The Role of the Mitochondrial Permeability Transition Pore in Cardioprotection

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I, Cara Hendry 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

Myocardial infarction is the largest cause of morbidity and mortality worldwide. Despite optimal treatment, patients have a mortality which approaches 12% at six months.

Reperfusion of the ischaemic myocardium is essential to salvage myocardium. However, reperfusion itself is harmful, with up to 40% of myocardial necrosis occurring at this time. This is known as “Lethal Reperfusion Injury”. Opening of the mitochondrial permeability transition pore (MPTP), a channel situated in the inner mitochondrial membrane is central to this process. In its quiescent state, the MPTP remains closed, but once open it becomes non-selectively permeable to solutes of up to 1.5 kDa, resulting in rapidly advancing necrotic cell death.

The molecular structure of the MPTP has not yet been fully determined, although cyclophilin D (Cyp D) has been shown to be essential to its function. Genetic ablation of Cyp D has been shown to result in delayed opening of the MPTP and resistance to myocardial damage after acute ischaemia-reperfusion injury. MPTP inhibition is cardioprotective, and may be achieved by a variety of means including ischaemic pre- and post-conditioning, and by pharmacological agents.

The aim of this thesis is to investigate the role of the MPTP (cyclophilin D) in cardioprotection from acute ischaemia-reperfusion injury.

In chapter 4 we investigate whether it is possible to protect the heart in mice deficient of cyclophilin D by applying a stronger ischaemic preconditioning stimulus to that previously investigated *in vivo.* We found that there was a

non-statistically significant trend towards a protective effect in cyclophilin D deficient mice which were subjected to a stronger IPC stimulus than that previously studied *in vivo.*

Chapter 5 explores whether it is possible to protect the heart whilst avoiding the deleterious extra-cardiac effects of cyclosporin A by sub-cellular targeting of cyclosporin A (CsA) to mitochondria in the first *in vivo* testing of a mitochondrial-specific form of CsA (mtCsA). We demonstrated that mtCsA administered at reperfusion did not protect the heart of wild type mice from ischaemia- reperfusion.

Chapter 6 examines the possibility of achieving cardioprotection independent of cyclophilin D. In this chapter, the effects of the matrix metalloproteinase inhibitor, Ilomastat, are studied. We confirmed that MMP inhibition was cardioprotective in wild type mice and also showed for the first time *in vivo* that administration of ilomastat can protect the heart from ischaemia-reperfusion in mice deficient in cyclophilin D.

In summary our data supports the hypothesis that it is possible to protect the heart from ischaemia – reperfusion in cyclophilin D deficiency by administration of an MMP inhibitor at reperfusion. We also showed a trend towards cardioprotection in cyclophilin D deficiency by administration of an increased IPC stimulus. Together, these data may suggest that necrotic cell death may not all be mediated by the MPTP, or possibly, that the MPTP may exhibit a threshold effect which is surmountable by an increase in inhibitory action of combined pharmacology or increase in IPC stimulus.

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List of abbreviations

The following is a list of abbreviations used in this thesis

AAR Area at risk

ACS Acute coronary syndrome

ADP Adenosine Diphosphate

ANT Adenine Nucleoside Translocase

ATP Adenosine Triphosphate

Bcl-2 B cell lymphoma- 2

CsA Cyclosporin A

Cyp D Cyclophilin D

DNA Deoxyribonucleic Acid

ECG Electrocardiogram

ECM Extra cellular matrix

IHD Ischaemic Heart Disease

IM Intramuscular

IP Intraperitoneal

IPC Ischaemic preconditioning

IPOC Ischaemic postconditioning

IS Infarct size

LAD Left Anterior Descending Coronary Artery

LRI Lethal Reperfusion Injury

LV Left ventricle

STEMI ST elevation myocardial infarction

MMP Matrix metalloproteinase

MPTP Mitochondrial Permeability Transition Pore

mtCsA Mitochondrial targeted cyclosporin A

PCR Polymerase Chain Reaction

RISK Reperfusion Injury Salvage Kinase

ROS Reactive Oxygen Species

RPM Revolutions per minute

SWOP Second window of protection

TAC trans-aortic constriction

TAE Tris Acetate EDTA

TIMP Tissue inhibitor of metalloproteinase

TPP Triphenyl phosphonium

TTC 2,3,5 Triphenyltetrazolium chloride

VDAC Voltage dependent anion channel

WMSI Wall motion score index

Chapter 1-Introduction

1.1 Epidemiology of Acute Myocardial Infarction

Ischaemic heart disease (IHD) is the leading cause of morbidity and mortality worldwide (1). It has previously been demonstrated that after coronary occlusion occurs, survival is directly related to the extent of cardiac muscle loss (2). The most important factor in salvaging myocardium is the restoration of flow to the affected coronary artery territory (reperfusion) (3-5). This may be achieved by mechanical (primary percutaneous coronary intervention) or pharmacological means (thrombolysis). The principal determinant of infarct size is the duration of ischaemia (6,7) and thus one of the major targets of treatment of myocardial infarction is to reduce the time to reperfusion after the onset of arterial occlusion. However, there is a “ceiling” effect in terms of reducing time to reperfusion: progress may be limited by a variety of factors which may be strategic (ie related to transport and treatment delays) or patient related (delay in symptom recognition before the call to help). Inevitably, even despite optimal treatment, some degree of cardiac damage does occur and the six month mortality rate of up to 12% reflects this (8). This high mortality rate is the driver behind the search to find interventions which may reduce infarct size further. In investigating this, it is important to understand the pathological processes involved in the myocardial cell death which occurs in the reperfused acute myocardial infarction.

1.2 Physiological changes in Acute Myocardial Infarction

Acute myocardial infarction is caused by the rupture of an atherosclerotic plaque, subsequent platelet activation and formation of occlusive thrombus in the affected coronary artery. This in turn results in cessation of delivery of oxygenated blood to the myocardium and the tissue becomes ischaemic. However the extent of cellular necrosis is not fully determined during the period of index ischaemia in the reperfused myocardium. Jennings in 1960 (9), first reported the pathological changes which take place after reperfusion of the circumflex artery in a canine model, describing what later became known as “lethal reperfusion injury”.

Although opening the occluded artery is essential to salvaging viable myocardium, the process of reperfusion itself leads to a variety of changes which take place at the cellular level which are deleterious to the myocardium. In fact, reperfusion itself accelerates the process of myocardial necrosis. This has been debated in the literature (10) - but the finding that interventions given at the time of reperfusion can reduce infarct size by up to 40%) (11-15), supports the theory that reperfusion itself creates a degree of myocardial damage, with the clear implication that tissue is “salvageable” during this time. This rapid process of cardiac damage is referred to as “Lethal Reperfusion Injury” (LRI), and it is defined as “injury caused by restoration of blood flow after an ischaemic episode leading to death of cells which were only reversibly injured during the preceding ischaemic episode”(16)**.**

1.3 The Effect of Ischaemia on the Myocardium

To fully understand lethal reperfusion injury, it is imperative to appreciate the effect of ischaemia itself on the myocardium.

The cessation of antegrade coronary flow results in a variety of changes at the cellular level- as a result of tissue hypoxia (due to lack of delivery of oxygenated blood) and failure of the nutrient (adenosine triphosphate (ATP) supply. The immediate effect of tissue hypoxia is failure of oxidative phosphorylation, with resultant failure of aerobic synthesis of ATP. Adenosine diphosphate (ADP), inorganic phosphate and reduced cofactors and coenzymes (eg NADH) then accumulate. Metabolism then switches to anaerobic glycolysis. This results in acidosis, both from lactate production and generation of hydrogen ions. The overall result is a rise in intracellular potassium and sodium and over-riding acidosis due to lactate production, reduced CO_2 efflux, and failure of the ATP driven H^+ pumps (17).

If the ischaemic insult persists beyond a critical duration, then the damage sustained becomes irreversible. This does not happen uniformly within the myocardium, but is incremental throughout the period of ischaemia beginning in the subendocardium and progressing to the epicardium (7). This was first described by Reimer and Jennings and this phenomenon is known as the “wavefront of cell death”.

The processes resulting in this cell death are described in brief overleaf.

The hallmark of irreversible damage is the loss of membrane integrity. This is the result of increased intra-cellular osmolarity creating an osmotic gradient, drawing water into the cell and causing it to become oedematous.

The cytosolic calcium concentration also rises during ischaemia. The sarcoplasmic reticulum cannot take up this redistributed calcium as there is inadequate phosphorylation of ATP. This results in increased calcium release further exacerbating the problem.

During ischaemia toxic lipid metabolites accumulate – eg acetyl- CoA, acyl CoA. Activation of phospholipase A2 causes breakdown of the phospholipid membrane and release of harmful free fatty acids some of which exert a direct toxic effect (18). Free fatty acids also promote generation of reactive oxygen species (ROS), by uncoupling oxidative phosphorylation.

Reactive oxygen species (ROS) are a group of compounds which, as their collective title suggests are a group of highly reactive oxygen derived free radicals or free radical precursors. If the production of reactive oxygen species exceed the capacity of cellular scavengers to remove them oxidative stress results. This has a direct toxic effect on the cell (19).

1.4 Lethal Reperfusion Injury

At the time of reperfusion there is a dramatic and rapid alteration in the cellular physiology. These changes are complex, and involve interaction of a number of harmful processes.

1.4.1 Restoration of physiological pH

Firstly, there is a rapid restoration of physiological pH. This occurs because lactate is washed out of the cell, and there is activation of the Na/HCO_3 symporter and Na/H exchange (20). The rise in pH which occurs is cardiotoxic, causing opening of the MPTP, hypercontracture of the cell and cell death (20). Studies have shown that perfusion of post-ischaemic hearts with acidic buffer reduces myocardial damage (22) in animal models, but this has not been borne out in clinical trials of eniporide, an Na^+/H^+ exchange inhibitor given at reperfusion in the setting of acute myocardial infarction (23).

1.4.2 ROS generation

Reoxygenation after an ischaemic insult to the myocardium has been shown to be directly harmful (24). It causes a degree of injury which is greater than the injury caused by ischaemia alone, and this has been referred to as “the oxygen paradox” (11,25,26). The mechanism for this is thought to be damage to mitochondria, which prohibits efficient transfer of electrons by complexes I and III of the electron transport chain, generating a huge burst of mitochondrial derived ROS, causing oxidative stress and resultant myocardial damage (27,28).

An additional effect of oxidative stress is that it limits availability of nitric oxide which itself has a cardioprotective effect (11).

Although it would appear that the role of ROS in myocardial damage seems undisputed, the literature is conflicted. There are several studies which appear to show benefit from treatment with ROS scavengers (29-31). In contrast, there are also a number of studies which appear to refute this (32,33).

It is clear that ROS have been shown to stimulate opening of the MPTP (34), and thus are likely to play some role in lethal reperfusion injury, but pharmacological modulation of this in humans is difficult due to the very short therapeutic window required to achieve a useful clinical effect and the inability to achieve sufficient levels of the antioxidant in the cardiomyocyte.

1.4.3 Rise in intracellular calcium

The accumulation of intracellular calcium which begins during ischaemia is further exacerbated by reperfusion. During ischaemia, anaerobic metabolism

is the source of ATP synthesis, and this produces lactate, acidifying the cytosol. In order to try to maintain physiological pH, the sarcolemmal Na^+/H^+ exchange mechanism is activated. The sodium ions are then exchanged for calcium ions by the Na^+/ Ca^{2+} exchanger (35). At the time of reperfusion, there is a rapid escalation in intracellular calcium content- this is due to damage to the sarcolemmal membrane and also dysfunction of the sarcoplasmic reticulum (11). A maladaptive response then occurs, whereby the calcium is taken up by the mitochondrial Ca^{2+} uniporter, driving calcium into the mitochondrial matrix (36). This, in turn, stimulates opening of the MPTP.

1.4.4 Rise in inorganic phosphates (Pi)

During the injurious ischaemic episode, inorganic phosphates accumulate within the mitochondrion. This is largely caused by a drop in the mitochondrial pH level, which leads inorganic phosphate to be taken up into mitochondria. This is a potent MPTP stimulant (202).

1.5 The Mitochondrial Permeability Transition Pore

1.5.1 What is the MPTP?

The mitochondrial permeability pore (MPTP) is a non-specific channel located in the inner mitochondrial membrane, which was first discovered by Hunter and Haworth in the 1970s (37-39). Its critical involvement in tissue damage occurring during myocardial ischaemia / reperfusion was revealed in the 1980s by Crompton (40-42). Under physiological conditions the pore remains closed. During ischaemia, due to inhibition by cellular acidosis, it remains

quiescent (43,44). The pore is sensitive to a number of stimuli as discussed above- alterations in calcium concentration, generation of reactive oxygen species, restoration of physiological pH and the presence of inorganic phosphate (45), conditions which are created within a few moments of reperfusion. When the pore opens it becomes freely permeable to solutes of up to 1.5 kDa including protons (38,46). The crossing of protons results in a rapid dissipation of the electrical gradient across the membrane and uncoupling of oxidative phosphorylation (failure of ATP generation) and also leads to reversal of the proton-translocating ATPase causing hydrolysis of ATP leading to profound ATP depletion (47). If the pore remains open, this will lead to rapidly advancing cell death by necrosis. At the same time, as proteins are unable to cross the membrane, an osmotic gradient is also generated between the cytosol and matrix, which causes the mitochondria to swell, rupturing the outer mitochondrial membrane and releasing cytochrome C (48). Cytochrome C then associates with apoptosis inducing factor and caspase-9, which induces the release of caspase-3 and onset of apoptosis. The pro-apoptotic members of the Bcl-2 (B-cell lymphoma-2) family are also upregulated and contribute to apoptotic cell death in this setting (223).

Importantly, the MPTP remains closed during myocardial ischaemia and opens only during the first few minutes of reperfusion (44), making it an ideal target for therapeutic manipulation in the setting of myocardial infarction.

1.5.2 Components of the MPTP

Despite much research, the precise structure of the MPTP remains elusive. Its function was initially thought to be the result of the interaction between a

number of subunits: namely cyclophilin D (Cyp D), the voltage dependent anion channel (VDAC) and adenine nucleotide translocase (ANT). Genetic studies have revealed that VDAC and the ANT play a non-essential role in pore function. The role of each, and the evidence for their involvement is detailed below.

1.5.2 Cyclophilin D

Cyclophilins are a group of proteins whose function is to catalyse the *cis-trans* isomerisation of peptidyl-prolyl bonds- hence being referred to as PPIases (peptidylprolyl *cis-trans* isomerases). Cyclophilin D, the mitochondrial isoform, is an 18kDa protein, and is located in the mitochondrial matrix (49). Its role in function of the MPTP was discovered after administration of cyclosporin A was found to be a potent inhibitor of the MPTP (50). Its identification as a PPIase took place shortly afterwards, by Halestrap's group, who observed that cyclosporin and its analogues inhibited a matrix peptidyl-prolyl *cis-trans* isomerase (PPIase) and subsequently purified it from rat livers, identifying it as cyclophilin D (51-53). Work by Baines confirmed that cyclophilin D was an essential component of the MPTP (13).

Ablation of the mouse *Ppif* gene which encodes cyclophilin D has enabled further understanding of its role in ischaemia-reperfusion injury (IRI). Mitochondria isolated from mice deficient in this gene have been shown to be resistant to the harmful effects of noxious stimuli including calcium overload and oxidative stress induced cell death (13), both of which are known to promote opening of the MPTP. Baines group also confirmed that MPTP opening was delayed in *Ppif* null mice and that they were protected to some

degree from IRI, with a significant reduction in the extent of infarction in comparison to wild type controls (13,54). Treatment with agents known to inhibit cyclophilins – Debio-25 (55), sanglifehrin A (56) have also been shown to inhibit the MPTP to a similar degree as *Ppif null* animals (57). As cyclophilin D is the binding site responsible for reduction in infarct size seen after ischaemia-reperfusion, these mice exhibit smaller infarct sizes and do not exhibit a further reduction in infarct size after administration of cyclosporin A (12).

1.5.3 Voltage Dependent Anion Channel (VDAC)

The voltage dependent anion channel (otherwise known as porin) is located in the outer mitochondrial membrane of all eukaryotic organisms (58). Mammals have three isoforms (VDAC- 1, 2 and 3). VDAC was formerly considered to be essential for function of the MPTP. In its closed state it is permeable to solutes of up to 1.5 kDa (as is the open MPTP), and when open it allows solutes of molecular weight of up to 5kDa to pass. However, if VDAC *was* an essential part of the MPTP it would be expected that closure of VDAC would result in closure of the MPTP, protecting the mitochondrion from calcium influx- but, as noted above the closed state of VDAC allows the same size of molecules to pass as the open MPTP. In fact the closed VDAC increases calcium influx into mitochondria, which would conversely result in MPTP opening (59). Initial data suggested that use of VDAC inhibitors also inhibited MPTP action, but these inhibitors were relatively non-selective in their site of action (60). Further data, in isolated mitochondria from mice lacking VDAC -1 has shown normal MPTP function, with inhibition by CsA (61). Experimental data by Baines et al, has demonstrated that cardiac fibroblasts lacking all three isoforms continue

to display MPTP activity in response to calcium and oxidative stress. Additionally, administration of cyclosporin A abolished this activity (62), refuting the previous suggestion that VDAC was a necessary component of the MPTP. It is more likely that VDAC instead plays a role in targeting of protective proteins such as Akt and protein kinase C.

1.5 4 Adenine Nucleotide Translocase

The adenine nucleotide translocase (ANT) is a small protein (approximately 300 amino acids long) which is located in the inner mitochondrial membrane. There are four isoforms of translocase, ANT-1and ANT-2, both of which are present in rodents, and ANT-3, which is present in humans but not rodents (63), and ANT-4 which is found in murine germ cells (64). The function of the translocase is to shuttle ATP and ADP across the inner mitochondrial membrane. Clues to its involvement in the function of the MPTP are that ATP and ADP directly inhibit the MPTP, but other nucleotides such as AMP, GDP and GTP, which don't pass via the ANT do not. The ANT has two conformations – C (cytosolic) and M (matrix). Bongkreic acid shifts the ANT to the M conformation, whilst atractyloside shifts it to the C conformation. Bongkreic acid has been shown to block the calcium induced MPTP (52), whilst atractyloside sensitises the MPTP response to calcium (65) in isolated mitochondria. This would be in keeping with a change in conformation of the ANT, from its resting state into the pore-forming cytosolic type at the time of reperfusion. In further support of this, studies in isolated cardiomyocytes have shown that bongkreic acid protects against oxidative stress (66), and atractyloside blocks the protective effect of nitric oxide donors (67). Additionally, ANT-1 interacts with cyclophilin D at contact sites between the

inner and outer mitochondrial membranes- this is where the pore is thought to be located (68), implicating ANT-1 in MPTP function.

Despite the evidence supporting its involvement in the MPTP, ANT is not thought to be an essential component. The fact that ANT-1, but not ANT-2 interacts with cyclophilin is one of the arguments against it being an essential component: the MPTP is found in most organs, yet ANT-1 is only found in striated muscle. Knock out studies have also drawn the exact role of ANT in pore function into question. Mice with both ANT-1 and ANT-2 knocked out (ie -/-) exhibit a typical pattern of calcium dependent pore action, and cyclosporin inhibits this (69). The calcium threshold for pore opening in the double knock-out was elevated, which does suggest some degree of ANT involvement, but this data demonstrates conclusively that the ANT does not play an *essential* role in function of the MPTP. It is, however, considered to play a regulatory role in pore function.

1.5.5 The mitochondrial phosphate carrier

Recent data has suggested that the mitochondrial phosphate carrier may regulate MPTP opening although the data is far from conclusive.

Data presented by Leung et al, shows that the phosphate carrier binds to cyclophilin D in a cyclosporin sensitive manner, and is also associated with the ANT, features which are suggestive of involvement in pore function (70). Its role has not yet been clearly established in pore function.

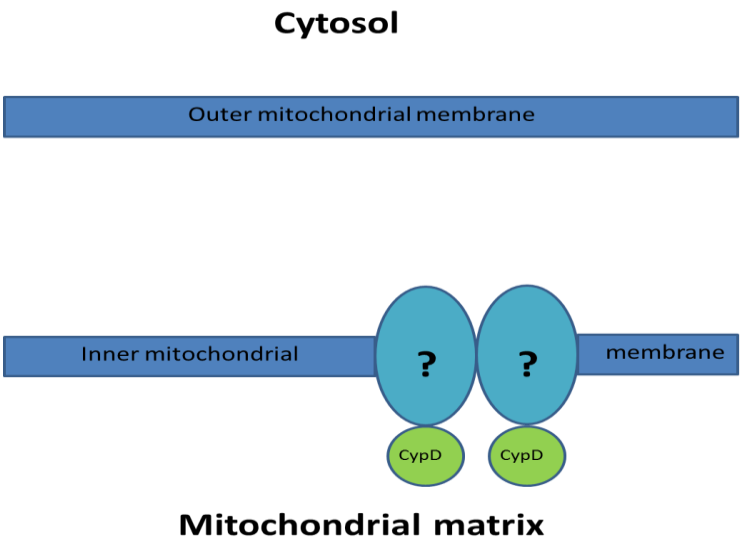


Figure 1.1 Schematic diagram of proposed structure of the MPTP

1.6 Biological function of the MPTP

The precise biological function of the MPTP remains unclear. Although genetic knock out studies initially suggested that loss of cyclophilin D exerts a positive effect on the myocardium, by protecting against ischaemia-reperfusion injury, data is emerging which suggests that genetic ablation of CypD has a long-term maladaptive effect. Elrod *et al* presented a study which explored the role of cyclophilin D in the heart, and found that animals deficient in cyclophilin D were unable to increase myocardial contractility in comparison to wild type controls after administration of the β-adrenergic agonist isoproterenol. After exposure to trans- aortic constriction (TAC), the CypD deficient animals developed severe left ventricular hypertrophy and cardiac fibrosis, with animals of this genotype showing increased propensity to develop heart failure when faced with mechanical pressure overload. This was also found when exercise in the form of swimming was used to assess the effect of physiological (exercise induced) hypertrophy – the CypD -/- genotype

had a significantly higher mortality from drowning due to fatigue. Histology showed a greater degree of hypertrophy and pulmonary oedema than their wild type counterparts. Further investigations showed that cardiac mitochondrial calcium content was elevated in mice deficient of cyclophilin D, and that there was an increase in the calcium threshold for MPTP opening. Cyclosporin A was seen to increase calcium efflux from mitochondria. Altered substrate utilisation was also noted, with increased levels of dehydrogenase activity with a net result of increased glucose in this series of experiments (71). Elrod's paper thus provides an important insight into the potential role of the MPTP in regulation of calcium metabolism and response to haemodynamic stress.

The MPTP does appear to have more than one role, however, mediating necrotic cell death after reperfusion of an ischaemic organ, and also in mediating protection by preconditioning and postconditioning .

The protection conferred by ischaemic preconditioning (discussed later in this chapter) is related to opening of the MPTP. In contrast to lethal reperfusion injury, IPC may be mediated by a transient opening of the MPTP, whereas in necrotic cell death, this is prolonged. This transient MPTP opening has been shown to be an essential part of IPC induced cardioprotection (72). Administration of MPTP blocking agents during preconditioning completely abolishes any protective effect. This effect is thought to result from mitochondrial uncoupling and subsequent reduction in calcium loading of mitochondria and increased mitochondrial ROS signalling (72).

1.7 Methods of cardioprotection: MPTP Inhibition

Infarct size is the strongest determinant of prognosis after acute myocardial infarction, and thus a wealth of research has been focussed on interventions to reduce infarct size. There is a degree of myocardial necrosis which is determined by the duration of ischaemia, but at the time of reperfusion, a substantial amount of damage occurs in tissue, which until then was salvageable - lethal reperfusion injury. The MPTP plays a major role in this cell death, and therefore interventions which inhibit its function have been the focus of much research.

Cardioprotective strategies such as ischaemic preconditioning (15,73) and postconditioning (74) (discussed in more detail below) have been shown to inhibit opening of the MPTP, and reduce cell death after ischaemia-reperfusion. In addition to this, drugs which inhibit MPTP function (75) have also been studied in detail and these are also discussed below.

1.8 Mechanical methods of cardioprotection

1.8.1 Ischaemic Preconditioning (IPC)

Ischaemic preconditioning is a phenomenon whereby the application of a period of non-lethal ischaemia to an organ prior to a sustained injurious ischaemic insult results in protection from necrotic cell death (76).

Murry et al in 1986 first reported in a canine model that brief periods of sublethal ischaemia administered to the circumflex artery before the onset of a prolonged ischaemic insult to the same vessel could protect against myocardial damage measured at four days post-reperfusion (76). These findings have been supported subsequently by a number of confirmatory

studies in a variety of animal models (54,77). Human cellular work using IPC in isolated cardiomyocytes (78) and superfused human myocardium (79) has also been successful in reducing cellular damage after an ischaemic insult.

There appear to be two periods during which the heart is maintained in a "preconditioned" state- the initial period, within 1-2 hours of the preconditioning stimulus (80), and a later "second window" of protection (SWOP), where the cardioprotective effect has been shown to recur 24 hours later and last for 2-3 days- described by some researchers as "delayed" or "late" IPC.

The mechanisms involved in IPC still remain elusive despite much research, but the signalling mechanisms involved are being discovered slowly over time.

The IPC stimulus causes release of a series of autacoids (eg bradykinin (81), adenosine (82) and opioids (83)) from the organ being preconditioned. Blockade of any one of these agents abolishes the protective effect of IPC. These autacoids are all thought to act in parallel with one another and act via G- protein coupled receptors.

More than one pathway for the protection of IPC has been proposed Inhibition of protein kinase C (PKC) has been shown to block the protective effect of all three substances (84-86), leading to the conclusion that PKC is a downstream point of convergence for the action of autacoids.

The autacoids also act via G protein-coupled receptors to trigger activation of signal transduction pathways involving PI3K- Akt- e NOS, Erk 1 /2, p38, JNK MAPK, JAK-STAT3, PKC/PKG (the signalling kinases) which act on downstream effectors such as the mitochondrial potassium- ATP channel,

MPTP, and ROS. These are referred to as pro-survival kinases. These then inhibit the MPTP (87), by a mechanism which to date is currently unknown.

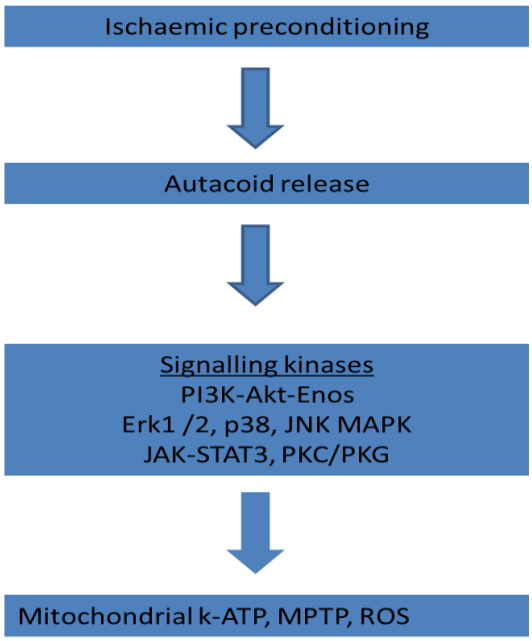


Figure 1.2 Simplified diagram of pathway resulting in the protective effect of ischaemic preconditioning.

Postconditioning achieves protection by similar means (87,88), and both pre- and postconditioning act via pathways which are convergent at the point of reperfusion- this is collectively referred to as the reperfusion injury salvage kinase (RISK) pathway (89).

A further pathway has also been found – which is referred to as the survival activating factor enhancement (SAFE) pathway (90). This has been reported to involve TNF- α activation, Signal Transducer and Activator of Transcription 3 (STAT3) and its actions are independent of the RISK pathway (91).

At the time of reperfusion after a sustained ischaemic insult, the MPTP is known to open. This is a sustained “high-conductance” opening, and this

event is a critical mediator of lethal reperfusion injury. However, the MPTP can open transiently, (as in IPC) and this can affect calcium efflux and mitochondrial calcium load (92). It has also been shown that transient pore opening can mediate mitochondrial ROS release (93) and may be triggered by mitochondrial uncoupling (94). These findings prompted further study, by Hausenloy et al, in order to determine whether transient opening of the MPTP could be a primary mediator of the cardioprotection afforded by IPC. The findings of this study in a Langendorff model of ischaemia-reperfusion confirmed that the cardioprotection of IPC, diazoxide and mitochondrial uncoupling were dependent on both MPTP opening and reactive oxygen species (72). This placed the MPTP in a crucial role for both IPC and lethal reperfusion injury.

This role has been confirmed in experimental data showing that administration of MPTP inhibitors during the IPC stimulus abolishes generation of mitochondrial ROS (95) and also cardioprotection (72). Additionally, mice deficient in cyclophilin D, an essential component of the MPTP do not appear to be protected after ischaemic preconditioning (54). Hausenloy et al confirmed that the protective effect of IPC was mediated by MPTP inhibition (15).

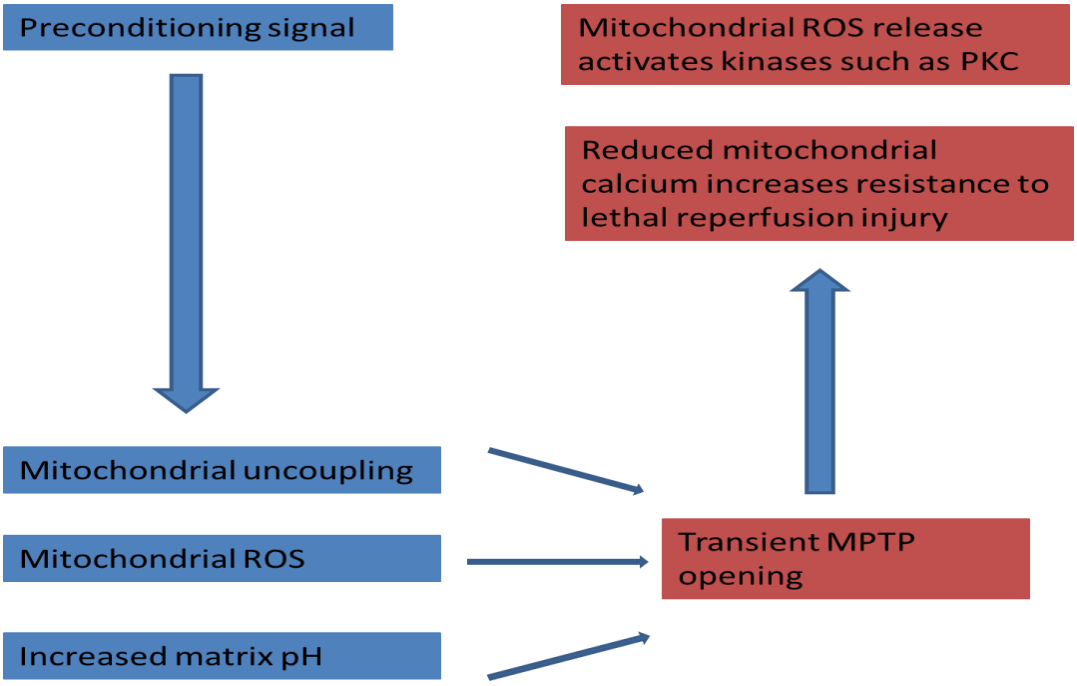


Figure 1.3 Simplified diagram of the role of transient MPTP opening as a mediator of the cardioprotection of IPC (reproduced with permission, Hausenloy *et al* (203)

The benefit of IPC in reduction of cardiac damage after sustained ischaemia countered the previous widely held belief that infarct size was fully determined during ischaemia and held huge potentially beneficial implications for the reduction in cellular injury sustained during an ischaemic event.

Although of great scientific importance, this data had one large drawback in terms of direct clinical application in treatment of acute myocardial infarction (AMI) –primarily because in the real world setting, the onset of myocardial infarction is usually unprecedented. However this method does have a potential role in situations where the onset of ischaemia is predictable- in vascular (96,97), gastro-intestinal (98), cardiac (99) and transplant surgery.

1.8.2 Ischaemic Postconditioning (IPOC)

In 2003, Zhao demonstrated in a canine model of reperfused myocardial infarction (with the LAD as the target vessel) that the application of three brief periods of ischaemia – reperfusion as a postconditioning stimulus immediately after reperfusion resulted in a reduction in infarct size measured at three hours (100). This data was supported in a variety of models (101) and was welcomed, as it lent new promise to future therapy for STEMI. In fact, after investigation in small numbers of patients, the use of ischaemic postconditioning (by inflating an angioplasty balloon for brief periods after restoration of coronary flow) in the catheter laboratory has been subsequently shown to reduce markers of myocardial injury, such as release of creatinine kinase and improve angiographic markers of reperfusion (blush grade) (102). However, the introduction of thrombectomy (manual aspiration of thrombus during treatment of acute myocardial infarction by primary angioplasty), has made it difficult to perform and assess the effect of postconditioning in the setting of primary angioplasty. To date no study of postconditioning has demonstrated a reduction in mortality. In addition, recent data, using myocardial salvage index, left ventricular ejection fraction, and infarct size by MRI as the outcome measures has been disappointing- with no improvement in any of these measures after three cycles of postconditioning in the setting of acute myocardial infarction (204). However, there is data utilising surrogate outcome measures, such as troponin and creatinine kinase release which do show benefit. To date the clinical trials have all had small patient numbers. There are also other studies, which are currently underway, such as the Danish Study of Optimal Acute Treatment of Patients with ST-elevation

Myocardial Infarction (DANAMI-3), which is currently recruiting, aiming for recruitment of 1000 participants, and is using magnetic resonance imaging to quantify infarct size out to three months after acute myocardial infarction.

1.8.3 Remote Ischaemic Preconditioning (RIPC)

The benefits of ischaemic conditioning may also be seen when a distant organ undergoes pre-emptive ischaemia. This is referred to as remote ischaemic preconditioning, and was initially described by Przyklenk who performed IPC in dogs and used the circumflex artery as the IPC stimulus, and the LAD as the vessel undergoing the prolonged ischaemic insult, with a significant reduction in infarct size (104). Perhaps this could be considered as “regional” IPC. However, this phenomenon has been shown in liver cells (105), and in the brain after limb ischaemia (106) and a reduction in renal and cardiac damage was observed after cross clamping the iliac arteries prior to repair of aortic aneurysm (107) – this has been termed remote IPC. Remote IPC has been shown to reduce troponin release in patients undergoing elective coronary intervention (108), surgery on children with congenital cardiac defects (109) and coronary artery bypass surgery (110). Recent data in humans has supported that inducing transient limb ischaemia by inflating a cuff at high pressure to a distant limb prior to complex cardiac surgery attenuates release of markers of myocardial damage (CK) and reduces length of intensive care stay (111).

Disappointingly, not all trials of RIPC have demonstrated beneficial effects (112,113) and none has translated to an improvement of survival. In fact, the most recent study investigating RIPC in patients undergoing high risk CABG

has shown an *increase* in the degree of myocardial injury in the group treated with RIPC (205).

It is clearly difficult to clearly define the benefit of the trials of interventions such as RIPC. There are a number of reasons for this. Firstly, the patient groups investigated are widely heterogeneous. Additionally, there has been no clinical trial carried out to determine the optimum conditioning protocol. It is possible that the protocol used widely at present – as described by Kharbanda et al (207), may be subtherapeutic in some groups of patients (206). Timing of RIPC stimulus in relation to the ischaemic episode is also critical, and varies amongst the clinical trials (208, 209). Anaesthetic agents used in the trials are also relevant. As discussed in chapter 3, the halothane anaesthetic agents have been shown to be cardioprotective in experimental infarction (180-182), and this may prevent any demonstration of protection. This is borne out by the fact that the studies showing a positive effect of RIPC have not used halothane anaesthesia (209, 210), and the studies showing no benefit have used the halothanes (208, 211). However, the issue remains far from clear.

Currently, the Effect of Remote Ischaemic Preconditioning on Clinical Outcomes in Patients Undergoing Coronary Artery Bypass Graft Surgery (ERICCA) study is being undertaken. This study is using a number of indicators to assess the potential benefit of IPC, such as mortality, major adverse cardiac events, troponin release and duration of stay in intensive care, amongst a variety of other indicators. The target for recruitment is over 1700 patients, which may permit demonstration of any potential treatment effect more clearly than previous small scale trials.

1.8.4 Remote Ischaemic Perconditioning

The term perconditioning refers to the application of an ischaemic stimulus to a remote organ after the onset of ischaemia and before reperfusion occurs. The most obvious potential clinical application of this is in the setting of acute myocardial infarction whereby inflation of a blood pressure cuff can be used as the stimulus, and can be applied before the patient even arrives at hospital. In the context of acute myocardial infarction (STEMI) trials have shown benefit in terms of myocardial salvage in patients with a large area of myocardium at risk, as measured a reduction in myocardial salvage index by SPECT (114), but the same study does not show any reduction in mortality or overall occurrence of heart failure. Thus far, there is no data which confirms an actual benefit in terms of overall improvement in left ventricular ejection fraction / heart failure / mortality after remote “perconditioning”. This is a major limitation of these trials and this is driven by the fact that the trials are not adequately powered to detect such differences. Subgroup analysis may suggest that in high- risk groups (ie those with an area at risk of over 35% of myocardium), left ventricular function may be improved by remote preconditioning (115). However, as with all subgroup analyses, these results must be interpreted with caution, and further studies are required to fully evaluate this method of cardioprotection in the setting of STEMI. On reviewing clinical trials underway in this regard, there are 8 studies described on www.clinicaltrials.gov ongoing at present to assess the effect of “conditioning” in acute myocardial infarction, reflecting the need for more data on this subject.

Author	Intervention	No of pts	Ischaemia duration	Timing	Outcome
Piot et al	Cyclosporin A[116]	58	< 5 h mean	Within 1 min of PPCI	Reduced CK release
Ma et al	Post-conditioning[103]	94	< 12 h	Within 1 min of PPCI	Reduced CK, improved TIMI-frame count & change in WMSI
Staat et al	Post-conditioning[102]	30	< 6 h	Within 1 min of PPCI	Reduced CK, Improved blush grade
Thibault et al	Post-conditioning[117]	38	< 6 h	Within 1 min of PPCI	Increased LV ejection fraction at 6 months
Laskey et al	Post-conditioning[118]	24	< 6 h	After flow wire	Improved CFR & ST- segment resolution
Yang et al	Post-conditioning[119]	41	< 6 h	30 secs	Reduced infarct size by SPECT, reduced CK
Thuny et al	Post-conditioning[222]	50	< 12 h	Within 1 Min of PPCI	Reduced infarct size at 48-72h post MI

Table 1.1 Successful conditioning treatments in humans with acute myocardial infarction treated by primary percutaneous coronary intervention

Key to abbreviations in Table 1.1:

h = hours, PCI = percutaneous coronary intervention, PPCI = primary percutaneous coronary intervention, CK = creatinine kinase, TIMI frame count = thrombolysis in myocardial infarction frame count, a measure of myocardial perfusion, WMSI = wall motion score index- a measure of left ventricular systolic function, LV = left ventricle, CFR = coronary flow reserve- a measure of microvascular function, ST- segment resolution = improvement in the

electrocardiogram indicative of reperfusion, SPECT = single photon emission computed tomography,

1.9 Non-mechanical cardioprotection

1.9.1 Pharmacological Cardioprotection

The role of pharmacological agents as conditioning-mimetics has been investigated extensively both in the laboratory and clinical setting of acute myocardial infarction. The earliest studies examined the role of inhibition of xanthine oxidase by allopurinol (120,121) in animal models. Further translational studies have been conducted with a myriad of agents including adenosine (122), erythropoietin (123,124) and lately, the most notable success is with the cyclophilin D inhibitor cyclosporin A (116), which is discussed in more detail in section 1.9.2.

A reduction in infarct size has been demonstrated by a variety of pharmacological agents known to inhibit the MPTP when administered at reperfusion. This includes the volatile anaesthetics (178-180) and cyclosporin A (116), amongst many others. The signalling cascades involved are the same as those recruited during ischaemic pre and post-conditioning (226).

1.9.2 Cyclosporin A

Cyclosporin A is an undecapeptide produced by the fungus *tolypocladium inflatum*. It was introduced clinically in the 1980s to combat rejection of transplanted organs in humans, and has been shown repeatedly to be partially cardioprotective in ischaemia-reperfusion injury (23-27). Its primary site of binding is cyclophilin D, which is known to be an essential component of the MPTP (125). It also inhibits the calcium- dependent phosphatase calcineurin, which is responsible for its suppressive effect on the immune system. The non-cardiac effects of this drug- nephrotoxicity, immunosuppression, potential cancer risk and very narrow therapeutic index (126) make it unattractive for ongoing therapeutic use. The benefit of cyclosporin A in reduction of myocardial damage in animal models has been well established[54], but in humans its use has been controversial (127,116)

Use of non-immunosuppressive (ie without calcineurin inhibition) cyclophilin D inhbitors (sanglifehrin A (54), Debio 025 (14), NIM811 (128)) in *in vivo* models of ischaemia-reperfusion injury also demonstrate significant cardioprotection, suggesting that cardioprotection is not mediated by calcineurin inhibition, but by inhibition of cyclophilin D.

However, calcineurin itself exerts an effect on the mitochondrion. Under normal resting conditions, mitochondria are maintained in a constant equilibrium of fusion and fission. Predominance of one or other states leads to either cell survival or cell death respectively. Calcineurin has been shown to be necessary for the process of mitochondrial fission, which is a well

described process in the death of this organelle (129). Dynamin- related protein-1 (Drp-1) is dephosphorylated in response to calcineurin activation, which occurs when the mitochondrion is exposed to a sustained rise in cytosolic calcium level and mitochondrial depolarisation, resulting in fission. Inhibition of the fission process by administration of the pharmacological Drp-1 inhibitor mitochondrial division inhibitor-1 (MDIVI-1) has been shown to delay MPTP opening and protect the heart from ischaemia-reperfusion injury (130). It is possible therefore, that cyclosporin A protects by both inhibition of calcineurin and cyclophilin D.

Cyclosporin A does remain a promising therapy in myocardial infarction, but at the price of its renal and immunosuppressive effect. One large scale clinical trial is currently recruiting patients with acute myocardial infarction to determine its effects when administered at reperfusion- the pilot data from this group suggests that there are potentially significant benefits to be seen in terms of infarct size reduction (116). The calcineurin- related effects could potentially be reduced by subcellular drug targeting. This is discussed in more detail in the next section.

1.10 Targeting drug to site of effect

In this thesis, we aim to explore the role of the MPTP in cardioprotection from ischaemia-reperfusion injury, and in this section, we discuss the mitochondrial-specific targeting of a drug known to inhibit cyclophilin D, the main component of the MPTP.

The use of therapeutic drugs is often substantially limited by the inability to access the site of action in adequate concentration to achieve its desired clinical effect. In the specific case of ischaemia-reperfusion injury, the most attractive target is not determined at organ or cellular level, but at a subcellular level. The organelle at the centre of the pathological changes is the mitochondrion, the function of which is a critical determinant of cell death after ischaemia-reperfusion (57). As such, this represents the major therapeutic target.

An ideal drug for cardioprotection therefore, would be one which maintains the cardioprotective effect of cyclophilin D blockade, whilst avoiding the deleterious effects of inhibition of extra mitochondrial cyclophilins and calcineurin.

1.10.1 Nanocarriers

By modifying a drug to facilitate its access to its site of action, one of the primary concerns is the maintenance of its therapeutic effect, which may be compromised by conjugating the drug to other molecules. The aim is that modifications would increase the treatment effect, but in trying to do so, it is possible that the drug could be rendered metabolically inactive. A group of

compounds known as nanocarriers have been developed which enable facilitated uptake of pharmacological agents to their target sites.

To gain selective access to mitochondria molecules require to be "mitochondriotropic"- ie they must be able to accumulate within the inner mitochondrial membrane despite its high membrane potential. Nanocarriers have been shown previously to enable specific uptake of drugs to mitochondria (131). A variety of molecules may be used for this purpose. Amongst these is the triphenylphosphonium cation.

1.10.2 The Triphenyl- phosphonium Cation

The triphenyl- phosphonium (TPP) cation was initially utilised as a method of exploring the electrical properties of phospholipid bilayers in organic chemistry. Latter years have shown these molecules to be invaluable in the investigation of mitochondrial function, with particular respect to measurement of the mitochondrial membrane potential (214).

In addition these molecules enable confirmation of mitochondrial localisation of labelled substances and facilitate accumulation of bioactive molecules and drugs within mitochondria. There are a number of examples of this in the literature (215).

In an attempt to selectively utilise the desirable aspects of cyclosporin A (inhibition of cyclophilin D), whilst potentially suppressing its deleterious effects (calcineurin and extra-mitochondrial cyclophilin inhibition), a unique mitochondrial- targeted cyclosporin A molecule (mtCsA) was developed by Professor David Selwood at University College, London.

This method of producing “mitochondriotropic” drugs is well described in the literature (132-134). The lipophilic triphenyl-phosphonium molecule is commonly used for this purpose as it can be conjugated to therapeutic agents and is effectively electrophoresed into the negatively charged mitochondria by utilising the membrane potential.

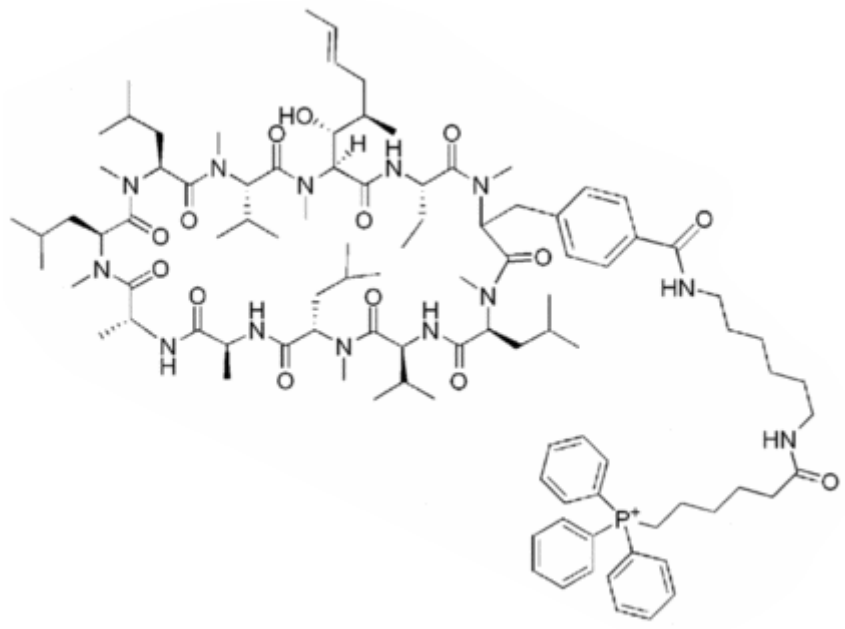


Figure 1.4 The triphenyl-phosphonium cation.

The mtCsA molecule was tested and found to inhibit cyclophilin D inside mitochondria preferentially over extra-mitochondrial cyclophilin A in an *in vitro* model. It was also seen to reduce cell death in hippocampal neurons which were exposed to oxygen and glucose deprivation- ie energy failure (135). The molecule could potentially have significant benefits *in vivo* in reducing myocardial damage in ischaemia-reperfusion, with the benefit of having a more selective site of action, avoiding calcineurin mediated effects and also allowing dose reduction due to increased selectivity.

The potential benefits in the clinical setting of acute myocardial infarction are obvious- delivery of a more efficacious drug to a specific site of action reducing the required dose and potential side effects, whilst maximising the degree of cardioprotection.

In chapter 5 of this thesis we set out in the first *in vivo* study of mitochondrial targeted cyclosporin A to test the hypothesis that its administration in an *in vivo* murine model of ischaemia-reperfusion will be cardioprotective.

1.11 Non-cyclophilin- D dependent methods of cardioprotection

The aim of this thesis is to investigate the role of the MPTP (cyclophilin D) in the myocardial damage which occurs after ischaemia-reperfusion. The focus of the introduction so far has been on interventions which aim to prevent or delay pore opening in order to reduce cardiac injury.

Cyclophilin D ablation is known to provide partial protection from ischaemia-reperfusion injury. However, a degree of cardiac damage still occurs, both in CypD knock- out mice exposed to I-R injury (13,54), and in wild type mice treated with MPTP inhibitors (12,14,136). This raises the possibility that there may be another pathway which results in cell death in this setting.

Recent evidence appears to support this theory. Roubille *et al* has published data which counters previous belief that it is not possible to protect the heart by ischaemic postconditioning after the first few minutes of reperfusion after an ischaemic insult, resulting in myocardial damage which is irreversible in the

absence of therapeutic intervention during this time (137). This paper shows that it is possible to achieve cardioprotection using a protocol of postconditioning applied at a period of up to *30 minutes* after reperfusion in an *in vivo* mouse model. This suggests that the time window during which cardioprotection can be achieved is substantially longer than was previously believed (138). The implication of this is that the cardioprotection in this setting may *not* all be MPTP mediated.

As discussed previously, the MPTP remains closed during ischaemia, but opens in the first few minutes of reperfusion (44), and most of the data published previously has suggested that after this time, the myocardium is not salvageable (137,139). However, experimental protocols investigating postconditioning exhibit widespread variations not only in timing of the IPOC stimulus, but also duration of index ischaemia and species. This, coupled with the strong bias of learned journals to report only positive data make it difficult to clearly establish protocols which have been previously tested (140).

This data provides an interesting challenge to what has been regarded to be a widely held belief that all necrotic cell death is pore mediated.

1.11.2 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are neutral zinc endopeptidases which are present throughout the body. They were first discovered approximately 40 years ago (141) and play an essential role in regulation of the continual process of degradation and synthesis of collagen within the extracellular matrix as part of an ongoing homeostatic process. MMPs possess the ability to degrade all components of the extracellular matrix. To date, 28 MMPs have been confirmed. They are grouped according to their proteolytic functions- eg gelatinases (MMP 2 and 9), collagenases (MMP 1, 8, 13), matrilysins (MMP 3, 10, 11), metalloelastases (MMP 12), stromelysins (MMP 7, 26) and membrane type MMPs (MMP 1-8).

The structure of MMPs is varied, although they possess similar characteristics. They are released as inactive zymogens and have a pro-peptide attached which protects the zinc activation site. The pro-peptide (which is highly conserved amongst the MMPs) is cleaved off by a variety of stimuli to reveal the active binding site and enable binding of the zinc ion. It has been shown that MMPs are involved in a number of pathological conditions- which involve inflammatory processes- rheumatoid arthritis (142), heart failure, cancer (143,144) and left ventricular remodelling in the aftermath of myocardial infarction (145-147).

MMP 2 and 9, the gelatinases, are capable of degrading gelatins and type IV collagen in basement membranes. They are both highly expressed within human myocardium (148).

MMPs are activated early in the time course of myocardial infarction- this has been confirmed in MMP 1 (absent in rodents (149)), 2 and 9 in both the area at risk and the remote area (150). Additionally, in an animal model of acute myocardial infarction, upregulation of matrix metalloproteinase activity has been shown to occur as early as 10 minutes after the onset of coronary occlusion (147). Within one minute of reperfusion in an ex vivo system, it has been shown that MMP -2 is upregulated, and is strongly correlated to mechanical dysfunction. In the same group of experiments, treatment with a known inhibitor of MMP activity (doxycycline) was shown to reverse this effect, demonstrating recovery of contractility (151). This raises important questions about the role of MMPs in ischaemia- reperfusion injury.

Preconditioning has also been shown to modify MMP activity (152,153), and in an hyperlipidaemic rat model, where the benefits of IPC are attenuated, administration of an inhibitor of MMPs has been shown to result in significant cardioprotection (154), which suggests that blocking MMP activity results in protection by a means which is disparate to that of IPC, and that these mechanisms may potentially be additive.

1.11.2.1 Regulation of Matrix Metalloproteinases

Transcription

MMPs are controlled on three main levels- transcription, activation of latent proenzyme and inhibition by TIMPs (tissue inhibitor of metalloproteinases).

A wide variety of stimuli have been shown to increase the synthesis of MMPs. This includes IL-1, IL-6, TNF- α, epidermal growth factor, platelet derived growth factor and CD40 (155).

1.11.2.2 Tissue Inhibitor of Metalloproteinases

Four TIMPs have been discovered in humans (TIMP1-4). They are the predominant method of intracellular regulation of matrix metalloproteinase activity. Each form of TIMP is encoded by a single gene. Under normal circumstances, TIMPs and MMPs act to maintain a constant state of equilibrium within the extra cellular matrix of the myocardium (156).

Dysregulation of this homeostatic mechanism –by upregulation of MMP activity, or downregulation of TIMP activity results in adverse remodelling within the extracellular matrix of the myocardium and causes disruption of the collagenous architecture.

Blockade of the matrix metalloproteinase system has been shown to reduce hepatic damage after ischaemia-reperfusion (157). Inhibition of MMPs has also been to reduce infarct size to a similar degree as that observed after preconditioning (158).

1.11.3 Pharmacological Inhibition of Matrix Metallproteinases

MMPs may be inhibited in a variety of ways. It is known that α-2 macroglobulin is a non – selective irreversible inhibitor, and has been described as the major plasma inhibitor of MMPs (159). Heparin is also known to be an exogenous inhibitor (160).

An extensive list of synthetic, so-called small molecule inhibitors of MMPs have been developed, but clinical trials of the MMP inhibitors have proven to be disappointing – many drugs have been shown to have troublesome side effects seen in phase II clinical trials, and as a result beneficial results have yet to be seen, with the exception of use of doxycycline which is used to treat periodontal disease. Interestingly, the development of side effects in these drugs appears to be cumulative and dose related: in the case of marimastat (tested in pancreatic cancer) the severe side effects developed after 56 days with a dose of 75mg twice daily and 199 days with a dose of 25mg once daily (161). This has also been demonstrated in animal models.

The most common side effect encountered appears to be the “musculoskeletal syndrome”, a condition whereby treated patients develop joint pains, stiffness and reduction in range of movement.

Ilomastat

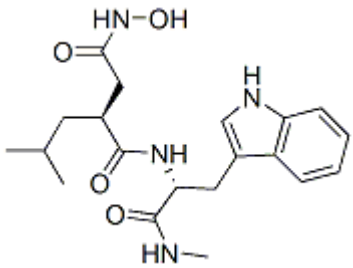


Figure 1.5 Ilomastat

Ilomastat (R)- N4 – Hydroxy-N1-[(S)-2-(1H-indol-3-yl)-1-methylcarbamoyl-ethyl]-2-isobutyl-succinamide (otherwise referred to as Galardin, GM6001) belongs to the group of MMP inhibitors referred to as hydroxamates. It has a broad spectrum of MMP inhibition, inhibiting MMP 1, 2, 9 and 12.

The hydroxamates act as chelators and also block ADAMs (A disintegrin and metalloprotease) proteins and TACE (tumour necrosis factor α convertase). They may potentially chelate other metalloproteins as they have a binding affinity to iron, nickel and copper.

The cardioprotective effect of ilomastat has been shown to persist even in a population of hyperlipidaemic rats (158) Hyperlipidaemia attenuates the effect of IPC, by a mechanism which is not fully understood, but has been proposed as being due to a reduction in cardiac nitric oxide availability (162,163) and increased apoptosis (164) . The finding that MMP inhibition can protect the heart in a model which is resistant to IPC raises the possibility that MMP inhibitor- induced cardioprotection is mediated by a pathway which is distinct to that of IPC, and thus *not* mediated by the MPTP.

In chapter six of this thesis, we set out to investigate whether the cardioprotection conferred by administration of ilomastat at the time of reperfusion is independent of cyclophilin D, a component of the MPTP.

Chapter 2

Summary, Aims Objectives and Hypotheses

2.1 Summary

The organelle at the centre of the processes resulting in cell death after myocardial ischaemia- reperfusion injury is the mitochondrion, and thus it is of great interest when therapeutic treatments to reduce the degree of cell death are being considered.

The central process which is involved in lethal reperfusion injury is the opening of the mitochondrial permeability transition pore (MPTP).

A variety of methods have been used to attempt to reduce this potentially reversible injurious process. These include mechanical methods such as ischaemic preconditioning (IPC) and ischaemic postconditioning (IPOC) and utilisation of cardioprotective drugs (eg cyclosporin A and its analogues). These interventions are targeted at inhibition of MPTP opening.

Mice deficient in cyclophilin D, which has been shown to be central to MPTP function, have been shown to be resistant to the effects of ischaemia-reperfusion (I-R), but nonetheless display a degree of myocardial damage after I-R injury, albeit much reduced in comparison to their wild type brethren. They appear to be resistant to further protection by standard IPC protocols. Preliminary, unpublished work, by Di Lisa et al, has questioned this, raising the possibility that these mice have a raised threshold for IPC, and that this may be overcome by increasing the IPC stimulus. In addition, the same group have shown (unpublished) work (Appendix) which shows that treatment with

agents known as “congenia inhibitors” (small molecule MPTP inhibitors) in combination with cyclosporin A results in improved calcium retention capacity (ie reduced MPTP opening) in comparison to cyclosporin A alone. In an *ex vivo* model, these agents also showed a better reduction in infarct size than cyclosporin A. Nakagawa also showed that in cyclophilin D deficient mice, the MPTP could be induced by administration of high doses of calcium, raising the possibility that the MPTP is subject to a threshold phenomenon (165).

The work detailed above questions whether there may a threshold effect of pore inhibition. In chapter four, we set out to investigate whether it is possible to protect the cyclophilin D deficient mouse by increasing the IPC stimulus.

Cyclosporin A is known to achieve its cardioprotective effect by binding to cyclophilin D in mitochondria, thus inhibiting the MPTP. However, its potential clinical utility is limited by its calcineurin- mediated (extra-mitochondrial) side effects of immunosuppression and deleterious effect on renal function. Targeting of cyclosporin A to mitochondria by use of nanocarrier particles could potentially achieve cardioprotection, whilst avoiding these detrimental effects, and could also potentially require smaller doses to achieve MPTP inhibition. In chapter 5, we investigate whether administration of a novel mitochondrial-targeted form of cyclosporin A is cardioprotective in its first *in vivo* use.

Data published by Ferdinandy’s group also highlights the possibility that the MPTP may not be the only pathway to cell death after ischaemia-reperfusion.

The ability to protect hyperlipidaemic hearts from this injury (which previously has not been possible by IPC - the strongest cardioprotective stimulus) by administration of the matrix metalloproteinase (MMP) inhibitor ilomastat suggests that its protective effect may not be mediated by inhibition of the MPTP. In chapter 6, we administer ilomastat in an *in vivo* model of ischaemia-reperfusion injury to mice deficient in cyclophilin D and their wild type counterparts to investigate whether it is possible to achieve cardioprotection by a mechanism independent of the MPTP.

2.2.1Hypotheses

2.2.1 Ischaemic preconditioning can protect the heart in the absence of cyclophilin D

2.2.1.1 Objectives

To determine whether mice deficient in cyclophilin D are amenable to cardioprotection by ischaemic pre- conditioning

2.2.1.2 Rationale

Cyclophilin D has been shown to be an essential component of the MPTP, which plays a central role in cell death in the aftermath of acute myocardial infarction. Genetic studies have confirmed that mice deficient in cyclophilin D are resistant to pore opening in response to calcium (166) and oxidative stress (13), and also exhibit smaller infarct sizes when exposed to ischaemia-reperfusion in the brain (167) and heart (54).

The reduction in myocardial damage seen in models of ischaemic preconditioning, pharmacological preconditioning as well as ischaemic

postconditioning have been shown to be mediated by inhibition of the MPTP (73,74,168).

Previous studies have shown that using standard IPC / IPOC protocols and established pharmacological preconditioning-mimetics it is not possible to reduce infarct size after ischaemia-reperfusion in the cyclophilin D deficient mouse (12,54).

However, mice deficient in cyclophilin D still display a degree of myocardial damage after myocardial ischaemia and reperfusion, suggesting that there may be another mechanism of myocyte damage, which is distinct to that involving cyclophilin D.

2.2.1 Hypothesis: Administration of a mitochondrial specific analogue of cyclosporin A will offer superior protection against myocardial damage in comparison to cyclosporin A

2.2.2 Objectives

To determine if it is possible to achieve superior cardioprotection by the administration of a novel mitochondria-selective form of cyclosporin A (mtCsA) in mice exposed to ischaemia-reperfusion.

2.2.3 Rationale

Opening of the MPTP has been shown to be a key step in the death of cells after ischaemia-reperfusion. Cyclophilin D is an essential component of the MPTP, and ablation of cyclophilin D is known to exert a protective effect on the heart exposed to ischaemia- reperfusion. The primary binding site of cyclosporin A is cyclophilin D, but it also binds to extra-mitochondrial forms of

cyclophilin (eg cyclophilin A) and also to calcineurin. This extra-mitochondrial binding is responsible for the negative effects of cyclosporin ie renal toxicity and immunosuppression.

It would therefore be desirable to target more specifically mitochondrial cyclophilin D in order to selectively inhibit the MPTP, whilst minimising effects on extra-mitochondrial cyclophilins.

Data presented previously by Maloutrie et al (135) has demonstrated that in a model of hippocampal neurons subjected to glucose and oxygen deprivation ie an “energy failure” model administration of a novel mitochondrial- selective form of cyclosporin A demonstrated enhanced cytoprotection in comparison to cyclosporin A.

2.3.1 Hypothesis: Inhibition of matrix metalloproteinases may protect the heart from ischaemia-reperfusion in the absence of cyclophilin D

2.3.2 Objectives

To confirm cardioprotection after administration of an MMP inhibitor at the time of reperfusion in an *in vivo* model of ischaemia-reperfusion injury.

To determine whether it is possible to protect the myocardium in mice devoid of cyclophilin D by treatment with an MMP inhibitor administered at the time of reperfusion.

2.3.3 Rationale

Cyclophilin D is an essential component of the MPTP as discussed previously. However, in mice devoid of cyclophilin D, exposure to ischaemia-reperfusion

still results in a degree of myocardial damage, although this is reduced in extent in comparison to mice retaining Cyp D.

The possibility therefore exists that there is another pathway distinct from cyclophilin D which results in part of the cellular damage which occurs after reperfused myocardial infarction.

Matrix metalloproteinases (MMPs) have long been the subject of study in the arena of acute myocardial infarction as their role in collagen turnover is an essential part of the remodelling process which occurs in STEMI, and a variety of other models of cardiac disease.

Study of MMPs by Etoh et al has revealed that MMP levels rise in the acute phase of myocardial infarction - and are detectable at 10 minutes of ischaemia (169), and also that administration of MMP inhibitors results in a degree of cardioprotection which is similar to that observed after IPC (154). Additionally, hyperlipidaemic rats (resistant to benefits of IPC) when exposed to ischaemia-reperfusion exhibited cardioprotection when treated with an MMP inhibitor (158).

This led us to question whether administration of an MMP inhibitor at the time of reperfusion would be cardioprotective in a manner which was independent of cyclophilin D.

Chapter 3 - Methods

3.1 General

In order to establish the role of the mitochondrial permeability transition pore in cardioprotection, a mouse model of *in vivo* ischaemia-reperfusion injury was selected. The unique genetic malleability of the mouse - in particular the availability of mice deficient in cyclophilin D (an essential component of the MPTP), enabled evaluation of whether cardioprotection after a variety of interventions was possible in these knockout mice in comparison to the wild type.

All experiments were carried out in accordance with the United Kingdom Home Office Guidance on the Operation of Animals for Scientific Procedures Act (A(SP)A)1986.

Mice were allowed water and feed ad libitum, and were exposed to standard 12 hour dark / light cycle.

All experiments were carried out on male mice to ensure maximum uniformity of infarct size, as data has recently been published which suggests that the infarct size and remodelling post myocardial infarction may vary between the sexes (170).

Male mice were bred in - house from a colony with a B6Sv129F1 background, which were deficient in Cyclophilin D (Cyp D -/-), provided to us by Baines et al (13). These had been back-crossed twice, which maintains a genetic background which has approximately 87.5% of B6Sv129F1 DNA. We used

cyclophilin D (Cyp D +/+) mice, created by breeding male with female homozygous +/+ mice as our wild type controls. Similarly, the Cyp D -/- mice were bred from homozygotes. Genotyping was carried out on a random basis to ensure correct allocation.

3.2 Chemicals and Drugs Used

Cyclosporin A (Tolypocladium inflatum) was supplied by Calbiochem (Merck, Nottingham, UK). Mitochondrial targeted cyclosporin A was synthesised and produced by Professor David Selwood (Head of Biological and Medicinal Chemistry Wolfson Institute for Biomedical Research, University College London). Ilomastat and TTC were supplied by Sigma Aldrich, UK. Anaesthetic drugs – ketamine, xylazine and atropine were obtained from Fort Dodge Animal Health Ltd (UK), Millpledge Veterinary (UK), and Lameln Pharmaceuticals (UK) respectively. PCR reagents were obtained from Qiagen (UK).

3.3 *In vivo* mouse ischaemia-reperfusion recovery model

In order to attempt to replicate the human situation of myocardial infarction and subsequent reperfusion, we sought to set up an *in vivo* model recovery model of ischaemia-reperfusion in the mouse. The intention was that this would enable non- invasive *in vivo* assessment of myocardial function and geometry, and permit serial measurements of changes over time using magnetic resonance imaging with gadolinium enhancement. This method of assessing ventricular geometry and infarct size has been well validated in mouse models of myocardial infarction (171-175).

Mice were anaesthetised using ketamine (75mg/kg) and medetomidine (1mg/kg) administered in a small volume (approx 0.4ml) using a short 27G needle (176).

The mouse was placed into a warmed cage, observed continuously and removed after three minutes, by which time it was adequately anaesthetised.

The hind limb reflex was then checked to confirm adequate depth of anaesthesia.

The neck and chest areas were then shaved using an electrical clipper, and cleaned with chlorhexidine solution.

The mouse was then positioned for intubation in the supine position on an electrical heating pad, with the upper limbs abducted and lower limbs extended and taped down.

The neck was extended by threading a suture behind the incisors and maintaining this under gentle tension, securing it to the heating pad with tape.

A rectal thermometer was lubricated with Vaseline and inserted to provide contemporaneous temperature measurements. Temperature was maintained at 37ºC (+/-0.5).

A small midline incision was made on the skin of the neck (1-1.5cm). Using blunt dissection, the salivary glands were separated and the paratracheal muscles exposed and split along the midline fascia and deflected laterally. They were retained in this position by a blunt hook stay on either side. This enabled direct visualisation of the trachea.

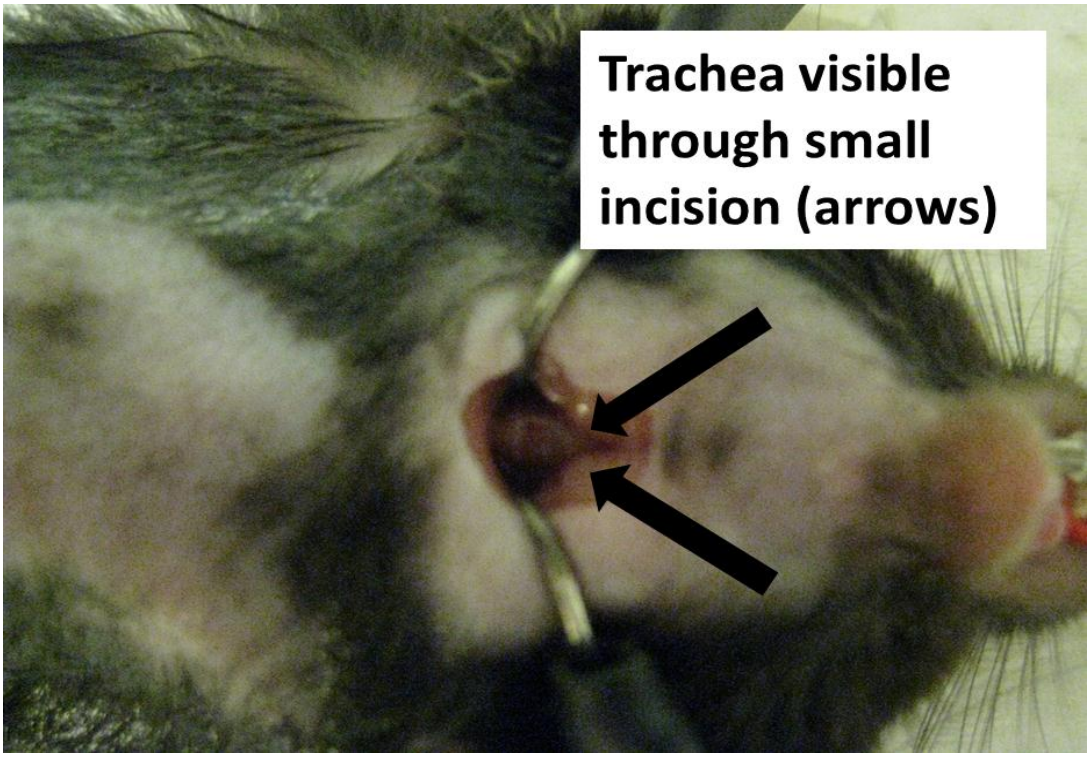


Figure 3.1 Visualisation of the trachea

A stainless steel cannula (Hugo Sachs, Germany) was then passed through the oral cavity and by direct visualisation through the cervical incision, into the trachea. The cannula was attached at its other end, to a plastic Y connector which was in turn connected to a ventilator (Minivent Mouse Ventilator, Hugo Sachs Electronik, Germany) set at 0.2 ml volume, 120 stroke/min with

supplemental oxygen supplied by an oxygen cylinder set at 1.5l/min. This was maintained under positive end expiratory pressure (PEEP) by immersing the exhaust from the ventilator in 3cm H_2O.

After establishing ventilation, a dose of buprenorphine was administered (0.05mg/kg IM), to ensure the animal was pain free post operatively.

The position of the mouse limbs was then altered to facilitate the surgery. The right hindlimb was first placed in an extended position and secured with tape. The left hindlimb was then placed over the right hindlimb and was also secured by tape. This position facilitates exposure of the heart and left anterior descending artery for cardiac surgery.

An oblique skin incision was made (approximately 1cm) reaching from the left sternal border to 1-2mm inferior to the left axilla (4^{th} intercostal space).

Blunt dissection was then used to separate the skin from connective tissue layers and the pectoral muscles were exposed. Pectoralis major and minor were then separated and held in place by hook stays taped down to the heating pad to maintain gentle tension.

The ribs were then opened at the 4^{th} intercostal space. Any bleeding was terminated by use of low temperature cautery. The chest was held open by a mini-Goldstein retractor.

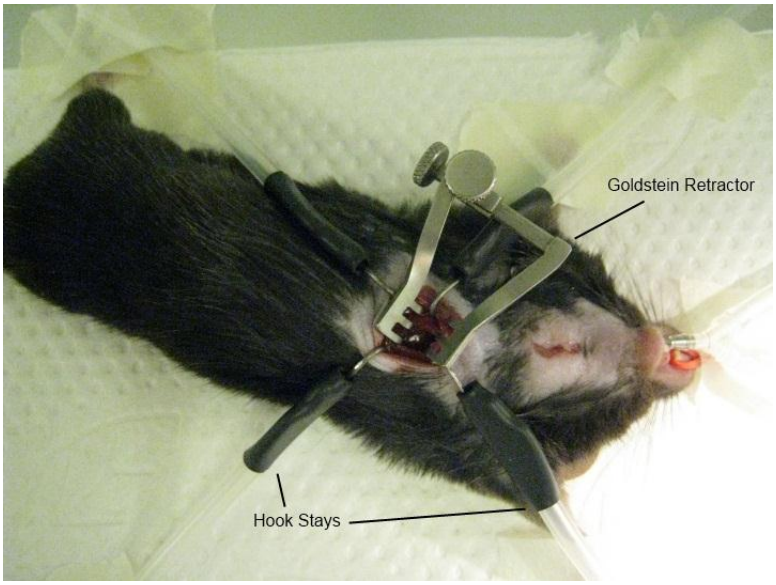


Figure 3.2 Use of retractors to expose the heart

The pericardium was teased apart and the heart exposed.

A suture (8/0 prolene) was hooked under the left anterior descending artery approximately 1mm distal to the tip of the left atrium.

The system described by Marber et al (177) was then used to temporarily occlude the left anterior descending coronary artery, using a short piece of PE-50 tubing threaded over the 8/0 prolene suture and using this to act as a snare. A short piece of PE-50 tubing (0.28 x 0.61mm) was also placed over the surface of the LAD to prevent it from being lacerated by the tightening of the suture. The distal tip of a pipette was then passed over the suture needle (tip first), and a short segment of PE 50 tubing was placed inside the pipette tip and this created a brake system (see figure overleaf).

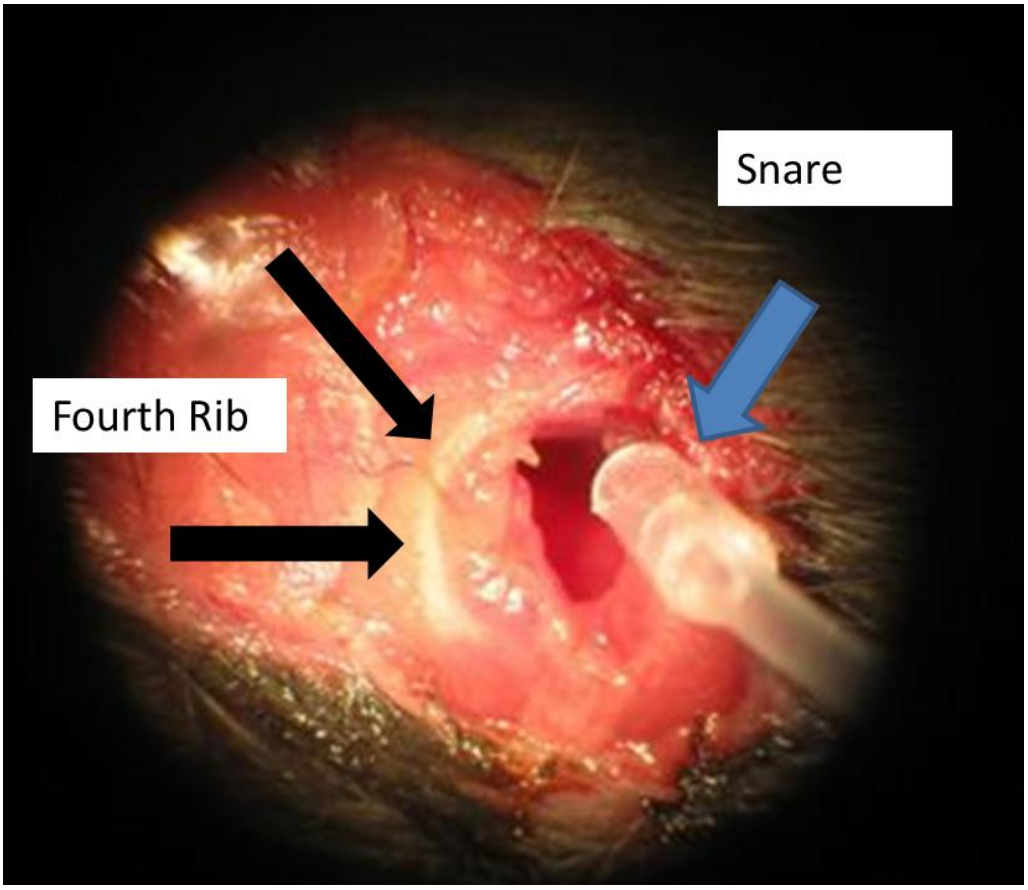


Figure 3.3 The snare arrangement

The heart was allowed to stabilise for fifteen minutes prior to occlusion of the LAD.

The suture was then tightened over the LAD for a period of thirty minutes.

The tubing was removed from the LAD to initiate reperfusion, and the prolene suture retained in position and loosely tied off.

Two interrupted 5/0 mersilk sutures were applied to close the ribs. The first (in the lateral position) was tightened first and before tightening the second suture, gentle pressure was applied to the thoracic cavity in order to expel any air and prevent formation of a large pneumothorax. The second suture was subsequently tightened.

The pectoral muscles were then gently replaced in their original position and the skin was closed with individual interrupted sutures (5/0 mersilk).

The wound was then cleaned with alcohol.

The anaesthetic was reversed with Atipamezole (5mg/kg IP).

The mouse was kept on the heated pad with supplemental oxygen until it recovered sufficiently, and was then returned to a warmed cage with access to water and diet, and was carefully supervised until it was fully ambulant.

A further IM dose of buprenorphine was administered at 6 and 18 hours post operatively.

3.4 Problems Encountered with the Ischaemia-reperfusion Recovery Model

In attempting to set up this model (not previously established in our centre) difficulties were encountered.

Using C57 mice to set up the model, firstly permanent infarction (where instead of using the snare arrangement, the LAD was occluded by tying off the prolene suture) was carried out to confirm that the animal could be safely recovered. The length of procedure from administration of anaesthesia approximated 30 minutes for this, and recovery was good. All sham operations (suture passed, but not tied off) survived.

However, with an initial stabilisation period of 35 minutes to standardise for three cycles of ischaemic preconditioning, and a sham operation, with a total anaesthetic time of 105 minutes, the operative survival was poor (10/24 mice). Despite a number of manoeuvres to improve this, including administration of warmed intraperitoneal saline at the start of the procedure to prevent dehydration resulting from prolonged open- chest procedure, the figure could not be improved upon.

Advice was sought from centres with an established recovery protocol and these centres used isoflurane as the anaesthetic of choice, both for induction and maintenance of anaesthesia, and also reported problems with survival when utilising injectable anaesthetic agents. We had initially elected to avoid the use of isoflurane in view of its cardioprotective properties (178-180). There

is a body of evidence supporting the fact that its mechanism of protection converges on the pathways involved in ischaemic preconditioning eg, inhibition of apoptosis, phosphorylation of protein kinase C, attenuation of ROS generation and delayed opening of the MPTP (178,181-183). This could potentially reduce the ability to detect a reduction in infarct size from a novel, potentially cardioprotective agent. However, in view of the problems with survival using injectable anaesthetic agents, isoflurane was used for subsequent procedures with 100% survival in the sham group with anaesthetic time of 105 minutes, and 71% in the ischaemia-reperfusion group.

Due to the extensive time taken to progress to this stage, and the limited time remaining to perform experiments, it was felt appropriate to switch to a non-recovery model in view of the need to perform further refinement of the technique in order to carry out a longitudinal study.

3.5 Non recovery model of ischaemia and reperfusion

Mice were anaesthetised with a combination of ketamine, xylazine and atropine, which was administered intraperitoneally. The total volume administered was 0.01ml/gram body weight. Final concentration of ketamine, xylazine and atropine was 10mg/ml, 2mg/ml and 0.06mg/ml respectively (54).

Once sufficient anaesthesia was achieved (confirmed by absence of the hindlimb reflex), animals were placed in the supine position, with the forelimbs abducted and taped down, and the hindlimbs extended and taped to a

warmed heating pad. The whiskers were also taped down to extend the neck (see fig below)

 .

Figure 3.4 Preparation and positioning for tracheotomy

Animals were then tracheotomised and ventilated using a rodent mini-ventilator (Type 845, Harvard Apparatus, Kent) at a stroke volume of 220 microlitres and ventilatory rate of 120/minute. Supplemental oxygen at a rate of 1.5l/min was administered.

Body temperature was monitored continuously using a rectal thermometer (Hanna, K couple HI 8757) and was maintained at 37ºC +/- 0.5º C.

Electrocardiographic monitoring was carried out using a standard three lead recording of limb lead I.

Figure 3.5 .In vivo set up (non-recovery model)

The external jugular vein and carotid artery were dissected out and cannulated with a length of PE-50 tubing for drug administration, (both of which had been shaped with a flame to create a finely tapered tip) and monitoring of intra-arterial blood pressure respectively.

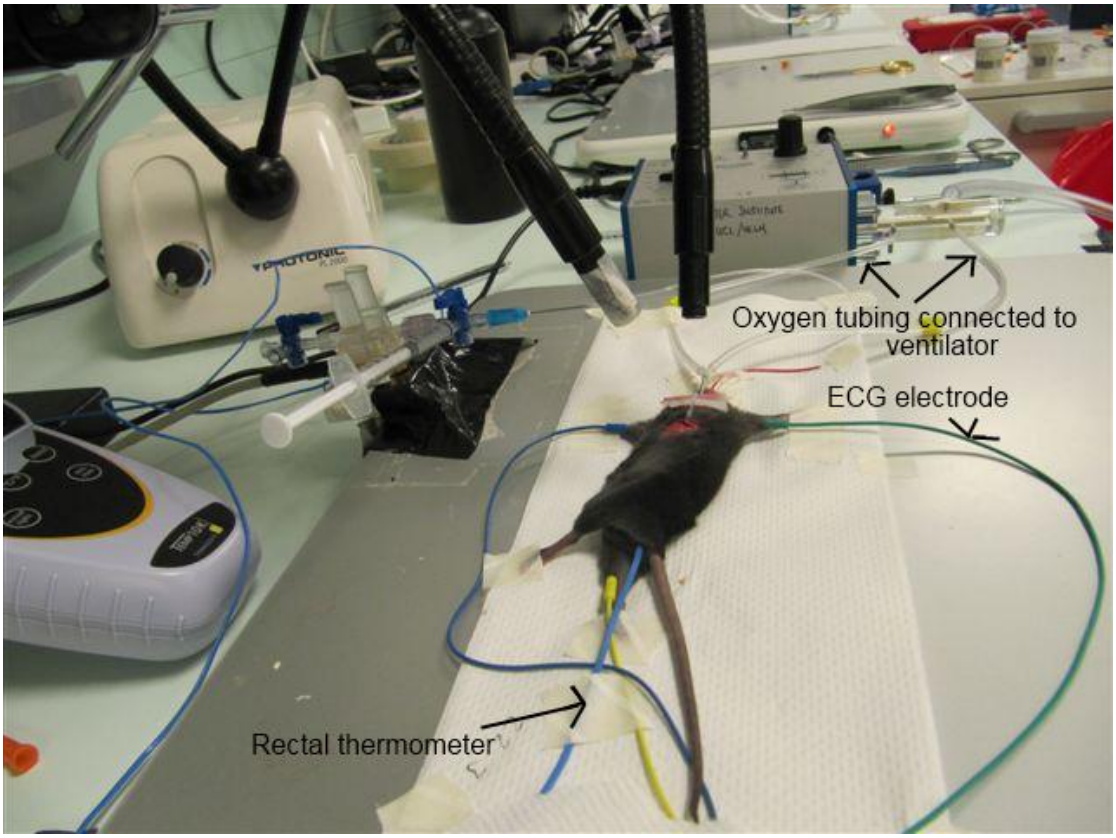


Figure 3.6 *In vivo* set up during experimental protocol

Both electrocardiogram and intra-arterial blood pressure were recorded using Chart 5 for Windows® software (AD Instruments, UK).

Using the method described by Fisher et al (177), animals were placed in position for thoracotomy. The skin was reflected and pectoralis major exposed and reflected back, as was pectoralis minor. A thoracotomy incision was made at the level of the 4th intercostal space, exposing both atria and ventricle beneath a thin layer of pericardium. The pericardium was gently teased apart using blunt forceps to expose the ventricle and the left anterior descending coronary artery (LAD) was seen as a bright orange vascular structure running from the atrium in a caudal direction to the apex of the ventricle.

An 8-0 prolene® synthetic monofilament (Ethicon, UK) suture was then passed around the LAD, at around 2mm from the inferior border of the atrium.

This was passed through a snare, fashioned from a short piece of PE 50 tubing tied to the end, and a D200 pipette tip with a piece of PE 50 tubing to act as a brake (see recovery model).

The animal was then maintained under anaesthesia for a maximum period of 35 minutes (varied according to experimental protocol- see figure 3.6). This period is known as stabilisation. The snare was then tightened for 30 minutes to achieve ischaemia, which was confirmed by a drop in mean arterial pressure (figure 3.9) and the presence of ST segment elevation / depression on the electrocardiogram (see figure 3.8).

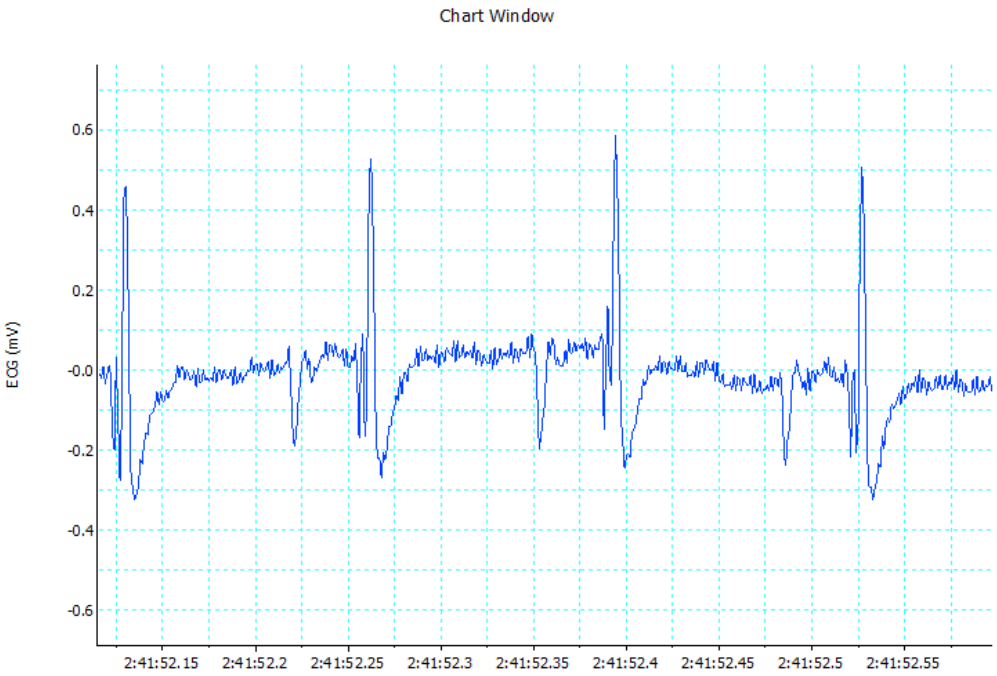


Figure 3.7 ECG during stabilisation

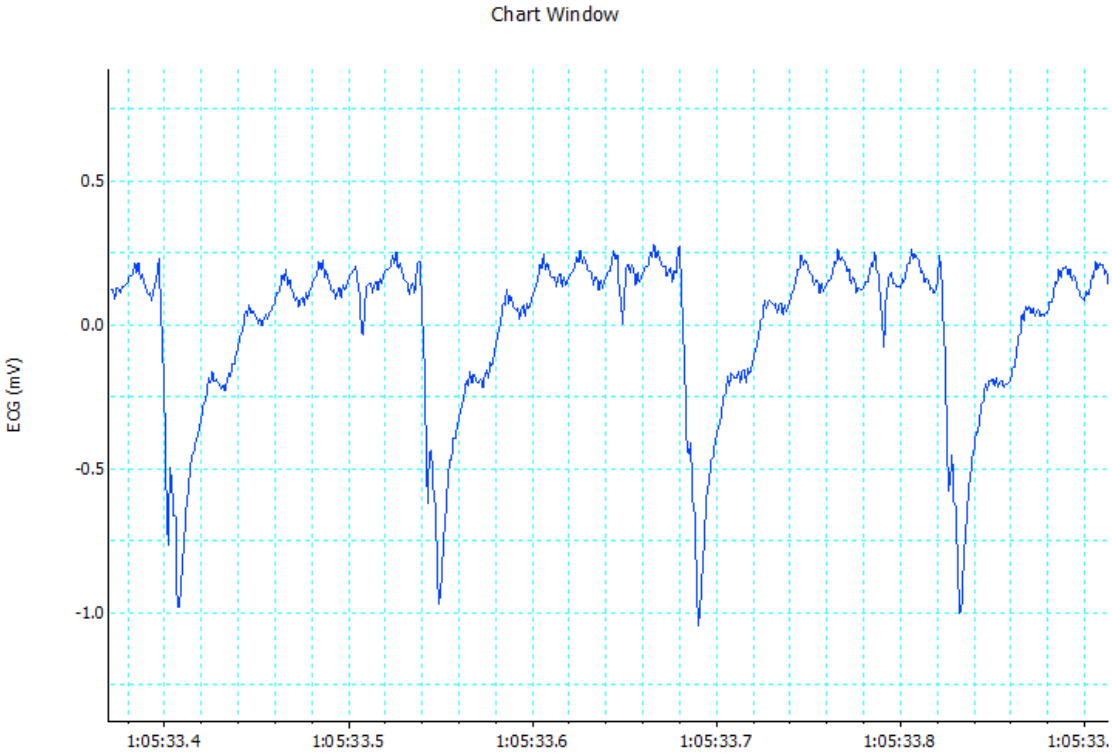
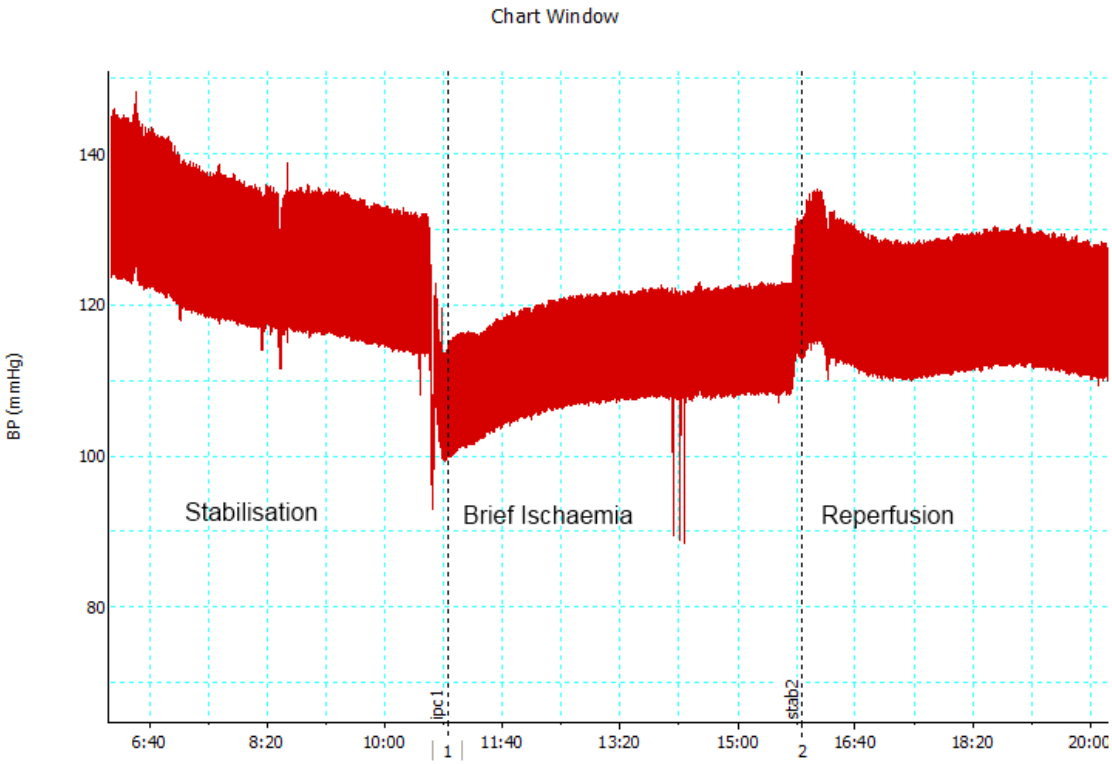


Figure 3.8 ECG during ischaemia

Figure 3.9 Expected blood pressure change during 5 minutes preconditioning



At the end of 30 minutes ischaemia, the snare was released and the LAD was reperfused for a period of two hours, after which time the heart was harvested and placed on ice.

A two hour reperfusion period was selected as it has previously been demonstrated in the murine ischaemia-reperfusion model to optimally demonstrate the infarct size with 2,3,5 triphenyltetrazolium chloride (TTC) staining (77).

3.6 Characterisation of the model

Commercially available C57BL6 black mice (Harlan, UK) were used to confirm that myocardial protection was achieved after a single five minute period of IPC prior to 30 minutes of sustained ischaemia, with two hours of reperfusion.

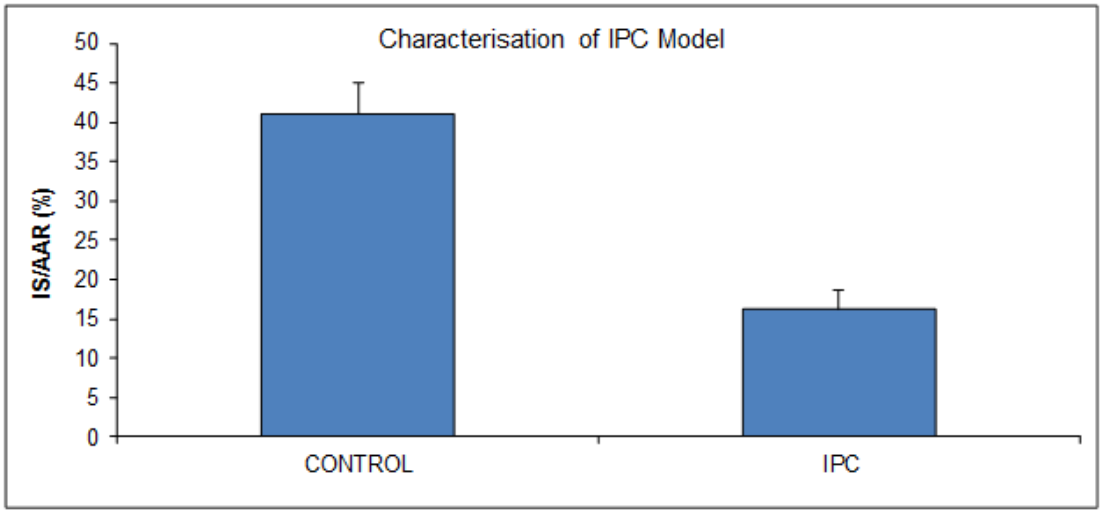


Figure 3.10 Cardioprotection achieved after one cycle of IPC in C57BL6 ($p<0.005$). N=5 in each group. Error bars denote SEM (standard error of the mean)

IS/AAR% = infarct size expressed as a % of the area at risk

3.7 Exclusion Criteria

Mice with any evidence of barbarism were excluded. Also, mice were excluded if there was absence of a drop in mean arterial blood pressure or if there was no change in electrocardiogram after the snare was applied.

Additionally, if the mean arterial pressure during the stabilisation period was <90mmHg, or if significant operative bleeding or the animal did not survive the 2 hour reperfusion period it was excluded.

3.8 Assessment of infarct size

Use of Evans Blue and 2, 3, 5 TTC

The heart was harvested from a midline incision and dissected out beneath the level of the aortic arch. To ensure an adequate length of aorta is obtained, it is recommended that the thymus is removed with the heart- within which the aortic arch resides. The connective tissue was dissected away to facilitate rapid cannulation of the aortic root (less than 3 minutes) and the heart was then perfused with 1ml of physiological (0.9%) saline in order to remove any residual blood. This was followed by injection of 10ml of 1% 2,3,5 triphenyltetrazolium chloride (TTC). TTC reacts with dehydrogenases to form a deep red (formazan) pigment. It is injected over 1-2 minutes in a small beaker to ensure staining of the external surface of the heart in order to delineate viable myocardium (stains red, infarcted areas remain unstained). Use of TTC in measuring infarct size is a standard, well validated method

(184). After this, the LAD suture was re-tied and 1ml of 0.5% Evans Blue was injected to determine the area at risk (AAR), delineated by absence of Evan's Blue dye. The area not at risk is stained with Evans Blue.

The heart was then blotted dry and weighed.

The heart was frozen at -20ºC for a minimum of 2 hours and then sectioned in the short axis from apex to the level of the suture, creating 5 slices of approximately 1mm thickness. The slices were then rinsed in physiological saline and bathed in 10% neutral formalin solution for 90 minutes at room temperature.

The right ventricle was then removed by careful dissection, leaving the left ventricle for analysis.

The slices were then placed sequentially between two transparent Perspex blocks and secured for photography. This was then scanned using a standard colour scanner using 1200 bit colour definition (Epson®) into a computer for analysis (Figure 3.10).

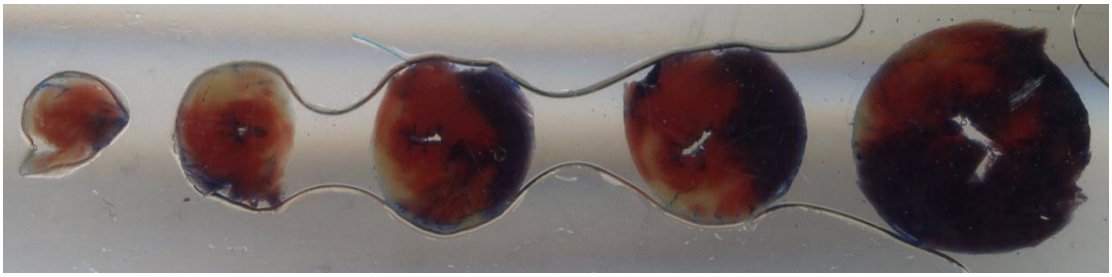


Figure 3.11 Typical example of stained heart before analysis

3.9 Image J

Image J software devised by the National Institute for Health, USA, (a public domain Java image processing domain which is freely available for use without license) was used to analyse the heart slices to determine the area at risk and infarct size.

Image J facilitates computerised planimetry of the ventricle by splitting the colourisation of photographed images into red and green wavelengths. Red is used to highlight the areas stained by Evans Blue and green to highlight the infarcted area. By altering the threshold, it is possible to accurately planimeter the surface area for each region (see figure 3.12). This is digitally measured by pixel counts for each slice and the totals for each slice are added to achieve the result for the entire left ventricle.

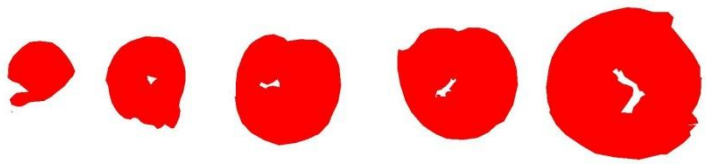


Figure 3.12 Outline of left ventricle.

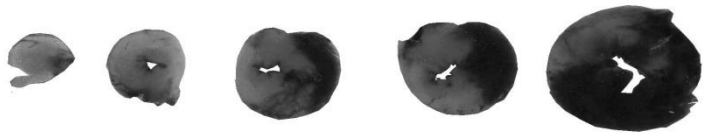


Figure 3.13 Red image to delineate the Evans Blue staining

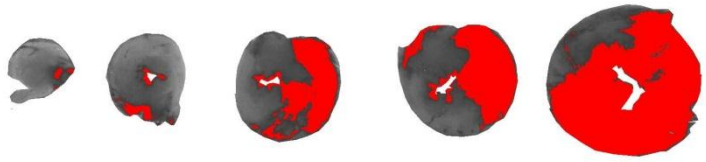


Figure 3.14 Highlighted areas show Evans Blue staining.

Figure 3.15 Green image delineating infarcted areas (pale).

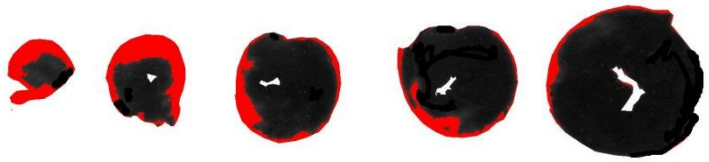


Figure 3.16 Automated planimetry highlighting areas of infarction

The area at risk (AAR) is expressed as a percentage of the left ventricle (AAR/LV%), and the infarct size is expressed as a percentage of the area at risk (IS/AAR%).

3.10 Genotyping

3.10.1 Digestion

Ear snips were taken from a random sample of mice at intervals to confirm correct genetic allocation. Each sample was placed in a 0.5 ml microcentrifuge tube and 180 µl of Direct PCR Lysis Reagent ® was added, along with 20µl of Qiagen proteinase K (Sigma). This was then mixed using a vortex and incubated at 55ºC overnight. The following morning each sample was vortexed for 15 seconds to ensure adequate mixing had taken place. This was then replaced in the heating block for 30-45 minutes and the temperature was then increased to 85ºC in order to denature the proteinase K, thus preventing continued lysis. The samples were then centrifuged for 10 seconds at 14,000 RPM to precipitate any hairs. 1 microlitre (µl) of lysate was used for each 20µl PCR reaction.

3.10.2 Polymerase Chain Reaction

All PCR reagents were kept on ice throughout. They were centrifuged and vortexed briefly before use. The reagents used are listed in the table below. A 'stock mix' of PCR reagents was prepared, which contained sufficient quantity for the number of samples present, a positive control and distilled water control.

Reagent	Volume per sample
Qiagen 10 x PCR buffer CL	2.0µl
10MM dNTPs	0.4µl
Primer 1 Exon 3F	0.2µl
Primer 2 Neo F	0.2µl
Primer 3 Exon 4R	0.2µl
Taq Polymerase	0.2µl
Distilled Water	15.8µl
Total	19µl

Table 3.1 Reagents for PCR

19µl of PCR 'stock mix' was then pipetted into fresh, labelled micro -PCR tubes, and to each of these, 1µl of DNA sample was added. The tubes were

all tapped gently, then centrifuged briefly, to ensure that the DNA was fully immersed in the reagent mixture. These tubes were then placed into the DNA engine (Peltier Thermal Cycler) and subjected to a saved protocol as follows:

Reaction time	Process
1. 95ºC	Double stranded DNA separation
2. 95ºC	Double stranded DNA separation
3. 59ºC	Annealing of primers
4. 72 ºC	DNA synthesis
5. 95 ºC	Double stranded DNA separation
6. 57 ºC	Annealing of primers
7. 72 ºC	DNA synthesis
8. Repeat stages 5-7 for 32 cycles	DNA Amplification
9. 72 ºC	DNA synthesis
10. 4 ºC	Storage

Table 3.2 Protocol for DNA amplification

3.10.3 Gel Electrophoresis

2% agarose gel was made up in Tris – Acetate - EDTA (TAE) buffer with 1µl of SYTO 60 ® (Invitrogen, UK)

A standard gel block (with a wide comb) was used, the edges of which were tightly secured with autoclave tape.

2.0 grammes of agarose powder was added to 100ml of 1 x TAE solution in a conical flask. This was rotated gently to dissolve the agarose. This solution was then microwaved on high power for 90 seconds. After cooling for approximately 5 minutes on a bench, 1µl of SYT060 was added, and rotated to mix. This was then aliquotted into the gel block and left to set for approximately 15 minutes.

After this time, the tape was removed from the set gel and placed in the central block of the Thermo apparatus. The gel combs were then gently removed, and the Thermo block was then filled to the fill line with TAE buffer.

The first well was then filled with a molecular weight reference ladder. 15µl of water was then added to the second well as a negative control PCR reaction. Each of the remaining wells were then filled with 15µl of the test samples. The lid was then replaced and the unit attached to a powerpack and set to run at 120V for 90 minutes.

The gels were analysed and recorded using the Li-Cor Odyssey® infra red imaging system. The images were exported as high quality JPEG files at 300 dpi (dots per square inch). An example demonstrating both cyclophilin D knock out and wild type genotypes is shown below.

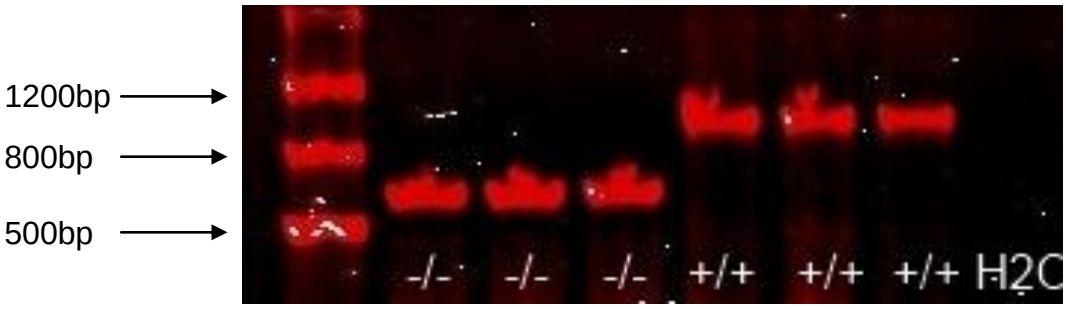


Figure 3.17 DNA electrophoresis

3.11 Statistics

Data was analysed using Prism software and one way ANOVA followed by Tukey's multiple comparison of means test. A P value of <0.05 was considered to be statistically significant. Results are expressed as means ±SEM.

Chapter 4- Ischaemic Preconditioning in The Cyclophilin D Deficient Mouse

4.1 Ischaemic Preconditioning Study

4.1.1 Background

As discussed in chapters one and two, cyclophilin D appears to be an essential component of the mitochondrial permeability transition pore, which has been shown to be a critical mediator of cell death after ischaemia-reperfusion (13).

Prior data has shown that it has not been possible to reduce infarct size in the mouse deficient in cyclophilin D when subjected to standard IPC/ IPOC / pharmacological preconditioning- mimetics (54).

Mice devoid of cyclophilin D when exposed to ischaemia – reperfusion exhibit a reduced infarct size in comparison to their wild type brethren. The level of infarct size resulting from this injurious insult is comparable to that of wild type animals treated with a cardioprotective stimulus (IPC/IPOC/cyclosporin A).

There is data which has been published in abstract form by Carpi *et al*, (Appendix 1.1) which may suggest that it is possible to protect the hearts of mice devoid of cyclophilin D by increasing the IPC stimulus to three cycles in an *ex vivo* model of ischaemia-reperfusion, and also further work (unpublished) by the same group (Contursi *et al*)- see appendix 1.2, which suggests that treatment with small molecule MPTP inhibitors “congenia

inhibitors" may result in a superior degree of cardioprotection in an *ex vivo* model than is achieved by administration of cyclosporin A (Appendix 1.2).

These findings challenge the widely held belief that once in a "protected" state either by IPC or genetic ablation of cyclophilin D the heart cannot be further protected against ischaemia-reperfusion- induced injury.

This has not been demonstrated previously in the literature, but raises the possibility that it may be possible to "condition" the heart to a greater extent than that achieved by genetic ablation of cyclophilin D, either by increasing the preconditioning stimulus, or by administration of novel pharmacological agents. If cyclophilin D knock-out animals can be protected from I-R injury, this raises the possibility that application of two protective stimuli in the clinical setting of myocardial infarction could provide significant benefit.

4.1.2 Aims

A series of experiments were conducted In order to test the following hypothesis:

Ischaemic preconditioning can protect the heart in mice deficient in cyclophilin D

In this chapter we have set out to assess if it is possible to demonstrate cardioprotection from ischaemia-reperfusion in mice devoid of cyclophilin D in response to an increase in the IPC stimulus in an *in vivo* model.

4.1.3 Methods

Both wild type and Cyp D deficient mice were subjected to thirty minutes of ischaemia followed by two hours of reperfusion. These were randomised into receiving either no IPC, a single cycle of IPC or three cycles of IPC.

Animals were anaesthetised and prepared for surgery as described in the non- recovery model in chapter 3. To reduce procedural time and minimise bleeding for this study the carotid artery was cannulated, but not the jugular vein. Thoracotomy was carried out and the pericardium was teased apart to expose the ventricle. An 8-0 prolene® suture was placed 1-2 mm beneath the caudal edge of the atrium. This was attached to a snare which was tightened over the left anterior descending artery to achieve periods of ischaemia and released to reperfuse the artery, as described in chapter 3.

Mice with any evidence of barbarism were excluded (n=1). Also, mice were excluded if there was absence of a drop in mean arterial blood pressure (n=1) or if there was no change in electrocardiogram after the snare was applied (n=0).

Additionally, if the mean arterial pressure during the stabilisation period was <90mmHg (n=2), or if significant operative bleeding or the animal did not survive the 2 hour reperfusion period it was excluded (n=1).

Firstly, in order to characterise the surgical model and confirm protection from a single cycle of ischaemic preconditioning a simple validation study was carried out.

4.2 Characterisation of the model

Commercially available C57BL6 mice (Harlan, UK) were used as they were more readily available than wild type mice, to develop the surgical model. I was able to demonstrate cardioprotection after a single 5 minute cycle of mechanical ischaemic preconditioning with n=5 in each group as demonstrated in chapter 3, figure 3.10.

4.3 Experimental Protocols

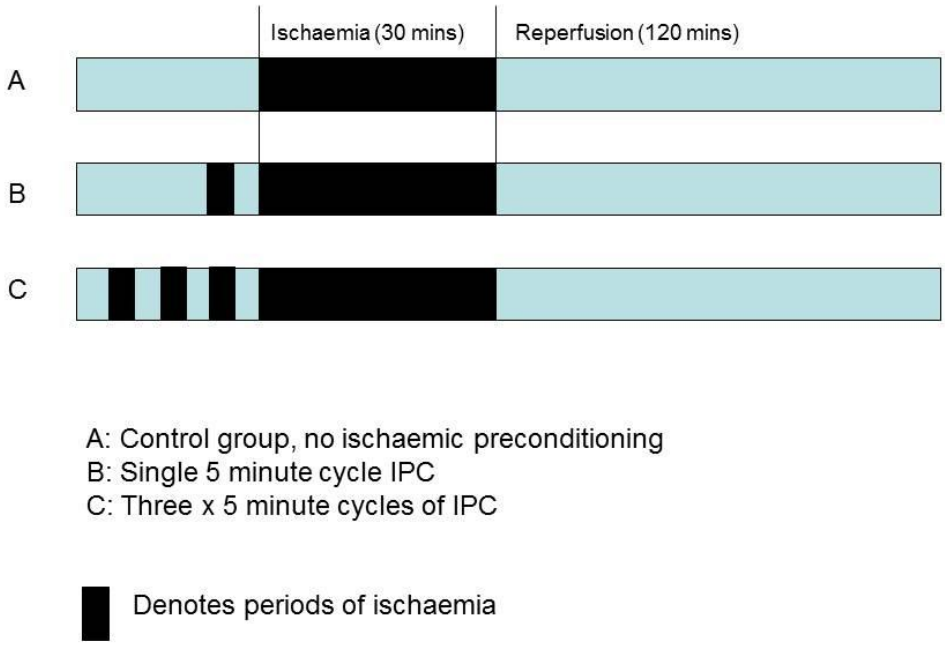


Figure 4.1 Experimental Protocols

Animals were anaesthetised and prepared for surgery as described in chapter three. All groups were subjected to thirty minutes of ischaemia and 2 hours of reperfusion preceded by one of the three protocols outlined above- a control group which had 35 minutes stabilisation prior to the 30 minutes ischaemia, a group which received a single 5 minute cycle of IPC within 5 minutes prior to the 30 minutes ischaemia, and finally, a group which underwent three alternating 5 minute cycles of IPC and LAD perfusion prior to the 30 minute ischaemia.

The above experiments were carried out on both cyclophilin D knock out and wild type mice.

Both genotype and experiments were randomised to in order to minimise intra-operator bias.
All experiments were conducted by a single operator (CH).

The physiological data relating to these experiments are detailed in the subsequent graphs and tables. For each of the graphs, the data shown represents the mean +/- SEM.

Physiological parameters (mean arterial pressure and heart rate) were recorded throughout the experimental protocol. In the graphs which follow, the timescale is non-linear, in order to highlight the changes in observations which occur during the short preconditioning cycles.

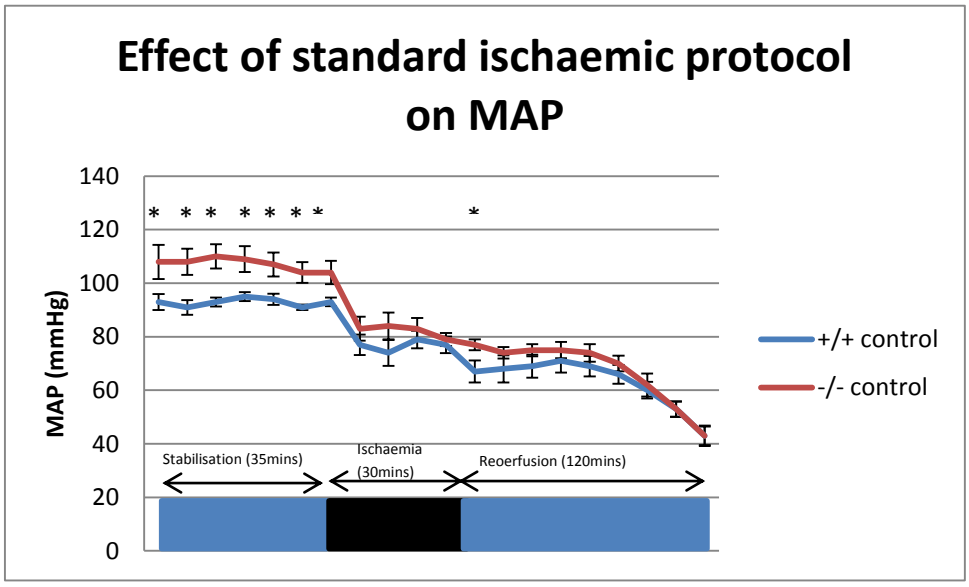
In each graph, the coloured bar describes the experimental protocol. The blue bars represent LAD perfusion whilst the black boxes indicate LAD ischaemia.

Notably, the trend of the mean arterial pressure was, in the control group, to remain stable during the stabilisation period, fall during ischaemia and then experience a drop at the time of reperfusion, then progressively decline until the end of the two hour protocol.

It was also noted that the MAP during stabilisation was significantly higher in the Cyp D knock-out mice (108 v 93 mmHg, $p<0.04$). This pattern persisted in the single cycle IPC data, but was not apparent in the data relating to the 3 cycle IPC protocol. This would suggest that this was not a true effect related to the genotype as it was not borne out in all experiments.

4.3.1 Results

Effect of standard (control) protocol on mean arterial pressure



* =P<0.05

Figure 4.2 Effect of standard ischaemic protocol on mean arterial pressure

The blue bars represent LAD perfusion whilst the black boxes indicate periods of LAD ischaemia.

MAP: Mean arterial pressure, measured in millimetres of mercury (mmHg)

+/+ = Wild type mice (blue)

-/- = Cyclophilin D deficient mice (red)

The data is displayed as the mean, with error bars to represent the standard error of the mean.

The above data demonstrates the blood pressure profile of the animals which were subjected to the standard ischaemic (control) protocol, with 35 minutes

of stabilisation followed by 30 minutes of ischaemia and 2 hours of reperfusion.

This data is presented in tabular format below on the next page. The blood pressure values shown are the mean arterial pressure recorded in millimetres of mercury. The given figure is the arithmetical mean.

Table 4.1 Blood pressure tables for standard ischaemic (control) protocol

Stabilisation Period

Time (mins)	N	5	10	15	20	25	30	35
MAP (mmHg) +/+	9	93 (3.01)	91 (2.74)	93 (1.7)	95 (1.68)	94 (2.08)	91 (1.0)	93 (1.59)
MAP (mmHg) -/-	9	108 (6.38)	108 (4.86)	110 (4.52)	109 (4.80)	107 (4.44)	104 (3.89)	104 (4.38)
P value		0.04	0.01	0.01	0.01	0.02	0.01	0.03

Ischaemia

Time (mins)	1	5	15	25
MAP (mmHg)+/+	77 (3.79)	74 (4.83)	79 (3.27)	77 (3.14)
MAP (mmHg)-/-	83 (4.49)	84 (5.01)	83 (4.04)	79 (2.44)
P value	0.24	0.11	0.29	0.44

Reperfusion

Time (mins)	1	5	10	15	30	60	90	120
MAP (mmHg)+/+	67 (4.10)	69 (5.03)	71 (4.43)	69 (3.84)	66 (3.53)	60 (3.10)	53 (2.79)	43 (3.46)
MAP (mmHg)-/-	77 (1.98)	75 (2.12)	75 (2.26)	74 (3.06)	70 (3.27)	62 (2.91)	53 (4.28)	43 (3.80)
P value	0.04	0.26	0.36	0.28	0.58	0.85	0.76	0.98

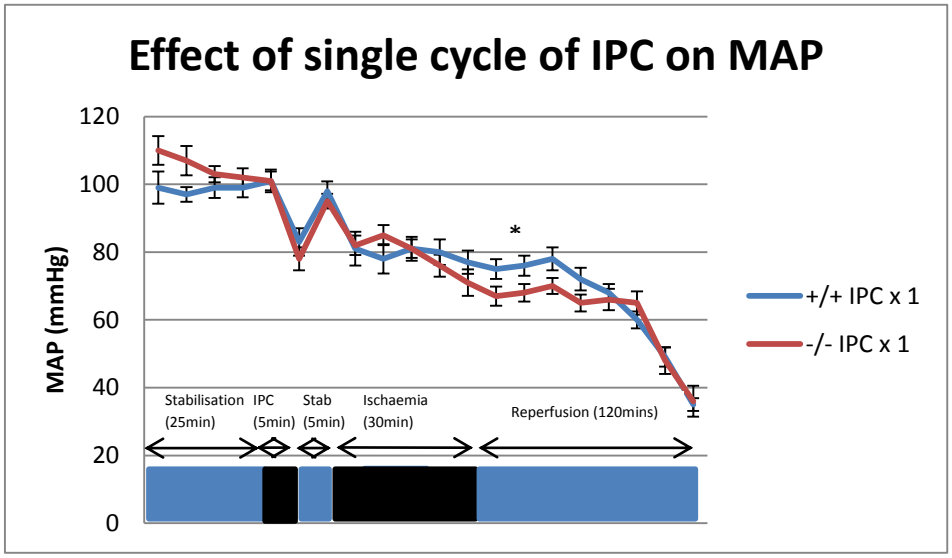
All data shown as mean +/- SEM

MAP = mean arterial pressure (millimetres of mercury)

+/+ = wild type

-/- = Cyclophilin D knock-out

4.4.1 Effect of single cycle of IPC on mean arterial pressure



*=P<0.05

Figure 4.3 Effect of single cycle of IPC on mean arterial pressure comparing the blood pressure response in wild type and Cyp D knock-out mice

The blue bars represent LAD perfusion whilst the black boxes indicate periods of LAD ischaemia.

MAP: Mean arterial pressure, measured in millimetres of mercury (mmHg)

+/+ = Wild type mice (blue)

-/- = Cyclophilin D deficient mice (red)

The data is displayed as the mean, with error bars to represent the standard error of the mean.

Figure 4.3 details the mean arterial pressures for the animals which were subjected to the single cycle of ischaemic preconditioning (referred to earlier as group B).

As with the standard protocol, there was a higher baseline MAP in the CypD knockouts.

The tables overleaf show the data presented above for the animals which were subjected to a single cycle of ischaemic preconditioning.

Table 4.2 Data tables for single cycle IPC protocol

Stabilisation

Time (mins)	N	5	10	15	20	25	30 IPC	35
MAP (mmHg)+/+	6	99 (4.74)	97 (2.15)	99 (3.05)	99 (2.82)	101 (2.77)	83 (4.08)	98 (2.84)
MAP (mmHg)-/-	9	110 (4.20)	107 (4.30)	103 (2.37)	102 (2.72)	101 (3.35)	78 (3.42)	95 (2.17)
P value		0.10	0.07	0.33	0.49	0.87	0.33	0.44

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
MAP (mmHg)+/+	81 (5.00)	78 (4.30)	81 (3.53)	80 (3.78)
MAP (mmHg)-/-	82 (2.86)	85 (2.98)	81 (2.72)	76 (3.24)
P value	0.87	0.21	0.88	0.36

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
MAP (mmHg)+/+	77 (3.45)	76 (2.94)	78 (3.35)	72 (3.34)	68 (2.54)	60 (2.48)	49 (2.82)	35 (1.87)
MAP (mmHg)-/-	71 (3.94)	67 (2.78)	68 (2.55)	68 (2.38)	70 (2.51)	65 (3.18)	48 (3.92)	36 (4.59)
P value	0.23	0.06	0.03	0.50	0.60	0.68	0.89	0.83

All data expressed as mean +/- SEM

MAP = mean arterial pressure (millimetres of mercury)

+/+ = wild type

-/- = Cyclophilin D knock-out

4.5.1 The effect of three cycles of IPC on Mean Arterial Pressure

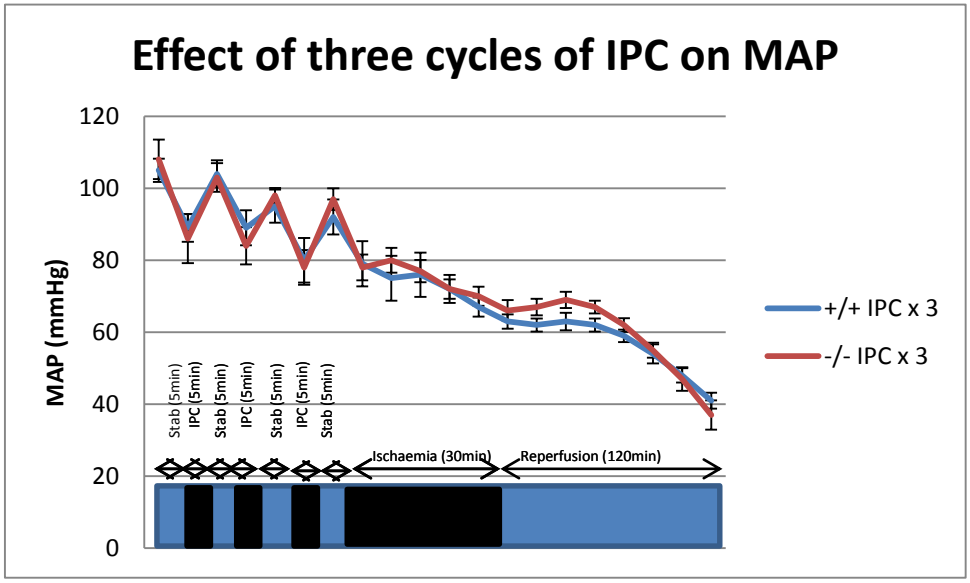


Figure 4.4 The data above demonstrates the blood pressure response to three cycles of ischaemic preconditioning

The blue bars represent LAD perfusion whilst the black boxes indicate periods of LAD ischaemia.

MAP: Mean arterial pressure, measured in millimetres of mercury (mmHg)

+/+ = Wild type mice (blue)

-/- = Cyclophilin D deficient mice (red)

The data is displayed as the mean, with error bars to represent the standard error of the mean.

The blood pressure response to three cycles of IPC was very similar in both wild type and cyclophilin D deficient mice.

Figure 4.7 demonstrates the temporal changes which occurred during the three cycles of IPC protocol. The initial blood pressure is satisfactory, and this drops in response to the first cycle of IPC. The pressure rises again, but does not fully recover to equate to the initial pressure. The next cycle of IPC follows a similar pattern with a large reduction in pressure confirming ischaemia, and again with reperfusion, the pressure improves, with partial recovery. Each preconditioning stimulus follows a similar pattern, and the mean arterial pressure is seen to undergo a graded decline. The period of index ischaemia results in a sustained reduction in MAP, and at reperfusion there is a further drop in pressure, due to the metabolic insult known as lethal reperfusion injury. There is a mild recovery of MAP after some time, and subsequently it gradually declines out to the two hour protocol end.

Haemodynamic differences observed

The graphic images appear to demonstrate that there is a significant difference in the initial mean arterial pressure recorded between the CypD knockout mice and the wild type. To test this hypothesis, a t test was carried out to compare the means of the initial MAP readings between all of the cyclophilin D deficient mice and all of the wild type mice undergoing the experimental protocol.

The results indicate that there was a statistically significant difference in MAP at the outset, with the mean for knock- out mice being higher than their wild type counterparts (109mmHg versus 99mmHg respectively).

However, during the remainder of the protocol the mean arterial pressure did not differ according to genotype, so this is not thought to be an important effect.

The data below demonstrates the values of mean arterial pressure recorded during the experimental protocol using three cycles of ischaemic preconditioning.

Table 4.3 Data tables for three cycles of IPC protocol

Stabilisation

Time (mins)	N	5	10 IPC	15	20 IPC	25	30 IPC	35
MAP (mmHg)+/+	6	105 (3.23)	89 (3.86)	104 (3.74)	89 (4.89)	95 (4.61)	80 (6.19)	92 (4.86)
MAP (mmHg)-/-	9	108 (5.47)	86 (6.80)	103 (3.98)	84 (5.17)	98 (2.09)	78 (4.83)	97 (3.00)
P value		0.67	0.70	0.76	0.51	0.69	0.84	0.34

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
MAP (mmHg)+/+	79 (6.28)	75 (6.27)	76 (6.14)	72 (3.89)
MAP (mmHg)-/-	78 (3.59)	80 (3.46)	77 (3.07)	72 (2.67)
P value	0.92	0.44	0.91	0.96

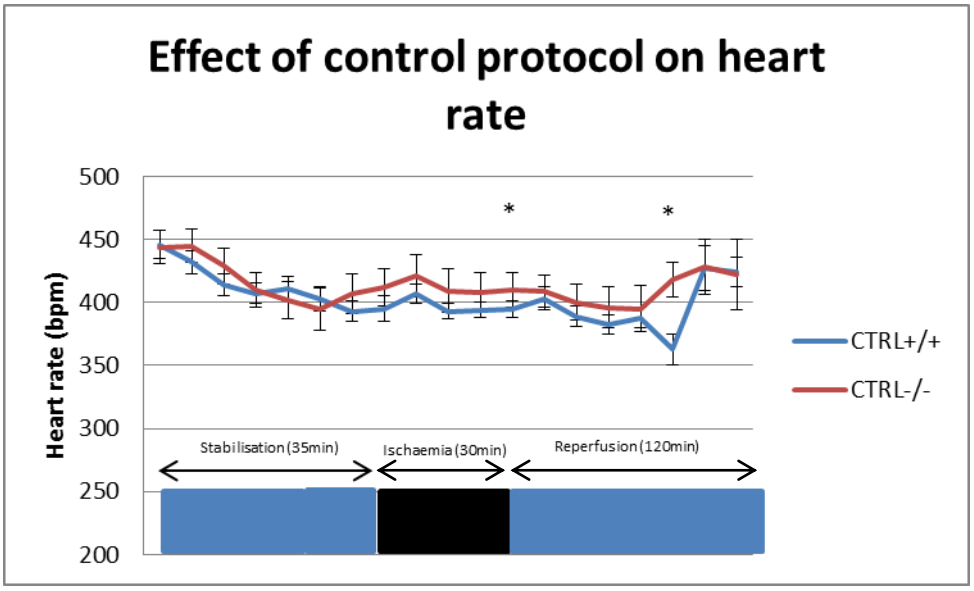
Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
MAP (mmHg)+/+	67 (2.65)	62 (1.99)	63 (1.82)	62 (2.43)	59 (1.75)	54 (2.65)	48 (1.98)	41 (2.23)
MAP (mmHg)-/-	70 (2.69)	67 (2.27)	69 (2.28)	67 (1.80)	62 (1.91)	55 (2.06)	47 (3.31)	37 (4.06)
P value	0.57	0.10	0.14	0.09	0.25	0.71	0.73	0.54

MAP= mean arterial pressure (millimetres of mercury), expressed as the arithmetical mean

+/+ = wild type mice, -/- = cyclophilin D knock-out mice

4.6.1 Effect of control protocol (no IPC) on heart rate profile



* = P<0.05

Figure 4.5 Heart rate profile in response to the control (no IPC) protocol in wild type and cyclophilin D- deficient mice

The blue bars represent LAD perfusion whilst the black boxes indicate periods of LAD ischaemia.

Heart rate is measured in beats per minute (bpm)

+/+ = Wild type mice (blue)

-/- = Cyclophilin D deficient mice (red)

The data is displayed as the mean, with error bars to represent the standard error of the mean.

The data is presented in tabular format overleaf.

Table 4.4 Heart Rate response to control protocol (no IPC) in wild type and cyclophilin D knock-out mice

Stabilisation

Time (mins)	N	5 mins	10 mins	15 mins	20 mins	25 mins	30 mins	35 mins
CTRL+/+ HR(bpm)	9	446 (11.15)	432 (8.95)	414 (8.91)	407 (8.14)	411 (10.07)	403 (9.43)	393 (8.23)
CTRL-/- HR(bpm)	9	418 (13.12)	413 (13.00)	397 (14.13)	400 (13.84)	398 (14.98)	387 (16.72)	400 (15.63)
P value		0.92	0.40	0.38	0.83	0.65	0.68	0.45

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
CTRL+/+HR (bpm)	395 (10.12)	407 (7.31)	393 (6.25)	394 (5.8)
CTRL-/-HR (bpm)	412 (14.93)	421 (17.14)	409 (17.25)	408 (15.63)
P value	0.34	0.46	0.35	0.07

Reperfusion

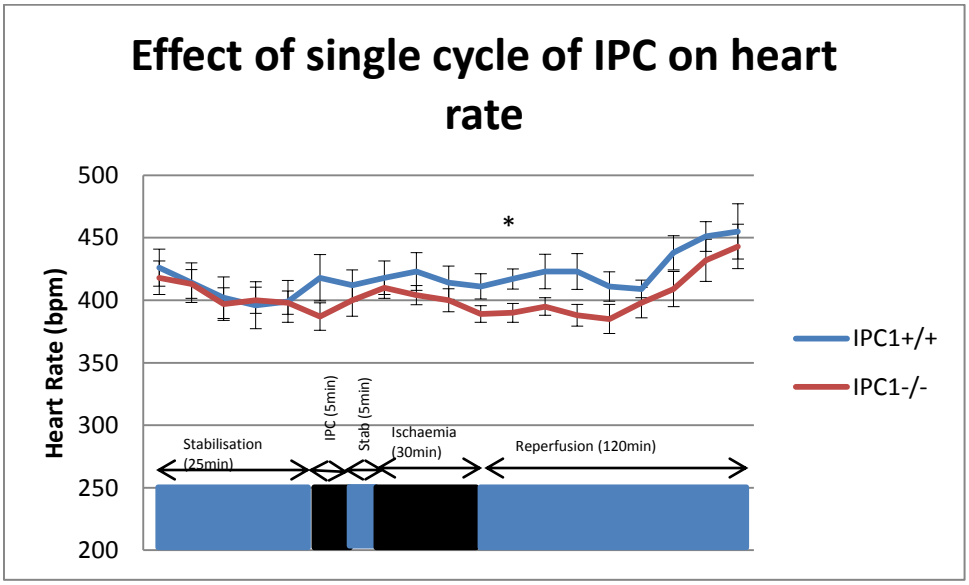
Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
CTRL+/+ HR(bpm)	395 (6.52)	403 (9.27)	389 (7.84)	382 (7.62)	388 (7.89)	363 (12.35)	427 (17.55)	424 (11.51)
CTRL-/- HR(bpm)	410 (13.56)	409 (12.90)	400 (14.04)	396 (16.34)	395 (18.54)	418 (13.92)	428 (22.03)	422 (28.18)
P value	0.03	0.07	0.05	0.14	0.47	0.03	0.38	0.68

HR = heart rate (beats per minute), expressed as the arithmetical mean.

+/+ = wild type mice

-/- = cyclophilin D knock-out mice

4.7.1 Effect of single cycle of IPC on heart rate profile



* = P<0.05

Figure 4.6 Heart rate profile in response to single cycle of IPC (wild type and cyclophilin D deficient mice).

The blue bars represent LAD perfusion whilst the black boxes indicate periods of LAD ischaemia.

Heart rate measured in beats per minute (bpm), expressed as the arithmetical mean.

+/+ = Wild type mice (blue)

-/- = Cyclophilin D deficient mice (red)

The data is displayed as the mean, with error bars to represent the standard error of the mean.

This data is presented in tabular format overleaf.

Table 4.5 Effect of single cycle of IPC protocol on heart rate

Stabilisation/IPC

Time (mins)	N	5 mins	10 mins	15 mins	20 mins	25 mins	30 mins	35 mins
IPC1+/+ HR(bpm)	6	426 (14.86)	414 (15.8)	402 (16.63)	396 (18.79)	399 (16.73)	418 (18.52)	412 (12.40)
IPC1-/- HR(bpm)	9	418 (13.37)	413 (11.56)	397 (13.01)	400 (10.36)	398 (9.35)	387 (10.94)	400 (12.87)
P value		0.71	0.96	0.81	0.85	0.80	0.17	0.50

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
IPC1+/+ HR(bpm)	418 (13.42)	423 (15.05)	414 (13.35)	411 (10.10)
IPC1-/-HR (bpm)	410 (8.49)	404 (7.70)	400 (9.18)	389 (6.56)
P value	0.62	0.29	0.35	0.08

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
IPC1+/+ HR(bpm)	417 (8.15)	423 (13.92)	423 (14.22)	411 (11.75)	409 (7.04)	438 (13.6)	451 (11.83)	455 (22.07)
IPC1-/- HR(bpm)	390 (7.56)	395 (6.97)	388 (8.72)	385 (11.66)	398 (12.10)	409 (14.04)	432 (16.88)	443 (17.81)
P value	0.03	0.08	0.05	0.14	0.47	0.17	0.38	0.68

HR = heart rate (beats per minute)

+/+ = wild type mice

-/- = cyclophilin D knock-out mice

4.8.1 The effect of three cycles of IPC on heart rate

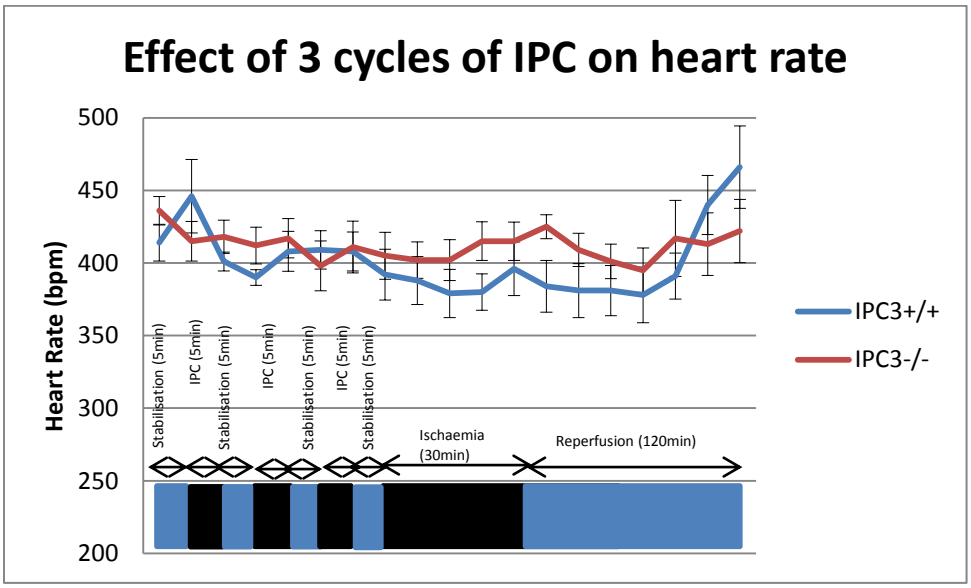


Figure 4.7 Heart rate response to three consecutive cycles of IPC in wild type and knock-out mice.

The blue bars represent LAD perfusion whilst the black boxes indicate periods of LAD ischaemia.

Heart rate measured in beats per minute (bpm)

+/+ = Wild type mice (blue)

-/- = Cyclophilin D deficient mice (red)

The data is displayed as the mean, with error bars to represent the standard error of the mean.

This data is shown in tabular format overleaf.

Table 4.6 Effect of three cycles of IPC on heart rate on wild type and cyclophilin D knock-out mice

Stabilisation/IPC

Time (mins)	N	5 mins	10 mins	15 mins	20 mins	25 mins	30 mins	35 mins
IPC3+/+ HR (bpm)	6	414 (12.63)	446 (25.36)	402 (6.58)	389 (5.35)	408 (13.78)	409 (13.22)	408 (13.46)
IPC3-/- HR (bpm)	9	436 (9.86)	415 (13.58)	418 (11.43)	412 (12.71)	417 (13.56)	398 (17.14)	411 (17.75)
P value		0.20	0.27	0.30	0.20	0.63	0.64	0.91

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
IPC3+/+HR (bpm)	392 (17.52)	388 (16.5)	379 (16.68)	390 (12.56)
IPC3-/-HR (bpm)	405 (16.23)	402 (12.54)	402 (14.01)	415 (13.32)
P value	0.59	0.52	0.30	0.22

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
IPC3+/+ HR (bpm)	396 (18.46)	384 (17.86)	381 (18.67)	381 (17.26)	378 (19.16)	391 (15.82)	440 (20.40)	466 (28.41)
IPC3-/- HR (bpm)	415 (13.27)	425 (8.25)	409 (11.46)	401 (11.89)	395 (15.30)	417 (26.19)	413 (21.66)	422 (21.78)
P value	0.40	0.10	0.20	0.28	0.50	0.47	0.41	0.24

HR = heart rate (beats per minute), expressed as the arithmetical mean.

+/+ = wild type mice

-/- = cyclophilin D knock-out mice

The heart rate profile shows common features in all three groups – initially the heart rate is high during stabilisation, and subsequently falls. During

ischaemia the heart rate rises as expected to maintain cardiac output, and this is persistent during the initial reperfusion phase. Later in reperfusion the heart rate falls slightly, to rise again at the end of 2 hours reperfusion as the blood pressure continues to fall.

4.9 Haemodynamics

In two of the experimental protocols it was noted that the initial mean arterial pressure was higher in cyclophilin D deficient than in wild type mice. This led us to compare the overall initial mean arterial pressure in all groups to assess if this was affected by genotype.

The mean MAP was 109mmHg in the CypD deficient group, and 98.5mmHg in the wild type mouse indicating a significant difference, with p of 0.008.

However, this difference was only present during the first recorded MAP and did not persist during the remainder of the experiment and for this reason it is unlikely to have had a significant impact on the results obtained.

4.10 Uniformity of area at risk (AAR) in IPC study

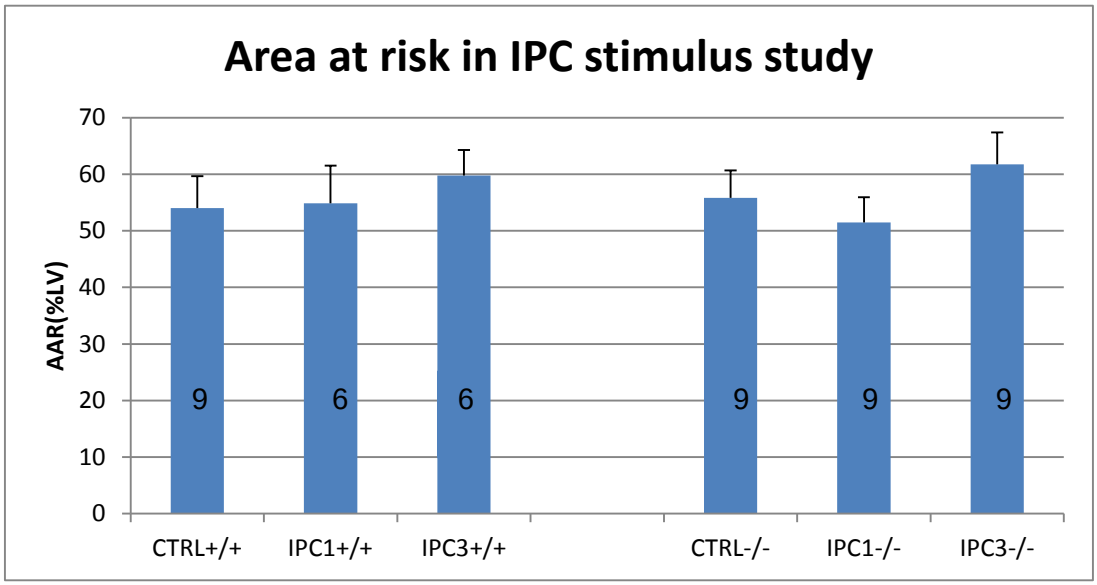


Figure 4.8 Area at risk expressed as a percentage of the left ventricle

AAR = Area at risk

%LV = percentage of left ventricle

The n number is denoted by the figure appearing in each data column.

As shown in the graph above, the area at risk was uniform between all groups within the ischaemic preconditioning study.

As discussed in the methods chapter, on measuring the infarct size, there are two main areas of myocardium identified: the area which stains blue with Evans Blue dye, and the area which does not. The area devoid of Evans' blue is the area which is subtended by the left anterior descending artery (LAD). This is referred to as the area at risk. It is well established that infarct size is influenced by the area at risk(76,185). The area at risk is a potential source of variation in infarct size and thus it is important to quantify it, to ensure there is no significant difference between the experimental groups.

4.11 Effect of increasing the ischaemic preconditioning stimulus

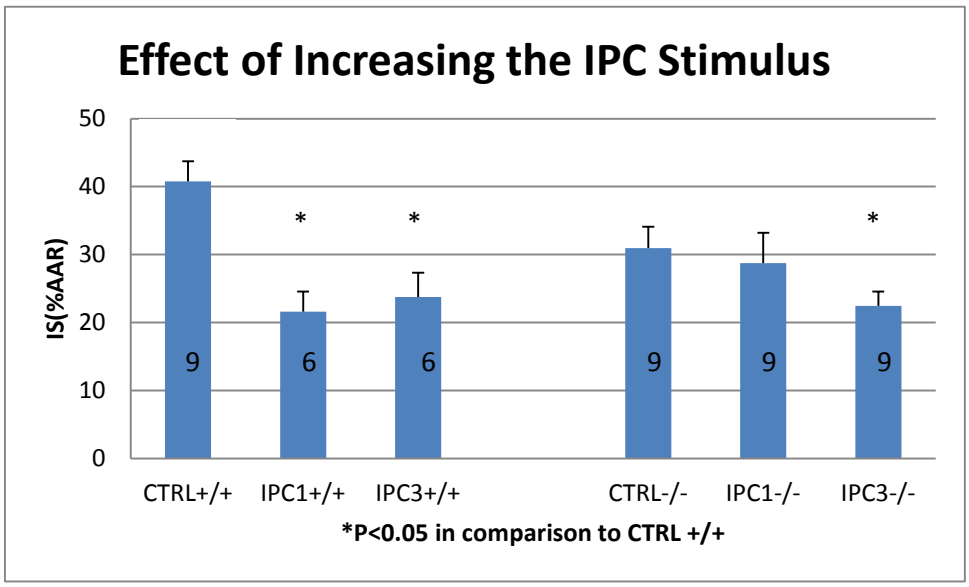


Figure 4.9 Effect of increasing the IPC stimulus on infarct sizes in wild type mice and controls.

The number of animals is displayed within the columns of data.

Key to Diagram:

CTRL refers to mice subjected to control protocol (ie no IPC)

IPC1 refers to mice subjected to a single IPC stimulus of 5 minutes

IPC3 refers to mice subjected to three episodes of five minutes IPC

The WT mice are referred to throughout the chapter as +/+

Mice deficient in cyclophilin D are referred to as -/-

	N	IS/AAR(%)	SEM
CTRL +/+	9	40.8	2.96
IPC1 +/+	6	21.6*	2.95
IPC3 +/+	6	23.7*	3.59
CTRL -/-	9	30.9	3.16
IPC1-/-	9	28.8	4.43
IPC3-/-	9	22.5*	2.12

Table 4.7 Infarct size expressed as a percentage of the area at risk in each treatment group

* = P<0.05 in comparison to wild type control protocol

The data above demonstrates that the application of a single IPC stimulus prior to 30 minutes of ischaemia results in a reduction of infarct size from 40.8% of the area at risk to 21.6% (P<0.05). The use of three IPC cycles did not appear to further reduce infarct size.

There was a trend towards a significantly smaller infarct size in the cyclophilin D deficient hearts in comparison to wild type (30.9% v 40.8% respectively). There was also a strong trend towards protection in the CypD deficient mice receiving 3 cycles of IPC, although this did not reach significance (30.9% v 22.5%). These findings are discussed overleaf.

4.12 Discussion

The aim of this chapter was:

1. **To test if the myocardium may be protected from ischaemia-reperfusion in cyclophilin D deficient mice by increasing the ischaemic preconditioning stimulus.**

Confirmation of protection in wild type mice

Firstly, the cardioprotective effect of ischaemic preconditioning by application of a single five minute cycle of IPC has been confirmed in wild type mice with a significant reduction in infarct size as shown in published data (54,177). Cardioprotection after three cycles of ischaemic preconditioning in wild type mice was also confirmed in keeping with the above publications (54,177).

There was no difference observed between the infarct sizes of the wild type mice receiving one and three cycles of ischaemic preconditioning, suggesting that once preconditioned, these hearts could not be protected further.

In the group of cyclophilin D knock-out mice, application of a single cycle of IPC did not reduce infarct size. This is expected as prior studies have shown that it has not been possible to protect Cyp D deficient mice from ischaemia-reperfusion injury when exposed to standard IPC protocols (54).

There was a strong trend towards a protective effect in the cyclophilin D deficient mice receiving three cycles of IPC. However, this did not reach statistical significance. It is possible that using larger experimental groups that protection may be seen. The statistical method of comparing multiple

measures to a single control may prevent a clear demonstration of such benefit.

In comparing the infarct size in the wild type mice and the cyclophilin D deficient mice subjected to ischaemia- reperfusion, there was no statistically significant difference between the groups with infarct size expressed as percentage of area at risk of 40.8% v 30.9% respectively (p=0.103). Prior data would suggest that a significant difference would be expected between these groups.

The control infarct size is in keeping with other series (54) and concurs with the infarct size found in C57BL/6 mice detailed as demonstrated in the characterisation of the model. It has been described in the literature that colony-specific variation of infarct size may occur (186), but as we used only one level of back-cross, then bred homozygotes, this would not be expected to incur a large variation in infarct size in our study.

Prior published data (from our centre) has shown a wild type control infarct size of 54%, and CypD deficient control infarct size of 27.9% (54). Baines described wild type control infarct size of 45% and CypD deficient controls of approximately 30% (13). In the experimental groups described in this chapter, the wild type control infarct size observed was 40.8%, and in the cyclophilin D deficient control mice 30.9%.

The infarct sizes obtained in the cyclophilin D deficient groups are all similar, but the “wild type” controls exhibit varied infarct sizes between studies.
It is important therefore to consider what exactly represented the “wild type”.

4.13 Importance of genetic background

In Baines paper, the wild type animal was a true wild type mouse, which was overexpressing cyclophilin D (+/+). In the paper by Lim et al, the “wild type” control was a B6129svF1, which was purchased from a commercial breeder (Harlan, UK).

Both wild type and cyclophilin D deficient animals used in the experiments described in this thesis were kindly provided by Baines et al. These were subjected to backcrossing with a C57BL6 background.

By this method CypD +/- (heterozygotes) are bred with one another and the homozygotes from each litter are discarded. Each subsequent litter results in an increase in the homogeneity of the genetic background. (see figure 4.4)

Genetic Background

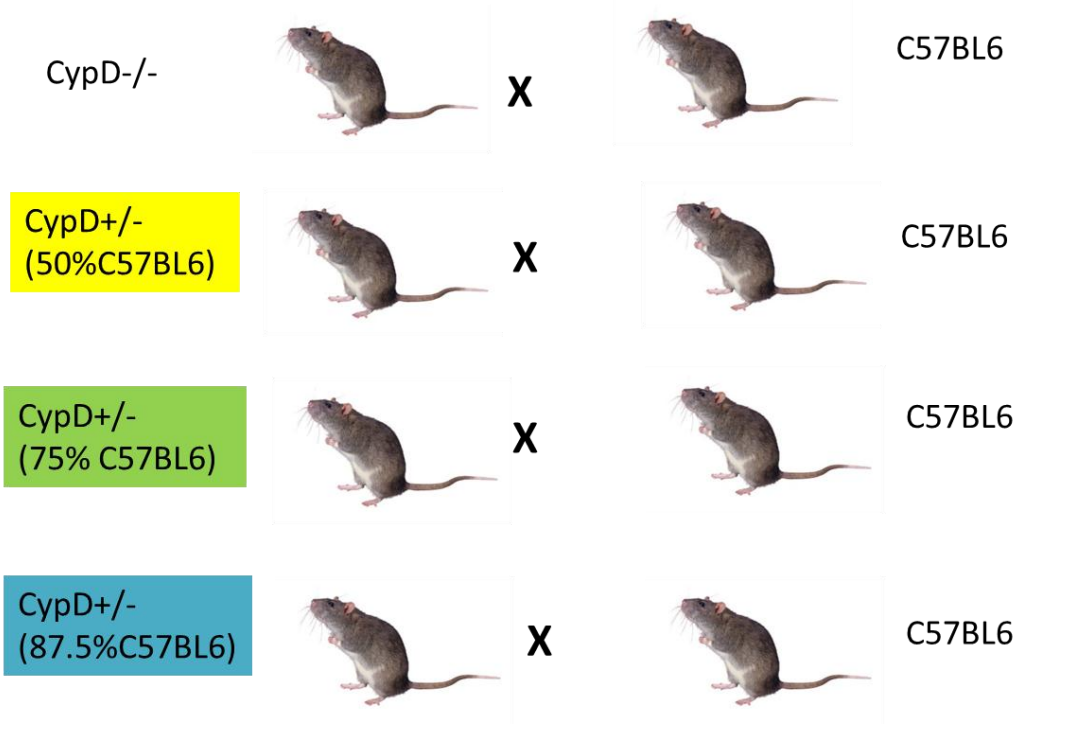


Figure 4.10 Backcrossing of mice with commercially available C57BL6 mice

At each level of backcrossing genotyping was carried out to identify homozygotes from heterozygotes. At each level heterozygotes are interbred with the C57BL6 mice.

The primary aim of backcrossing is to minimise the amount of genome-wide heterozygosity. At the third level of backcross, this is limited to 12.5% (ie 100-87.5%) (187).

In this thesis this was the level of backcrossing utilised. The heterozygotes from the third backcross were then interbred with one another to produce homozygous mice.

The table below demonstrates the genotypes obtained after breeding heterozygotes with one another (table 4.5)

CypD Allele	**+**	**-**
+	+/+	+/-
-	+/-	-/-

Table 4.8 Genotypes obtained after interbreeding of heterozygotes.

It has been described in the literature that there is a significant degree of diversity in the amount of myocardial damage which results from an ischaemic stimulus amongst mice with differing genetic background. Marber's group have described a very significant difference in the volume of infarct measured in a Langendorff model of global ischaemia. In this paper, the measured infarct sizes vary from 24% in one C57BL6 wild type mouse (MKK3) to 65.8% in another C57BL6 based wild type mouse (MAPKAPK2). This is borne out by previous data showing that in a model of transient forebrain ischaemia (188) and in situ mouse heart undergoing ischaemia-reperfusion (189) that different strains exhibit differing vulnerability to ischaemia.

This data make it unsurprising that the control infarct sizes in this study varied between Baines group and previous data from our unit, as the genetic background was differing in all three groups.

In order to clarify this issue further, increasing the n number in each group may be one option to determine a true difference between the groups, or comparing a smaller number of groups to control is another method which may facilitate the demonstration of a difference. It was our aim to minimise the

number of animals used in the experiments to comply with Home Office regulations. To increase the ischaemic stimulus (ie duration of ischaemia) in all groups would be another method of increasing the infarct size and potentially demonstrating important differences between the groups.

4.14 Was the IPC stimulus sufficient?

It would have been desirable to assess the effect of a further increase in the IPC stimulus, but due to localised damage to the left anterior descending artery occurring resulting in increased infarct size and mortality as described in the problems section, I did not find this to be feasible using the model described. Unfortunately, this is a limitation of this *in vivo* murine surgical model. There have been other methods of achieving LAD ischaemia, described by Eckle *et al* (212). It is possible that adoption of a different surgical model may result in less localised trauma to the LAD, potentially enabling more cycles of IPC to be administered.

Alternatively, to further test this hypothesis, the experiments could potentially be carried out in a larger animal model, by using an increased number of IPC stimuli. However, in view of the genetic malleability of mice, they are by far the most practical option when investigating the effect of ablation of a particular gene.

To further explore the effects of IPC in the cyclophilin D deficient mouse, it would be interesting to investigate whether signalling pathways known to be involved in IPC (190) are activated after three cycles of IPC, which could potentially indicate that there is a threshold effect. If there was found to be activation of the RISK pathway, this might prompt further experiments, perhaps in a mouse Langendorff model, with a larger n number. This is further discussed in the next section.

4.15 Should the sample size be increased?

Using Stata® software (version 10.0), a power calculation was carried out to assess whether a larger cohort would be required to achieve statistical significance. This demonstrated that to detect a 40% reduction in infarct size (ie infarct reduction from 46% to 28% based on previous literature), with a confidence level of 0.05, n of 12 in each group would be required.

To detect a 40% reduction in infarct size in the cyclophilin knock out group (from 28% to 17%) the desired n number would be 30 in each group.

This was considered, but due to both the limited availability of both knock out and wild type animals it was not felt to be feasible. Additionally, we felt that to do so would not be in keeping with the basic principles of the Guidance on the Use of Animals (Scientific Procedures) Act 1986, which encourages reduction of animal use where possible.

4.16 Problems Encountered

In this study, problems were encountered with the surgical model.

Although administration of one cycle of IPC with recovery of blood pressure and resolution of ECG changes was straightforward, when three cycles of IPC were undertaken, it was noted that in a number of cases (n=5), by the third cycle the arterial pressure and ECG did not resolve.

Examination of the LAD and left ventricle under the dissecting microscope showed that the myocardium remained pale and the LAD did not appear to refill. It was assumed that local damage to the LAD had occurred, which would result in an increase in the duration of ischaemia. In view of this, the animals falling into this category were excluded from the final analysis.

Hearts from 3 of these animals were examined and the infarct size found after 2 hours reperfusion was found to be consistent with this theory, with a mean infarct size (expressed as % of the area at risk) of 49.79% (higher than the control infarct size).

Additionally, increased mortality was noted (n=6, including the 5 mice which had obvious evidence of no-reflow) in the mice randomised to three cycles of IPC. These mice were excluded from the overall analysis as they did not reach the pre-determined two hour reperfusion end point, at which time the infarct size was measured.

As discussed in chapter one evidence is emerging to suggest that the MPTP, or more precisely, cyclophilin D, is unlikely to be the end effector of a pathway to myocardial damage. This was first considered because genetic ablation of cyclophilin D does not completely abolish myocardial damage in the setting of ischaemia-reperfusion. Further data by Nakagawa et al showed that cells from cyclophilin D deficient mice responded normally to a variety of apoptotic stimulants (r- Bid, Bad and r- Bax). Additional findings from this paper were that it was possible to induce the MPTP by administration of high doses of calcium, this would support the idea that inhibition of the MPTP is a threshold effect (165). This theory is further supported by research showing that in a model of muscular atrophy following denervation, cyclophilin D deficiency did not protect against muscular atrophy in response to activation of apoptotic pathways after denervation (191) by division of the sciatic nerve.

Further, unpublished work, described in Appendix 1.1 demonstrates cardioprotection by three cycles of IPC in an *ex vivo* model of ischaemia-reperfusion. In this series of experiments, not only was the Langendorff *ex vivo* model used- there was also an increase in the duration of ischaemia, which may highlight any difference between the groups by increasing the control infarct size. This data did show a reduction in infarct size by application of three cycles of IPC in the CypD deficient mouse.

The experiments described in this chapter did not support the hypothesis that ischaemic preconditioning can protect the heart in the absence of cyclophilin D.

4.17 Suggested areas for future study

Proposed future study would include assessment of signalling pathway activation during each protocol to demonstrate upregulation of these survival kinases (eg ERK 1 /2, AKT, MAPK) by Western blotting techniques. If there is upregulation of this pathway with a higher number of IPC cycles, this may suggest that, in fact there is a protective effect, but that our model is too insensitive to detect it.

A cellular model could also be used, for example a model of hypoxia-reoxgenation with repeated episodes of hypoxia to mimic the effect of ischaemic preconditioning. Both these models have significant limitations in their potential clinical applicability – the Langendorff *ex vivo* isolated perfused heart model, although preferred in comparison to cellular models, has the disadvantage of requiring differing concentration of drugs, and the full biological effect of drug is not assessed as neural and humoral signals are not appreciated by this method.

It would also be useful to carry out a dose – response curve to determine whether it would be possible to increase the duration of ischaemia further in order to improve the likelihood of detecting a difference between groups. In order to minimise animal use, this was not performed due to the low numbers of animals available.

There are alternative methods of achieving experimental LAD occlusion described in the literature, for example the “hanging weights” system described by Eckle *et al* (211). This system is reported to potentially reduce

the localised damage to the LAD as a result of the application of ischaemia, but no formal comparison of these methods has been carried out. For future experiments, this model could potentially be taken into consideration for use as an alternative.

Chapter 5

Mitochondrial Targeted Cyclosporin A

A Pharmacological Intervention Study

5.1 Background

Cyclosporin A (CsA) is known to protect the myocardium from ischaemia-reperfusion injury, and as such is the focus of much research into treatment of acute myocardial infarction. Cyclosporin A has a high binding affinity for the cyclophilins (peptidyl-prolyl isomerases (*PPIases*)). This effect is mediated by inhibition of cyclophilin D, which is an essential component of the MPTP. The MPTP plays a central role in the cell death which occurs after reperfused myocardial infarction.However, the cardioprotective effects of CsA are limited *in vivo* by its effect on the extra-mitochondrial cyclophilins (cyclophilin A and B). The interaction of CsA and cyclophilin A inhibits the calcium- dependent phosphatase, calcineurin.

The main therapeutic use of cyclosporin A at present is in immunosuppression, an effect which results from its binding to cyclophilin A. However, this also results in a variety of non-desirable clinical effects- renal and hepatic toxicity, as well as increased susceptibility to cancers.

Targeting of cyclosporin to mitochondria should in theory abolish these extra-mitochondrial effects mediated by calcineurin inhibition, whilst maintaining inhibition of peptidyl-prolyl isomerase (PPIase) activity, and thus MPTP inhibition.

It is also theoretically possible that therapeutic effect may be possible in a lower dose range if the drug has increased affinity for its binding site, which could also be clinically beneficial, by avoidance of dose related side effects.

Previous data has shown that in isolated mitochondria and hippocampal neurons deprived of glucose and oxygen administration of a novel mitochondrial- targeted mtCsA was cytoprotective (135).

In this chapter, the aim was to determine in the first *in vivo* experiment whether mtCsA would be cardioprotective in animals subjected to ischaemia-reperfusion.

In this chapter, we carried out the first *in vivo* experiments utilising a novel mitochondrial targeted form of cyclosporin A (mtCsA) created by Professor David Selwood (Wolfson Institute for Biomedical Research, University College London).

5.2 Aims

To prove the hypothesis: Administration of a mitochondrial specific analogue of cyclosporin A at reperfusion will offer superior protection to the myocardium from ischaemia-reperfusion in wild type mice when compared to standard CsA.

5.3 Methods

Animals were prepared and anaesthetised as described in chapter 3. In this chapter the jugular vein was dissected out and cannulated with P10 tubing in order to administer medication to the central circulation safely. In each of the experimental groups a fifteen minute stabilisation period was observed before the snare was tightened over the left anterior descending artery for a period of thirty minutes (index ischaemia). One minute prior to reperfusion, the drug to which the animal was randomised was infused slowly through the cannula to ensure bioavailability at the time of reperfusion. The snare on the coronary artery was then released. Two hours of reperfusion followed.

Drug was prepared by dissolving in 1% cremophor EL/ethanol solution and reconstituted with physiological saline. This was aliquotted into individual doses, frozen and was thawed in a water bath at 37ºC and sonicated both whilst being reconstituted and again after thawing for five seconds prior to administration to aid dissolution (volume 0.1ml).

5.4 Experimental Protocols

As the formulated mitochondrial targeted cyclosporin A (mtCsA) had not previously been administered *in vivo*, a selection of doses were used in order to create a dose response curve. Given the increased specificity of the drug for mitochondria, it was felt that doses lower than the conventional dose of cyclosporin used in animal models (10mg/kg) (54) should be used for initial investigations. On an arbitrary basis the doses 0.2mg/kg, 1mg/kg and 5mg/kg were selected for use.

Mice with evidence of barbarism were excluded (n=0). Also, mice were excluded if there was absence of a drop in mean arterial blood pressure (n=1) or if there was no change in electrocardiogram after the snare was applied (n=0).

Additionally, if the mean arterial pressure during the stabilisation period was <90mmHg (n=2), or if significant operative bleeding or the animal did not survive the 2 hour reperfusion period it was excluded (n=1).

Mice were randomised into eight groups as follows:

1. Wild type vehicle (WT VEH)
2. Wild type cyclosporin A 10mg/kg (WT CsA)
3. Wild type mitochondrial targeted cyclosporin A (0.2mg/kg) (WT mt 0.2)
4. Wild type mitochondrial targeted cyclosporin A (1mg/kg) (WT mt1)
5. Wild type mitochondrial targeted cyclosporin A (5mg/kg) (WT mt 5)
6. Cyp D knock out vehicle (KOVEH)
7. Cyp D knock out cyclosporin A (KOCSA)
8. Cyp D knock out mitochondrial targeted cyclosporin A (1mg/kg) (KO mt1)

5.5 Results

Physiological effect of pharmacological inhibition of cyclophilin in wild type mice

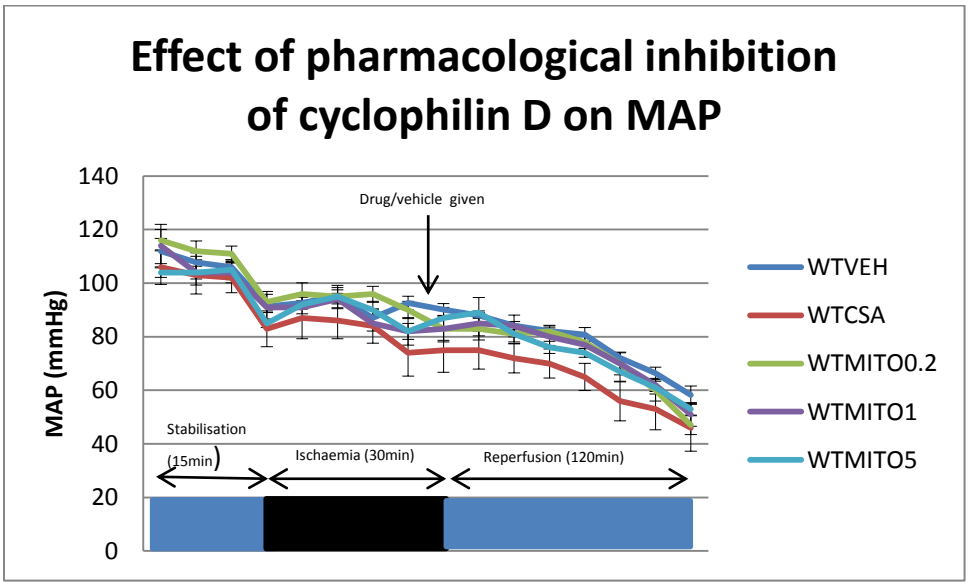


Figure 5.1 Effect of pharmacological inhibition of cyclophilin D on mean arterial pressure

MAP = mean arterial pressure (millimetres of mercury), expressed as mean value +/- standard error of the mean

Black bar denotes LAD ischaemia, Blue bar denotes LAD perfusion

WTCsA = Wild type treated with cyclosporin A

WTMITO 0.2 = Wild type treated with 0.2mg/kg mitochondrial targeted CsA

WTMITO 1 = Wild type treated with 1mg/kg mitochondrial targeted CsA

WTMITO 5 = Wild type treated with 5 mg/kg mitochondrial targeted CsA

Table 5.1 Effect of pharmacological inhibition of cyclophilin D on MAP

Stabilisation

Time (mins)	N	5 stab	10 stab	15 stab
VEH MAP (mmHg)	6	112 (4.66)	108 (3.60)	106 (2.76)
CsA MAP (mmHg)	6	106 (6.42)	103 (7.03)	102 (5.52)
P value VEH v CSA		0.45	0.51	0.45
mt0.2MAP (mmHg)	6	116 (4.00)	112 (3.71)	111 (2.89)
P value VEH v m0.2		0.55	0.43	0.25
mt1 MAP (mmHg)	6	114 (7.92)	104 (4.74)	104 (3.82)
P value VEH v Mt1		0.85	0.52	0.70
mt5 MAP (mmHg)	4	104 (1.78)	104 (2.39)	105 (3.66)
P value VEH v mt5		0.25	0.46	0.75

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
VEH MAP (mmHg)	91 (2.23)	93 (2.28)	95 (3.08)	87 (3.35)
CsA MAP (mmHg)	83 (6.69)	87 (7.73)	86 (6.77)	84 (6.39)
P value VEH v CSA	0.22	0.44	0.20	0.66
mt0.2MAP (mmHg)	93 (3.91)	96 (4.18)	95 (4.21)	96 (2.80)
P value VEH v m0.2	0.65	0.47	0.99	0.08
mt1 MAP (mmHg)	91 (4.81)	91 (2.46)	94 (3.49)	85 (2.94)
P value VEH v Mt1	0.90	0.58	0.71	0.69
mt5 MAP (mmHg)	85 (1.60)	92 (1.03)	95 (1.25)	90 (2.84)
P value VEH v mt5	0.10	0.80	0.99	0.53

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
VEH MAP (mmHg)	93 (2.59)	88 (1.09)	84 (1.56)	82 (2.13)	81 (2.58)	72 (1.87)	66 (2.20)	58 (3.36)
CsA MAP (mmHg)	74 (8.64)	75 (7.08)	72 (5.47)	70 (5.41)	65 (5.06)	56 (7.42)	53 (7.76)	46 (8.77)
P value VEH v CSA	0.04	0.05	0.04	0.05	0.01	0.03	0.07	0.18
mt0.2MAP (mmHg)	90 (3.20)	83 (4.26)	81 (3.67)	82 (2.02)	78 (2.40)	70 (4.26)	60 (3.99)	47 (3.51)
P value VEH v mt0.2	0.57	0.21	0.40	0.96	0.49	0.59	0.14	0.04
mt1 MAP (mmHg)	82 (5.16)	85 (4.62)	84 (4.06)	80 (3.26)	77 (3.37)	70 (2.02)	62 (2.41)	51 (4.37)
P value VEH v Mt1	0.09	0.56	0.91	0.58	0.32	0.45	0.14	0.23
mt5 MAP (mmHg)	82 (2.99)	89 (5.63)	81 (2.99)	76 (2.29)	74 (1.65)	67 (3.79)	61 (2.40)	53 (2.21)
P value VEH v mt5	0.02	0.91	0.26	0.09	0.11	0.22	0.12	0.32

VEH = wild type treated with vehicle only

CsA = wild type treated with 10mg/kg of cyclosporin A

mt 0.2 = Wild type treated with 0.2mg/kg mitochondrial targeted CsA

mt 1 = Wild type treated with 1mg/kg mitochondrial targeted CsA

mt 5 = Wild type treated with 5 mg/kg mitochondrial targeted CsA

5.6 The effect of pharmacological inhibition of cyclophilin D on mean arterial pressure in cyclophilin D deficient mice

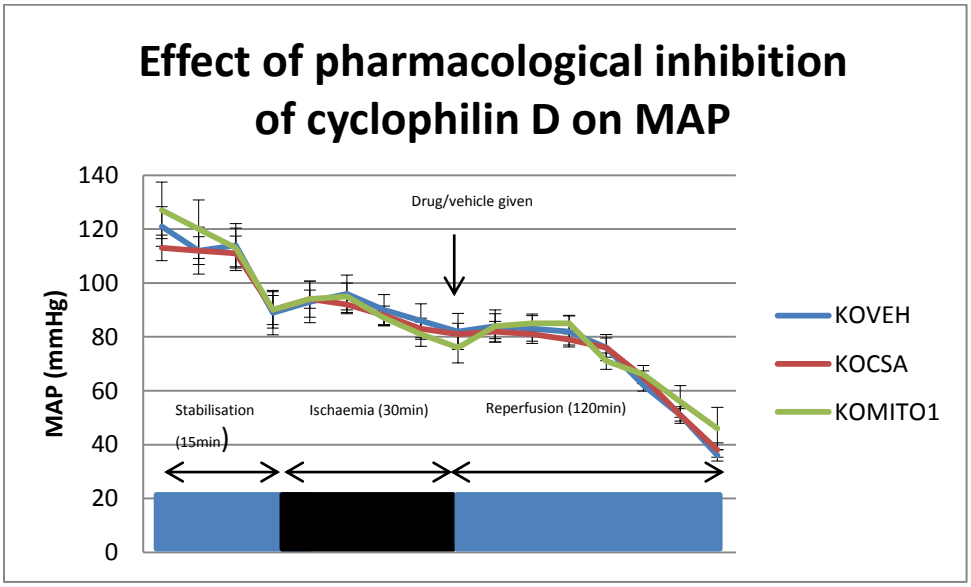


Figure 5.2 Effect of administration of cyclophilin D inhibitors on mean arterial pressure (MAP) during the experimental protocol in cyclophilin D knock- out mice

Black bar denotes LAD ischaemia

Blue bar denotes LAD perfusion

MAP expressed as mean +/- standard error of the mean (in millimetres of mercury)

KOVEH = knock-out treated with vehicle

KOCSA = knock-out treated with 10mg/kg cyclosporin A

KOMITO 1 = knock-out treated with 1mg/kg mitochondrial targeted CsA

The data shown above is demonstrated in tabular format overleaf.

Table 5.2 Effect of pharmacological inhibition of cyclophilin D on MAP in CypD deficient mice

Stabilisation

Time (mins)	N	5 stab	10 stab	15 stab
VEH MAP (mmHg)	6	121 (7.32)	112 (8.69)	114 (8.02)
CsA MAP (mmHg)	6	113 (4.74)	112 (5.15)	111 (6.39)
P value VEH v CSA		0.36	0.89	0.78
Mt1MAP (mmHg)	6	127 (10.52)	120 (10.83)	113 (7.40)
P value VEH v Mt1		0.62	0.61	0.98

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
VEH MAP (mmHg)	89 (8.25)	93 (7.70)	96 (6.96)	90 (5.74)
CsA MAP (mmHg)	90 (5.37)	94 (3.42)	92 (3.34)	88 (3.46)
P value VEH v CSA	0.92	0.97	0.66	0.79
Mt1MAP (mmHg)	90 (6.73)	94 (6.70)	95 (4.97)	87 (2.88)
P value VEH v Mt1	0.88	0.97	0.91	0.69

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
VEH MAP (mmHg)	86 (6.32)	84 (5.98)	83 (5.47)	82 (5.74)	76 (4.87)	62 (2.06)	51 (3.12)	36 (2.08)
CsA MAP (mmHg)	83 (3.91)	82 (3.79)	81 (2.60)	79 (2.07)	76 (3.55)	65 (2.33)	51 (2.32)	38 (2.69)
P value VEH v CSA	0.65	0.75	0.69	0.61	0.96	0.36	0.83	0.54
Mt1MAP (mmHg)	81 (4.48)	84 (4.6)	85 (2.93)	85 (3.01)	71 (3.04)	66 (3.33)	56 (5.88)	46 (7.88)
P value VEH v Mt1	0.58	0.99	0.72	0.71	0.41	0.31	0.42	0.21

All data expressed as mean value +/- SEM (in parentheses)

VEH = knock-out treated with vehicle

CSA = knock-out treated with 10mg/kg cyclosporin A

MITO 1 = knock-out treated with 1mg/kg mitochondrial targeted CsA

5.7 Effect of pharmacological inhibition of cyclophilin D on heart rate in wild type mice

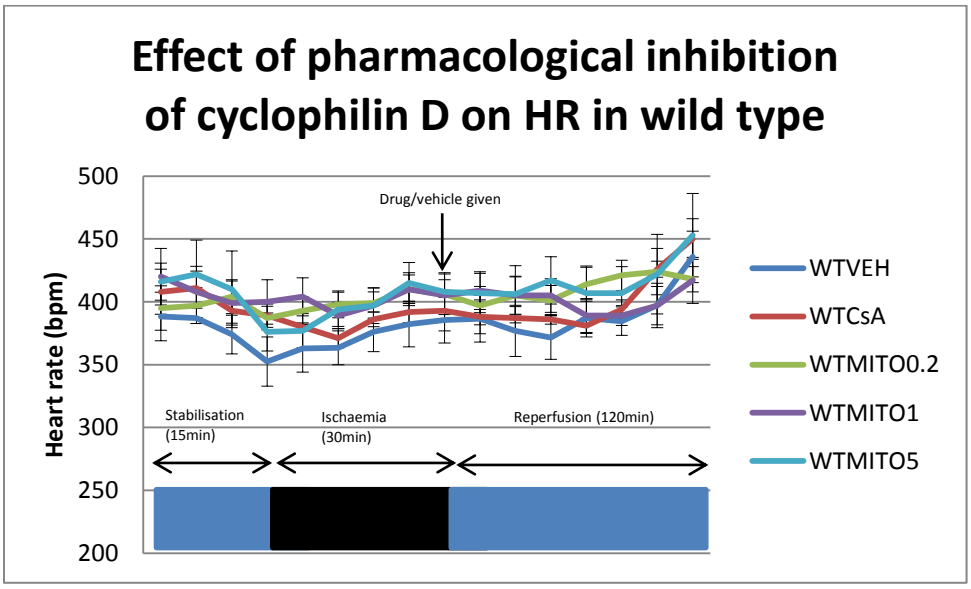


Figure 5.3 Effect of pharmacological inhibition of cyclophilin D on heart rate (measured in beats per minute) during ischaemia-reperfusion.

Key to Figure 5.3

Black bar denotes LAD ischaemia, Blue bar denotes LAD perfusion

Data expressed as mean value +/- standard error of the mean

WTCsA = Wild type treated with cyclosporin A

WTMITO 0.2 = Wild type treated with 0.2mg/kg mitochondrial targeted CsA

WTMITO 1 = Wild type treated with 1mg/kg mitochondrial targeted CsA

WTMITO 5 = Wild type treated with 5 mg/kg mitochondrial targeted CsA

This data is shown in tabular format overleaf.

Table 5.3 Effect of pharmacological inhibition of Cyp D on HR in WT

Stabilisation

Time (mins)	N	5 stab	10 stab	15 stab
VEH HR (bpm)	6	388 (19.31)	387 (12.72)	374 (15.37)
CsA HR (bpm)	6	408 (17.87)	411 (13.46)	393 (10.08)
P value VEH v CSA		0.50	0.24	0.37
mt0.2HR (bpm)	6	395 (17.62)	397 (14.15)	404 (13.44)
P value VEH v mt0.2		0.81	0.63	0.17
mt1 HR (bpm)	6	420 (22.54)	408 (20.32)	399 (17.55)
P value VEH v mt1		0.31	0.38	0.30
mt5 HR (bpm)	4	416 (14.74)	422 (26.99)	410 (30.54)
P value VEH v mt5		0.35	0.21	0.27

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
VEH HR (bpm)	352 (19.55)	363 (18.80)	363 (13.50)	376 (15.84)
CsA HR (bpm)	389 (9.34)	380 (3.14)	371 (7.55)	386 (5.87)
P value VEH v CSA	0.20	0.46	0.65	0.64
mt0.2HR (bpm)	387 (9.45)	393 (10.83)	398 (10.62)	399 (12.01)
P value VEH v 0.2	0.16	0.21	0.07	0.30
mt1 HR (bpm)	400 (17.68)	404 (15.17)	389 (10.43)	397 (10.87)
P value VEH v mt1	0.10	0.13	0.18	0.32
mt5 HR (bpm)	376 (15.14)	377 (13.60)	394 (13.58)	397 (14.30)
P value VEH v mt5	0.44	0.62	0.17	0.41

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
VEH HR (bpm)	382 (18.06)	387 (18.55)	377 (20.26)	372 (17.23)	387 (15.04)	384 (11.27)	397 (17.62)	436 (20.48)
CsA HR (bpm)	392 (8.43)	388 (13.44)	387 (9.15)	386 (3.81)	381 (6.13)	394 (7.52)	426 (6.34)	450 (16.25)
P value VEH v CSA	0.68	0.96	0.69	0.51	0.76	0.54	0.22	0.62
mt0.2HR (bpm)	410 (11.30)	397 (15.32)	405 (14.97)	401 (11.99)	414 (13.59)	421 (12.15)	424 (18.40)	418 (10.06)
P value VEH v mt0.2	0.23	0.70	0.30	0.20	0.22	0.048	0.31	0.46
mt1 HR (bpm)	410 (13.06)	409 (14.97)	405 (15.30)	405 (13.43)	389 (13.44)	389 (7.87)	397 (15.32)	417 (18.36)
P value VEH v mt1	0.25	0.39	0.30	0.17	0.92	0.79	0.99	0.50
mt5 HR (bpm)	415 (16.22)	407 (15.68)	406 (22.78)	417 (18.76)	407 (21.43)	407 (21.04)	422 (31.61)	453 (33.12)
P value VEH v mt5	0.26	0.49	0.38	0.12	0.45	0.34	0.48	0.66

VEH = wild type treated with vehicle

CsA = Wild type treated with cyclosporin A

mt0.2 = Wild type treated with 0.2mg/kg mitochondrial targeted CsA

mt1 = Wild type treated with 1mg/kg mitochondrial targeted CsA

mt5 = Wild type treated with 5 mg/kg mitochondrial targeted CsA

5.8 The effect of pharmacological inhibition of cyclophilin D in Cyp D knock- out mice on heart rate response to ischaemia-reperfusion

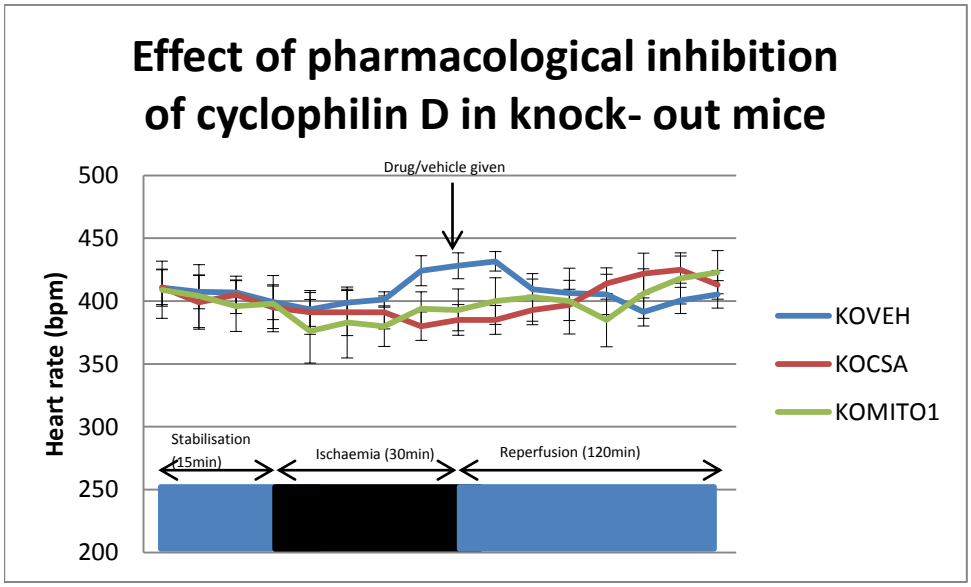


Figure 5.4 Effect of pharmacological inhibition of cyclophilin D on heart rate (beats per minute) in cyclophilin D deficient mice subjected to ischaemia-reperfusion

Black bar denotes LAD ischaemia

Blue bar denotes LAD perfusion

Data expressed as mean value +/- standard error of the mean

KOVEH= knock-out treated with vehicle

KOCsA = knock-out treated with 10 mg/kg cyclosporin A

KOMITO 1 = knock-out treated with 1mg/kg mitochondrial targeted CsA

The above data is presented in tabular format overleaf.

Table 5.4 Effect of pharmacological inhibition of CypD on heart rate in cyclophilin D deficient mice

Stabilisation

Time (mins)	N	5 stab	10 stab	15 stab
VEH HR (bpm)	6	411 (14.84)	407 (13.51)	407 (9.96)
CsA HR (bpm)	6	411 (13.88)	399 (21.23)	405 (14.82)
P value VEH v CSA		0.98	0.76	0.91
Mt1HR (bpm)	6	409 (22.82)	404 (25.13)	396 (20.20)
P value VEH v mt1		0.94	0.91	0.64

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
VEH HR (bpm)	399 (13.87)	394 (13.48)	399 (9.48)	401 (6.04)
CsA HR (bpm)	395 (16.94)	391 (17.49)	391 (18.45)	391 (13.05)
P value VEH v CSA	0.84	0.91	0.73	0.48
Mt1HR (bpm)	398 (22.34)	376 (25.24)	383 (28.29)	383 (16.12)
P value VEH v mt1	0.98	0.55	0.59	0.21

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
VEH HR (bpm)	424 (11.86)	432 (7.79)	410 (7.99)	407 (10.15)	405 (16.03)	391 (11.18)	401 (10.35)	405 (10.87)
CsA HR (bpm)	380 (11.33)	385 (11.53)	393 (11.67)	397 (12.37)	414 (12.52)	422 (16.28)	425 (10.82)	413 (11.34)
P value VEH v CSA	0.02	0.007	0.27	0.55	0.69	0.15	0.14	0.66
Mt1 HR (bpm)	394 (13.30)	400 (18.64)	403 (18.90)	400 (26.28)	385 (21.22)	406 (19.69)	418 (20.34)	423 (17.20)
P value VEH v mt1	0.12	0.13	0.75	0.80	0.45	0.51	0.44	0.39

VEH= knock-out treated with vehicle

CsA = knock-out treated with 10 mg/kg cyclosporin A

Mt1 = knock-out treated with 1mg/kg mitochondrial targeted CsA

5.9 Haemodynamic effects of pharmacological inhibition of cyclophilin D

The data above demonstrates that the blood pressure response during this experimental protocol is similar within all experimental groups- including both cyclophilin D deficient and wild type genotypes.

The mean arterial pressure tends to drop slightly after the first recording made during the stabilisation phase. This is likely due to the stress involved in being anaesthetised. It then remains stable during the stabilisation phase and drops markedly at the onset of ischaemia (it is one of the findings which confirms ischaemia). It then drifts downwards slightly during the period of ischaemia. Due to the administration of the fluid bolus containing the experimental drug or vehicle, there is a rise in the observed MAP at the beginning of the reperfusion phase. The effect of this is brief, and the MAP gradually declines as the protocol continues to its two hour completion, as observed in the preconditioning experiments of chapter four.

The heart rate responses were similar within all groups during the protocol. This remained constant throughout the protocol, but towards the end of the experiments there was a tendency of the heart rate to rise slightly, as part of the haemodynamic response to heart failure.

5.10.1 Area at risk in wild type mice

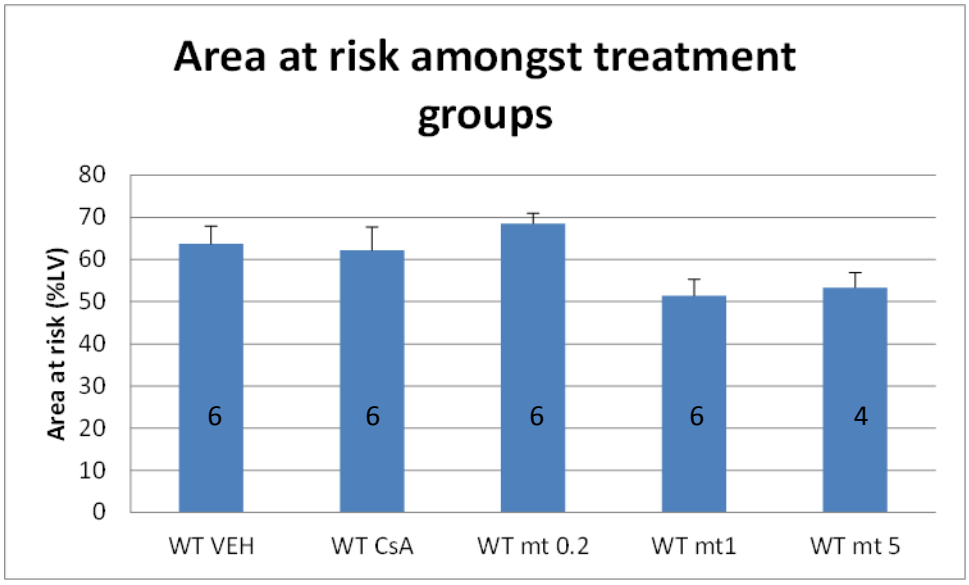


Figure 5.5 Area at risk in wild type mice subjected to administration of cyclophilin D inhibitors at the time of reperfusion

Area at risk is expressed as a percentage of the left ventricle, and mean value displayed +/- standard error of the mean

N for each group is displayed in each column.

WT VEH= wild type receiving vehicle only

WT CsA = wild type receiving 10mg/kg of cyclosporin A

WT mt0.2 = wild type receiving 0.2 mg/kg of mitochondrial targeted CsA

WT mt 1 = wild type receiving 1mg/kg of mitochondrial targeted CsA

WT mt 5 = wild type receiving 5mg/kg of mitochondrial targeted CsA

The area at risk did not vary significantly amongst the groups.

5.10.2 Area at risk in cyclophilin D knock out mice

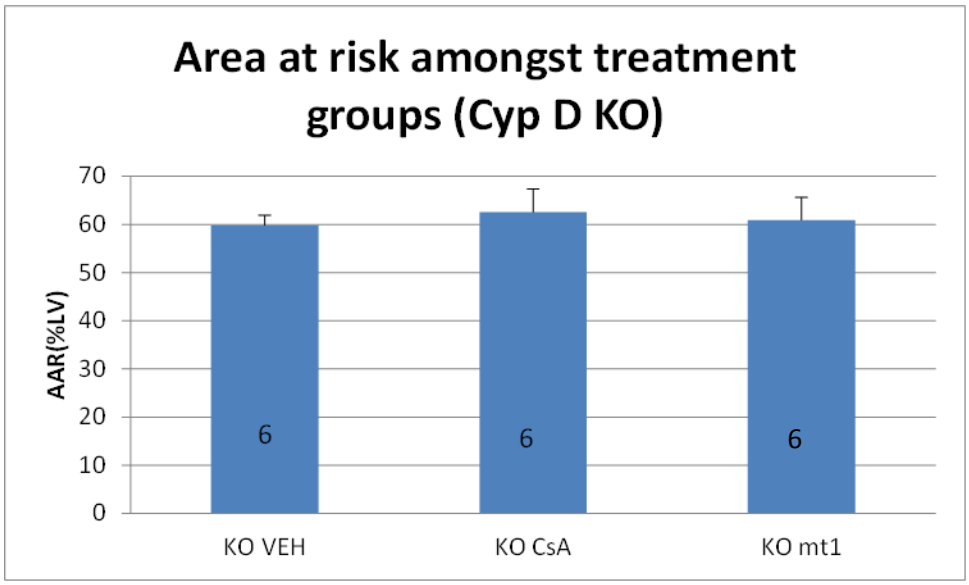


Figure 5.6 Area at risk expressed as a percentage of the left ventricle.

Data expressed as the mean value +/- standard error of the mean. The n number of each group is displayed in the corresponding column.

KO VEH = knock-out receiving vehicle

KO CsA = knock out receiving 10 mg/kg cyclosporin A

KO mt1= knock-out receiving 1 mg/kg of mitochondrial-targeted cyclosporin A

As shown above, the area at risk was uniform amongst treatment allocations.

5.11 Effect of cyclophilin D inhibition on infarct sizes after ischaemia-reperfusion in wild type mice

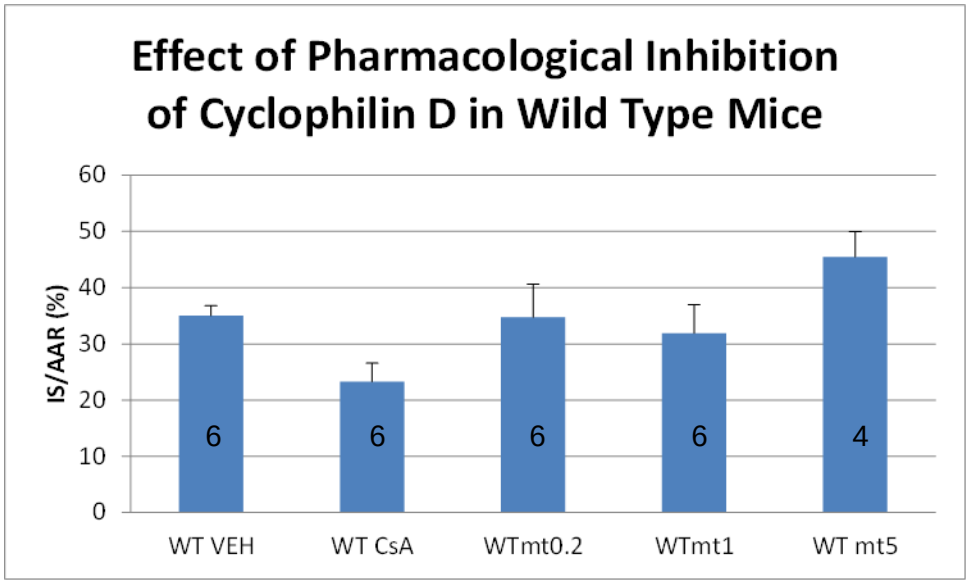


Figure 5.7 Infarct sizes obtained after administration of cyclophilin D inhibitors to wild type mice at reperfusion

IS/AAR(%) = infarct size expressed as a percentage of the area at risk

WT VEH= wild type receiving vehicle only

WT CsA = wild type receiving 10mg/kg of cyclosporin A

WT mt0.2 = wild type receiving 0.2 mg/kg of mitochondrial targeted CsA

WT mt 1 = wild type receiving 1mg/kg of mitochondrial targeted CsA

WT mt 5 = wild type receiving 5mg/kg of mitochondrial targeted CsA

The n number in each group is displayed in the corresponding column.

Effect of pharmacological inhibition of Cyp D on infarct size

	IS/AAR (%LV)	SEM
WT vehicle	35.04	1.74
WT cyclosporin A	23.26	3.33
WT mt CsA 0.2	34.71	5.93
WT mtCsA 1	31.89	5.06
WT mtCsA 5	45.46	4.46

Table 5.5 Effect of pharmacological inhibition of cyclophilin D on infarct size in wild type mice

WT vehicle = wild type treated with vehicle

WT cyclosporin A = wild type treated with cyclosporin A

WT mtCsA 0.2= wild type receiving 0.2 mg/kg of mitochondrial targeted CsA

WT mtCsA 1 = wild type receiving 1mg/kg of mitochondrial targeted CsA

WT mtCsA 5 = wild type receiving 5mg/kg of mitochondrial targeted CsA

The data above demonstrates that there was a strong, but not significant trend towards protection in the group treated with CsA in comparison to vehicle (35% v 23%, P>0.05). The groups receiving the lower doses of mitochondrial specific CsA exhibited infarct sizes comparable to vehicle controls. The group receiving the highest dose of mtCsA showed a higher infarct size (not significant) than control, which could suggest that the treatment is actually harmful at higher doses.

5.12 Effect of pharmacological inhibition of cyclophilin D on infarct size in cyclophilin D deficient mice

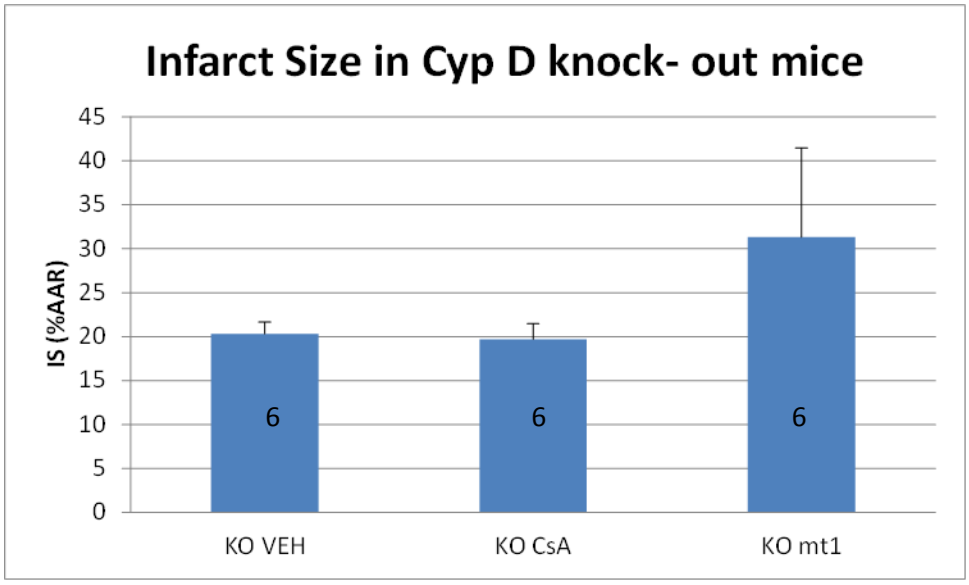


Figure 5.8 Infarct size obtained after cyclophilin D knock- out mice received pharmacological inhibition of cyclophilin at the time of reperfusion

IS/AAR(%)= Infarct size expressed as a percentage of the left ventricle

N number for each group displayed in the corresponding column

KO VEH = knock-out receiving vehicle

KO CsA = knock out receiving 10 mg/kg cyclosporin A

KO mt1= knock-out receiving 1 mg/kg of mitochondrial-targeted cyclosporin A

The data above shows that there was no difference in infarct size measured after 30 minutes ischaemia in cyclophilin D deficient mice treated with CsA. The administration of mitochondrial-targeted CsA resulted in an apparent (non- significant) increase in infarct size observed.

Effect of pharmacological inhibition of cyclophilin D on infarct size in knock- out mice subjected to ischaemia-reperfusion

	KO vehicle	KO cyclosporin A	KO mitoCsA
IS/AAR(%LV)	20.32	19.70	31.32
SEM	1.33	1.76	10.15

Table 5.6 Effect of pharmacological inhibition of cyclophilin D in knock out mice

IS = infarct size

AAR = area at risk

SEM = standard error of the mean

KO vehicle = knock- out receiving vehicle

KO cyclosporin A = knock- out receiving cyclosporin A

KO mito CsA = knock-out receiving mitochondrial targeted cyclosporin A

There was no change in infarct size observed after the administration of cyclosporin A in comparison to vehicle control (20% v 20%, P>NS) to Cyp D deficient mice. However, when mitochondrial-targeted CsA was given to Cyp D deficient mice, there was an increase in infarct size observed.

5.11 Discussion

This group of experiments was designed to test the hypothesis that administration of a novel mitochondrial targeted form of cyclosporin A at the time of reperfusion in a mouse model of ischaemia-reperfusion injury would be cardioprotective.

From the data represented in fig 5.7, it can be seen that there was no evidence of cardioprotection in wild type mice from mitochondrial -targeted cyclosporin administered at doses of 0.2mg/kg, 1mg/kg and 5mg/kg just prior to the onset of reperfusion.

As part of this chapter we administered cyclosporin A as a positive control. In this series of experiments, when analysed as a group by Tukey's analysis of multiple means (assuming equal variances) this did not demonstrate a statistically significant reduction in infarct size. There was a strong trend to a reduction in infarct size, which, when analysed by Student's T test did appear to be significant, with a p value of <0.05. However, when comparing multiple groups to a single control, the T test would not be an appropriate statistical test to use. This demonstrates the dilution effect of multiple comparisons when trying to demonstrate statistical significance.

In the cyclophilin D knock- out mice, there was no significant difference between the groups. As expected, there was no difference between the Cyp D knockouts receiving vehicle and those receiving cyclosporin A. The effect of cyclosporin A is mediated by via cyclophilin D, and prior data supports the fact that cyclophilin D deficient animals treated with CsA do not show a reduction in infarct size after ischaemia-reperfusion (54).

To minimise the use of animals, in the Cyp D knock -out group only one dose of mitochondrial targeted cyclosporin A was tested. The mid-range dose of 1mg/kg was arbitrarily selected for use in this experiment. Also, use of a mitochondrial - targeted molecule would not be expected to confer any reduction in infarct size in this group for the same reason. It was interesting to note that the infarct size was observed to be higher in the group treated with mitochondrial targeted cyclosporin A. This raises the possibility that the treatment may be harmful.

Localisation of mitochondrial specific cyclosporin A to site of action

To investigate whether the mtCsA reached its intended site of action in mitochondria, after terminal anaesthesia with 160mg/kg pentobarbitone, *ex vivo* hearts were perfused with mtCsA labelled with100nM chloromethyl-X-rosamine (Mito-tracker Red- which labels respiring mitochondria) in a Langendorff model using C57BL6 (Harlan, UK) mice by Dr Sean Davidson (Hatter Institute).

Twenty micromoles per litre of blebbistatin (an inhibitor of actin–myosin cross-bridge cycling) was added to prevent contraction and movement (201).

Fluorescence was then excited at 840nm and multi-photon imaging was carried out using a Zeiss 510 NLO microscope. Fluorescence was strongly localised to cardiomyocyte mitochondria as shown in figure 5.9 below

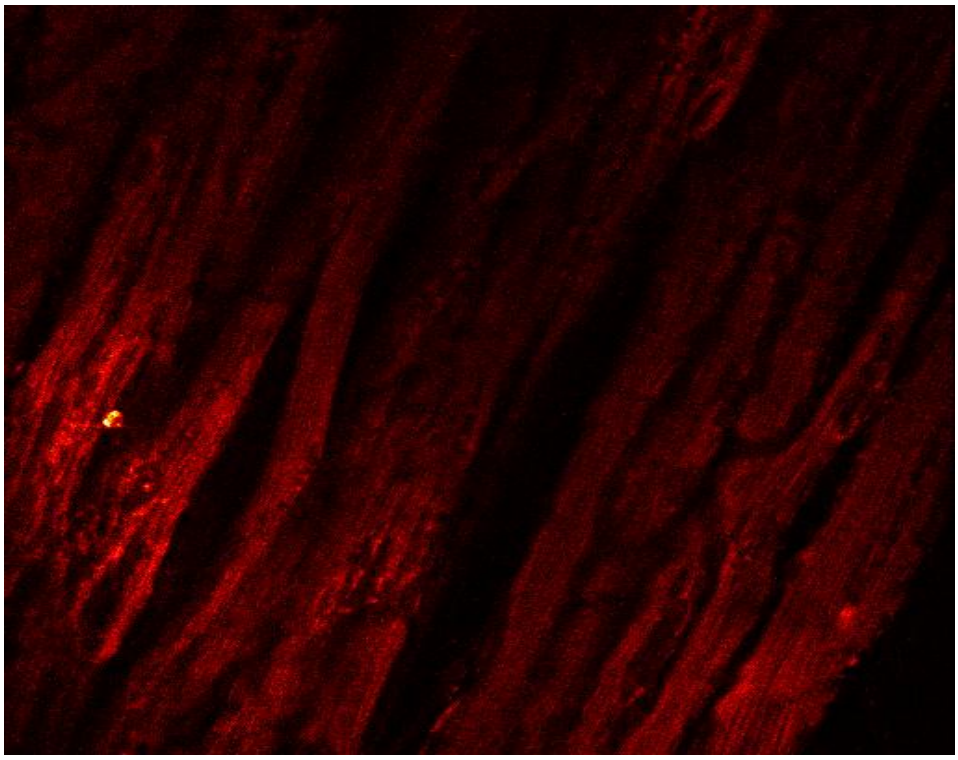


Figure 5.9 Fluorescence of cardiomyocyte with loaded with 100 nano-molar mtCsA – rosamine

(Reproduced with kind permission of Dr Sean M Davidson (Hatter Institute, UCL)

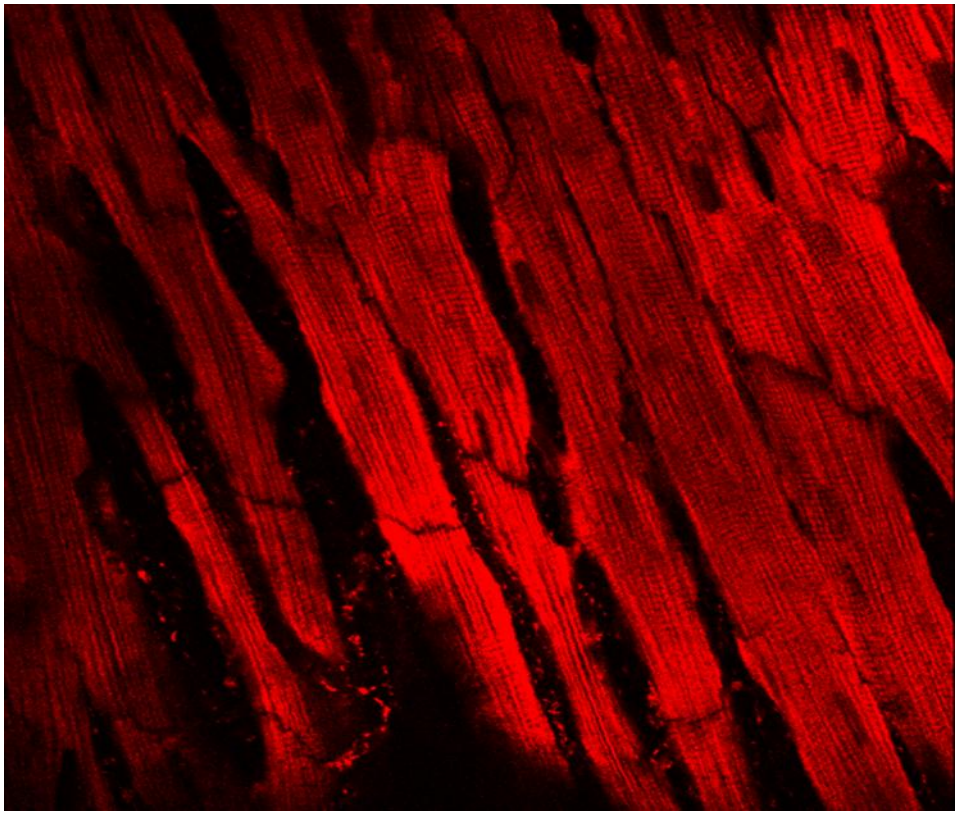


Figure 5.10 Fluorescence in heart loaded with TMRM (tetra-methylrhodamine-methyl ester), which labels respiring mitochondria.
Reproduced with kind permission of Dr Sean M. Davidson (Hatter Institute, UCL).

The images above demonstrate that the fluorescence of the mtCsA is approximately 50% of that in the TMRM assay, ie mtCsA has been taken up into approximately 50% of respiring mitochondria, thus confirming that it has reached its desired site of action.

In this chapter the pharmacological effect of a novel, potentially cardioprotective agent has been studied in both wild type and cyclophilin D knock- out mice which have been subjected to myocardial ischaemia and reperfusion.

Cyclosporin A has a high degree of affinity for the Cyclophilins. These are proteins which possess peptidyl-prolyl isomerase activity, and leads them to be referred as “PPIases” as discussed in chapter 1. This is an essential process required for protein folding *in vivo.* Cyclosporin A inhibits PPIase activity in its bound state. Cyclophilin A and B are located in the cytosol and endoplasmic reticulum respectively, whilst cyclophilin D is located within mitochondria. The function of these proteins varies widely: CypA is involved in translocation of apoptosis inducing factor to the nucleus and protection from oxidative stress, whilst CypB suppresses the apoptosis involved with oxidative stress and altered calcium metabolism. The binding of cyclosporin A to CypA results in a drug-protein complex which inhibits calcineurin (a calmodulin and calcium- dependent phosphatase) (82). This process is responsible for the immunosuppressive effect of cyclosporin A, causing inhibition of interleukin-2, tumour necrosis factor -α, interleukin-3, interleukin-4, CD 40L, granulocyte – macrophage colony stimulating factor and interferon-ɣ (194). In 1990, Halestrap and Davidson revealed that CsA inhibited the opening of the MPTP by binding to cyclophilin D (52). Cyclophilin D was later confirmed as the molecular target of cyclosporin A in 1996 (53).

Genetic ablation of cyclophilin D in mice by Baines has since confirmed that CypD is the cellular target of cyclosporin A, and that it is essential for the function of the MPTP (213).

Cyclosporin A, as discussed in chapter 1 exerts a cardioprotective effect on the myocardium when administered prior to reperfusion. This has been reproduced in many models and in a variety of settings (54, 213). However, its deleterious effects on the immune system and renal function make it undesirable for clinical use. Its effect in clinical trials has also been disappointing limited (112, 216).

The potential benefits of targeting cyclosporin A to mitochondria, and thus increasing the selectivity to cyclophilin D are increased potency and abolition of these cyclophilin A/ calcineurin inhibitory mediated side effects of cyclosporin which involve immunosuppression, renal and hepatic toxicity and increased susceptibility to cancers.

The synthesis of a novel mitochondrial selective form of cyclosporin A (mtCsA) thus held much promise. The novel mitochondrial selective cyclosporin A (mtCsA) molecule was created by Professor David Selwood (University College London), by conjugating cyclosporin A with the triphenyl-phosphonium cation. The resulting compound (mtCsA) then accumulates into the mitochondria by electrophoresis down the proton gradient of the mitochondrial membrane.

In vitro testing of this agent described by Maloutrie *et al* demonstrated positive results, with a reduction in cellular necrosis seen in hippocampal neurons subjected to deprivation of glucose and oxygen, which was superior to that of

cyclosporin A. Additionally, mtCsA was shown not to inhibit calcineurin, thereby potentially avoiding the deleterious effects of cyclosporin A (135).

The series of experiments detailed in chapter 5 represented the first *in vivo* testing of the mtCsA molecule. There had been no previous work to confirm its utility in this setting, nor its bioavailability at the desired site of action. The doses selected for use were lower than the experimental dose of cyclosporin A on the basis that as the molecule was more specific to mitochondria, the dose required would be lower to achieve the desired protective effect. We did attempt a dose- response curve to assess the optimal drug dose for use, but found that none achieved any benefit in terms of reduction in infarct size. It is possible therefore that all doses were either too low to achieve any clinical effect, or conversely, were administered at toxic doses. The experiments carried out by another researcher in our centre using an *ex vivo* model (Fig 5.10) did later confirm that the mtCsA did reach the desired site of action. The *in vivo* effects of mtCsA however, have not demonstrated any benefit in terms of reduction in infarct size, despite being tested at a variety of doses.

The potential reasons behind this are multiple-

1. The cyclophilin D binding affinity was not maintained due to structural modifications occurring during the manufacturing of the mtCsA molecule.

2. Recent data has shown that the mtCsA molecule required a higher dose of mtCsA to block the MPTP than CsA itself (192)

3. The drug may have a low therapeutic index, resulting in administration of toxic doses

4. The uptake of the drug, administered one minute prior to reperfusion via the internal jugular vein *in vivo* may be insufficient to enter mitochondria by the time of pore opening.

In considering the above scenarios- and taking each in turn, it is possible that the altered structure of the mtCsA could potentially reduce the cyclophilin D binding affinity as a result of the manufacturing process. The method of chemical linkage from the triphenyl-phosphonium cation to CsA uses “position-3”, which is required for the insertion of CsA into CypD. As a result, there is the potential for sterical prevention of binding to cyclophilin D. However, switching to other mitochondrial- targeting agents, such as Rosamine, for linkage, had similar effect, with similar reduction in binding affinity, suggesting that the TPP molecule was not directly responsible for this effect. It has been proposed that the actual linker from mtCsA to CypD may have undergone a conformational change resulting in folding back on itself, maximising hydrophobic interactions, this reducing access to the binding sites

on cyclosporin A. This is borne out by the findings detailed by Dube, whereby the adoption of a shorter (ether) linking molecule in combination with the TPP molecule has improved the binding affinity of a novel form of mtCsA (192).

A reduction in binding capacity of mtCsA for cyclophilin D has been confirmed in later experiments by Dube *et al.* (192).

The suggestion of toxic dose administration, although possible, is unlikely given the findings of Dube *et al,* discussed below.

The late drug uptake *in vivo* may remain a possibility, but given that cyclosporin A when reconstituted in an identical manner reaches its site of action in identical circumstances, is unlikely. Additionally, the mtCsA was shown to be present in mitochondria, in an *ex vivo* model (fig 5.9 and 5.10).

A recent publication by Dube *et al*, has discussed the reason for therapeutic failure of the initial mtCsA and examines the effect of a new molecule, which possesses an 18- fold increase in the cyclophilin D affinity in comparison to the molecule we tested, and 12- fold increase in MPTP inhibition. The binding affinity of the original mtCsA was studied and was noted to be 1/30 of the binding affinity for cyclosporin A (192). This would suggest that a lack of binding affinitity for cyclophilin D is the primary reason for the failure of mitochondrial- specific CsA (mtCsA) to protect the heart from ischaemia-reperfusion injury.

Proposed future work would involve administration of the second generation mitochondrial targeted cyclosporin A to in an *in vivo* mouse model of ischaemia-reperfusion, and demonstrating a dose response curve in order to

determine the most effective dose with which to achieve cardioprotection. To confirm the molecule reaching the site of action, it would be useful to repeat the experiments of Dr Davidson, using mito-tracker red labelled with the second generation mtCsA.

A direct comparison to cyclosporin A could then be carried out to demonstrate whether the molecule shows an enhanced degree of cardioprotection in an *in vivo* setting in comparison to cyclosporin A.

There is great potential utility of a novel mitochondrial- specific cyclosporin. However, the effect of mtCsA in our experiments was disappointing for the reasons above. However, the recently developed second generation molecule does show promise for future use, although much work is required to refine its role in cardioprotection.

Chapter 6

Non MPTP pathways to cardioprotection

Hypothesis: Inhibition of matrix metalloproteinases may protect the heart from ischaemia- reperfusion in cyclophilin D deficiency

6.1 Background

As discussed in chapter one, matrix metalloproteinases have generated a great deal of interest over the last decade. They have been shown to be closely involved in the processes which occur in the aftermath of myocardial infarction- not only in the setting of the chronic remodelling process post myocardial infarction (145,149), but also in the acute stage of ischaemia and reperfusion (151).The gelatinases MMP 2 and 9 are highly expressed within human myocardium. As their name suggests, they degrade gelatins and also type IV collagen, which is present in basement membranes. MMP 2 and 9 have been shown to be upregulated in the myocardium early in the course of myocardial infarction- both in the area at risk and remote myocardium (150).MMP-2 has been shown to be upregulated by oxidative stress (217) and phosphorylation (218). Both these factors are closely implicated in the processes involved in ischaemia – reperfusion.Administration of a non-selective inhibitor of MMPs has been shown to result in recovery of contractility in an *ex vivo* model of ischaemia-reperfusion (151).

MMP-2 has also been shown to cleave troponin I in an *ex vivo* model of ischaemia-reperfusion injury, and both troponin I release and the resulting mechanical dysfunction has been shown to be reduced by administration of doxycycline (a non-selective inhibitor of MMPs) (195).It is possible therefore,

that inhibition of matrix metalloproteinase activity could be used to modify the maladaptive response of ischaemia-reperfusion injury which occurs on opening the occluded coronary artery in the setting of acute myocardial infarction.

To test the hypothesis that cardioprotection could be achieved by a non – MPTP dependent mechanism, an MMP inhibitor (ilomastat) was administered in an isolated heart preparation using a Langendorff apparatus (see fig 6.1 overleaf). These isolated heart experiments (*ex vivo)* were carried out by another researcher in our group (Dr Robert Bell, Hatter Institute, UCL). As this group of experiments involved a model of global ischaemia, the measurement of infarct size differs; the infarct size is expressed as a percentage of the left ventricle (as opposed to percentage of the area at risk in the *in vivo* experiments).

Key to diagram overleaf:

WT Ctrl = Wild type exposed to control protocol

WT iPoC = Wild type treated with ischaemic post- conditioning

KO Ctrl = Cyclophilin D knock-out treated by control protocol only

KO iPoC = Cyclophilin D knock-out treated with ischaemic postconditioning

KO Ilomastat = Cyclophilin D knock-out treated with ilomastat (0.25 micromol/L)

6.2 Effect of Ilomastat in an Isolated Mouse Heart Model

(Data presented courtesy of RM Bell)

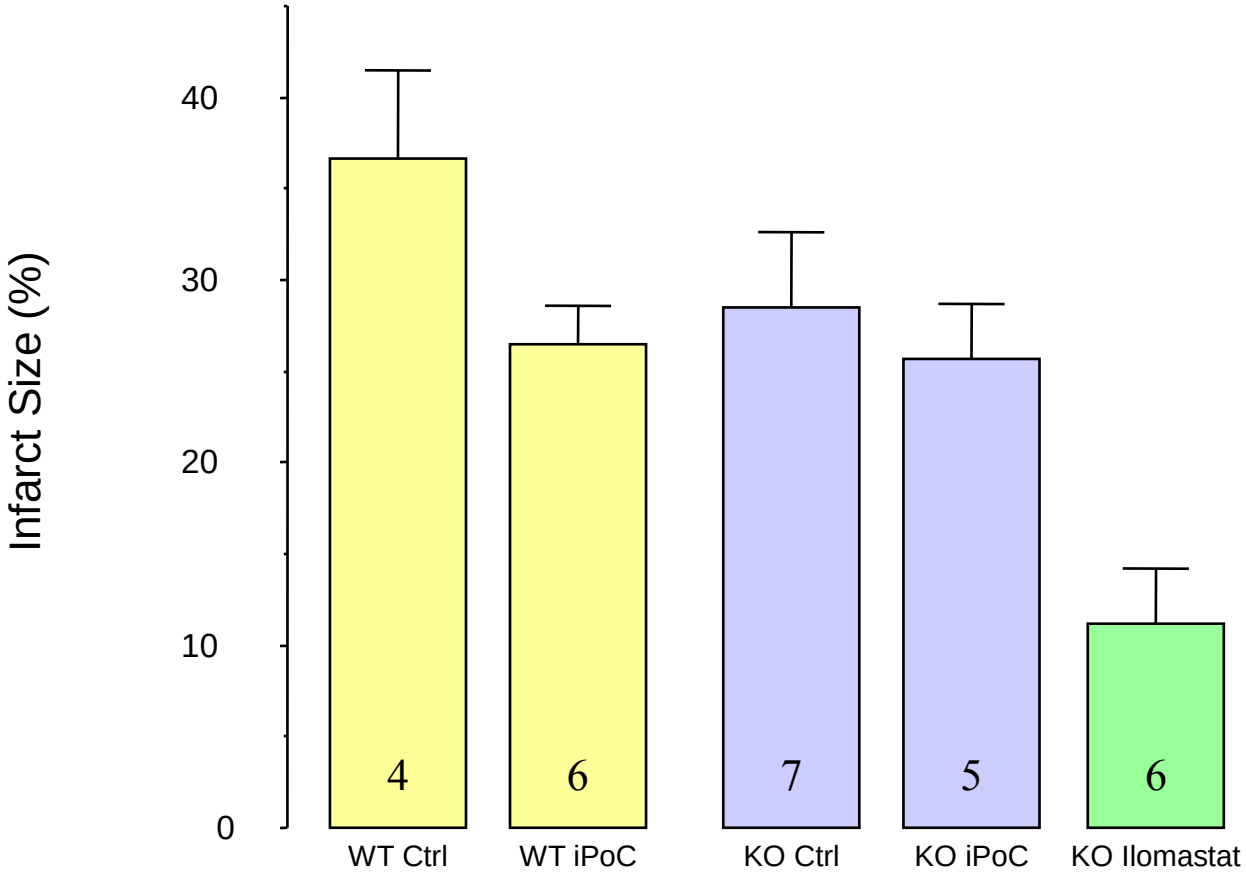


Figure 6.1 Effect of ischaemic postconditioning and inhibition of matrix metalloproteins. Data expressed as mean +/- SEM. Infarct size expressed as percentage of left ventricle

6.3 Aims

In order to assess more fully the effect of matrix metalloproteinase inhibition a series of experiments were conducted in the *in vivo* model of ischaemia-reperfusion.

6.4 Effect of Matrix Metalloproteinase Inhibition in an *in vivo* model of ischaemia – reperfusion

6.4.1 Methods

The series of experiments detailed in figure 6.1 demonstrate that administration of an MMP inhibitor resulted in a significant reduction in infarct size both in wild type and in cyclophilin D knock- out mice.

In order to test the hypothesis that ilomastat would be cardioprotective *in vivo* in the absence of cyclophilin D a set of experiments were conducted as follows.

Both wild type and CypD deficient mice were exposed to a standard period (30 minutes) of ischaemia. Those randomised to IPC underwent a single 5 minute cycle of IPC prior to ischaemia.

Ilomastat or vehicle control was administered via a central vein less than one minute prior to reperfusion.

Ilomastat was administered at a dose of 6 µmol/kg (154) based on the total dose administered by Ferdinandy's group as 4 separate boluses. The drug was dissolved in 1% cremophore/ethanol solution and sonicated for ten seconds and was then frozen until the day of use. The aliquots of drug were

then placed in a water bath at 37 degrees Celsius until they were defrosted. They were then sonicated prior to use to aid dissolution, and injected via a P10 cannula placed in the jugular vein one minute prior to reperfusion of the left anterior descending artery.

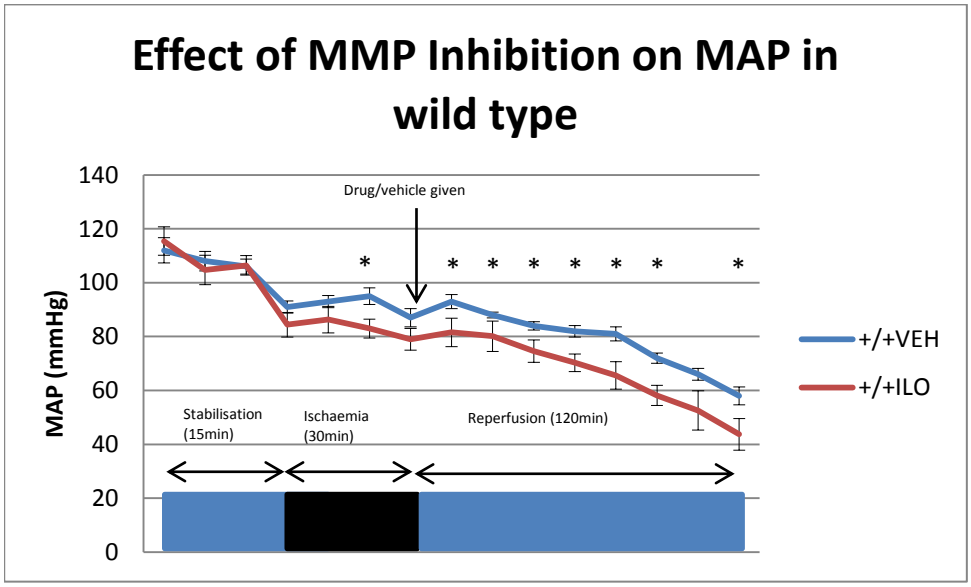
6.4.2 Randomisation

Both wild type and cyclophilin D deficient mice were used for this experiment. The treatments were randomised into 6 groups as follows (n=6 in each group):

1. Wild type mice receiving vehicle only
2. Wild type mice receiving ilomastat
3. Cyclophilin D knock- out mice receiving vehicle only
4. Cyclophilin D knock- out mice receiving ilomastat
5. Wild type mice receiving vehicle plus a single 5 minute cycle of IPC
6. Wild type mice receiving ilomastat plus a single 5 minute cycle of IPC

6.5 Results

Physiological Effect of Inhibition of Matrix Metalloproteinases



* = P<0.05

Figure 6.2 Effect of ilomastat given at reperfusion on mean arterial pressure in wild type mice exposed to 30 minutes ischaemia and two hours reperfusion.

Data is expressed as the mean value +/- SEM.

Blue bars denote periods of LAD perfusion, back bars denote LAD ischaemia.

MAP = mean arterial pressure (millimetres of mercury).

+/+ VEH = wild type mice receiving vehicle only

+/+ ILO = wild type mice receiving ilomastat

This data is shown in tabular format overleaf.

Table 6.1 Effect of MMP Inhibition on MAP in wild type mice

Stabilisation

Time (mins)	N	5 stab	10 stab	15 stab
+/+VEH MAP(mmHg)	6	112 (4.66)	108 (3.60)	106 (2.76)
+/+ILO MAP(mmHg)	6	115 (5.28)	105 (5.49)	106 (3.65)
P value		0.64	0.66	0.93

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
+/+VEH MAP(mmHg)	91 (2.23)	93 (2.28)	95 (3.08)	87 (3.35)
+/+ILO MAP(mmHg)	84 (4.57)	86 (4.97)	83 (3.51)	79 (4.05)
P value	0.20	0.27	0.02	0.15

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
+/+VEH MAP(mmHg)	93 (2.59)	88 (1.09)	84 (1.56)	82 (2.13)	81 (2.58)	72 (1.87)	66 (2.20)	58 (3.36)
+/+ILO MAP(mmHg)	82 (5.30)	80 (5.64)	75 (4.16)	70 (3.30)	66 (5.14)	58 (3.74)	53 (7.26)	44 (5.91)
P value	0.09	0.20	0.01	0.05	0.02	0.01	0.09	0.05

MAP = mean arterial pressure (millimetres of mercury).

+/+ VEH = wild type mice receiving vehicle only

+/+ ILO = wild type mice receiving ilomastat

Effect of MMP Inhibition on MAP in knock-out mice

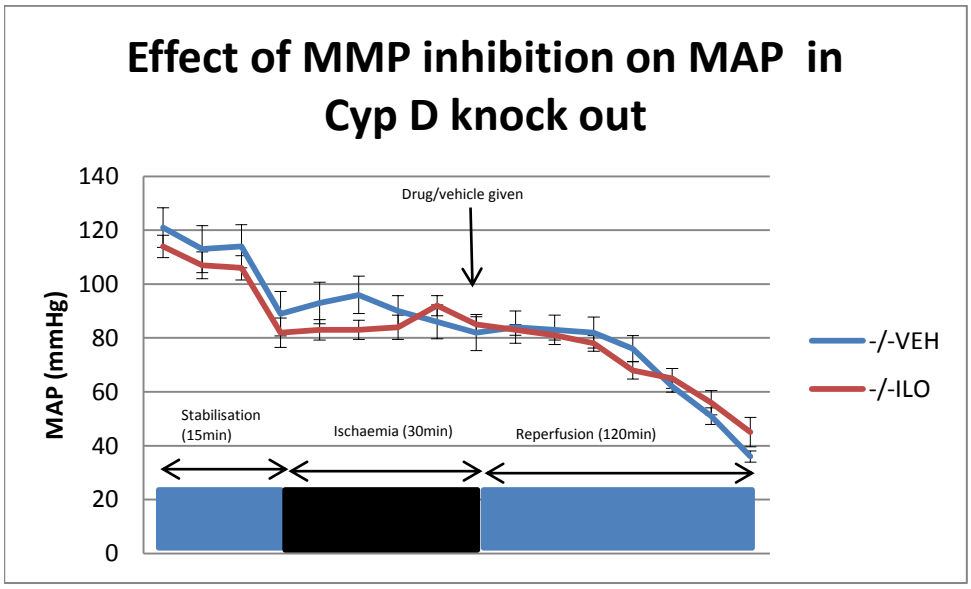


Figure 6.3 Effect of ilomastat bolus on mean arterial pressure in cyclophilin D knock- out mice exposed to 30 minutes of ischaemia and two hours reperfusion.

Data is expressed as the mean value +/- SEM.

Blue bars denote periods of LAD perfusion, back bars denote LAD ischaemia

MAP = mean arterial pressure (millimetres of mercury).

-/- VEH = cyclophilin D knock-out mice receiving vehicle only

-/- ILO = cyclophilin D knock-out mice receiving ilomastat

This data is shown in tabular format overleaf.

Table 6.2 Effect of MMP inhibition on MAP in Cyclophilin D deficient mice

Stabilisation

Time (mins)	N	5 stab	10 stab	15 stab
-/-VEH MAP(mmHg)	6	121 (7.32)	113 (8.69)	114 (8.02)
-/-ILO MAP(mmHg)	7	114 (4.15)	107 (4.94)	106 (4.51)
P value		0.42	0.55	0.38

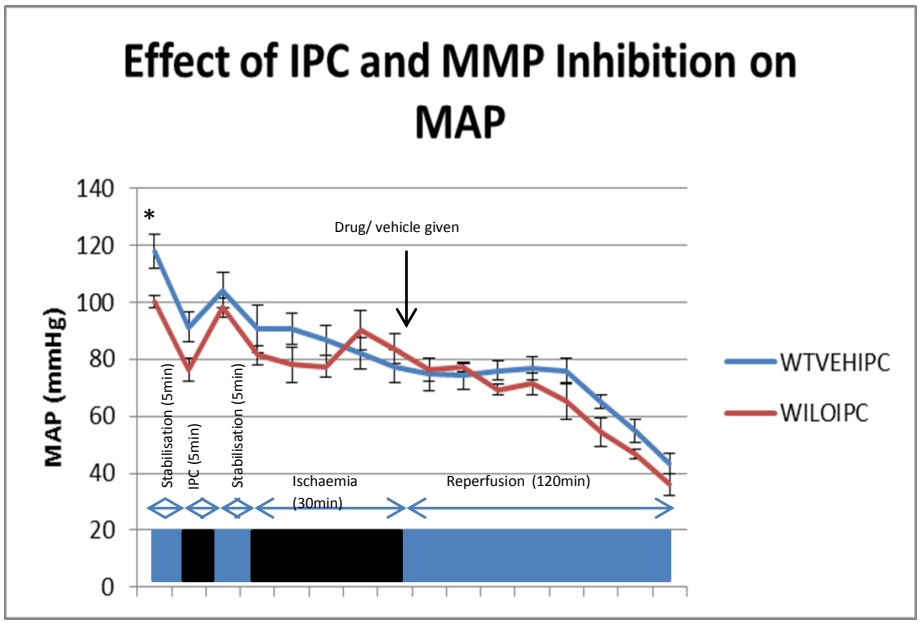
Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
-/-VEH MAP(mmHg)	89 (8.25)	93 (7.70)	96 (6.96)	90 (5.74)
-/-ILO MAP(mmHg)	82 (5.47)	83 (3.81)	83 (3.54)	84 (4.49)
P value	0.51	0.21	0.10	0.43

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
-/-VEH MAP(mmHg)	86 (6.32)	82 (5.98)	84 (5.47)	83 (5.74)	82 (4.87)	62 (2.06)	51 (3.12)	36 (2.08)
-/-ILO MAP(mmHg)	91 (3.76)	85 (1.96)	83 (1.72)	81 (2.87)	78 (3.23)	65 (3.72)	56 (4.52)	45 (5.46)
P value	0.43	0.88	0.72	0.48	0.18	0.60	0.36	0.17

Effect of IPC and MMP inhibition on Mean Arterial Pressure



***** = P<0.05

Figure 6.4 Combined effect of IPC and MMP inhibition on mean arterial pressure in wild type mice exposed to a single cycle of IPC, 30 minutes of ischaemia followed by 2 hours of reperfusion

WTVEHIPC= wild type subjected to IPC and administration of vehicle at reperfusion

WTILOIPC = wild type subjected to IPC and administration of the MMP inhibitor ilomastat at reperfusion

MAP = mean arterial pressure measured in millimetres of mercury (mmHg).

Figures shown as mean +/- SEM.

This data is shown in tabular format overleaf.

The bar in the x axis demonstrates periods of ischaemia (black) and reperfusion (blue). The timescale is non- linear to highlight the changes occurring during ischaemia.

Table 6.3 Effect of IPC and MMP inhibition on Mean Arterial Pressure

Stabilisation

Time (mins)	N	5 stab	10 IPC	15 stab
+/+VEHIPC MAP(mmHg)	6	118 (5.95)	91 (5.21)	104 (6.22)
+/+ILOIPC MAP(mmHg)	5	100 (2.08)	76 (4.09)	98 (3.21)
P value		0.043	0.06	0.47

Ischaemia

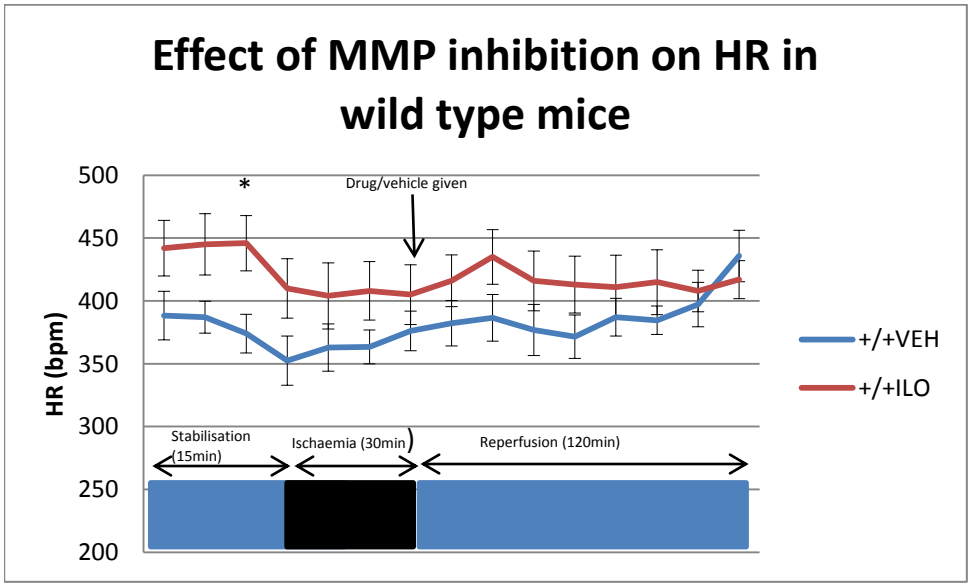
Time (mins)	1 I	5 I	15 I	25 I
+/+VEHIPC MAP(mmHg)	91 (8.46)	91 (5.34)	87 (5.25)	82 (5.38)
+/+ILOIPC MAP(mmHg)	81 (3.38)	78 (6.03)	77 (3.84)	90 (7.02)
P value	0.42	0.13	0.21	0.33

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
+/+VEHIPC MAP(mmHg)	77 (5.67)	74 (4.67)	76 (3.43)	77 (3.92)	76 (3.92)	65 (2.38)	55 (4.02)	43 (3.41)
+/+ILOIPC MAP(mmHg)	84 (5.24)	77 (1.53)	69 (1.76)	71 (3.93)	65 (6.44)	54 (4.84)	47 (1.86)	36 (4.00)
P value	0.43	0.64	0.17	0.35	0.16	0.03	0.16	0.17

In the tables above +/+ refers to wild type mice, ILOIPC refers to mice treated with a single cycle of IPC and ilomastat administered at reperfusion. VEHIPC relates to those mice treated with a single cycle of IPC and vehicle administered at reperfusion.

Effect of MMP inhibition on heart rate in wild type mice



* = P<0.05

Figure 6.5 Effect of ilomastat on heart rate in wild type mice subjected to 30 minutes of ischaemia and two hours of reperfusion.

Data expressed as mean +/- SEM

Blue bars denote periods of LAD perfusion, back bars denote LAD ischaemia

HR (bpm) = heart rate (beats per minute)

+/+ VEH = wild type mice receiving vehicle only

+/+ ILO = wild type mice receiving ilomastat

The bar in the x axis demonstrates periods of ischaemia (black) and reperfusion (blue). The timescale is non- linear to highlight the changes occurring during ischaemia.

This data is shown in tabular format overleaf.

Table 6.4 Effect of MMP inhibition on heart rate in wild type mice

Stabilisation

Time (mins)	N	5 stab	10 stab	15 stab
+/+VEH HR (bpm)	6	388 (19.32)	387 (12.72)	374 (15.37)
+/+ILO HR (bpm)	6	442 (22.19)	445 (24.47)	446 (22.02)
P value		0.09	0.06	0.02

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
+/+VEH HR (bpm)	352 (19.54)	363 (18.80)	363 (13.50)	376 (15.84)
+/+ILO HR (bpm)	410 (23.67)	404 (26.31)	408 (23.18)	405 (23.67)
P value	0.09	0.23	0.12	0.33

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
+/+VEH HR (bpm)	382 (18.06)	386 (18.56)	377 (20.26)	372 (17.23)	387 (15.04)	385 (11.27)	397 (17.62)	436 (20.48)
+/+ILO HR (bpm)	416 (20.58)	435 (21.8)	416 (23.78)	413 (22.61)	411 (25.45)	415 (25.83)	408 (16.54)	417 (15.15)
P value	0.24	0.12	0.24	0.17	0.44	0.30	0.67	0.46

HR (bpm) = heart rate (beats per minute)

+/+ VEH = wild type mice receiving vehicle only

+/+ ILO = wild type mice receiving ilomastat

Effect of MMP inhibition and IPC combined on heart rate in wild type mice

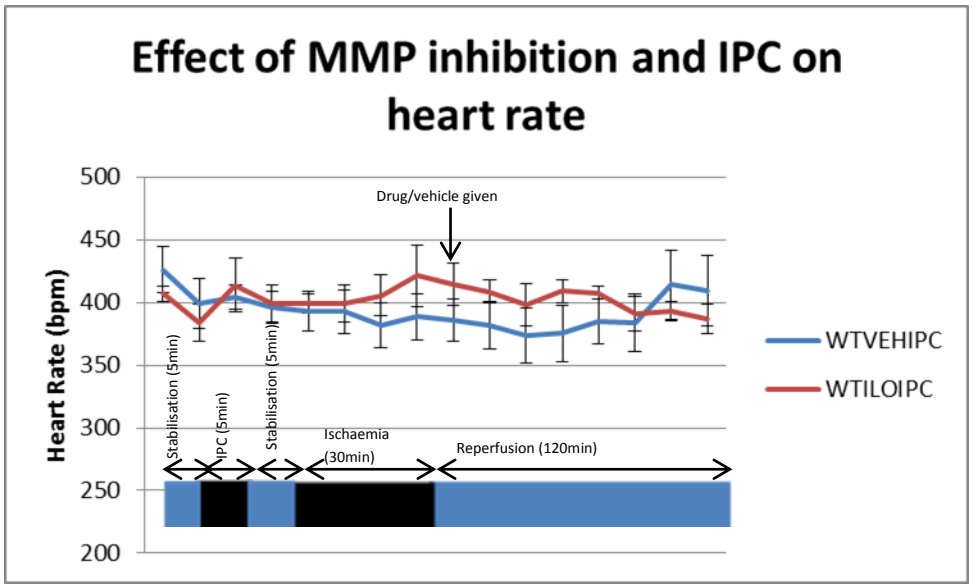


Figure 6.6 Response of heart rate to combination of IPC and MMP inhibition in wild type

Key to diagram

WTVEHIPC= Wild type animal treated with a single cycle of ischaemic preconditioning and vehicle administered one minute prior to reperfusion

WTILOIPC= Wild type animal treated with a single cycle of ischaemic preconditioning prior to ilomastat administered one minute prior to reperfusion.

The bar in the x axis demonstrates periods of ischaemia (black) and reperfusion (blue). The timescale is non- linear to highlight the changes occurring during ischaemia.

This data is shown overleaf in the corresponding data tables.

Table 6.5 Effect of IPC and MMP inhibition on heart rate in wild type

Stabilisation

Time (mins)	N	5 stab	10 IPC	15 stab
+/+VEHIPC HR (bpm)	6	426 (18.29)	399 (20.20)	405 (9.72)
+/+ILOIPC HR (bpm)	5	407 (6.43)	384 (15.06)	414 (21.66)
P value		0.51	0.63	0.66

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
+/+VEHIPC HR (bpm)	396 (13.06)	393 (16.07)	393 (17.10)	382 (17.60)
+/+ILOIPC HR (bpm)	399 (14.77)	399 (7.69)	399 (14.57)	406 (16.19)
P value	0.88	0.79	0.82	0.42

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
+/+VEHIPC HR (bpm)	389 (17.98)	382 (18.78)	374 (21.89)	376 (22.64)	385 (18.08)	383 (23.10)	414 (27.20)	410 (28.12)
+/+ILOIPC HR (bpm)	421 (24.59)	409 (9.39)	398 (17.06)	409 (9.45)	408 (5.24)	391 (14.01)	393 (7.62)	387 (11.59)
P value	0.32	0.37	0.50	0.35	0.42	0.84	0.62	0.60

+/+ VEHIPC= Wild type animal treated with a single cycle of ischaemic preconditioning and vehicle administered one minute prior to reperfusion

+/+ ILOIPC= Wild type animal treated with a single cycle of ischaemic preconditioning prior to ilomastat administered one minute prior to reperfusion.

Effect of MMP inhibition on heart rate in CypD deficient mice

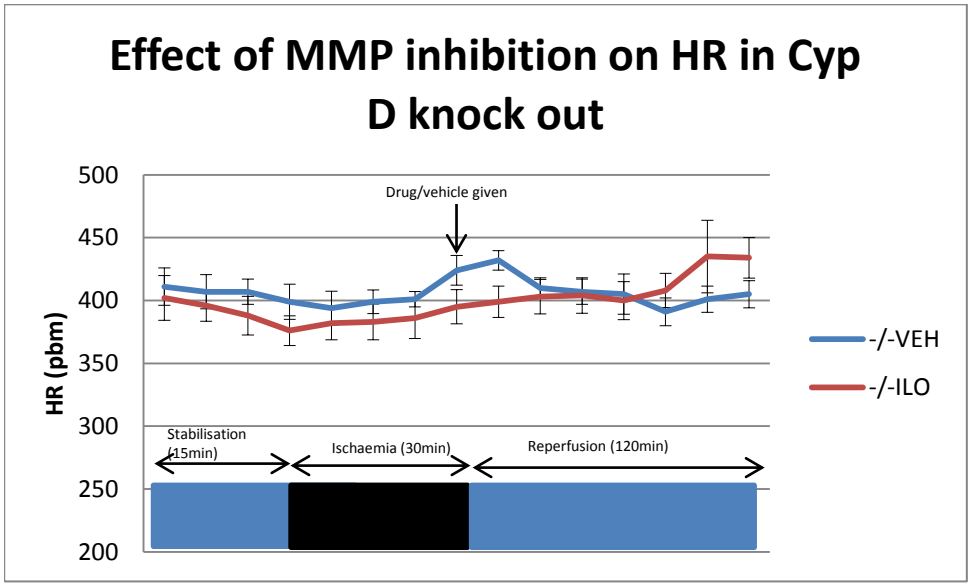


Figure 6.7 Effect of ilomastat on the heart rate profile of cyclophilin D knock-out mice subjected to ischaemia-reperfusion

Data is expressed as the mean value +/- SEM.

On the x axis blue bars denote periods of LAD perfusion, back bars denote LAD ischaemia

HR (bpm) = heart rate (beats per minute).

-/- VEH = cyclophilin D knock-out mice receiving vehicle only

-/- ILO = cyclophilin D knock-out mice receiving ilomastat

This data is presented in tabular format overleaf.

Table 6.6 Effect of MMP inhibition on heart rate in CypD deficient mice

Stabilisation

Time (mins)	N	5 stab	10 stab	15 stab
-/-VEH HR(bpm)	6	411 (14.84)	407 (13.51)	407 (9.96)
-/-ILO HR(bpm)	7	402 (17.79)	396 (12.63)	388 (15.40)
P value		0.72	0.56	0.35

Ischaemia

Time (mins)	1 I	5 I	15 I	25 I
-/-VEH HR(bpm)	399 (13.87)	394 (13.48)	399 (9.48)	401 (6.04)
-/-ILO HR(bpm)	376 (11.72)	382 (13.22)	383 (14.33)	386 (16.30)
P value	0.22	0.57	0.41	0.46

Reperfusion

Time (mins)	1 R	5 R	10 R	15 R	30 R	60 R	90 R	120 R
-/-VEH HR(bpm)	424 (11.86)	432 (7.79)	410 (7.99)	407 (10.15)	405 (16.03)	391 (11.18)	401 (10.35)	405 (10.87)
-/-ILO HR(bpm)	395 (13.62)	399 (12.53)	403 (13.73)	404 (14.12)	400 (15.11)	408 (13.66)	435 (28.92)	434 (16.10)
P value	0.15	0.06	0.73	0.90	0.82	0.39	0.34	0.20

-/- VEH = cyclophilin D knock-out mice receiving vehicle only

-/- ILO = cyclophilin D knock-out mice receiving ilomastat

6.6 Haemodynamic effects

The blood pressure and heart rate responses observed during this group of experiments was similar to that found during the preceding chapters, with a marked drop in mean arterial pressure confirming onset of ischaemia and progressive drop in pressure during the period of reperfusion to the protocol end.

Heart rate remained stable initially, and then rises during the stress of ischaemia and subsequently stabilises, finally rising towards the protocol end.

6.7 Area at Risk

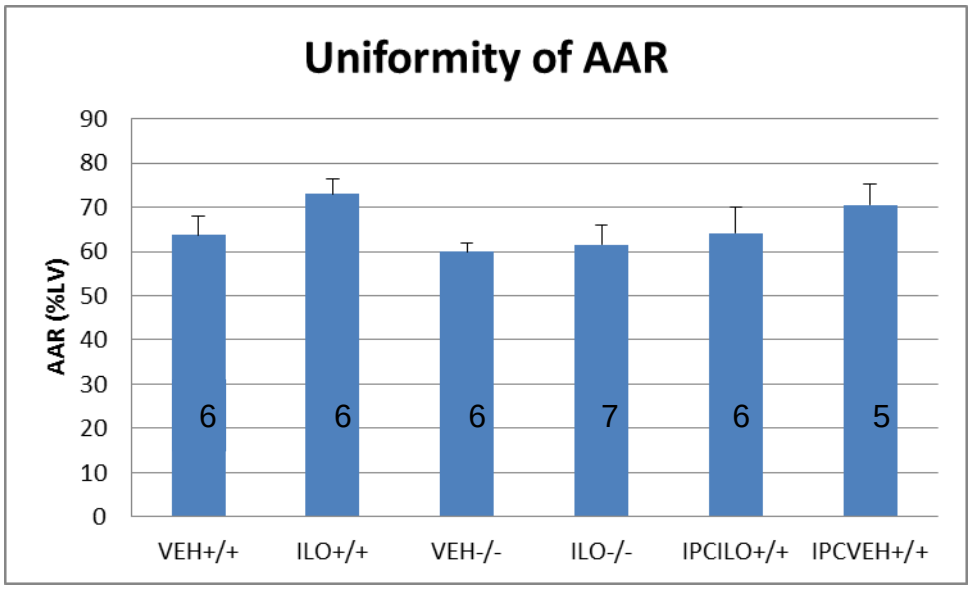


Figure 6.8 Uniformity of area at risk amongst all treatment allocations

Area at risk expressed as mean value +/- SEM, as a percentage of the left ventricle. N numbers are displayed within the corresponding data columns.

VEH +/+ = wild type mice receiving vehicle only

ILO +/+ = wild type mice receiving ilomastat

VEH -/- = Cyp D knock-out mice receiving vehicle only

ILO -/- = Cyp D knock-out mice receiving ilomastat

IPCILO +/+ = wild type mice receiving IPC and ilomastat

IPCVEH+/+ = wild type mice receiving IPC and vehicle

The area at risk was uniform for all groups as demonstrated above.

6.8 Effect of MMP inhibition of on Infarct Size

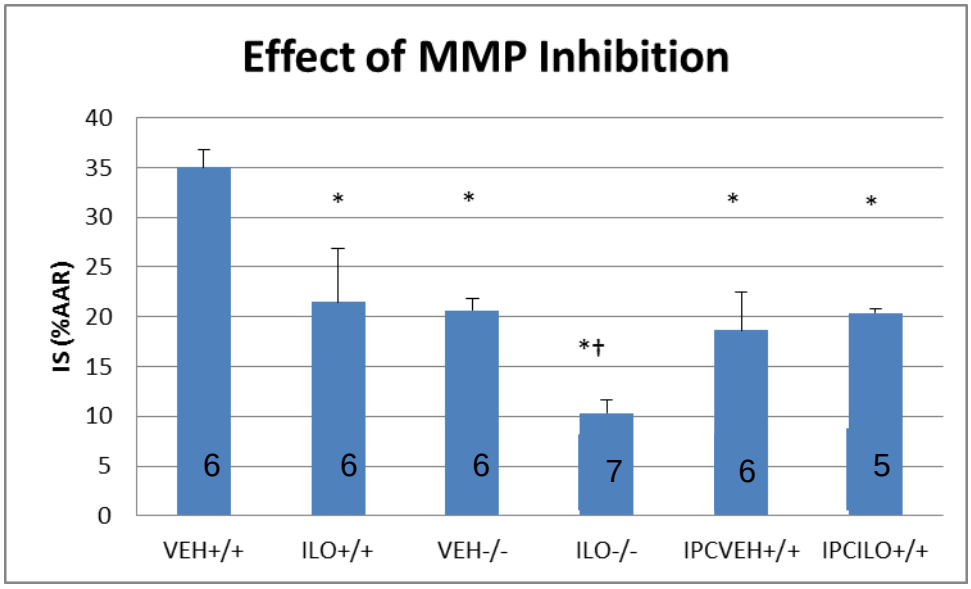


Figure 6.9 Effect of matrix metalloproteinase inhibition on infarct size in both wild type and cyclophilin D deficient mice

The infarct size is expressed as a percentage of the area at risk

The n number for each group is displayed in the corresponding data column.

VEH +/+ = wild type mice receiving vehicle only

ILO +/+ = wild type mice receiving ilomastat

VEH -/- = Cyp D knock-out mice receiving vehicle only

ILO -/- = Cyp D knock-out mice receiving ilomastat

IPCILO +/+ = wild type mice receiving IPC and ilomastat

IPCVEH+/+ = wild type mice receiving IPC and vehicle

The * denotes a statistically significant difference ($p<0.05$) in comparison to wild type receiving vehicle control

† denotes a statistically significant difference ($p<0.05$) in comparison to cyclophilin D knock out receiving vehicle control

Effect of MMP inhibition of on Infarct Size

	IS/AAR (%LV)	SEM
+/+ Vehicle	35.04	1.74
+/+ Ilomastat	21.51*	5.31
-/- Vehicle	20.66*	1.18
-/- Ilomastat	10.35*†	1.31
+/+ Vehicle & IPC	18.65*	3.87
+/+ IPC and Ilomastat	20.36*	0.43

Table 6.7 Effect of MMP inhibition on infarct size

+/+ refers to wild type mice

-/- refers to cyclophilin D knock-out mice

* = $p<0.05$ in comparison to +/+ vehicle control

†= $p<0.05$ in comparison to -/- vehicle control

The data above shows a significant reduction in infarct size after 30 minutes ischaemia in wild type animals treated with ilomastat in comparison to those receiving vehicle (35% v 22%, $P<0.05$).

CypD deficient mice exposed to the same ischaemic protocol exhibited smaller infarct sizes than their wild type counterparts. The administration of ilomastat to CypD knock-outs resulted in a significant reduction in infarct size when compared with those receiving vehicle alone (20.7% v 10.4%, $P<0.05$).

Co-administration of ilomastat and IPC did not result in further reduction in infarct size in comparison to either IPC or ilomastat alone.

6.9 Discussion

Matrix metalloproteinases (MMPs) play a central role in the maintenance of the integrity of the extracellular matrix. There exists a constant equilibrium between collagen synthesis and breakdown, mediated by the up- and down-regulation of MMP activity and that of tissue inhibitors of metalloproteinases (TIMPs). All MMPs contain zinc ($Zn^{2+)}$ at their active binding site.

There is strong evidence to show that modification of this continuous process occurs in the setting of acute myocardial infarction, and a variety of other cardiac diseases (170,193,198). In the setting of MI, upregulation of MMP activity has been shown to occur within 10 minutes of the onset of ischaemia (147). In an *ex vivo* system, activation has been confirmed within one minute of reperfusion, and this is strongly related to mechanical dysfunction. This effect has been shown to be reversed by administration of a broad spectrum MMP inhibitor (doxycycline) (151).

The process of ischaemic preconditioning has also been shown to modify the activity of MMPs (152,153). Interestingly, in the instance of hyperlipidaemic rats, where the benefits of IPC are inhibited, MMP inhibition has demonstrated the ability to protect the heart from ischaemia-reperfusion injury (154). This could potentially suggest that the cardioprotection achieved by administration of MMP inhibitors may occur by a mechanism which is distinct to that afforded by IPC.

Data demonstrating attenuation of cardiac damage in experimental myocardial infarction by inhibition of matrix metalloproteinases is plentiful (149,154) and as such these agents are of great interest to scientists and

clinicians alike in the search for agents which can protect the human heart in the setting of reperfused myocardial infarction, a pharmacological role which has to date remained unfilled (199). The pharmacological inhibitors of MMPs belong to the class hydroxamates, which inhibit MMPs by binding of hydroxamic acid to the Zn^{2+} site of MMP.

Ilomastat belongs to the hydroxamates, and has a broad range of MMP-inhibition, which includes MMP 1, 2, 9 and 12. In our experiments, we used ilomastat in a dose which equalled the summative dose administered by Ferdinandy's group, demonstrating cardioprotection. We showed that not only did ilomastat protect the heart from ischaemia – reperfusion in wild type mice, but this protection was independent of an effect the MPTP- ie it occurred in mice which were deficient of cyclophilin D. This has not been demonstrated previously *in vivo.* Furthermore, we also showed that inhibition of MMP activity did not result in delayed opening of the MPTP, confirming that the protective effect was independent of MPTP inhibition.

The additive benefit of ilomastat to MPTP inhibition is a very attractive clinical concept, and raises the possibility of either lone administration (of ilomastat), and the potential of co-administration with MPTP blocking agents such as cyclosporin A to gain added benefit, which may result in a substantial reduction in infarct size, not yet seen, even with cyclosporin A in human myocardial infarction.

What has not been uncovered by our investigations is the precise mechanism by which ilomastat protects the heart. Future investigations will involve the measurement of MMP and TIMP levels over the time frame of ischaemia and reperfusion, and the time frame involved in the reduction of MMP activity. MMPs do appear to be essential for the trigger phase of preconditioning by adenosine, bradykinin and opiates (219-221).

Previous clinical trials have been set up in a variety of clinical disease processes (159) including cancer models (161), periodontal disease (200) and wound healing (201) to identify the clinical potential of inhibition of matrix metalloproteinase activity. The clinical utility of these agents has been limited by the side effects of long term administration in clinical trials – primarily due to development of the musculoskeletal syndrome.

In a review of some ten years ago, this very issue was discussed (155), and the authors concluded that the likely role of MMP inhibition was as part of combination therapy on a long term basis to treat heart failure in the aftermath of myocardial infarction. However, on the basis of the data we have obtained, there could possibly be a place for MMP inhibition administered as a single dose at the time of reperfusion to attenuate lethal reperfusion injury. Single dosing could potentially abolish the deleterious effects on the musculo-skeletal system which have been the thorn in the side of the MMP inhibitors, as this appears to be related to their longer term administration (200).

In this chapter we set out to test the hypothesis that administration of a matrix metalloproteinase inhibitor at reperfusion would be cardioprotective in a manner which was independent of cyclophilin D.

We have confirmed that there is significant attenuation of myocardial damage after ischaemia-reperfusion in cyclophilin D deficient mice in comparison to wild type.

This series of experiments also shows that administration of the matrix metalloproteinase inhibitor ilomastat at the time of reperfusion results in significant cardioprotection in wild type mice subjected to ischaemia and reperfusion.

We have also confirmed the cardioprotective effect of ischaemic preconditioning in wild type mice exposed to ischaemia-reperfusion.

We have demonstrated a significant attenuation in the degree of myocardial damage after ischaemia-reperfusion in cyclophilin D deficient mice treated with a matrix metalloproteinase inhibitor.

We have shown that when an inhibitor of MMPs was administered to wild type mice exposed to ischaemic preconditioning there was no further reduction in infarct size than with IPC alone.

This data confirms the hypothesis that administration of a matrix metalloproteinase inhibitor at the time of reperfusion protects the heart in the absence of cyclophilin D.

As expected from previous published work we found that ilomastat protected wild type hearts from ischaemia-reperfusion (154).

We confirmed previous data from our centre, and others, that IPC also confers a cardioprotective effect in wild type mice (experimental data in chapter 4 (54, 77, 189, 196, 200, 201)).

We also found that ischaemic preconditioning (IPC) did not have an additive effect to matrix metalloproteinase inhibition in wild type mice subjected to ischaemia-reperfusion. This was expected, as published data has demonstrated that IPC modifies MMP activity (152,197). Also, if there is a conditioning “threshold” then this would be met by one or other method, but no added benefit would be derived from application of two differing stimuli- it has been shown that application of IPC or pharmacological pore inhibition does not reduce infarct size in mice lacking cyclophilin D (54). One confounder to this is that in hypercholesterolaemic rats, where there is no clear benefit from IPC, cardioprotection could be achieved by administration of MMP inhibitors, suggesting that the mode of protection by IPC and MMP inhibition differs (154).

The finding that ilomastat administration resulted in a significant reduction in infarct size in the cyclophilin D deficient mouse would be in keeping with the mode of protection achieved by ilomastat being independent of an effect on function of the MPTP. This is a novel finding, which challenges previous data which to date has suggested it is not possible to protect the heart of the cyclophilin D deficient mouse (12,54).

This data suggests therefore, that there is a non-MPTP mediated pathway to cardioprotection, which has the potential for clinical utility in the setting of acute myocardial infarction.

Timing of MPTP Opening Using Confocal Laser Microscopy

To investigate its mode of action further ilomastat was administered to isolated cardiomyocytes exposed to laser light- triggered reactive oxygen species. MPTP opening was not altered in comparison to controls, whereas cyclosporin A (used as a positive control) did show delayed MPTP opening (data not shown). This suggests that the mode of action of ilomastat is independent of an effect on pore function. (The confocal laser experiments were carried out by Dr Robert Bell and Dr Sean Davidson, Hatter Institute, UCL).

This data strongly supports that the protective effect of ilomastat is independent of MPTP inhibition. However, the mechanism by which it achieves cardioprotection is unknown.

6.10 Suggestions for Future Study

"Late" Administration of Ilomastat

A further series of experiments could be conducted to assess whether administration of ilomastat after the time period when the MPTP is known to open (within the first few minutes if reperfusion) would potentially provide further confirmatory evidence that this protective process is non- pore mediated. However, the proposed non-MPTP pathway may also require intervention within the first few minutes of reperfusion, so a result showing no protection with "late" administration of ilomastat is not essential in demonstrating independence from PTP-related protection.

Signalling mechanisms involved

The signalling method resulting in the cardioprotection observed with ilomastat is unclear. Ilomastat has been shown to inhibit MMP activity, but exactly how this results in cardioprotection is not known. Investigation of the role of the reperfusion injury salvage kinase (RISK) pathway, which is central and upstream to the protection of MPTP inhibition would perhaps reveal a possible mechanism of protection. Suggested study would involve use of the Western blotting technique to determine whether there was an increase in phosphorylation of kinases, such as that found in IPC.

Assay to determine MMP and TIMP levels

Confirmation of reduction in MMP activity would be essential to guide further study into the cardioprotective effect of ilomastat. Thus, measurement of the

baseline gelatinase levels (MMP- 2 and 9) would be of value as well as the post treatment levels. The effect of ilomastat on tissue inhibitor of metalloproteinases (TIMPs) would also be of great interest.

Chapter 7

Discussion and Conclusions

The role of this thesis was to evaluate further the role of the mitochondrial permeability transition pore in cardioprotection from ischaemia-reperfusion injury.

The primary focus was to assess whether it is possible to improve cardioprotection by a variety of means- increasing the IPC stimulus, improving the sub-cellular selectivity of cyclosporin, and finally, by assessing whether cyclophilin D is essential in cardioprotection.

7.1 Summary of findings

In chapter 4, using an *in vivo* murine model of ischaemia- reperfusion injury, we examined the effect of increasing the IPC stimulus on the infarct size sustained in both wild type and cyclophilin D deficient mice.

We have confirmed previous data which demonstrated that wild type mice could be protected by a single 5 minute cycle of IPC.

Increasing the IPC stimulus to three cycles of 5 minutes IPC and ischaemia-reperfusion continued to demonstrate a cardioprotective effect, but this was not significantly different to that observed with a single cycle of IPC in wild type mice.

Cyclophilin D deficient mice had a lower observed infarct size in comparison to wild type mice which corresponds to literature suggesting that they are in an inherently protected state. In this chapter, this was not statistically significant, although there was a trend to smaller infarct sizes in CypD knock-outs. Experiments detailed in chapter 6, however, did confirm a significantly smaller infarct size in cyclophilin D deficient animals.

In cyclophilin D deficient mice there was no reduction in infarct size after exposure to a single 5 minute cycle of IPC in comparison to control protocol. This is in keeping with published data on this subject.

In cyclophilin D knockouts, after increasing the IPC stimulus to three cycles of 5 minutes IPC, there was no statistically significant cardioprotective effect observed. However, there was a trend towards protection in this group.

The statistical methods employed were very rigorous as discussed in chapter 4. If fewer groups had been compared, it is possible that protection of the

cyclophilin D deficient heart may have been demonstrated with three cycles of IPC.

Further experiments to investigate this could potentially compare a smaller number of experimental groups, which may improve the statistical ability to demonstrate a reduction in infarct size. Alternatively, further increasing the number of cycles of preconditioning could be an option. However, due to the mechanical limitations of repeatedly snaring and reperfusing the left anterior descending artery causing localised trauma and no-reflow, it was felt not to be technically possible in this model.

From the experimental data presented in chapter 4, we can conclude that there is a trend towards cardioprotection of the cyclophilin D deficient heart with an increase in the IPC stimulus, but in our data this did not reach statistical significance.

In chapter 5, we investigated whether it was possible to enhance the cardioprotective effect of cyclosporin A *in vivo* by targeting the drug at a sub-cellular level to mitochondria, by using a novel mitochondrial-targeted form of cyclosporin A (mtCsA) which was administered at the time of reperfusion.

Despite previous published data showing very encouraging results in cellular models (135), we were unable to demonstrate any protection against ischaemia-reperfusion injury in the *in vivo* setting.

The potential reasons for this are numerous. In the absence of prior *in vivo* work using the molecule, we were faced with selecting an arbitrary dose for administration. We selected three doses, all of which were a fraction of the

dose used for conventional cyclosporin A. This was based on the assumption that increased selectivity would mean more active drug would reach the target site (ie cyclophilin D). The drug was also difficult to reconstitute- we used the same method employed in making cyclosporin A – reconstituting with a solution of cremophore and ethanol, and sonicating to aid dissolution.

Ethanol itself has been shown to be cardioprotective in a number of experimental models (224). It is possible that its use as a vehicle may prevent demonstration of a protective effect by a therapeutic substance, as the vehicle itself would delay MPTP opening. Evidence suggesting that this effect was not important is provided by the fact that control infarct sizes were similar to those observed in the other chapters with identical durations of ischaemia.

Later experiments carried out by Dube *et al* (192) revealed the reason for the lack of efficacy- the binding affinity of the mtCsA molecule was dramatically reduced by the process used to achieve mitochondrial selectivity. The sterical modifications required in order to make the molecule mitochondrial- selective caused shielding of the active zinc-binding site preventing it binding to cyclophilin as expected.

The same paper detailed above uses another novel form of mtCsA and cellular work has shown that it has improved MPTP inhibition in comparison to cyclosporin A, and is biologically active at much lower doses. This could potentially have a future in reducing the infarct size after ischaemia-reperfusion.

In vivo work is required to test the true potential of this new form of mitochondrial targeted cyclosporin A, which may hold some promise in the

treatment of STEMI- but any human use of this drug will be dependent on satisfactory pre-clinical data, and safety data from phase I-IV clinical trials, which would involve a process lasting years.

In chapter 6, we investigated the potential role of non-MPTP pathways in ischaemia-reperfusion by administering a matrix metalloproteinase inhibitor (ilomastat) at the time of reperfusion. This was administered to both wild type and mice deficient in cyclophilin D to firstly confirm previous data demonstrating its cardioprotective effect (in wild type), and to ascertain whether it was possible to protect the myocardium from ischaemia in mice lacking cyclophilin D, a major component of the MPTP.

These experiments confirmed that wild type mice were protected by ilomastat, with a significant reduction in infarct size from 35% to 22% (P=<0.05).

We also showed that ilomastat administered at the time of reperfusion resulted in a significant reduction in infarct size in the cyclophilin D deficient mouse. Infarct size was reduced from 21% to 10% by ilomastat (P=<0.05). This data would suggest that ilomastat may protect by a method which is not dependent on cyclophilin D inhibition.

To further investigate this theory, isolated wild type cardiomyocytes were exposed to laser-light triggered reactive oxygen species and the effects of cells treated by ilomastat were compared with those treated with cyclosporin A (used as a positive control). The cyclosporin A treated cells had delayed pore opening, whereas the ilomastat-treated cardiomyocytes did not, indicating that MPTP function is not affected by treatment with ilomastat.

This data would suggest that the cardioprotective effect of ilomastat is not mediated by inhibition of MPTP function, as MPTP function is attenuated due to the congenital absence of cyclophilin D, which is its major constituent. This is a new finding- demonstrated in two different experimental models in our centre, and suggests that there exists a pathway mediating protection from lethal reperfusion injury which is pore independent. This area holds substantial potential for further research and pharmacological studies.

We have therefore demonstrated in this thesis that it is possible to protect the cyclophilin D deficient heart *in vivo*. The experiments of chapter 4 which showed a non-significant trend to protection with an increase in the IPC stimulus suggested, but did not prove, this hypothesis. The experiments in chapter 6 showed that there was significant protection of the CypD deficient heart subjected to treatment with the matrix metalloproteinase inhibitor Ilomastat. The mechanism for protection by Ilomastat has not yet been elucidated. However, the confocal microscopy experiments by Bell and Davidson which show that Ilomastat does not delay MPTP opening would appear to suggest that its protection is not pore-mediated. This raises the possibility that there is a pathway to cardioprotection from necrotic cell death which is not MPTP dependent.

Chapter 5 investigated whether it was possible to maintain cardioprotection whilst avoiding the deleterious effects of cyclosporin A by targeting the drug to its desired site of action in mitochondria and found that the molecule investigated did not protect the heart against ischaemia-reperfusion. However,

it is possible that mitochondrial-targeting through nanoparticle technology may increase the ability of cyclosporin A to protect the heart in future.

7.2 Potential Clinical Implications

The reduction in infarct size obtained by administration of the MMP inhibitor ilomastat could potentially represent a significant step forward in the understanding of lethal reperfusion injury and the processes involved in cell salvage.

There may be significant cardioprotection achieved when administered alone, but co-administration of this agent with pore inhibitors, such as cyclosporin A could potentially result in an even greater reduction infarct size in comparison to cyclosporin alone.

7.2.1 Adjunct to primary angioplasty

In the setting of acute STEMI (ST segment elevation myocardial infarction), administration of ilomastat, either alone or in combination with the MPTP inhibiting effect of cyclosporin A could potentially result in a very large reduction in infarct size due to the synergistic actions of pore inhibition and MMP inhibition as found in our experimental data. This could potentially be large enough to finally translate into the real, measurable clinical benefit, which to date has eluded researchers in this field.

7.2.2 Elective cardiac surgery

In a similar manner, in the setting of elective high risk cardiac surgery or percutaneous coronary intervention, this drug could potentially be administered prior to reperfusion, bypass or angioplasty, potentially limiting the myocardial damage occurring at that time. As noted above co-

administration with pore inhibitor (cyclosporin A) could potentially yield a superior clinical benefit to lone administration, as ilomastat appears to have additive benefit to MPTP inhibition.

7.3 Limitations and Suggestions for Future Studies

As the data contained in this thesis relates to a murine model of ischaemia-reperfusion injury, there are a number of limitations.

In keeping with the legislation in the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, we have minimised the quantity of animals used in each experiment. In the case of the experiments of chapter 4, we were unable to answer fully the research question, as to do so would have required use of a large number of animals to demonstrate a negative result, and we did not feel that this was scientifically or ethically justified.

The major limitation of use of animal models are that, as found in many clinical trials of myocardial infarction that the data obtained may not be directly applicable to human patients with multiple co-morbidities. This has been a substantial reason for failure of translation into new clinical therapies for myocardial infarction to date.

This thesis did not re-evaluate the novel version of mitochondrial targeted cyclosporin A, as this was not available at the end of the study period. It would have been helpful to determine if the new method of manufacturing with a differing linkage method to the triphenylphosphonium cation, which facilitates mitochondrial selectivity, demonstrated cardioprotection *in vivo*. This molecule

could potentially protect the heart at lower doses than those required for protection with conventional cyclosporin A.

Investigating the mechanism of the cardioprotection afforded by matrix metalloproteinase inhibition would be a very interesting and essential part of the experiments provoked by this research. The finding of enhanced cardioprotection above that seen with inhibition of the mitochondrial permeability pore prompts a series of experiments.

Confirmation of a significant reduction in MMP activity in response to administration of ilomastat would confirm that MMP reduction is one possible mechanism of the cardioprotective effect of ilomastat.

It would also be useful to assess the effect of MMP inhibition on the signalling mechanisms involved in cardioprotection, eg the RISK pathway.

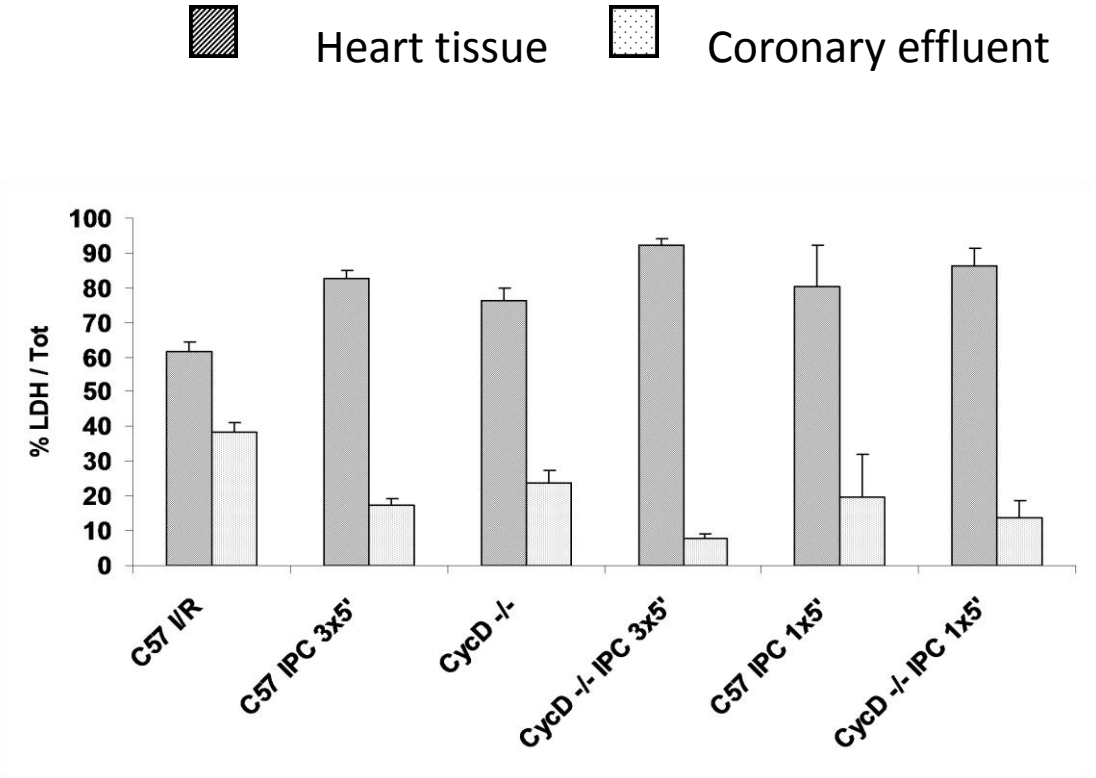
Additional experiments could include assessment of the effect of MMP inhibition on caspase levels and mitochondrial morphology to determine if the pathway being inhibited is apoptosis.

Confirmatory studies in a larger animal model would be desirable, and if successful, the ultimate test of ilomastat would be in administration to humans being treated for acute myocardial infarction. A useful tandem study would be to assess the benefit in co-administration with cyclosporin A in acute myocardial infarction, measuring traditional measures of cardiac damage such as high sensitivity troponin, brain natriuretic peptide and left ventricular function by magnetic resonance imaging, as well as mortality both in the short and long term.

Appendix

1.1 Ex vivo data showing cardioprotective effect of increased IPC stimulus

The following data has been reproduced from unpublished work by Andrea Carpi, Roberta Menabò, Emy Basso, Paolo Bernardi and Fabio Di Lisa. In this work, an *ex vivo* model of ischaemia- reperfusion has been used. 40 minutes of ischaemia was used with a variety of IPC protocols (no IPC, 3 x 5 minutes IPC, 5 x 5 minutes IPC), followed by 15 minutes of reperfusion in both C57 and cyclophilin D deficient mice. The outcome measure was lactate dehydrogenase release. The results are demonstrated below.



The absence of CypD resulted in a significant decrease of LDH release induced by post-ischemic reperfusion (23.9±3.7% vs 41±4.7% of total LDH content in *CypD-/-* and WT mice, respectively). As expected the 3 cycle IPC protocol resulted in a high degree of protection that however was significantly enhanced by CypD ablation (17.2±2% vs 7.7±1.6% in WT and *CypD-/-*, respectively). The increase in IPC protection was decreased when the less robust protocol of 1 cycle IPC was applied (19.9±12.1% vs 13.7±4.9 in WT and *CypD-/-*, respectively) and appeared to be rather specific. ∗ = $p<0.05$

1.2

Identification and characterisation of novel inhibitors of the MPTP and their efficacy in cardiac infarction.

Contursi, Ballarini, Fancelli, Carpi, Di Lisa et al have also studied the effects of administration of small molecular weight inhibitors of the MPTP (referred to as “Congenia Inhibitors”). They studied the effects of administration of these agents to purified human liver mitochondria, and measured the calcium retention capacity, which is a surrogate marker for MPTP opening.

The calcium retention capacity of purified liver mitochondria is determined by measuring the point at which pulse loaded calcium is released from the mitochondria (detected by fluorescence). MPTP inhibition results in a delay in release of calcium.

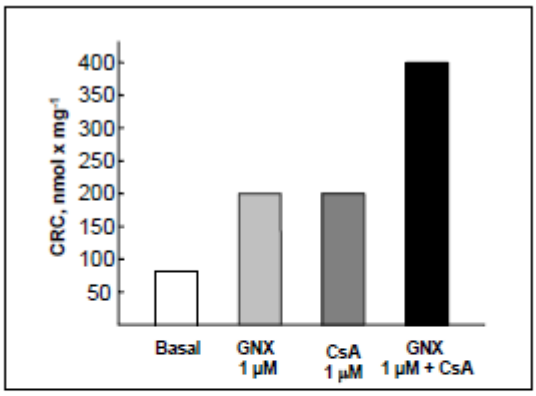


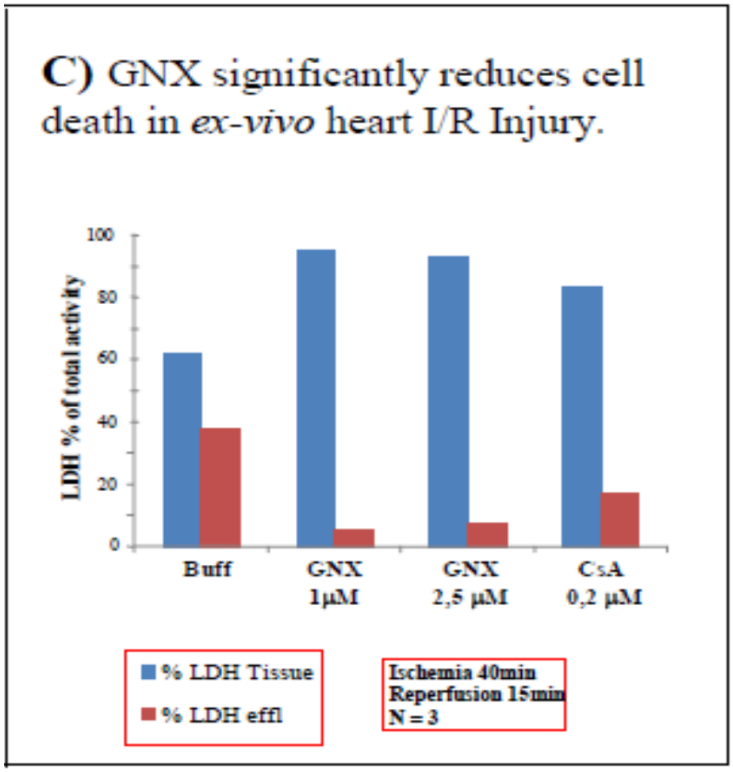
Figure 3

Congenia Inhibitors Are additive with CsA.

The maximum CRC obtainable with CsA is seen at 1 µM. Addition of Congenia compounds shows that the CRC can be increased when used in combination with CsA. These data suggest that the Congenia compounds have a different molecular target to CsA.

Reproduced from Contursi et al (unpublished). The congenia inhibitor is referred to as GNX. The above diagram demonstrates that the congenia inhibitor has similar effect on calcium retention to cyclosporin A, but also that there is an additive effect with cyclosporin A resulting in further delay in calcium release from mitochondria.

The congenia inhibitor has also been shown to be protective in an *ex vivo* model of ischaemia-reperfusion injury (see overleaf).



Reproduced from Contursi *et al,* (unpublished). In this *ex vivo* model, the congenia inhibitor reduces release of lactate dehydrogenase, a measure of myocardial damage.

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