vasodilatory capacity and thereby blunt myocardial blood flow during stress, leading to hypoperfusion and ischaemia (220;352;353). Evidence for ongoing subclinical myocardial ischaemia is supported by a clinical study which found abnormal levels of circulating serum cardiac troponins in 50% of HCM subjects, with a trend to more severe disease characteristics in that group (354). Observations from nuclear imaging and cardiac magnetic resonance imaging studies confirm impaired hyperaemic blood flow and an association between chronic

ischaemia and myocardial fibrosis (355-357) . It is likely that the abnormal myocardial perfusion caused by microvascular dysfunction leads to myocardial ischaemia-related myocyte death and subsequent replacement by fibrotic tissue (225). A therapy which could modulate myocyte resistance to ongoing episodes of ischaemia could interrupt this process and preserve myocyte viability.

A key role for either of these agents could include a reduction in cardiomyocyte death due to episodes of ischaemia-reperfusion injury, leading to a reduction in replacement with fibrotic tissue. The evidence currently would suggest that reduction of ischaemic damage could improve the clinical picture. Data from MRI studies suggest strongly that the degree of fibrosis in HCM positively correlates with the degree of left ventricular impairment, and progression to adverse clinical outcomes such as the development of atrial fibrillation, life-threatening arrhythmias and progression to overt heart failure, as might be expected (358-361).

What role might ciclosporin have, and what evidence is there that it is useful?

Its role in ameliorating ischaemia-reperfusion injury by inhibition of the mPTP is well established in cellular (63;65;278;280;343) and ex-vivo models (362;363). In humans, cyclosporine has been shown to protect via delaying mPTP opening in a laser model of cardiomyocyte oxidative stress (64), and

in an ex-vivo atrial trabecular model of simulated ischaemia-reperfusion (364). Additionally, clinical MRI data confirms that, used an adjunct to emergency percutaneous coronary intervention for acute myocardial infarction, cyclosporine reduces final infarct mass (365). These findings are present acutely at day 5 post-infarction, and persist through longer-term follow up at 6 months (366), and lend support to the notion that the agent has a role in modifying the effects of myocardial ischaemia-reperfusion injury.

Are there any other potential benefits of the drug?

Calcineurin, a calcium-regulated phosphatase, plays a critical role in the pathogenesis of HCM. Administration of cyclosporine, which is a known inhibitor of calcineurin, has been shown to prevent disease in mice that were genetically predisposed to develop HCM as a result of aberrant expression of tropomodulin, myosin Light chain-2, or fetal beta-tropomyosin proteins (367). Ciclosporin had a similar effect in a rat model of pressure-overload hypertrophy, so could theoretically have an anti-hypertrophy role in HCM (368). However, on a cautionary note, some investigators have shown that in a mouse form of HCM, ciclosporin A treatment actually augmented hypertrophy, possibly due to a shared hypertrophic signalling pathway which, paradoxically, triggered myocyte growth.(369;370) So whilst cyclosporine may offer a protective effect from reduction of myocyte damage during periods of ischaemia, the long term safety in terms of anti or pro-hypertrophy

effects are unknown. In addition, cyclosporine is an immunomodulatory and immunosuppressive drug, which requires ongoing therapeutic level monitoring, and carries with it a risk of infection. These factors mean that it is probably not a practical long-term therapy solution for the majority of HCM patients; at least not until any disease modification effect is well established.

Is atorvastatin therapy a viable alternative?

The role of statins in primary and secondary prevention of coronary artery disease is well established, and discussed elsewhere. What we do know about atorvastatin is that it is the most widely used HMG-CoA reductase inhibitor, and that it is safe and well tolerated, even at the intensive therapy dose of 80mg daily (115). One key role for atorvastatin in HCM could lie with the modification of the myocyte response to episodes of ischaemia-reperfusion injury. Atorvastatin is well established as an agent that can cardioprotect via activation of the prosurvival RISK pathway (72). In a rat cardiomyocyte model identical to that used above, activation of the PI(3)K-Akt prosurvival kinase pathway inhibited opening of the mPTP, and confirmed the important link between the survival kinases and the pore (65). This could explain the differential prolongation in cell survival times seen in this study, with cyclosporine perhaps acting more immediately and directly at pore level and resulting in more established pore inhibition, whilst atorvastatin confers its protection acting via a signalling pathway. This could be the reason behind it being relatively less protective (than

cyclosporine) in this study, when the stress stimulus was applied within a matter of minutes following drug exposure.

Pharmacological inhibition of components of the RISK pathway, to better delineate the signalling pathways involved, has been previously described in this cellular model (65). Repeating these experiments with further cell groups, including groups in the presence of the inhibitors would allow further elucidation of the signalling pathways involved.

Is atorvastatin inducing NO-mediated inhibition of the mPTP?

Enhanced eNOS expression is a key component of statin-induced cardioprotection, and statin cardioprotection cannot be elicited in eNOS deficient models (72;312) Statin-induced cardioprotection is mediated by eNOS mRNA stabilisation (309-311), through the blocking of geranylgeranylation of small GTPases in the heart (254;311), and additionally may be mediated by increased eNOS phosphorylation, induced by the PI3K-Akt cascade (72;313).

Both presence and activity of eNOS and iNOS have been shown to be crucial in animal studies, with inhibition of either or both abrogating cardioprotection. At the cellular level, experimental studies in rat myocyte models showed that pretreatment with an NO donor induces a modest, sustained mitochondrial depolarization and protects cardiomyocytes from ischaemia-reperfusion injury , with a concomitant reduction in cytosol and mitochondrial calcium levels. Binding with cytochrome oxidase, NO

interacts with the mitochondrial electron transport chain and limits mitochondrial activity during the ischaemic insult by causing slight mitochondrial depolarisation (340;347;348). Enhanced bioavailability of NO, secondary to enhanced eNOS and iNOS activity induced by atorvastatin, may thus inhibit pore opening, and this mechanism could be operating in this model (344;345).

Are there any other proven benefits to atorvastatin used in the setting of HCM?

Both simvastatin and atorvastatin have been shown to prevent, reduce and reverse cardiac hypertrophy and fibrosis in transgenic animal models of HCM, with a concomitant reduction in the levels of cardiac fibrosis (263;264) Clinical studies in humans have demonstrated that statin therapy leads to reduction in left ventricular mass in HCM patients with angina, hinting that it may be of benefit in this high-risk cohort of patients (261). Other pilot studies of statin use in HCM patients have been inconclusive or unhelpful, mostly due to study design, drug dose or patient compliance issues (371;372). The investigators have also probably looked for the wrong end-points, using crude echocardiographic markers of LV mass rather than more contemporaneous detailed MRI assessments of mass and function, coupled with stress perfusion data to delineate and quantify ischaemic burden (225;265). However, atorvastatin probably does have additional positive structural effects on the myocardium, as evidenced by improved extracellular

remodelling indices in patients with high-risk coronary disease (373) , in addition to a potential anti-hypertrophy role. These potential avenues need to be investigated by further detailed MRI fibrosis and stress perfusion studies.

Cardioprotection during acute coronary syndromes in HCM patients

As outlined above, HCM and coronary disease frequently occur together, and confer a particularly adverse prognosis (241;242) . Evidence that the pharmacological cardioprotection pathways being investigated in “normal” subjects also exist in the setting of HCM is favourable in that HCM subjects with acute coronary syndromes could potentially benefit from interventions proven in non-HCM settings. In this study, the role of the mPTP, central to the control of myocyte death (56), has been established in the setting of HCM ,and the potential of drug therapy to modify and reduce myocyte injury through its inhibition has been demonstrated. As with all cardioprotective interventions, pharmacological and non-pharmacological, the challenge of translating these into findings into clinical practice remains huge (27;374).

Periprocedural intervention

Perioperative cardioprotection has been discussed elsewhere, but another potential exploitation of this cardioprotective mechanism might be in the setting of HCM patients undergoing procedures where myocardial damage

could be predicted. Specifically this would include the settings of cardiac surgery or percutaneous coronary intervention for coronary artery disease. Around 5% of patients with HCM will require surgical intervention by means of surgical myectomy, for severe left ventricular outflow tract disease (202). The application of a cardioprotective agents to reduce myocardial ischaemia-reperfusion injury associated with the mechanical effects of surgical handling during myectomy, and from the cardiopulmonary bypass insult would be a potential therapeutic avenue, and current studies of pharmacological intervention with statins in cardiac surgery appear promising, especially in reducing early postoperative adverse outcomes (138;164-166;170;171;174;177).

Limitations

One potential criticism of this data would include the issue of genetic heterogeneity of the sample. No genetic subtypes had been identified amongst the study patients, and it is clearly possible that, in some genetic HCM subtypes, the outlined cardioprotective mechanisms may be impaired or absent due to the underlying genetic lesion. However, it is not current practice to perform detailed genetic analysis on these patients, and study design mandating this would yield data that is not necessarily applicable in normal clinical practice. Indeed, data which applies to “all comers” is potentially more clinically valuable in a condition with significant and

expanding heterogeneity (197), currently running at 1000 mutations in 13 or more genes (196;198;199).

Additionally, it is accepted that the numbers of cell groups are relatively small, although cell yields did steadily improve with continued repeated operator exposure to the isolation process. During the course of the study, preparations were made to move the isolation process closer to the point of surgery, to further assist with gaining higher-yield isolations. However, the main strength of this data is that it has been gained through the use of human cells, isolated from live patients.

Conclusion

The present study demonstrates, for the first time in an isolated human ventricular myocyte model, that the mPTP is functional in the setting of human HCM. Additionally, its opening can be delayed by ciclosporin and atorvastatin, both agents know to have a cardioprotective effect in non-HCM settings. The ability to modify cardiomyocyte response to ischaemia-reperfusion injury could play a role not only in acute cardioprotection during acute coronary syndromes or during surgical/percutaneous interventions, but also in long-term disease modification in HCM, where the presence of impaired coronary reserve and hypoperfusion is well proven. Ultimately, such a therapy could reduce the progression of cell death and myocardial fibrosis, shown to be associated with an adverse long-term clinical outcome.

Chapter Seven: DISCUSSION

An overview of the findings

This thesis has described three experimental investigations aimed at elucidating cardioprotective mechanisms in the human myocardium. The experimental protocols have examined pharmacological cardioprotection in the setting of “normal”, diabetic and HCM-affected hearts. Each investigative avenue has sought to confirm the applicability of the existing animal data to the setting of human cardioprotection. The main investigative effort has been to establish whether pharmacological activation of the Reperfusion Injury Salvage Kinase (RISK) enzymes and nitric oxide synthase isoforms is possible using Atorvastatin, and whether this confers cardioprotection against ischaemia-reperfusion injury. Studies also examined the effects of Atorvastatin with regards to mitochondrial permeability transition pore inhibition in the setting of hearts affected by hypertrophic cardiomyopathy, which are even more vulnerable to the effects of ischaemia than “normal” hearts, and chronically affected by repeated episodes of ischaemia-reperfusion injury.

Chapter four described experiments relating to acute cardioprotection of human atrial myocardium using Atorvastatin administered at the point of reoxygenation, following a period of *ex-vivo* hypoxia-reoxygenation injury.

For the first time in human myocardium, it was demonstrated that Atorvastatin can protect human atrial myocardium as evidenced by a marked improvement in recovery of contractile function. This cardioprotective effect was similar in magnitude to that afforded in previous studies of human myocardium by ischaemic preconditioning techniques (270), administration of pharmacological agents (83), and ischaemic postconditioning (82) . All these techniques have a shared pharmacological basis in that they have all been shown to activate the RISK pathway (287), in which key elements are the activation of the PI3-kinase and Erk-1/2 protein kinases (286). By using specific inhibitor agents it has now been shown that this pathway is mechanistically important in the acute cardioprotection seen with Atorvastatin in humans. Specifically, inhibition of PI3-kinase by the inhibitor agent LY294002 abrogated protection, as did inhibition of Erk-1/2 kinase by UO126. Statin-induced cardioprotection has also been associated with activation of nitric oxide synthase (NOS) isoforms, and this was also investigated in the studies presented in this thesis. Statins have been shown to stabilise eNOS mRNA (309-311), and phosphorylation of eNOS directly by the PI3K and Akt signalling cascades may increase cardioprotective eNOS activity (313). In this experimental setting, the addition of L-NAME at re-oxygenation, a non-specific NOS inhibitor abrogated protection, as did the iNOS-specific inhibitor 1400W. Despite previous studies suggesting a higher threshold for achieving protection in diabetic patients (304), data from a

small diabetic subgroup suggests that this cardioprotective effect is also applicable even with underlying diabetes.

From the host of animal data available, the most consistent finding using Atorvastatin in this setting is activation of the RISK pathway and NOS signalling systems (72;106;291) and these new findings confirm the key role of both the RISK pathway and NOS in the mediation of Atorvastatin-induced acute cardioprotection in humans.

Chapter five focused on assessing whether an acute dose of Atorvastatin can re-capture cardioprotection, where chronic background statin therapy has been administered. Previous animal studies have shown that the cardioprotective effects of statins reduce after acute administration, due to activity of a house-keeping phosphatase, PTEN (189) . PTEN effectively reduces the phosphorylation states of the RISK enzymes back to their baseline state to avoid chronic cell dysfunction and hypertrophy (375). This loss of cardioprotection can be averted, in animal models, by giving an additional high dose of statin prior to experimental myocardial infarction (180) Recent clinical studies have shown that an additional “top-up” dose of Atorvastatin can be used to reduce periprocedural myocardial injury following percutaneous coronary intervention as evidence by reduced biomarker release (123;190), and this study sought to suggest a potential mechanism to explain this and similar findings. In this study, administration of a 160mg dose of Atorvastatin 12 hours prior to hypoxia-reoxygenation

injury recaptured cardioprotection in *ex-vivo* human atrial myocardium explanted from patients undergoing cardiac surgery, as evidenced by a significant improvement in recovery of contractile function. Again, the potential roles of RISK pathway enzymes and NOS isoforms were assessed using inhibitor agents. Interestingly, neither inhibition of PI3-kinase by LY294002 nor inhibition of Erk-1/2 MAP kinase by UO126 abrogated cardioprotection here. It is possible that the RISK cascade had already had its effect by the time of simulated reperfusion in this experimental setting, having been administered some 12 hours prior. Perhaps the inhibition at the point of simulated reperfusion was simply too late. However, both the NOS inhibitors (L-NAME and 1400w) abrogated protection. It is suggested that Atorvastatin can induce a form of pharmacological preconditioning (delayed, or second-window cardioprotection) in which iNOS is a particularly important mediator (332-338), and previous workers have elucidated that eNOS has an early role in activating a protective cascade, with iNOS being the effector later on (341). However, these experimental findings support the contention that both NOS isoforms are active in eliciting delayed cardioprotection, and that they are both important during the reperfusion phase (342).

Chapter 6 described an investigation into possible cardioprotective mechanisms in the setting of hypertrophic cardiomyopathy (HCM). HCM is the most common inherited cardiovascular disorder, affecting up to 1 in 500 people (195-197), caused by mutation-induced dysfunction of the

sarcomere. We have demonstrated for the first time that the opening of the mitochondrial permeability transition pore (mPTP), a critical determinant of reperfusion injury (56;376), occurs in the setting of HCM. This work was performed on human cardiomyocytes harvested from patients undergoing cardiac surgery to relieve left ventricular outflow tract obstruction, and used a standardised laser insult to induce oxidative stress within the cells. Importantly, inhibition of the mPTP was possible using ciclosporin A as a positive control, an agent known to inhibit pore opening in animal models and non-HCM human atrial tissue (64;280;362). Additionally, Atorvastatin was also able to delay mPTP opening when administered to HCM-affected cells prior immediately prior to oxidative stress, which may be due to a direct effect on the mPTP, or as a result of activation of prosurvival signalling pathways. Moreover, the study has confirmed that the mPTP is a potential therapeutic target for cardioprotection in the setting of HCM, and the implications of this will be discussed further below.

Clinical implications

A common uniting theme in all these investigations is the ability of Atorvastatin to induce cardioprotection in human myocardium in a variety of settings, via subtly differing pathways. The ability of Atorvastatin to cardioprotect acutely seems to be due to activation of the RISK pathway, in particular PI3-kinase, Erk-1/2, and this is in keeping with the wealth of experimental animal data (72;285). Additionally, the roles of both eNOS and

iNOS are strongly implicated in acute Atorvastatin-induced cardioprotection, possibly as effectors, and this is also in keeping with previously published animal data (72;311). Atorvastatin is in wide clinical use, is safe and well-tolerated (115). When given at high dose, it rapidly reaches pharmacologically active serum levels (116), and due to its lipophilic nature enters endothelial and other cells rapidly (179). It would be an ideal pharmacological agent to study more closely following administration to patients suffering from ST-elevation myocardial infarction, and could be given in the prehospital setting (at home or in the ambulance). Even where direct transfer to a heart attack centre is undertaken, there is still a delay prior to mechanical reperfusion being established. By administering en-route to hospital, the drug would have reached a pharmacologically active serum level, and thus be present in the circulation and be able to activate the RISK pathway once forward flow in the infarct related artery has been achieved by the interventional cardiologist performing primary PCI. The ultimate aim of this pharmacological adjunctive therapy would be improved myocardial salvage and a reduction in final infarct size, translating into better long-term outcomes. A unique contribution to this field is confirmation of the ability of Atorvastatin to cardioprotect via activation of the RISK pathway. This could explain the underlying pharmacological mechanism responsible for the positive findings of the clinical studies of Atorvastatin use in reducing periprocedural myocardial injury in the settings of both PCI (102;123) and cardiac surgery (174).

The ability of high-dose Atorvastatin administration to recapture cardioprotection in the setting of chronic background statin therapy extends the potential clinical benefits further. Whether being used as an adjunct to reperfusion by primary PCI, or as an upstream therapy to protect patients undergoing elective revascularisation procedures, these studies have established that the down-regulating effects of PTEN can be overcome in human myocardium by a high-dose of Atorvastatin. Given that current international guidelines recommend the use of chronic statin therapy as a secondary prevention measure in all patients with coronary artery disease, the majority of patients undergoing PCI or CABG should already be taking a statin. High-dose Atorvastatin reloading may be a means by which we are able to offer a simple, safe drug intervention to recapture protection from periprocedural injury, and reduce adverse event rates. The findings in chapter 5 may contribute a scientific basis for the results of recent clinical trial of cardioprotection during PCI (190).

The mechanisms by which HCM causes damage to the myocardium are multifactorial and complex, and recent insights including advanced imaging and coronary techniques highlight the role of chronic spells of ischaemia-reperfusion injury within the diseased heart (224;225). Additionally, there are currently no medical therapies which can be offered to HCM patients which actually modify the disease – treatments only alleviate symptoms. The potential for Atorvastatin to be used to cardioprotect acting via the mPTP could have clinical applications in the acute setting of myocardial

infarction, as HCM and coronary disease frequently co-exist (240;241;243). Additionally it could have a role in reduction of periprocedural events in HCM patients undergoing PCI, CABG, surgical myectomy and percutaneous septal ablation. There is also some data that support an anti-hypertrophy effect of statin therapy, although clinical trials have proved inconclusive. However, demonstrating the functionality of the mPTP as a determinant of cell death, and the ability to inhibit it using Atorvastatin in the setting of HCM is a step forward in understanding the mechanisms of cardioprotection potentially available in this condition. This therapeutic avenue could possibly be used to modify the heart's response to chronic ischaemia-reperfusion injury in HCM, perhaps even reducing fibrosis long-term.

Future directions

A common problem in research in the field of cardioprotection has been a hasty switch from small laboratory studies to large, heterogeneous clinical trials, often with serious design flaws which reduce the likelihood of scientifically robust and clinically meaningful results (374;377). Whilst testing a cardioprotective agent in human heart tissue is a crucial translational step between bench and bedside, further basic science investigations could be used to better delineate the optimum cardioprotective effect of Atorvastatin. Initially, further experiments using the human atrial model could be used to establish dose-response data for Atorvastatin, as in

animal models a therapeutic ceiling was reached, and further increases in dosing above 50 µM/L did not confer any additional protection (72). As high-dose Atorvastatin in the animal models sometimes results in transient hypotension, it is important that supra-therapeutic doses are not given which may confer haemodynamic instability (and thus potentially worsen outcome in the setting of acute coronary syndrome) where no additional cardioprotective benefit is possible. Other statin agents should also be assessed, as cardioprotection in humans may be a class effect, particularly using agents of a similar lipophilic nature to Atorvastatin.

Confirming that these findings are applicable in human ventricular muscle, the key clinical target of cardioprotection, could be achieved by using an isolated ventricular cardiomyocyte model using excised right ventricular outflow tract tissue similar to that used by Verma et al to investigate Pravastatin (323). Additionally, the mPTP work could usefully be repeated with these cells, and this would extend our understanding of the interaction of Atorvastatin and the mPTP outside the setting of HCM. A natural progression of this work would also include using hypoxia rather than laser-induced oxidative stress, which would allow an investigation into cell survival in this setting, and whether this can be optimised in cells exposed to Atorvastatin.

To establish the role of Atorvastatin with respect to increased eNOS expression, Western blotting or standard calibrated reverse-transcripted

polymerase chain reaction (PCR) techniques could be used to assess eNOS mRNA expression. Additionally, a direct measurement of NOS activity can be performed using commercially available radiochemistry equipment. Similar work has been performed in human right atrial appendages harvested during cardiac surgery to assess the effects of angiotensin-converting enzyme inhibitors (378) and β-adrenoceptor agonists (379-381). Some preliminary work using snap-frozen right atrial appendage tissue was commenced in this field, but requires much technical optimisation before meaningful data can be reliably obtained.

In the setting of HCM, it would be valuable to cross back over to investigations using atrial tissue, and use explanted atrial trabeculae to confirm improved functional recovery with Atorvastatin following hypoxia-reoxygenation injury. These patients require full cardiopulmonary bypass for their surgery, and thus could potentially consent for right atrial appendage harvest to allow these experiments to be undertaken. Potential underlying prosurvival cascades could be further assessed using RISK pathway inhibitors alongside Atorvastatin in either atrial tissue at reoxygenation, or by adding them to the cellular isolates. As discussed above, hypoxia studies could also be performed in the isolated cardiomyocytes to assess the impact of Atorvastatin on cell survival.

Only when full delineation of the cellular mechanisms and optimal target doses are established should a formal prospective clinical study be designed, with special care to target an appropriate clinical setting, with careful patient selection, coupled with a proper assessment of myocardial salvage and area at risk, probably best determined using advanced magnetic imaging techniques.

Conclusion

In summary, these experiments have investigated the role of Atorvastatin with respect to acute cardioprotection of the human heart. The key unique contribution to this field has been to provide a crucial translational step between bench and bedside, furthering our understanding of how Atorvastatin provides a cardioprotective effect in human cardiac tissue.

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