CHARACTERISATION OF TEMPERATURE PRECONDITIONING OF ADULT RAT VENTRICULAR MYOCYTES

Thesis Submitted for the Degree of

Doctor of Philosophy

at the University of Leicester

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June 2012

Characterisation of temperature preconditioning of adult rat ventricular myocytes

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Temperature preconditioning is a relatively novel cardioprotective intervention, demonstrated to protect *ex vivo* isolated rat hearts against ischaemia- reperfusion injury. For the first time, the effect of temperature preconditioning on isolated ventricular myocytes was investigated in this study. This was followed by characterisation of the molecular mechanisms involved in temperature preconditioning.

Temperature preconditioning (16°C) was found to be cardioprotective in isolated adult rat ventricular myocytes enhancing contractile recovery and preventing calcium dysregulation after metabolic inhibition and re-energisation (simulated ischaemia-reperfusion). Temperature preconditioning also preserved mitochondrial function by delaying the pathological opening of the mitochondrial permeability transition pore (mPTP) in a model of reperfusion injury.

For the first time, reactive oxygen species (ROS) are shown to be released from the mitochondria exclusively during the hypothermic episodes of the temperature preconditioning protocol. This was characterised using a mitochondrially targeted ROS biosensor and ROS release was observed during the brief bursts to 16°C during temperature preconditioning. A ROS scavenger (MPG) significantly attenuated ROS accumulation during temperature preconditioning and consequently abolished the temperature preconditioning-induced protective delay in mPTP opening.

Western blot analysis revealed temperature preconditioning phosphorylation of the pro-survival kinase ERK1/2. ERK1/2 activation was shown to be downstream of ROS release as the presence of a ROS scavenger during temperature preconditioning completely blocked ERK1/2 activation. The cardioprotective effects of temperature preconditioning on mPTP opening were completely lost by inhibiting ERK1/2 activation. Thus mitochondrial ROS release and ERK1/2 activation are both necessary to signal the cardioprotective effects of temperature preconditioning in cardiac myocytes.

Publications

Paper

Bhagatte, Y., Lodwick, D., Storey, N.M. (2012) Mitochondrial ROS production and subsequent ERK phosphorylation are necessary for temperature preconditioning of isolated ventricular myocytes. *Cell Death & Disease. Manuscript accepted.*

Abstracts

Bhagatte, Y. and Storey N.M. Investigating the effects of temperature preconditioning on isolated ventricular myocytes. *Proceeding of the Young Physiologist Symposium, 2009. Poster presentation.*

Bhagatte, Y. and Storey N.M. The cardioprotective effects of temperature preconditioning on isolated ventricular myocytes. *Proceeding of the Biophysical Society 55th annual meeting, 2011. Poster presentation.*

Bhagatte Y and Storey NM. Mitochondrial ROS production and ERK activation are necessary for temperature preconditioning of isolated ventricular myocytes. *Proceeding of the BSCR autumn meeting, 2011.poster presentation.*

Acknowledgements

Firstly, a huge thanks to my supervisor Dr Nina Storey for her continued support and encouragement throughout my PhD. Thank you to Prof Nick Standen for having the confidence in my abilities and encouraging me to undertake this PhD. I would also like to thank my committee members Prof Andrew Tobin and Dr John Mitcheson for their valuable contributions and feedback. I would like to pay special thanks to Dr Noel Davies, Dr Carl Nelson, Dr Richard Rainbow, Dr Matthew Perry, Dr Rebecca Stratton and Dr Jenny Brignell for numerous informative discussions, invaluable advice and mentoring over the past 3 and a half years. Thank you to Dr Dave Lodwick for his generous gift of the cp-YFP ROS biosensor which proved to be most useful. Many thanks to Mrs Dianne Everitt for her technical support and for making my time in the lab enjoyable. Finally, thank you to all my friends and family for their kind words of encouragement which have helped me to get to this stage. I am grateful to the British Heart Foundation for funding this project.

Abbreviations

ADP	adenine diphosphate
AM	acetoxymethyl ester
AMP	adenine monophosphate
AMPK	AMP-activated protein kinase
ANT	adenine nucleotide translocate
ATP	adenine triphosphate
BSA	bovine serum albumin
cAMP	cyclic AMP
CAT	carboxyatractyloside
CHD	coronary heart disease
COXIV	cytochrome C oxidase subunit IV
cpYFP	circularly permuted yellow fluorescent protein
CsA	cyclosporine A
CyP-D	cyclophilin-D
DHE	dihydroethidium
DMEM	Dulbecco's minimum essential medium
DNP	dinitrophenol
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ETC	electron transport chain
FBS	fetal bovine serum
GSK-3β	glycogen synthase kinase 3β
H_2O_2	hydrogen peroxide

IPC	ischaemic preconditioning
IPost	ischaemic postconditioning
LVDP	left ventricular developed pressure
MEM	minimum essential medium
MI	metabolic inhibition
mK_{ATP}	mitochondrial ATP-sensitive potassium channel
MPG	mercaptopropionyl glycine
mPTP	mitochondrial permeability transition pore
mt-cpYFP	mitochondrially targeted circularly permuted yellow fluorescent protein
NADH	nicotinamide adenine dinucleotide
NCX	Na^+/Ca^{2+}-exchanger
NHE 1	sodium/hydrogen exchanger 1
NO	nitric oxide
PBS	phosphate buffered saline
PCI	percutaneous coronary intervention
PI3K	phosphatidylinositol-3-OH kinase
PIA	A_1 receptor-selective agonist R(-)-N^6-(2-phenylisopropyl) adenosine
PiC	mitochondrial phosphate carrier
PKG	protein kinase G Cyclic guanosine monophosphate
PMCA	plasmalemmal Ca^{2+}-ATPase
PPIase	peptidylprolyl isomerase
RISK	reperfusion injury salvage kinase
ROI	regions of interest
ROS	reactive oxygen species
$SarcK_{ATP}$	sarcolemmal ATP-sensitive potassium channels
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase

SfA	sanglifehrin A
SNAP	*S*-nitroso-*N*-acetyl penicillamine
SOD	Cu/Zn-superoxide dismutase
SR	sarcoplasmic reticulum
TMRM	tetramethyl rhodamine methyl ester
TNFα	tumour necrosis factor alpha
TP	temperature preconditioning
VDAC	voltage-dependent anion channel
Xan Oxi	xanthine oxidase
Xan	xanthine

Contents

1 Introduction

1.1 Coronary heart disease

Coronary heart disease (CHD) refers to the condition in which an atherosclerotic plaque causes narrowing of a coronary blood vessel resulting in impaired blood flow. Plaques are composed of fatty deposits (atheroma), cholesterol and calcium amongst other substances and harden over time. This limits the flow of oxygen rich-blood to the myocardium and can cause pain and discomfort known as angina pectoris. The condition can be exacerbated if the plaque ruptures as this can cause a blood clot to form on the surface of the plaque. If the resultant clot is large enough to cause total artery occlusion this will cause the downstream region of the heart to be deprived of blood. This is referred to as a heart attack or myocardial infarction. Lack of oxygen and metabolic substrates to the myocardium for prolonged periods can cause irreversible damage, resulting in the development of an infarction. The size of the infarction will be proportional to the duration of ischaemia, therefore in order to salvage the ischaemic myocardium it is imperative to restore blood flow to the ischaemic region swiftly. Myocardial cell death poses a serious problem since cardiac myocytes are unable to divide to replace dead cells. On the contrary, dead myocytes are replaced with fibroblasts which can impair the myocardium's pumping efficiency, leading to heart failure or life-threatening arrhythmias (Giordano, 2005; Swynghedauw, 1999). Since the entire body is dependent on the supply of oxygen rich-blood provided by the rhythmical pumping action of the heart, an impairment of the contractile function of the heart, even for a short period, can be potentially fatal or result in significant

morbidity. Thus prevention and effective treatment of CHD is a major heath care goal and the subject of intense research.

Over the past fifty years, mortality rates from CHD have continuously decreased since 1961, at which time it was responsible for a quarter of all deaths in the UK. This is due to a combination of improved treatment and greater awareness of risk factors, leading to healthier lifestyle choices. Nonetheless, mortality rates from CHD are still alarmingly high and CHD represents the most common cause of death in the UK (Allender *et al.*, 2008). In 2008, around one in five male deaths and one in eight female deaths were caused by the disease – totaling approximately 88,000 deaths. Further, CHD is also the most common cause of premature death in the UK and was responsible for over 28,000 premature deaths in 2008. It is believed that approximately 124,000 individuals suffer a heart attack in the UK each year. A total of 1.5 million people in the UK have experienced a heart attack, of which over 900,000 are under the age of 75. The prevalence of angina pectoris in the UK population is even higher and is placed at 2.1 million people. The prevalence of CHD (angina and myocardial infarction) in the UK is 2.7 million people; of which nearly 1.6 million are under the age of 75 (Scarborough *et al.,* 2010). Although this figure has progressively decreased over the past five decades and the UK has one of the fastest falling rates in Europe, rates are still relatively high compared to other western European countries (Rayner *et al.*, 2009). Thus, there remains a strong need for continued research for the treatment and prevention of CHD.

In addition to the human burden (death and disability) caused by CHD, the disease also has major economic costs to society. These costs can be divided into direct costs to the

healthcare system and indirect costs in lost production due to death or disability. In 2006 it is estimated that CHD cost the UK healthcare system £3.2 billion, and that the total cost to the UK was £9 billion. Of this total cost, around 36% was due to direct healthcare costs, 43% to productivity losses, and 21% to the informal care of people with CHD (Scarborough *et al.*, 2008).

1.2 Myocardial ischaemia-reperfusion injury

The most common outcomes of CHD are angina pectoris and myocardial infarction. The symptoms of angina are severe tightness in the chest which may spread to the arms, neck, jaw, back and stomach. Individuals may also experience a shortness of breath. Angina can be bought on by a number of factors, but most notably an increase in physical activity. The pain caused by angina can be subsided through rest and the administration of glyceryl trinitrate spray or tablets. While angina is a serious condition most sufferers have a good quality life and can continue with normal daily activities (Lilly, 1998, Beattie, 1999). In contrast, myocardial infarction brought about by thrombotic occlusion of a major coronary artery is a more serious condition. It establishes an immediate risk of potentially fatal ventricular fibrillation, or longer term heart failure through irreversible myocardial damage. For individuals who suffer a myocardial infarction, approximately 30% die before reaching hospital. For the remainder, prognosis is dependent on the time it takes to restore blood flow to the ischaemic myocardium. Following an acute myocardial infarction, early reperfusion represents the most effective way of minimising myocardial infarct and improving clinical outcome (Thuny *et al.*, 2012). This is best achieved by either thrombolytic therapy or primary percutaneous coronary intervention (PCI). It is reported that

mortality rates were twice as high in patients treated 4-6 hours after the onset of symptoms, compared to those treated within 1-2 hours (An international randomized trial comparing four thrombolytic strategies for acute myocardial infarction. The GUSTO investigators. 1993). Thus the sooner the offending occlusion is removed, the greater the benefits to the myocardium and prognosis of long term survival of the patient. The irreversible damage caused to the myocardium as a result of acute myocardial infarction is attributed to ischaemia-reperfusion injury. As mentioned above, this damage can be minimised by early reperfusion. However, the restoration of blood flow to previously ischaemic myocardium can paradoxically result in additional cardiac damage, thereby reducing the beneficial effects of myocardial reperfusion. This is a phenomenon known as myocardial reperfusion injury and represents an area of great interest for a large community of researchers (Yellon & Hausenloy, 2007). It is hoped that understanding the pathophysiology of myocardial reperfusion injury may lead to the development of adjunctive therapies to improve prognosis of patients for whom reperfusion therapy is delayed.

Myocardial ischaemia brought about by blocked coronary blood flow is sufficient to cause cardiomyocyte injury and death and therefore must be removed as a matter of urgency. Studies conducted as early as 1986 demonstrated that the myocardium was capable of tolerating short-term ischaemia without enduring irreversible damage. However, ischaemia beyond 20 minutes was found to cause irreversible damage and death to the myocardium, despite reperfusion (Murry *et al.*, 1986). Myocardial ischaemia is characterised by a lack of perfusion of oxygen and metabolic substrates to the heart tissue. As a consequence, the major source of ATP production for energy

metabolism, mitochondrial oxidative phosphorylation, stops, leading to a rapid decline in cellular ATP concentration with a concomitant rise in ADP, AMP and P_i. Under these low oxygen conditions anaerobic glycolysis can provide limited amounts of ATP with associated lactate and H^+ accumulation (Allen & Orchard, 1987). However this process of energy generation is insufficient to meet the high energy demands of ventricular myocytes and cardiac contractility rapidly ceases. Secondary to ATP depletion is a major disturbance in ionic homeostasis as ATP depletion impairs the activity of ATP-dependent ion pumps. Ionic homeostasis is tightly regulated and is critically important for the contractile function of cardiomyocytes. Contraction is governed by the cardiac action potential and can be modulated by changes in the amplitude and duration of the action potential (Allen and Orchard, 1987). The cardiac action potential is initiated by the pacemaker activity of sinoatrial node cells which then spreads as a wave of depolarization through gap junctions throughout the myocardium. The depolarization activates voltage-gated Na^+-channels causing an influx of Na^+-current which induces a rapid depolarization of the cell membrane. This facilitates the opening of L-type Ca^{2+}-channels which cause Ca^{2+} influx and further calcium release from the sarcoplasmic reticulum as the entered Ca^{2+} activates ryanodine receptors. This process is termed Ca^{2+}-induced Ca^{2+}-release and results in a rapid elevation in cytosolic Ca^{2+} concentration, which is of fundamental importance for cardiac contraction. The plateau phase of the ventricular action potential is a result of equilibrium between Ca^{2+} influx and K^+ efflux and is diminished by the inactivation of voltage-gated Ca^{2+} channels, along with increased K^+ efflux through the activation of voltage-gated K^+ channels. As the action potential repolarises the inward-rectifier K^+ (I_{k1}) current increases moving membrane potential towards resting voltages (Michael *et al.*, 2009).

The rise in intracellular Ca^{2+} during the plateau phase of the action potential is critical to mediating excitation-contraction coupling and activates the contractile machinery of ventricular myocytes (Eisner *et al.*, 2000). Ca^{2+} binds to the troponin c component of the myofilaments, moving the troponin complex away from the actin binding site. This allows binding of the myosin head to the actin filament which uses ATP hydrolysis to pull the actin filament towards the center of the sarcomere, causing contraction. Relaxation is initiated by the diffusion of Ca^{2+} from the myofilaments back to the cytosol. The Ca^{2+} must then be removed from the cytosol and the main mechanisms by which this occurs are through the actions of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, the plasmalemmal Ca^{2+}-ATPase (PMCA) and the sarcolemmal Na^{+}/Ca^{2+}-exchanger (NCX). The actions of the SR Ca^{2+}-ATPase and the PMCA are dependent on ATP hydrolysis to drive the movement of Ca^{2+}, while the NCX harnesses the energy stored in the electrochemical gradient of Na^{+}. The NCX permits the flow of Na^{+} down its concentration gradient across the plasma membrane in exchange for the countertransport of Ca^{2+}. The stoichiometry of the NCX is $3Na^{+}/Ca^{2+}$ and therefore ion transport via the NCX is electrogenic. This means that the direction of ion transport via the NCX can reverse depending on the net electrochemical driving force. Under resting conditions, when the membrane is polarized, the NCX operates in the forward mode permitting Na^{+} influx in exchange for Ca^{2+} efflux. However, during the action potential Na^{+} influx via the activation of voltage-gated Na^{+} channels results in membrane depolarization which causes the NCX to operate in reverse mode (Sher *et al.*, 2008). This results in Ca^{2+} influx and is believed to contribute to the observed plateau during the ventricular action potential. Finally, repolarization of the membrane potential once again favors forward mode NCX transport and this contributes to cardiac relaxation

through the removal of cytosolic Ca^{2+}. Thus under normal conditions, a wide array of ion transport mechanisms function in consort to maintain ionic gradients that are responsible for action potential generation which underlies cardiac contractility.

During myocardial ischaemia severely depleted ATP levels stimulate glycolysis through the activation of phosphofructokinase. In the absence of oxygen, oxidative phosphorylation is inhibited causing myocytes to respire glucose anaerobically resulting in very little ATP generation. As a byproduct of anaerobic glycolysis lactic acid accumulates causing intracellular acidosis. This eventually inhibits phosphofructokinase thereby preventing anaerobic glycolysis and also inhibits mitochondrial fatty acid metabolism. Additionally, the low pH stimulates activation of the Na^+/H^+ antiporter resulting in intracellular Na^+ accumulation. Na^+ influx also occurs via Na^+/HCO_3 cotransporters and persistent (non-inactivating) Na^+ channels. Under normal conditions intracellular Na^+ concentration is tightly regulated through the action of the Na^+/K^+-ATPase which acts to maintain a low cytoplasmic Na^+ concentration. However, during ischaemia the regulatory role of this pump is impaired owing to reduced ATP concentrations, resulting in a rise of intracellular Na^+ concentration, which can cause the NCX to operate in reverse mode leading to a rise in intracellular Ca^{2+} concentration worsening a state of impaired ionic homeostasis. Previous studies have demonstrated a sustained rise of intracellular Ca^{2+} during ischaemia, which is secondary to Na^+ accumulation and is in close temporal association with the onset of myocardial rigor contracture (Ruiz-Meana *et al.*, 2000; Siegmund *et al.*, 1994). A critically low intracellular ATP concentration (< 100µM) is believed to underlie the development of rigor, essentially independent of Ca^{2+}. This event marks

the beginning of cytosolic Ca^{2+} accumulation and represents a key event in the progression of ischaemic injury (Dekker *et al.*, 1996).

Elevated intracellular Ca^{2+} concentrations combined with depleted ATP levels induced by prolonged ischaemia result in a progressive decline in cellular integrity. This occurs through the activation of phospholipases and Ca^{2+}-activated proteases (calpains) in a background in which ATP-dependent repair mechanisms are inhibited through a lack of ATP (Halestrap, 2010). Elevated intracellular Ca^{2+} also causes alterations in the sensitivity of contractile proteins causing impaired contractility despite elevated cytosolic Ca^{2+}. Ultrastructural features of ischaemic myocytes include swelling of the mitochondria, SR and cytoplasm, along with margination and clumping of nuclear chromatin (Buja, 2005). Prolonged ischaemia can result in irreversible myocyte injury characterised by flocculent mitochondrial morphology, rupture of the sarcolemma and necrotic cell death. There may also be a role for apoptotic cell death as translocation of BAX to the mitochondria has been demonstrated in ischaemic myocytes, leading to mitochondrial outer membrane permeabilisation and subsequent cytochrome c release (Capano & Crompton, 2006). However, the contribution of apoptosis to myocardial ischaemic damage will be determined by the rate and magnitude of ATP depletion since programmed cell death, as induced by apoptosis, is an ATP-dependent process.

Prolonged myocardial ischaemia is detrimental resulting in irreversible cardiomyocyte injury and myocardial infarct development. This can lead to myocardial remodeling in order to cope with increased stress placed on the remaining viable ventricular mass. Controlled remodeling can sometimes lead to normalization of wall stress. However,

remodeling in response to excessive wall stress can lead to fixed structural dilation of the ventricle and heart failure (Buja, 2005). Therefore limitation of infarct development and cardiomyocyte death has been an area of intense investigation for some time and has revealed the benefits of early reperfusion on preventing ischaemia induced damage.

Reperfusion as a therapeutic intervention undoubtedly limits myocardial damage caused by ischaemia, with the proportion of myocardial salvage directly related to the timing of the intervention. Reperfusion following a short period of ischaemia will result in very little, if any, myocardial damage although the myocardium may initially exhibit impairment in contractile function known as stunning (Bolli, 1990). However given sufficient time this will recover and no adverse long term effects will persists. In contrast, reperfusion following a prolonged period of ischaemia can have deleterious effects exacerbating the damage caused to the myocardium during ischaemia. This phenomenon is termed reperfusion injury and culminates in the death of cardiac myocytes that were viable immediately prior to myocardial reperfusion. In this manner reperfusion injury adds to myocardial injury thereby increasing the size of the myocardial infarct (Yellon & Hausenloy, 2007). The majority of cell death caused by the combined effects of ischaemia-reperfusion injury is necrotic and is accompanied by the release of Troponin 1 and intracellular enzymes such as lactate dehydrogenase, which together with infarct size can be used to determine the extent of damage. A small proportion of the damage is attributed to apoptotic cell death and is limited to the periphery of the infarct.

Elevated intracellular Ca^{2+} concentration is a significant factor underlying many of the deleterious effects associated with myocardial ischaemia-reperfusion injury. In reperfused cardiomyocytes in which mitochondrial energy production is recommenced, elevated cytosolic Ca^{2+} concentration rapidly changes due to various Ca^{2+} transporting mechanisms. Large quantities of Ca^{2+} are actively pumped into the SR by the re-energised SR Ca^{2+}-ATPase (SERCA), greatly reducing the cytosolic Ca^{2+} concentration. Typically, the SR would subsequently rapidly release the excessive Ca^{2+} load back into the cytosol due to the opening of ryanodine receptor release channels. This process occurs at a high frequency giving rise to Ca^{2+} oscillatory shifts between the cytosol and the SR (Piper *et al.*, 2006). Evidence suggests that the repetitive spikes in cytosolic Ca^{2+} concentrations are responsible for uncontrolled contractile activation in reperfusion-induced Ca^{2+} contracture (Schafer *et al.*, 2001). Several studies have demonstrated that reducing either oscillatory Ca^{2+} elevations or Ca^{2+} sensitivity at the time of reperfusion greatly reduced the development of hypercontracture in reoxygenated isolated myocytes and infarct size in reperfused hearts *in vivo* (Schafer *et al.*, 2001; Siegmund *et al.*, 1997; Garcia-Dorado *et al.*, 1992). Further, Ca^{2+} overload caused by waves of SR Ca^{2+} release at the time of reperfusion are thought to be the mechanism that underlies some types of arrhythmias associated with ischaemia-reperfusion injury. Delayed after depolarisations are often observed in cardiac myocytes as a result of reperfusion-induced arrhythmias and constitute a form of ischaemia-reperfusion injury (Piper *et al.*, 2003; Spencer & Sham, 2003). Reperfusion arrhythmias can originate as a consequence of chemical changes associated with opening and reperfusion of a coronary artery. The chemical changes are thought to

modulate cellular ionic homeostasis, giving rise to arrhythmias (Jurkovicova & Cagan, 1998).

Elevated cytosolic Ca^{2+} concentration in cardiac myocytes represents a key factor which underlies many of the pathophysiological changes associated with ischaemia-reperfusion injury. Therefore, it is not surprising to learn that preventing or reducing Ca^{2+} overload has cardioprotective effects and significantly reduces the damage caused by ischaemia-reperfusion injury. Elevations in cytosolic Ca^{2+} concentration can be greatly reduced by opening of sarcolemmal ATP-sensitive potassium channels (sarcK_{ATP}). Opening of sarcK_{ATP} channels occurs in response to a decrease in cellular ATP concentrations and causes failure of the action potential and hyperpolarization of the resting membrane potential by increasing K+ conductance, clamping the resting membrane potential close to the K-equilibrium potential. Failure of the action potential reduces Na^{+} and Ca^{2+} influx through voltage-gated ions channels. Additionally, membrane hyperpolarization would also serve to inhibit reverse mode NCX operation, thereby preventing Ca^{2+} influx via this mechanism (Sher *et al.*, 2008). In support of the above mentioned, Baczko *et al* demonstrated that pharmacological inhibition of sarcK_{ATP} channel with HMR1098 was associated with increased Ca^{2+} loading in isolated myocytes during re-oxygenation. Moreover, it was demonstrated that sarcK_{ATP} channel activation during re-oxygenation, with the openers pinacidil or P-1075, was sufficient to hyperpolarize membrane potential and significantly reduce reperfusion-induced Ca^{2+} loading of ventricular myocytes and contractile dysfunction (Baczko *et al.*, 2005). Opening of sarcK_{ATP} has also been shown to decrease Ca^{2+} loading during simulated ischaemia (metabolic inhibition), through shortening of action

potential duration which occurs as a result of accelerated phase 3 repolarisation (Noma, 1983). Rodrigo *et al* demonstrated that treatment of isolated myocytes with the sarcK_{ATP} channel opener, diazoxide, significantly decreased the time to contractile failure during metabolic inhibition, indicating accelerated action potential failure compared to untreated control myocytes. Early activation of sarcK_{ATP} served to decrease Ca^{2+} loading and may have conserved cellular ATP levels through reduced activity of the contractile machinery (Rodrigo *et al.*, 2004). Thus the beneficial effects of sarcK_{ATP} in the setting of ischaemia-reperfusion injury are two-fold; sarcK_{ATP} opening can reduce Ca^{2+} loading during ischaemia and reperfusion by causing membrane hyperpolarization and secondly, it can serve to conserve cellular ATP concentration during ischaemia by causing accelerated action potential failure.

From a clinical perspective, myocardial reperfusion injury manifests as four different types of cardiac dysfunction, three of which have been mentioned above. Namely, these are myocardial stunning, lethal irreversible injury and reperfusion arrhythmias. A fourth type of reperfusion injury is known as the coronary no-reflow phenomenon, in which perfusion through a given segment of the coronary circulation is inadequate, despite the absence of vessel obstruction. Prior temporary occlusion of the artery is a prerequisite condition for no-reflow and persistent no-reflow has been associated with increased mortality and a high incidence of myocardial infarction (Abbo *et al.*, 1995).

Strong evidence suggests that a major determinant of reperfusion-induced injury is the irreversible opening of mitochondrial permeability transition pore (mPTP) (Halestrap, 2010). For this reason, the mPTP is presently a major focus of research and a target for therapeutic intervention to minimize reperfusion injury.

1.3 The mitochondrial permeability transition pore

The mPTP is a non selective, large conductance pore of the mitochondrial inner membrane that is thought to open under conditions of excessively high (pathological) matrix Ca^{2+} concentration (Hunter & Haworth, 1979). However, elevated matrix Ca^{2+} alone is not sufficient to trigger irreversible mPTP opening and must be accompanied by adenine nucleotide depletion, high inorganic phosphate (Pi) concentration and oxidative stress (Halestrap, 2010; Crompton & Costi, 1988). These factors are known to modulate the sensitivity of the mPTP to Ca^{2+} and therefore make its opening more likely. The opening of the mPTP increases the permeability of the otherwise impermeable mitochondrial inner membrane, allowing all small molecular weight molecules (< 1.5 kDa) to equilibrate across the mitochondrial inner membrane. However, a high concentration of proteins is retained within the matrix and this causes water to be drawn into the matrix by osmosis causing mitochondrial swelling. Consequently the cristae unfold to maintain the structural integrity of the mitochondrial inner membrane, but the pressure exerted by matrix swelling can not be tolerated by the outer membrane and causes it to rupture. This leads to the release of cytochrome c and other pro-apoptotic proteins that has the potential to cause apoptotic cell death if ATP levels are maintained sufficiently high enough. However irreversible mPTP opening is more likely to lead to necrotic cell death as a result of severe disruption to metabolism and ionic homeostasis. mPTP opening leads to mitochondrial uncoupling as a result of dissipation of the pH gradient and mitochondrial membrane potential which are required to drive oxidative phosphorylation. Thus ATP synthesis is halted. Further, under these conditions mitochondria can consume ATP through reversal of the F_1F_0 ATP synthase, resulting in

severely depleted ATP levels which underlie necrotic cell death (Campanella *et al.*, 2008). In short, irreversible mPTP opening leads to cell death and is strongly believed to be the cause of reperfusion injury.

Prolonged ischaemia increases intracellular Ca^{2+} and Pi concentrations, while causing depletion of adenine nucleotides. These are factors which promote mPTP opening but an overriding inhibitory factor prevails during ischaemia preventing opening. This factor is low intracellular pH produced as a consequence of anaerobic glycolysis and most probably inhibits mPTP opening by competing with Ca^{2+} at the trigger site (Haworth & Hunter, 1979). Reperfusion of the previously ischaemic myocardium causes intracellular pH to return to normal over a period of 2-3 minutes as lactic acid leaves the cell and pH regulatory transporters are activated (Vandenberg *et al.*, 1993). The early stages of reperfusion are also associated with increased mitochondrial matrix Ca^{2+} uptake and excessive ROS production. Reperfusion allows the resumption of respiration through the delivery of oxygen and therefore ATP production is recommenced. This reenergizes mitochondria and stimulates Ca^{2+} uptake. The exact source of ROS production observed during reperfusion is unknown but involves molecular oxygen interacting with a reduced electron carrier to produce superoxide. The conditions which prevail in the reperfused myocardium are exactly those which favor mPTP opening and experimental data confirms opening to coincide during the early stages of reperfusion (Griffiths & Halestrap, 1995). Thus mPTP opening at the time of myocardial reperfusion represents a critical mediator of cardiomyocyte death in the setting of ischaemia-reperfusion injury.

1.4 The molecular mechanism and composition of the mPTP

Mitochondrial swelling as a result of change in the permeability of the mitochondrial inner membrane, referred to as the 'mitochondrial permeability transition', was first described in the 1950's (Tapley, 1956; Hunter & Ford, 1955). Later studies conducted in the 1970's and 1980's demonstrated the existence of the mPTP which could reversibly open to induce the mitochondrial permeability transition. At present, much is known about the regulation of the mPTP but the molecular structure of the pore remains undefined.

Cyclosporine A (CsA) is an immunosuppressant drug that has proven to be key to the elucidation of the molecular mechanism involved in mPTP opening. In 1988 CsA was shown to inhibit Ca^{2+}-dependent mPTP opening in heart mitochondria (Crompton & Costi, 1988). This discovery paved the way for Halestrap and Davidson to demonstrate that CsA inhibits mPTP opening by inhibiting the activity of an 18kDa protein, cyclophilin-D (CyP-D) (Halestrap *et al.*, 1997). This was later corroborated by the demonstration that a number of CsA analogues such as 6-methyl-Ala-CsA, 4-methyl-Val-CsA, *N*-methyl-4-isoleucine-CsA and D-3-methyl-Ala-4ethyl-Val-CsA also bind CyP-D and inhibit mPTP opening (Griffiths & Halestrap, 1991; Waldmeier *et al.*, 2002; Hansson *et al.*, 2004; Gomez *et al.*, 2007). The role of CyP-D in mPTP opening was confirmed beyond doubt by the generation of CyP-D knockout mice. These animals were found to exhibit increased tolerance to Ca^{2+} induced mPTP opening and behaved identically to control mitochondria treated with CsA (Baines *et al.*, 2005; Basso *et al.*, 2005). CyP-D has peptidylprolyl *cis-trans* isomerase activity which can be activated by Ca^{2+}. It is believed that under pathological conditions in which matrix Ca^{2+} is elevated,

that CyP-D facilitates a Ca^{2+}- triggered conformational change in a membrane protein to induce mPTP opening. However, the identity of the membrane component responsible for forming the pore is currently undefined, but a number of candidate molecules have been suggested and these will be briefly discussed below. It should also be noted that mPTP opening can occur independently of CyP-D if the stimulus is large enough, thus implying that CyP-D only plays a regulatory role in mPTP opening.

It was first proposed in 1990 that the adenine nucleotide translocase (ANT), which is located on the mitochondrial inner membrane, might represent the pore forming component of the mPTP (Halestrap & Davidson, 1990). This proposal was supported by the demonstration that mPTP sensitivity could be modulated by specific ligands of the ANT. Enhanced mPTP opening was observed by the presence of carboxyatractyloside (CAT) and adenine nucleotide depletion. In contrast, mPTP opening was inhibited by a second ligand of the ANT, bongrekic acid (BKA), in addition to ATP and ADP. CAT and BKA are known to induce two distinct conformations of the ANT, both of which have opposing effects on mPTP opening. Further support for ANT as a core component of the mPTP was provided by the demonstration that the ANT could bind to an immobilized fusion protein between GST-CyP-D, and that this interaction could be prevented by pre-treatment with CsA (Woodfield *et al.*, 1998). However, work conducted by Kokoszka *et al.* in 2004 using mice in which two isoforms of the ANT were genetically inactivated revealed that the ANT is not a structural component of the mPTP. This study reported that mitochondria lacking ANT could still be induced to undergo the mitochondrial permeability transition in a CsA-sensitive manner; although more Ca^{2+} than normal was required to activate the mPTP. Additionally it was reported that mPTP opening could not be modulated by ANT ligands suggesting that the ANT

plays a purely regulatory role in mPTP opening (Kokoszka *et al.*, 2004). An alternative view may be to assume that the ANT can form the pore but in its absence other proteins serve this function (Halestrap, 2010).

Increasing evidence implicates the mitochondrial phosphate carrier (PiC) as a key player in mPTP formation (Leung *et al.*, 2008). It is well established that phosphate sensitizes pore opening to Ca^{2+} and until recently this effect was believed to be attributed to the ability of phosphate to enhance Ca^{2+} uptake while maintaining matrix pH (Petronilli *et al.*, 1993). However, recent evidence presented by Halestrap's group suggests that phosphate may enhance mPTP opening by binding to the PiC (Leung *et al.*, 2008). This group demonstrated that PiC co-immunoprecipitated with GST-CyP-D and that this binding was prevented by CsA but increased by oxidative stress and to a lesser extent by CAT, both of which sensitize pore opening to Ca^{2+} concentration. The binding was also found to be prevented by pre-treatment of mitochondria with oxidized ubiquinone (UQo) and Ro 68-3400 which are known inhibitors of the mPTP. Further UQo, Ro 68-3400, and *N*-ethylmaleimide (NEM), an established inhibitor of the PiC, were all shown to inhibit mitochondrial phosphate transport with the same potency as they inhibited mPTP opening. They also reported co-immunoprecipitation of the ANT with PiC but found that this association was insensitive to CsA. This led the authors to propose that the ANT may regulate mPTP opening through an association with the PiC (Leung *et al.*, 2008). Further evidence in support of the PiC as the pore forming component of the mPTP comes from the demonstration in HeLa cells that knockdown of PiC reduces their sensitivity to staurosporine induced apoptosis (Alcala *et al.*, 2008) which is believed to be mediated by mPTP opening (Tafani *et al.*, 2001), whereas overexpression of PiC induced apoptosis (Alcala *et al.*, 2008). However,

despite such strong evidence in support of the PiC as the pore forming component of the mPTP it was recently demonstrated that genetic ablation of the PiC using siRNA, resulting in 65-80% knockdown, did not decrease mPTP opening. This could be interpreted as a lack of involvement of the PiC in mPTP formation but on the other hand, it is possible that the remaining 20-35% PiC is sufficient to cause maximal mPTP opening (Varanyuwatana & Halestrap, 2012). Thus, definitive proof for the involvement of the PiC in pore formation remains to be demonstrated. This awaits the demonstration of the effects of total knockdown of the PiC on mPTP opening, or alternatively the demonstration that the reconstituted PiC can function as the mPTP.

It was previously believed that the voltage-dependent anion channel (VDAC) was a pore forming component of the mPTP. This belief stemmed from the demonstration that under certain conditions VDAC could be co-purified with the ANT (McEnery *et al.*, 1992) and that GST-CyP-D could pull down VDAC from detergent solubilized heart mitochondria (Crompton *et al.*, 1998). However a role for VDAC in mPTP formation was ruled out by the demonstration that VDAC knockout in mice did not alter mPTP formation or sensitivity (Krauskopf *et al.*, 2006; Baines *et al.*, 2007). The peripheral benzodiazepine receptor, now more commonly referred to as the mitochondrial translocator protein of the outer membrane (TSPO) was also shown to associate with the ANT (McEnery *et al.*, 1992), although its role in mPTP formation is regarded as controversial since there are conflicting reports about the effects of TSPO ligands on mPTP opening (Li *et al.*, 2007; Obame *et al.*, 2007; Schaller *et al.*, 2010). It is suggested that hexokinase II (HK-II) may play a regulatory role in mPTP formation since it was demonstrated that glycogen synthase kinase 3β (GSK-3β) could phosphorylate VDAC1,

resulting in decreased binding of HK-II to mitochondria and reduced inhibition of mPTP opening (Pastorino *et al.*, 2005).

1.5 Inhibiting mPTP opening protects against ischaemia-reperfusion injury

Since mPTP opening is considered a critical event in reperfusion injury it raises the hypothesis that inhibition of pore opening should be cardioprotective. Indeed this was first demonstrated in 1991 by Crompton's group who reported that CsA could protect isolated cardiac myocytes against reoxygenation injury (Nazareth *et al.*, 1991). This was subsequently demonstrated in the Langendorff perfused heart by Griffiths and Halestrap in 1993 (Griffiths & Halestrap, 1993) and later by a range of investigators using several different models of ischaemia and reperfusion, and a range of indicators to assess injury including haemodynamic function, enzyme release and infarct size (Hausenloy *et al.*, 2002; Hausenloy & Yellon, 2003; Argaud *et al.*, 2005). The cardioprotective effects of CsA are now widely accepted and this was confirmed by a recent proof of concept clinical trail in humans in which it was demonstrated that CsA treatment improved recovery of patients undergoing PCI for the treatment of coronary thrombosis (Piot *et al.*, 2008). The mechanism of mPTP inhibition by CsA is believed to occur as a result of CsA binding to CyP-D, thereby preventing CyP-D binding to the pore forming component of the mPTP (possibly PiC) to induce pore opening. Sanglifehrin A (SfA) is another mPTP inhibitor which also offers protection of the heart against ischaemia-reperfusion injury (Clarke *et al.*, 2002). Like CsA, SfA also targets CyP-D to prevent pore opening but its mechanism of action differs from CsA. SfA prevents pore opening by inhibiting the PPIase activity of CyP-D, therefore the conformational

change in the pore forming component of the mPTP is not facilitated, resulting in mPTP inhibition. Further evidence in support for the central role of mPTP opening in reperfusion injury comes from the demonstration that CyP-D knockout mice have increased tolerance to the damaging effects of ischaemia-reperfusion injury (Baines *et al.*, 2005; Hausenloy *et al.*, 2010). These studies also highlight the critical role of CyP-D in mPTP regulation and confirm inhibition of CyP-D activity as the mechanism of action of CsA and SfA. However, targeting CyP-D as a means of achieving cardioprotection is not ideal since mPTP opening can still occur if the stimulus is sufficiently great. Moreover, targeting CyP-D is likely to result in inhibition of other cyclophilins such as CyP-A which can lead to inhibition of calcineurin and have direct effects on heart function (Periasamy, 2002). Additionally, the use of CsA is likely to have unwanted immunosuppressive effects (Schreiber & Crabtree, 1992) and although this problem can be overcome through the use of non-immunosuppressant CsA analogues, or use of the unrelated drug SfA, there is still the concern as to what other side effects will come about as a result of inhibition of the PPIase activity of cyclophillins. To address this potential problem Malouitre *et al.* recently described the use of a mitochondrially targeted CsA derivative which specifically targets mitochondrial CyP-D and protects against necrotic cell death in a neuroblastoma cell line (Malouitre *et al.*, 2009). More recently, the same group demonstrated protection against mPTP opening using this drug in isolated cardiomyocytes and reported greater protection over that induced by normal CsA (Dube *et al.*, 2012). Therefore this mitochondrially targeted CsA derivative has the potential to be of greater therapeutic benefit than normal CsA, but requires further investigations before this can be said for certain.

CyP-D represents a means by which to inhibit mPTP opening and cell death in the reperfused myocardium but inhibition of CyP-D activity can lead to secondary problems. For this reason CyP-D is not the ideal pharmacological target for mPTP inhibition but unfortunately at present, it is the only confirmed component of the mPTP for which appropriate inhibitors exist. Therefore until the molecular composition of the mPTP is resolved, which would lead to pharmacological targeting to inhibit the pore forming component of the mPTP, an alternative approach to inhibit mPTP opening is to manipulate those factors which sensitise pore opening to Ca^{2+}. Oxidative stress is one of the most potent activators of mPTP opening and reducing oxidative stress at the time of myocardial reperfusion is cardioprotective. Propofol is a general anesthetic with cardioprotective properties, which are attributed to the reactive oxygen species (ROS) scavenging effects of the agent. Propofol has been shown to inhibit mPTP opening in both isolated mitochondria (Sztark *et al.*, 1995) and in mitochondria from propofol treated hearts (Javadov *et al.*, 2000). Furthermore, the cardioprotective effects of propofol have been demonstrated in the Langendorff perfused rat heart and this was found to be associated with reduced mPTP opening on reperfusion (Javadov *et al.*, 2000). The knowledge that ROS scavenging can have cardioprotective effects led to the development of MitoQ, a ROS scavenger that is mitochondrially targeted through a positively charged hydrophobic moiety (Adlam *et al.*, 2005). This is especially useful since it is known that increases in matrix ROS promote mPTP opening, possibly through modifications of thiol groups on the ANT (McStay *et al.*, 2002), while cytosolic ROS may contribute to cardioprotection through the activation of protective signaling pathways (Liu *et al.*, 2008). As expected, MitoQ was found to significantly decrease heart dysfunction, cell death and mitochondrial

damage following ischaemia-reperfusion (Adlam *et al.*, 2005). Therefore, reducing reperfusion induced oxidant stress likely represents an effective way to limit myocardial damage. Alternatively targeting the correction of low intracellular pH at reperfusion can also protect hearts by inhibiting mPTP opening. Administration of cariporide, a Na^+/H^+ exchanger 1 (NHE1) inhibitor has been shown to protect the heart against reperfusion injury (Mentzer *et al.*, 2003). Additionally, AVE-4890, a specific NHE1 inhibitor was also found to protect Langendorff perfused hearts against ischaemia-reperfusion injury and this effect was found to be associated with reduced mPTP opening (Javadov *et al.*, 2008). Pyruvate is an extremely potent cardioprotective agent and evidence suggests that this effect may stem from its ability to maintain lower pH during reperfusion. However, pyruvate is also known to have free radical scavenging properties and so its cardioprotective effects are most likely the result of multiple factors which ultimately inhibit mPTP opening and maintain mitochondrial integrity and energy generation (Mallet *et al.*, 2005). Pyruvate has been shown to improve cardiac function in a manner which correlated with mPTP inhibition (Kerr *et al.*, 1999). In summary, strong evidence suggests that manipulation of the factors which regulate mPTP opening results in protection against ischaemia-reperfusion injury, and that this effect is associated with reduced mPTP opening at reperfusion. Thus irreversible mPTP opening appears to be the central determinant of cell fate in the setting of ischaemia-reperfusion injury and cardioprotection can be achieved by inhibiting mPTP opening at the time of myocardial reperfusion.

1.6 Ischaemic preconditioning

The damaging effects of ischaemia-reperfusion injury on the myocardium have been known for several decades and attention was first directed to the problem in animal laboratories in the 1970's. A number of interventions were used in an attempt to reduce infarct size but the protection observed was modest (10-20%) and the reproducibility of protection was poor (Yellon & Downey, 2003). However, in 1986 Murry *et al.* described the phenomenon of ischaemic preconditioning (IPC) in canine hearts, which resulted in profound protection of the myocardium against ischaemia-reperfusion injury. In its initial description IPC involved subjecting the myocardium to four cycles of 5 minutes of ischaemia followed by 5 minutes of reperfusion prior to an index ischaemic episode of 40 minutes. The results of this intervention were staggering as infarct size in preconditioned hearts was found to be reduced to 75% of that observed in control hearts, measured four days after reperfusion. However, the investigators reported that IPC only served to slow the progression of cell death during ischaemia, since it was found that IPC could not significantly reduce infarct size when the index ischaemic episode was increased to 3 hours (Murry *et al.*, 1986). The protective effects of IPC is bi-phasic with an immediate protection manifesting immediately following the preconditioning stimulus, referred to as classic or early preconditioning, which last for 1-4 hours depending on species and experimental differences (Murry *et al.*, 1991; Sack *et al.*, 1993). A second window or delayed protection then reappears 24 hours later and lasts for up to 72 hours (Baxter *et al.*, 1997). However, although delayed protection induces more prolonged protection than that arising immediately following the preconditioning stimulus, the protection is less robust (Yellon & Downey, 2003).

The seminal report of IPC prompted huge interest and was immediately successful due to the robustness of the phenomenon. The cardioprotective effects of IPC were consistently reproduced by other investigators and this endogenous protective mechanism was subsequently shown to exist in all species investigated including, mouse, rat, rabbit, feline, sheep, monkey and most importantly humans (Yang *et al.*, 2010). The cardioprotective effects of IPC in the clinical setting are limited to procedures in which myocardial ischaemia is predetermined and as such IPC is not amenable for the treatment of acute myocardial infarction or for patients surviving cardiac arrest. However, interestingly the cardioprotective effects of IPC can be simulated by pre-infarction angina which may represent a form of IPC. Studies have demonstrated that pre-infarction angina improves myocardial salvage and is associated with reduced infarct size development following ischaemia-reperfusion injury (Lonborg *et al.*, 2012; Ahmed *et al.*, 2012). The discovery of IPC represents a major milestone in the ongoing battle against ischaemia-reperfusion injury as it was the first demonstration that cardioprotection was indeed possible. Moreover, elucidation of the signaling pathways involved in IPC has led to the discovery of various pharmacological agents which can elicit the protective effects of IPC when applied before ischaemia. The mechanism of protection of IPC has been studied extensively and a key finding was the elucidation of the different elements of the protective IPC response. These include receptors that act as the trigger mechanism, signal transduction pathways that act as mediators and end effectors which ultimately are responsible for the increased tolerance of the preconditioned myocardium to ischaemia-reperfusion injury (Yellon & Downey, 2003). A second important finding was the discovery that although IPC is applied prior to ischaemia, it exerts a protective

effect at the time of myocardial reperfusion by inhibiting effectors of cell death (Garcia-Dorado *et al.*, 2006; Hausenloy *et al.*, 2005a). This led to a shift of attention to the reperfusion phase as target to intervene and harness the protection induced by IPC. However, a lack of complete understanding of the molecular mechanisms involved in the phenomenon has until present hindered the translation of basic research on IPC into clinical practice.

1.7 Ischaemic postconditioning

As mentioned above the clinical application of IPC is limited because of the need to intervene prior to ischaemia and therefore an interventional strategy applied at the reperfusion stage would offer greater clinical potential. In this regard, over the past 20 years a number of pharmacological agents have been suggested to reduce myocardial injury when administered at reperfusion based on experimental data, but unfortunately the translation of cardioprotection when investigated in humans has been disappointing. An apt example of this was the use of Ca^{2+} channel antagonists which, when applied at reperfusion was found to significantly decrease myocardial infarct size in experimental studies (Klein *et al.*, 1989). However, the corresponding clinical study in humans yielded disappointing negative results (Boden *et al.*, 2000).

In 2003, Vinten-Johansen's group described ischaemic postconditioning (IPost), a simple and efficient interventional strategy that was found to significantly reduce infarct size in the canine heart when applied at the onset of reperfusion (Zhao *et al.*, 2003). The protective maneuver involved applying three cycles of 30 seconds of reperfusion interspersed with 30 seconds of ischaemia at the onset of reperfusion, following a prolonged ischaemic episode. Myocardial damage was assessed after 3

hours of reperfusion and IPost was found to reduce infarct size by 44% of that observed in the control group. Furthermore, the protective effects were found to be comparable to IPC and no statistical difference in infarct size from IPC and IPost hearts was found. The timing of the strategy (applied at reperfusion) made IPost an attractive and clinically relevant intervention which was quickly implemented by several independent investigators in patients undergoing primary PCI (Laskey, 2005; Ma *et al.*, 2006; Yang *et al.*, 2007). These studies confirmed the cardioprotective effects of IPost and more importantly demonstrated that IPost has beneficial effects in humans causing a significant reduction of final infarct size (Staat *et al.*, 2005). Since its initial description IPost has been confirmed as a protective strategy comparable to IPC in several animal species including rats, rabbit and mice (Bopassa *et al.*, 2006; Kin *et al.*, 2004; Tsang *et al.*, 2004). These animal studies have been fundamental to elucidating the complex molecular mechanisms involved in IPost and are vital to the identification of pharmacological agents that can mimic the protection afforded by IPost. Although the beneficial effects of IPost have been clearly demonstrated in the human myocardium, the practicality of this invasive mechanical strategy is limited to the setting of primary PCI (Thuny *et al.*, 2012). In the case of patients where reperfusion is achieved by pharmacological thrombolysis, IPost cannot be performed. Thus, pharmacological postconditioning represents an attractive alternative with wider clinical relevance that could be applied alongside existing reperfusion strategies.

1.8 Therapeutic hypothermia

It was first recognized in the late 1970s that hypothermia could be used therapeutically to limit the damage caused to the myocardium by ischaemia-

reperfusion injury. Abendschein *et al.* demonstrated in dogs that the ischaemic myocardium could be protected by whole-body hypothermia, resulting in a 40% reduction in infarct size compared to normothermic control hearts, measured 5 hours after coronary artery occlusion (Abendschein *et al.*, 1978). This pioneering study highlighted the potential of hypothermia, applied during ischaemia, to limit infarct size caused by ischaemia without reperfusion. Subsequently, Haendchen *et al.* demonstrated that the benefits of hypothermia on limiting infarct size also extended to dogs with reperfused myocardial infarcts (Haendchen *et al.*, 1983). Since then the cardioprotective effects of hypothermia have been shown to be present in several animal species and hypothermia is now proposed to be one of the most potent cardioprotective strategies available. Hypothermia localised to the myocardial risk zone early after the onset of coronary occlusion salvaged 18% of the risk region and significantly decreased area of infarct in rabbit studies (Hale & Kloner, 1998). More recently it was demonstrated that myocardial hypothermia by endovascular cooling and cold saline infusion decreased infarct size in pigs when administered during ischaemia (Gotberg *et al.*, 2008). Therapeutic hypothermia is used clinically to protect the heart against ischaemia in a number of surgical procedures such as surgery for coronary bypass or valve repair and transplantation (Darbera *et al.*, 2011; Dixon *et al.*, 2002; Miki *et al.*, 1998).

Studies conducted as early as the 1980's determined the effects of hypothermia on metabolic rate and found a decrease in temperature to be associated with a reduction in the utilisation of high-energy phosphates and glucose (Jones *et al.*, 1982; Simkhovich *et al.*, 2004)(). It is proposed that a 1^{o}C drop in body temperature slows metabolic rate by approximately 8% (Polderman, 2009). Consequently, hypothermic treatment of the

ischaemic myocardium results in increased myocardial ATP preservation during ischaemia and upon reperfusion (Ning *et al.*, 1998). This hypothermia-induced alteration of mitochondrial bioenergetics was initially suggested to be the underlying factor causing cardioprotection. However, recent studies have demonstrated the activation of survival signalling cascades induced by therapeutic hypothermia, suggesting an alternate mechanism for protection. In two separate studies conducted by Shao *et al.*, therapeutic hypothermia was used to protect isolated cardiomyocytes against reoxygenation injury. This protection was demonstrated to involve the activation of PKC_{ϵ} and nitric oxide synthase in a chick cardiomyocyte study using a therapeutic hypothermic temperature of 25^{o}C (Shao *et al.*, 2007). Subsequently, a murine cardiomyocyte study revealed the involvement of increased phosphorylation of Akt and HSP27 in the protective signalling cascades of therapeutic hypothermia (32^{o}C) (Shao *et al.*, 2010). Further, a study conducted by Ning *et al.* revealed a hypothermia-induced alteration in the expression of 6 genes related to apoptosis, including an increased expression of the anti-apoptotic Bcl-2 homologue *Bcl-x* and a decreased expression of the proapoptotic Bcl-2 homologue *Bak.* These findings led the authors to suggest that modifications in the signalling pathways for apoptosis may underlie protection by hypothermia (Ning *et al.*, 2002). Moreover, a recent study conducted by Yang *et al.* using isolated rabbit hearts revealed a hypothermia-induced preservation of ERK activity during ischaemia which was found to be essential for the infarct-limiting effects of hypothermia (Yang *et al.*, 2011). Thus, it has emerged that the beneficial effects of hypothermia in the setting of ischaemia-reperfusion injury involve more than a simple alteration in mitochondrial bioenergetics, but rather include the activation of several protective signalling pathways and survival proteins.

Previous studies suggest that the optimal time to implement hypothermia to achieve maximum protection in the context of ischaemia-reperfusion injury is prior to the onset of ischaemia. Hale and Kloner discovered a close relationship between the temperature measured in the myocardium immediately prior to the onset of ischaemia and the size of a developed infarction as a consequence of coronary artery occlusion. They found that lower myocardial temperatures strongly correlated with smaller infarcts (Hale *et al.*, 1997). In order to translate the benefits of hypothermia in the setting of acute myocardial infarction, where the onset of myocardial ischaemia has already commenced, several studies investigated the effects of hypothermia when implemented after the onset of ischaemia. These studies revealed that protection could still be achieved but to a lesser extent compared to when hypothermia was applied prior to ischaemia. Also, the infarct-sparing effects of hypothermia applied during ischaemia were found to be time dependent; decreased protection was observed if hypothermia was applied later into ischaemia (Miki *et al.*, 1998; Hale *et al.*, 1997; Dae *et al.*, 2002). Following on, studies subsequently showed that the beneficial effects of hypothermia where lost when hypothermic treatment was started at the end of ischaemia or early reperfusion (Maeng *et al.*, 2003; Otake *et al.*, 2007)(). Therefore the literature suggests that hypothermia grants greatest cardioprotection when applied prior to the ischaemic insult.

The use of therapeutic hypothermia to treat acute MI is surrounded with potential problems and therefore these issues need to be thoroughly addressed before it can be routinely used in conjunction with existing reperfusion strategies. Mild hypothermia can induce shivering as an unwanted side effect which can counteract the induction of hypothermia and additionally result in unfavourable increases in metabolic rate and

oxygen consumption. However, in humans this problem has been overcome through the use of pharmaceutical intervention (Mokhtarani *et al.*, 2001). Other potential problems of moderate to deep hypothermia are the increased risk of bleeding events and infection and hyperglycaemia (Hale & Kloner, 2011). A more relevant practical limitation of therapeutic hypothermia to treat acute myocardial infarction is the time taken to reach the target temperature. Early reperfusion is crucial to myocardial salvage and hence nothing should delay reperfusion strategies. This limitation was realised during COOL-MI (Cooling as an Adjunctive Therapy to Percutaneous Intervention in Patients With Acute Myocardial Infarction), a randomised multicentre clinical trial aimed at assessing the effectiveness of utilising hypothermia to limit myocardial infarct size. This study evaluated the beneficial effects of reducing core body temperature to <33^{o}C prior to reperfusion. This was achieved by positioning an endovascular cooling catheter in the inferior vena cava through which cold saline was circulated, thereby cooling blood as it passed along the catheter. The study failed to demonstrate an overall hypothermia-induced reduction in myocardial infarct size compared to control. However, a major limitation of the study was that the target temperature was only achieved in a minority of patients at the time of PCI. The median reduction in core temperature was found to be approximately 1^{o}C, which was much less than anticipated (O'Neill, 2003). Another obstacle which can potentially prevent the clinical use of hypothermia is that hypothermia should not reduce the efficacy of the thrombolytic therapy. *In vitro* studies have demonstrated that hypothermia adversely affected the thrombolytic action of tissue plasminogen activator (TPA) (Shaw *et al.*, 2007). Of course, this is not an issue when PCI is the reperfusion strategy employed to remove coronary occlusion.

1.9 Temperature preconditioning

In 2007, Khaliulin *et al.* described a novel use of hypothermia to achieve cardioprotection using a protocol they termed 'temperature preconditioning' (TP) which combines the principles of ischaemic preconditioning and therapeutic hypothermia. TP involves exposing the heart to brief periods of hypothermic perfusion (26^{o}C) interspersed by periods of normothermic perfusion (37^{o}C) for three cycles prior to prolonged ischaemia. The cardioprotective effects of TP were compared against the gold standard of cardioprotection as achieved by IPC and the authors reported that the protective effects of TP were greater. Langendorff perfused isolated rat hearts were subjected to 25 minutes of global ischaemia followed by 60 minutes of reperfusion after which time the cardioprotective effects of TP and IPC were assessed. Both TP an IPC were found to improve haemodynamic recovery, reduce arrhythmias and necrotic damage (assessed by lactate dehydrogenase release) compared to control hearts, with the greatest improvements observed in TP hearts. Preischaemic ATP and creatine phosphate levels were analysed and TP was found to increase both compared to controls. In contrast IPC only increased creatine phosphate levels. At reperfusion total adenine nucleotide levels were measured and found to be significantly higher in TP and IPC hearts compared to controls, implying better preservation of energy metabolism (Khaliulin *et al.*, 2007).

TP is a relatively novel cardioprotective intervention and as such its cardioprotective mechanisms are not completely understood. Further, unlike IPC, the cardioprotective effects of TP are yet to be reproduced in humans and its feasibility for use in the clinical setting needs to be assessed. A potential practical limitation preventing the use

of TP in the clinical setting would be the ability to achieve and maintain the heart hypothermic using only short bursts of cold perfusion in a closed chest situation (Ghouri *et al.*, 2007). The study of Khaliulin *et al.* was performed in isolated rat hearts devoid of contact with warm organs such as the lungs, making reductions in myocardial temperatures relatively easy. Therefore TP may not be a clinically relevant intervention but its discovery is important since it provides the opportunity for a possible alternative mechanism for the treatment of ischaemia-reperfusion injury. Elucidation of the signalling events associated with TP could lead to the development of more effective pharmacological interventions that limit the detrimental effects of ischaemia-reperfusion injury.

1.10 Cardioprotection and mPTP inhibition

Irreversible mPTP opening at the time of myocardial reperfusion results in ATP depletion, mitochondrial swelling and necrotic cell death (Halestrap, 2010). The role of mPTP opening as a critical mediator of cell death in the setting of ischaemia-reperfusion injury is supported by multiple lines of evidence. Firstly, the factors which prevail in the reperfused myocardium are exactly those which promote mPTP opening. These are mitochondrial Ca^{2+} accumulation, excessive oxidative stress, adenine nucleotide depletion, high phosphate accumulation and restoration of neutral pH (Halestrap, 2010; Crompton *et al.*, 1987). Secondly, mPTP opening has been demonstrated to specifically open during the first few minutes of myocardial reperfusion and its opening closely correlates with the restoration of normal pH (Griffiths & Halestrap, 1995; Di Lisa *et al.*, 2001; Kim *et al.*, 2006). Thirdly, pharmacological inhibition of mPTP opening using compounds such as CsA and SfA

results in protection against ischaemia-reperfusion injury (Griffiths & Halestrap, 1993; Hausenloy *et al.*, 2002; Hausenloy & Yellon, 2003; Argaud *et al.*, 2005). Finally, CyP-D (an essential component of the mPTP) knockout mice sustain smaller infarcts following ischaemia and reperfusion (Baines *et al.*, 2005).

Further evidence in support of the notion that mPTP opening is the central determinant of myocyte death from ischaemia-reperfusion injury comes from the demonstration that both IPC and IPost inhibit mPTP opening during the first few minutes of reperfusion. The connection between IPC and mPTP inhibition was first made by Yellon's group in 2002. It was demonstrated that IPC and the preconditioning mimetic diazoxide significantly reduced infarct size compared to controls in the isolated rat heart subjected to ischaemia-reperfusion injury. However, this protection was abolished by the presence of the mPTP opener atractyloside. It was further demonstrated that diazoxide increased tolerance of mitochondria to Ca^{2+}-induced mPTP opening (Hausenloy *et al.*, 2002). Subsequently, the same group demonstrated that hypoxic preconditioning increased the tolerance of isolated myocytes to oxidative stress-induced mPTP opening (Hausenloy *et al.*, 2004). Additional evidence comes from the work of Javadov *et al.* who used the entrapment of titriated deoxyglucose technique in isolated perfused rat hearts to directly demonstrate that IPC reduced mPTP opening during the early stages of reperfusion (Javadov *et al.*, 2003). Further, in 2006 multiphoton microscopy was used to directly demonstrate less mPTP opening in IPC hearts, using mitochondrial membrane depolarisation as a surrogate marker of mPTP opening (Matsumoto-Ida *et al.*, 2006).

There is substantial evidence linking IPC induced cardioprotection to reduced mPTP opening at the onset of myocardial reperfusion and similar evidence exist with regards to IPost and mPTP opening. Mitochondria isolated from IPost hearts were found to have increased tolerance to Ca^{2+}-induced mPTP opening, suggesting that the cardioprotective effects of IPost are attributable to mPTP inhibition at reperfusion (Argaud *et al.*, 2005). In support of this it was found that CyP-D deficient mice exhibited increased tolerance to ischaemia-reperfusion injury and that this protection could not be enhanced by IPost. This suggests that IPost elicits its cardioprotective effect through inhibiting mPTP opening (Lim *et al.*, 2007). In this regard, pharmacological postconditioning agents which are administered at the time of myocardial reperfusion and mimic the protective effects of IPost have also been demonstrated to inhibit mPTP opening. An example of this is the volatile anaesthetic isofluorane, which when applied at reperfusion was found to protect against reperfusion injury by preventing mPTP opening (Feng *et al.*, 2005).

Unsurprisingly, the cardioprotection induced by TP has also been demonstrated to be linked to mPTP inhibition. Mitochondria were isolated after 3 minutes of reperfusion from control, IPC and TP hearts and subsequently assayed for mPTP opening by the addition of Ca^{2+}. It was found that IPC mitochondria displayed a 2-fold decrease in the amplitude and rate of swelling, whilst TP mitochondria displayed a 4-fold decrease, compared to control. These investigators suggested that this increased tolerance of TP mitochondria to mPTP opening may underlie the greater protective effects, as assessed by several parameters, of TP over IPC as found in their investigation (Khaliulin *et al.*, 2007).

Extensive evidence demonstrates that numerous cardioprotective interventions inhibit the opening of the mPTP at the onset of reperfusion and this underlies protection against ischaemia-reperfusion injury. The mPTP is therefore implicated to be a common end effector of cardioprotective interventions and it would not be unreasonable to hypothesise that all protective interventions inhibit mPTP opening. The signalling pathways and mechanism involved in IPC, IPost and TP that ultimately inhibit mPTP opening are not well understood, but the signalling proteins that have been demonstrated to be involved suggest a common cardioprotective pathway may exist. These signalling factors which mediate mPTP inhibition at reperfusion will be briefly discussed below.

1.11 Cardioprotective mediators of mPTP inhibition

The reperfusion injury salvage kinase (RISK) pathway constitutes a group of pro-survival protein kinases, which when activated at reperfusion induce cardioprotection. The RISK pathway recruits activation of phosphatidylinositol-3-OH kinase (PI3K)-Akt and p42/p44 extra-cellular signal-regulated kinases (ERK1/2) pro-survival kinase cascades at the time of myocardial reperfusion, which has been demonstrated to be sufficient to induce cardioprotection. Moreover it has also been demonstrated that both IPC and IPost recruit the activation of RISK at reperfusion (Hausenloy *et al.*, 2005a); implicating the RISK pathway as a common cardioprotective signalling pathway which unites both protective interventions. This finding also implies that although IPC is implemented prior to index ischaemia resulting in the transduction of protective signalling pathways; equally important for IPC induced protection are the signalling pathways that are recruited at the time of myocardial reperfusion. A range of

pharmacological agents which mimic the protective effects of IPC and IPost when administered at reperfusion have also been shown to recruit RISK pathway activation. Such drugs are described as 'pharmacological postconditioning' agents as they exert a cardioprotective effect at reperfusion and examples include insulin (Jonassen *et al.*, 2001), transforming growth factor β1 (TGF-β1) (Baxter *et al.*, 2001), urocortin (Brar *et al.*, 2000) and bradykinin (Bell & Yellon, 2003). The activation of RISK has been linked to mPTP inhibition (Bopassa *et al.*, 2006; Juhaszova *et al.*, 2004; Zhao & Vinten-Johansen, 2006), however, the mechanisms through which this inhibition is mediated is currently unclear. A number of mechanisms have been suggested, implicating the involvement of PKC_{ϵ} and mitochondrial ATP-sensitive K^+ channels (mK_{ATP}) (Costa *et al.*, 2006), GSK3β (Juhaszova *et al.*, 2004), inhibition of BAX translocation to mitochondria (Tsuruta *et al.*, 2002) and activation of mitochondrial hexokinase II (Pastorino *et al.*, 2005), although no consensus has been reached (Halestrap, 2010).

The survivor activating factor enhancement (SAFE) pathway is another prosurvival pathway shown to be recruited at the time of myocardial reperfusion. The recruitment of SAFE pathway has been shown to lessen reperfusion injury-induced cardiomyocyte death and protects independently of the activation of the RISK pathway (Lecour, 2009). The prosurvival SAFE pathway involves activation of the cytokine tumour necrosis factor alpha (TNFα) and signal transducer and activator of transcription-3 (STAT-3) and recent evidence suggests that IPost confers its cardioprotective effects via activation of this pathway. Lacerda *et al* demonstrated that TNF or STAT-3 knockout hearts were not amenable to the infarct limiting effects of IPost. Further, this group demonstrated that treatment of hearts with TNFα (TNFα postconditioning) mimicked the protective effects of IPost and that this protection was not abrogated by

the presence of wortmanin (PI-3Kinase/Akt inhibitor) or PD98059 (ERK 1/2 inhibitor), implying a lack of RISK involvement in the protective effects induced by TNFα postconditioning. However, TNFα induced protection was abrogated by the administration of AG490, a STAT-3 inhibitor. Moreover, western blot analysis demonstrated that STAT-3 phosphorylation was increased in both IPost and TNFα postconditioned hearts (Lacerda *et al.*, 2009). Similarly, evidence has also been presented implicating TNFα as a fundamental signalling mediator in IPC. Genetic ablation of the TNFα gene was sufficient to abrogate the protective effects of classical IPC. Moreover, TNFα administration alone was sufficient to mimic protection afforded by IPC (Smith *et al.*, 2002). Therefore, the literature suggests that SAFE pathway activation is recruited by both IPC and IPost, and may work alone or in parallel with the RISK pathway during early reperfusion to induce cardioprotection. Furthermore, as with the RISK pathway, the end effector of the SAFE pathway remains to be identified. However, the mPTP has been implicated as a likely convergent point and end effector of both the SAFE and RISK pathways (Lecour, 2009).

The role of Akt-GSK3β has been investigated with regards to TP and was not found to play a major role, since increased phosphorylation of either protein was not detected. However, the authors mentioned that a role for these kinases in TP could not be ruled out since phosphorylation could be transient (Khaliulin *et al.*, 2007). The involvement of ERK1/2 in TP is yet to be investigated and was therefore examined in the current study to see if RISK pathway activation could underlie the reported effects of TP on mPTP inhibition.

An alternative mechanism by which cardioprotective interventions may inhibit mPTP opening is through attenuation of oxidative stress at the time of myocardial reperfusion, which is a major cause of reperfusion injury (Bolli *et al.*, 1988; Vanden Hoek *et al.*, 2000). Evidence suggests that restoration of pH and excessive oxidative stress at reperfusion may be more important than mitochondrial Ca^{2+} accumulation for promoting mPTP opening (Kim *et al.*, 2006; Ruiz-Meana *et al.*, 2006). In this regard IPC, IPost and TP have all been demonstrated to be associated with reduced oxidative stress at reperfusion. IPC was shown directly to decrease oxidative stress at reperfusion in the isolated rat heart using lucigenin-enhanced chemiluminescence compared to controls (Crestanello *et al.*, 1996). Subsequent studies corroborated this finding in rat myocytes (Narayan *et al.*, 2001) and chick myocytes (Vanden Hoek *et al.*, 2000) with reduced production of oxidative stress observed in hypoxic preconditioned myocytes at reperfusion. In the initial study which described IPost, decreased oxidative stress in the IPost canine heart was reported, measured 1 hour into reperfusion (Zhao *et al.*, 2003). This finding was later confirmed by several studies including one conducted by Sun *et al.* who demonstrated reduced ROS in a cardiomyocyte study (Sun *et al.*, 2005). Similarly, TP has also been shown to reduce oxidative stress at reperfusion as assessed by protein carbonylation (Khaliulin *et al.*, 2007). Thus, attenuation of oxidative stress at reperfusion may represent a universal mechanism by which hearts are protected against reperfusion injury. Reduced oxidative stress would decrease the chance of mPTP opening, resulting in enhanced bioenergetic recovery and less myocardial death. mPTP opening is also inhibited by acidic pH and evidence exists suggesting that both IPC (Hausenloy *et al.*, 2007) and IPost (Fujita *et al.*, 2007) delay the restoration of pH at reperfusion. The mechanism through which this

transient acidosis is achieved is unclear but it is proposed that this may be crucial to cardioprotection by permitting the activation of survival kinases and inhibiting mPTP opening (Fujita *et al.*, 2007; Cohen *et al.*, 2007). The effects of TP on restoration of pH at reperfusion have not been investigated and so it is currently unknown whether this is a mechanism of mPTP inhibition that operates in TP hearts.

1.12 Overview of the project

In contrast to IPC and IPost, TP is a relatively novel cardioprotective intervention and as such the signalling pathways and mechanisms involved in TP-induced cardioprotection are not well characterised. At the time of writing this Thesis only three papers have been published with regards to TP, all of which were conducted in the Langendorff perfused isolated rat heart (Khaliulin *et al.*, 2007; Khaliulin *et al.*, 2010; Khaliulin *et al.*, 2011). The aims of this project were to investigate the effects of TP on isolated rat ventricular myocytes, followed by an examination of the signalling pathways and molecular effects induced by TP. Working with isolated myocytes was particularly useful as it allowed the use of fluorescent dyes to measure cytosolic Ca^{2+} levels and mPTP opening. Further, it allowed the determination of kinase activation immediately following administration of a cardioprotective intervention.

Initial investigations focused on characterising the protective effects of TP on isolated ventricular myocytes by assessing recovery of contractile function and Ca^{2+} homeostasis following metabolic inhibition and reenergisation (simulated ischaemia-reperfusion injury).

Having observed a protective effect of TP on isolated myocytes I next investigated the effects of TP on pathological irreversible mPTP opening – a critical mediator of lethal

reperfusion injury. This was investigated using two different cardiomyocyte models of reperfusion injury and represents the first time the effects of TP on mPTP opening has been investigated in the intact myocyte. All previous work in this regard has been conducted on isolated mitochondria. In addition to investigating the effects of TP on irreversible mPTP opening, the effects of TP was also investigated on transient mPTP flickering, for which evidence exists implicating it as a mediator of IPC induced protection (Hausenloy *et al.*, 2010).

The final set of experiments conducted in this project focused on the signalling pathways involved in mediating TP induced cardioprotection. ROS production has previously been demonstrated to be crucial for TP and in this Thesis I characterise the timing of ROS involvement during the TP stimulus using a superoxide biosensor. Further, protein kinase inhibitors and western blot analysis was used to determine the involvement of the RISK pathway protein, ERK1/2, in TP induced cardioprotection.

2 Materials and methods

2.1 Isolation of adult rat ventricular myocytes

Adult male Wistar rats (200-350g) were sacrificed by cervical dislocation in accordance with the UK Animals (Scientific Procedures) Act, 1986. The heart was rapidly excised and placed in nominally Ca^{2+}- free Tyrode solution titrated to pH 7.4 with NaOH, containing; (in mM) 135 NaCl, 6 KCl, 0.33 NaH_2PO_4, 5 Na pyruvate, 10 glucose, 1 $MgCl_2$, 10 HEPES. Within 1 minute the heart was cannulated at the aorta and retrogradely perfused using a Langendorff apparatus for 6 minutes at a constant flow rate of 10 ml/min with Ca^{2+}- free Tyrode at 37^{o}C, vigorously bubbled with 95% 0_2 / 5% $C0_2$. The heart was then perfused with Ca^{2+}- free Tyrode solution containing collagenase type I (0.4 units/ml), protease type XIV (approximately 2.7 units/ml) and 0.05% bovine serum albumin (BSA) for 7 to 10 minutes, to digest the heart. This was followed by perfusion for 3 minutes with Tyrode solution (containing 2mM $CaCl_2$) to remove the enzymes. The atria were removed and isolated ventricular myocytes were obtained by cutting the ventricular mass in two and gently agitating in Tyrode solution at 37^{o}C, in a shaking water bath. The Tyrode solution (containing isolated myocytes) was decanted and collected in a flask approximately every 3 minutes and fresh Tyrode solution was added to the remaining ventricular tissue. This process was repeated until the ventricular tissue had almost completely disintegrated. The Tyrode solutions containing isolated myocytes were sieved (0.2mm opening size) to remove any undigested tissue and placed in a test tube where myocytes were left to settle for 10 minutes at room temperature (18 - 23^{o}C). The myocytes were washed twice by gravity settling the myocytes in a test tube, removing the Tyrode solution and re-suspending

myocytes in fresh Tyrode solution. This isolation procedure is well established (Rodrigo *et al.*, 2002) and typically yielded 70 – 90% viable rod shaped myocytes. Following isolation myocytes were stored and cultured in media 199 supplemented with ITS+3 (insulin, transferrin, sodium selenite, linoleic acid, oleic acid and bovine serum albumin)(2mg/ml), carnitine (2mM), creatine (5mM), taurine (5mM), T3 (1.5pM), penicillin and streptomycin (100 units) at 37°C and 5%CO_2 (Bell & McDermott, 2000; Xu *et al.*, 2006; Ellingsen *et al.*, 1993).

2.2 Metabolic inhibition and reenergisation

In this study a metabolic inhibition (MI) solution was used to simulate ischaemia. MI solution was composed of substrate-free Tyrode solution (Tyrode solution without glucose and pyruvate), cyanide (2mM) and iodoacetic acid (1mM). Substrate-free Tyrode solution was titrated to pH 7.4 using NaOH and contained; (in mM) 140 NaCl, 6 KCl, 0.33 NaH_2PO_4, 10 sucrose, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES. Cyanide inhibits electron transfer at complex IV in the mitochondria and iodoacetic acid blocks glycolysis by alkylation of the active site of glyceraldehyde-3-phosphate dehydrogenase. These factors combined with the lack of metabolic substrates, glucose and pyruvate, prevents ATP generation and generates oxidative stress in myocytes to simulate ischaemia (Webster *et al.*, 1994). Isolated myocytes were superfused with MI solution for 7 minutes followed by 10 minutes of reenergisation by superfusion with Tyrode solution to simulate reperfusion injury. Perfusion of myocytes with Tyrode solution following MI washes away the metabolic poisons and re-introduces myocytes to metabolic substrates (glucose and pyruvate). This allows mitochondrial respiration to restart resulting in the production of ATP.

2.3 Temperature preconditioning of isolated ventricular myocytes

For the purposes of temperature preconditioning (TP), 1ml of isolated ventricular myocytes in Tyrode solution (approximately 0.4million cells) was placed in an Eppendorf, in a water bath to reach a final temperature of 16°C for a 2 minute period. This was followed by incubation in a water bath at 37^{o}C for 3 minutes; for two cycles. The final temperature in the Eppendorf was measured using a thermistor (TH-30; Dagan Corporation). The TP protocol is illustrated in figure 2.1. Myocytes were temperature preconditioned in this manner for all experiments with the exception of work involving characterisation of the role of ROS in TP, using the mt-cpYFP superoxide biosensor. In the case of the latter, TP was conducted within the superfusion chamber, mounted onto the microscope stage, by superfusing myocytes with Tyrode solution cooled to 16^{o}C for 2 minutes and 37^{o}C for 3 minutes; for two cycles.

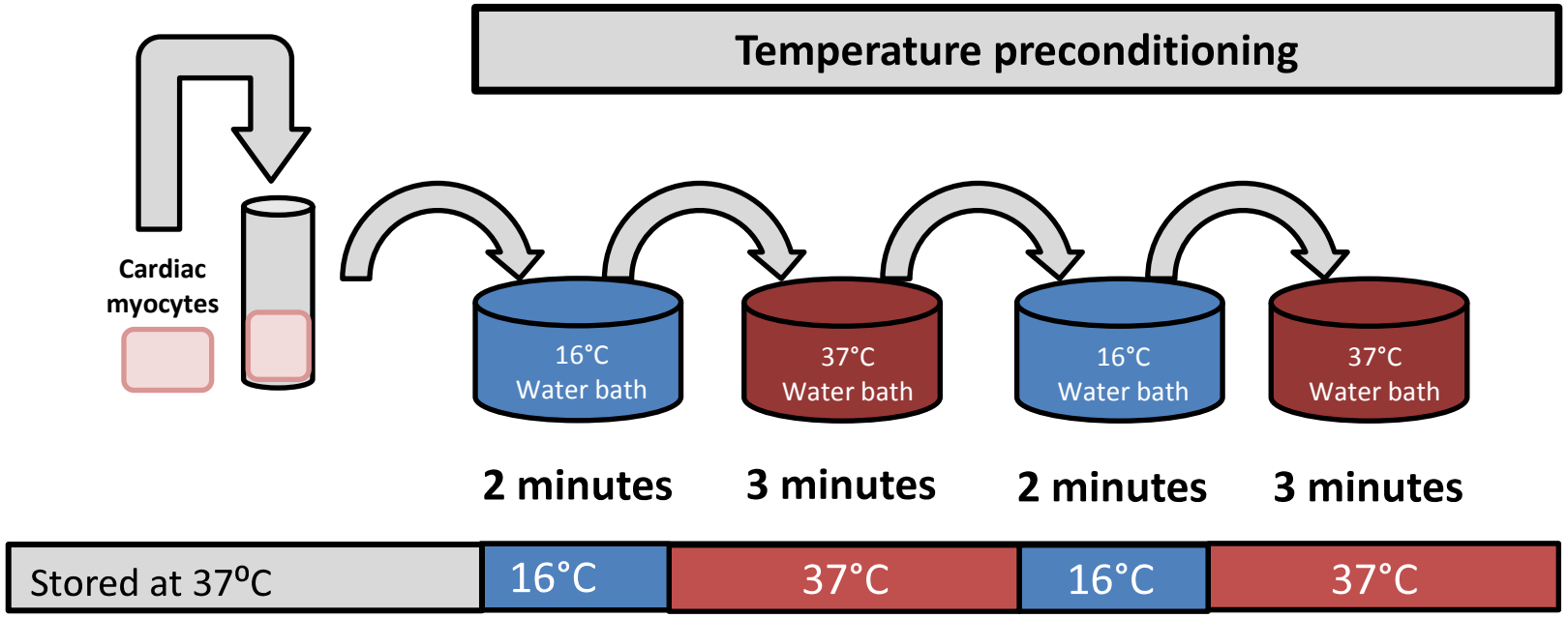

Figure 2.1: protocol used for temperature preconditioning of isolated ventricular myocytes.

Following isolation isolated myocytes were stored in an incubator at 37°C and 5%CO_2. 1ml of isolated ventricular myocytes in Tyrode solution was transferred between water baths of 16°C and 37°C to achieve temperature preconditioning of isolated myocytes. In initial characterisation experiments, hypothermic temperatures of 26°C and 21°C were used in place of 16°C to realise the optimal hypothermic temperature for temperature preconditioning-induced cardioprotection.

2.3 Measurement of myocytes contractile recovery

Isolated ventricular myocytes were place in 300µl Perspex chamber which was mounted onto the stage of a Nikon inverted light microscope. The myocytes were left to settle for 10 minutes before superfusion of myocytes within the chamber was started at a rate of 4.5ml/min, at 33^{o}C ±2^{o}C. The temperature and rate of perfusion were kept constant for all experiments. The chamber was set up for electrical field stimulation (Physiological Stimulator, Farnell) to be delivered to the chamber via two parallel platinum electrodes (0.25mm) fixed on opposite side of the diagonal shaped chamber. The rate of stimulation was set at 1Hz, with a pulse width of 5ms at 150% of threshold voltage (approximately 15 – 30V) and myocytes were paced at this rate throughout each experiment. A charge coupled device (CCD) camera was attached to an eyepiece of the microscope and was linked to a black and white monitor. The monitor in turn was linked to a DVD recorder. This set up allowed myocytes within the chamber to be viewed and recorded throughout the duration of an experiment. Prior to MI, myocytes were superfused with substrate-free Tyrode solution and viable rod shaped myocytes that were synchronously contracting in response to electric field stimulation were identified and marked on the monitor. The contractile response of these myocytes was then tracked throughout 7 minutes of superfusion with MI and 10 minutes of reenergisation, achieved by superfusion with Tyrode solution. Those myocytes that were contractile upon 10 minutes of reenergisation were considered to have recovered their contractile function. Myocytes that did not respond to electrical field stimulation or asynchronously contracted were deemed not to have recovered contractile function. For the purposes of TP, myocytes were temperature preconditioned prior to being placed in the chamber for settling. For DNP

preconditioning myocytes were superfused within the chamber for 5 minutes in substrate-free Tyrode solution containing DNP (50μM) followed by 5 minutes in substrate-free Tyrode solution immediately prior to MI. For CsA treatment, myocytes were settled in the chamber in Tyrode solution containing CsA (4μM). Furthermore, CsA (4μM) was added to all the superfusing solutions throughout the experiment. Figure 2.2 illustrates the MI and reenergisation protocol used to simulate ischaemia and reperfusion and indicates when respective conditioning protocols were administered.

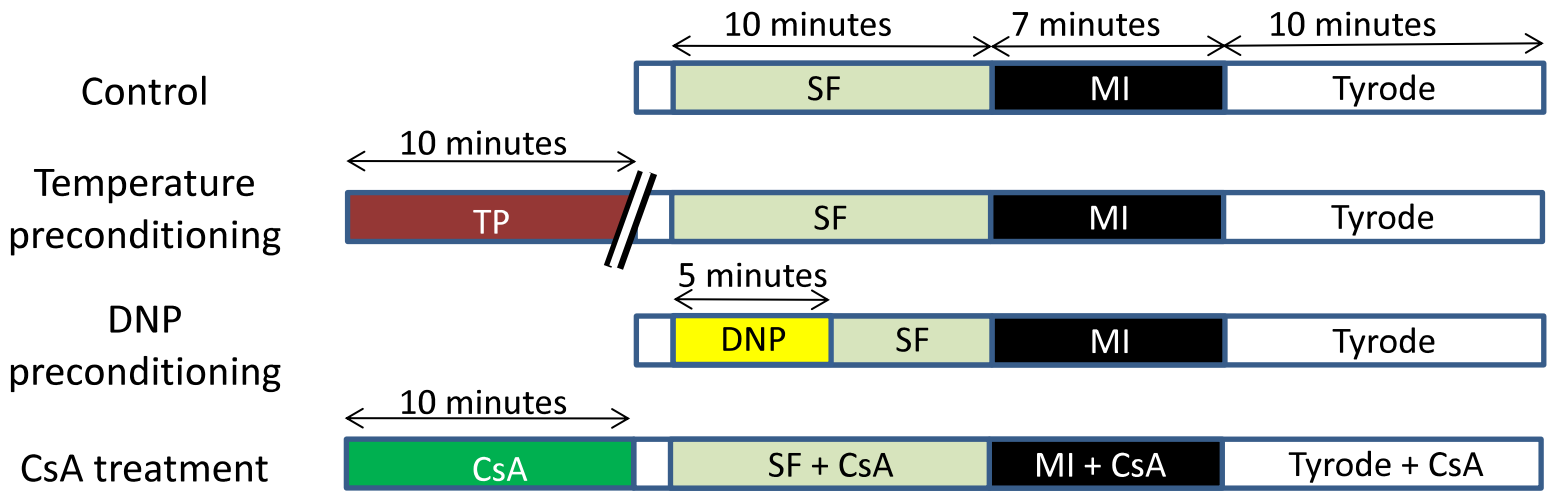

Figure 2.2: protocol for MI reenergisation experiments used to asses the effects of simulated ischaemia-reperfusion injury on contractile recovery.

Control experiments; ventricular myocytes were exposed to 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation with Tyrode solution. Contractile recovery was assessed at 10 minutes of re-energisation. Temperature preconditioning (TP) was conducted prior to experiment and a 10 minute gap existed between the end of TP and the start of experiment. In positive control experiments myocytes were preconditioned with DNP (50µM) or treated with CsA (4µM).

2.4 Fluorescence imaging

Isolated ventricular myocytes were placed in the chamber of a Nikon (Eclipse TE200) inverted epifluorescence microscope. Myocytes were superfused with Tyrode solution at a rate of 4.5ml/min at 34^{o}C (Dagan). For fluorescence imaging myocytes were viewed with an inverted microscope fitted with a 20 X, 40 X oil immersion or 60 X oil immersion objective. Myocytes were excited at the appropriate wavelength using a monochromator (PTI deltaRAM) and emission was measured using a CCD camera (Photometrics Cascade: 512B; Roper Scientific, Inc.). EasyRatioPro software was used for all analysis (PTI).

2.4.1 Measurements of intracellular calcium following metabolic inhibition and reenergisation

For calcium imaging isolated ventricular myocytes in Tyrode solution were loaded with the acetoxymethyl ester (AM) form of the ratiometric calcium sensitive dye Fura-2 (2.5µM) (Invitrogen) for 20 minutes at room temperature. Following loading, myocytes were washed in Tyrode solution to remove any remaining extracellular dye. The fura-2 loaded myocytes were placed in a superfusion chamber mounted onto the stage of an inverted microscope and left to settle for 10 minutes. Thereafter superfusion of Tyrode solution at a rate of 4.5ml/min and electrical field stimulation at 1 Hz (research stimulator, Harvard apparatus) was commenced and persisted for the duration of the experiment. All rod shaped myocytes that responded to electrical field stimulation at the start of the experiment were included in the study. Myocytes were imaged with a 20 X fluorescence objective and were alternately excited at 340nm and 380nm. Emission was recorded at >520nm using a CCD camera. The camera allowed

real time changes in myocytes fluorescence to be tracked and this was plotted as traces of fluorescence intensity against time. This produced line graphs that represented changes in fluorescence intensity from regions of interest (ROI) placed around myocytes for the duration of an experiment. Defining ROI was a function of the PTI EasyRatioPro software and ROI were placed around all rod shaped myocytes that responded to electrical field stimulation at the start of the experiment. Data was sampled at either 0.1Hz or 1Hz. Sampling at 0.1Hz was not quick enough to measure individual calcium transients and therefore this resulted in an aliasing effect, produced by the slow sampling of rapid transients. This manifested as slowly oscillating calcium levels that can be used as an indicator of calcium homeostasis and contractile function (Rodrigo & Samani, 2008). A calcium calibration was conducted to convert arbitrary fluorescence units into estimated cellular calcium (nM) concentration. This was achieved by exposing fura-2 loaded and ionomycin (10µM) treated myocytes to a high calcium solution (10mM) followed by exposure to a calcium-free solution (EGTA, 15mM). The Grynkiewicz equation was used to estimate free calcium:

$$[Ca^{2+}]_{free} = K_d{}^{EGTA} \times \frac{[R - R_{min}]}{[R_{max} - R]} \times \frac{F^{380}max}{F^{380}min}$$

R is the ratio of 510 nm emission intensity with excitation at 340 nm, to 510 nm emission intensity with excitation at 380 nm; R_{min} is the ratio at zero free Ca^{2+}; R_{max} is the ratio at saturating Ca^{2+} (i.e., 10mM); $F^{380}max$ is the fluorescence intensity with excitation at 380 nm, for zero free Ca^{2+}; and $F^{380}min$ is the fluorescence intensity at

saturating free Ca^{2+} (Grynkiewicz et al., 1985). Plotting the log of $[Ca^{2+}]_{free}$ (x-axis) versus the log of $[(R - R_{min})/(R_{max} - R)] \times (F^{380}max/F^{380}min)\}$ yields a straight line, the x-intercept of which is the log of K_d.

2.4.2 Measurements and model of photo-damage induced mPTP opening

HeLa cells in extracellular solution (135mM NaCl, 6mM KCl, 10mM HEPES, 1mM $MgCl_2$, 2mM $CaCl_2$ pH 7.4) or isolated ventricular myocytes in Tyrode solution were loaded with the fluorescent dye tetramethyl rhodamine methyl ester (TMRM) (5µM) at 37^{o}C for 15 minutes, followed by a wash in Tyrode solution to remove excess extracellular dye. The cells were placed in a superfusion chamber mounted onto the stage of an inverted microscope and superfusion of extracellular solution for HeLa cells or Tyrode solution for myocytes persisted for the duration of the experiment. TMRM loaded cells were imaged using a 60 X oil immersion objective, typically yielding 20 HeLa cells or 2 - 8 myocytes in a field of view which were continuously excited at 546nm and the emission measured using a long pass filter at >560nm at a rate of 0.5 Hz (25ms exposure) . ROI were placed around all viable cells in the field of view and fluorescence changes were plotted as line graphs over time.

TMRM is a lipophilic cation which selectively accumulates in mitochondria due to the negative mitochondrial membrane potential. The high concentration of TMRM within the mitochondria causes autoquenching of the fluorescent signal and therefore prior to illumination TMRM loaded myocytes exhibit relatively low fluorescence. When excited at 546nm, TMRM causes the generation of free radicals from within the mitochondria which induce mPTP formation (Hausenloy *et al.*, 2004). Consequently,

mitochondrial depolarisation occurs and TMRM moves out of the mitochondria into the cytosol which is reflected by an increase in the fluorescent signal. Loss of mitochondrial membrane potential can be visualised in real time using a fluorescence microscope and is a dependable reflection of mPTP formation. In this model the role of TMRM is two-fold: firstly to produce TMRM-induced oxidative stress, and secondly to serve as a marker by which its localisation communicates the state of the mPTP.

Isolated ventricular myocytes were loaded with TMRM (5μM) prior to viewing and illumination. Under control conditions (no pre-treatment of myocytes), myocytes were continuously illuminated until mPTP opening had occurred (indicated by redistribution of TMRM into the cytosol). The time taken to induce mPTP formation was recorded and this was compared to the time taken to induce mPTP formation in ventricular myocytes that were preconditioned or pharmacologically treated.

2.4.3 Measurements and protocol for xanthine and xanthine oxidase-induced mPTP opening

The photodamage-induced mPTP opening assay described above used TMRM both as a reporter of mitochondrial membrane potential and as an inducer of mPTP opening by free radical formation. The results from this assay were confirmed by a second assay in which TMRM was used solely to report changes in mitochondrial membrane potential.

Isolated ventricular myocytes in Tyrode solution were loaded with TMRM (50nM) at 37^{o}C for 15 minutes, followed by a wash in Tyrode solution to remove excess extracellular dye. TMRM loaded Myocytes were placed in a superfusion chamber mounted onto the stage of a Nikon (Eclipse TE200) inverted microscope and left to

settle for 10 minutes. Thereafter Xanthine (2mM) and xanthine oxidase (20mU) in Tyrode solution (pH 7.4) was superfused onto the myocytes to provide ROS for induction of mPTP opening. Depolarisation of the mitochondrial membrane potential was reported by decrease in TMRM fluorescence as the dye diffused from the mitochondria and the cell. TMRM loaded myocytes were imaged using a 60 X oil immersion objective, typically yielding 2 -8 myocytes in a field of view which were continually excited at 546nm at a rate of 0.2 Hz and the emission at >560nm was measured. In this assay TMRM-induced photodamage was minimised by using a low concentration of TMRM (50nM) and secondly by using a low sampling rate (0.2 Hz) to decrease TMRM excitation. ROI were placed around rod shaped myocytes in the field of view and fluorescence changes were plotted as line graphs over time.

2.4.4 Measurements of non-pathological, transient mPTP flickering – calcein cobalt quench assay

Image-IT ™ LIVE Mitochondrial Transition Pore Assay Kit (Invitrogen) was used for the detection of mPTP flickering. Isolated ventricular myocytes in Tyrode solution were loaded with calcein-AM (1µM) and cobalt chloride (1mM) for 15 minutes at room temperature, followed by a wash in Tyrode solution to remove excess extracellular dye. Myocytes were placed in a chamber mounted onto the stage of a Nikon (Eclipse TE200) inverted microscope and left to settle for 2 minutes at approximately 34^{o}C. Calcein-cobalt chloride loaded myocytes were imaged using a 60 X oil immersion objective, typically yielding 2 -8 myocytes in a field of view which were excited at 488nm and the calcein fluorescence emission collected using a long pass filter at >505nm. During the course of an experiment three images of calcein fluorescence

were measured at times 0 minutes, 10 minutes and 30 minutes from rod shaped myocytes. When mPTP flickering occurs calcein is redistributed to the cytoplasm and quenched by cobalt which is measured as a decrease in fluorescence (Hausenloy *et al.*, 2004; Petronilli *et al.*, 1999). ROI were placed around all viable myocytes to monitor fluorescence changes. These were then plotted as line graphs over time as a function of the PTI EasyRatioPro software and fluorescence changes at time 30 minutes were expressed as a percentage of the initial fluorescence value at time 0 minutes.

2.4.5 Measurements of mitochondrial ROS in 48 hour infected mt-cpYFP expressing myocytes

For measuring mitochondrial ROS formation during TP, ventricular myocytes were infected with adenovirus encoding mt-cpYFP for 48 hours (Wang *et al.*, 2008). Ventricular myocytes were cultured in media 199 supplemented with ITS+3 (2mg/ml), carnitine (2mM), creatine (5mM), taurine (5mM), T3 (1.5pM), penicillin and streptomycin (100 units) at 37°C and 5%CO_2. After 48 hours approximately 70% of myocytes were infected. For experiments, myocytes were gravity settled and re-suspended in Tyrode solution (warmed to 37^{o}C). Myocytes were placed in a superfusion chamber mounted onto the stage of a Nikon (Eclipse TE200) inverted microscope and left to settle for 10 minutes at approximately 34^{o}C. TP of myocytes within the chamber was achieved by temporarily turning off the temperature controller (set to 37^{o}) and superfusing myocytes with Tyrode solution that was cooled to 16^{o}C. For measurements of mt-cpYFP fluorescence, myocytes were excited at 488nm and the mt-cpYFP fluorescence emission was collected using a long pass filter at >515nm. mt-cpYFP infected myocytes were imaged using a 60 X oil immersion

objective and images were collected at a rate of 0.2Hz (100ms exposure time). ROI were placed around viable rod shaped myocytes in the field of view and fluorescence changes were plotted as line graphs over time.

2.4.6 Measurements of mitochondrially loaded carboxy snarf to detect pH changes

To measure changes in mitochondrial pH, 48 hour cultured (uninfected) myocytes were loaded with carboxy snarf-AM (10µM) for 10 minutes at 37^{o}C, followed by incubation at room temperature for a further 4 hours. This dye loading protocol favours selective loading of carboxy snarf into mitochondria (Takahashi *et al.*, 2001) and allowed investigation of whether mitochondrial pH changes during the temperature preconditioning protocol. Carboxy snarf loaded myocytes were imaged using a 60 X oil immersion objective and were excited at 488nm. Emission was collected using a long pass filter at >560nm and images were collected at a rate of 0.2Hz. ROI were placed around viable rod shaped myocytes in the field of view and fluorescence changes were plotted as line graphs over time.

2.5 Confocal microscopy - Characterisation of mt-cpYFP in 48 hour infected CHO cells and myocytes

Confocal microscopy was used for characterising the mt-cpYFP superoxide biosensor in both Chinese hamster ovary (CHO) cells and isolated myocytes. mt-cpYFP infected (48 hours) cells were placed in a superfusion chamber and mounted on the stage of a Olympus IX70 confocal microscope where they were imaged using a 60 X oil immersion objective and images were collected at a rate of 0.2Hz (100ms exposure time). For measurements of mt-cpYFP fluorescence, infected CHO cells and myocytes were

illuminated using the 488nm emission line of an Argon laser (Melles Griot) and fluorescence was measured by use of a 515nm long-pass filter. For co-localisation analysis, 48 hour infected myocytes were loaded with MitoTracker deep red (500nM) (Invitrogen) for 20 minutes at room temperature and then re-suspended in Tyrode solution to wash away excess dye. For measurement of MitoTracker deep red fluorescence, myocytes were illuminated using the 633nm emission line of a red helium-neon laser (Melles Griot) and fluorescence was measured by use of a 660nm long pass filter.

2.6 Transfection, amplification and harvesting of superoxide biosensor adenovirus

Mitochondrially-targeted cpYFP (provided by Dr Heping Cheng) was sub-cloned into pENTR-1A (Invitrogen) and recombined with pAd-CMV-V5-DEST (Invitrogen) to produce a recombinant adenoviral genome. Adenovirus particles were produced by transfection of human embryonic kidney (HEK)293T cells with Pac-1 digested DNA using Lipofectamie 2000 (Invitrogen). After 72 hours in culture at 37°C and 5% CO_2 the formation of viral plaques indicated successful transfection. Amplification of the virus was achieved by adding infected HEK293T cells to flasks (T75cm^2) of non-infected HEK293T cells that were approximately 50-60% confluent. The cells were left in culture for approximately 2-3 days, as determined by the appearance of viral plaques, and then re-harvested for further amplification. The amplification process was repeated for several cycles ultimately generating 16 flasks (T75cm^2) containing virally infected HEK293T cells from which the virus was harvested and then separated into 100µl aliquots and stored at -80^{o}C.

2.7 Tissue culture methods

Three stable cell lines were used in this study; HeLa cells, HEK293T cells and CHO cells. HeLa cells were maintained in Dulbecco's minimum essential medium (DMEM) containing GlutaMAX-1, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HEK293T cells were maintained in DMEM containing GlutaMAX-1 with sodium pyruvate and 4500 mg L-1 glucose, 10% FBS and 1% penicillin/streptomycin. CHO cells were maintained in minimum essential medium (MEM) α containing ribonucleosides, deoxyribonuleosides and L-glutamine, 10% FBS, 1% fungizone and 1% penicillin/streptomycin.

Once confluent, flasks containing cells was rinsed with cell dissociation buffer (PBS based) and harvested with 0.05% trypsin. The trypsin was removed and cells were resuspended into fresh medium. Cells were incubated at 37°C and 5%CO_2.

2.8 Preparation of protein samples for analysis of phosphorylated ERK1/2 (p44/p42 MAPK)

Isolated ventricular myocytes (approximately 0.4 million in 1ml Tyrode solution) were collected by centrifugation at 14,000 g for 1 minute. The supernatant was removed and myocytes were resuspended in 200µl of ice cold lysis buffer (20 mM Tris-base, pH 7.4, 1% (v/v) Triton® X-100, 10% (v/v) glycerol, 137 mM NaCl, 2 mM EDTA and a 1:100 dilution of a protease inhibitor cocktail (Sigma)). The myocytes were briefly vortexed and placed in an orbital shaker at 4^{o}C for 20 minutes followed by centrifugation at 28,000 g for 5 minutes. Aliquots of the supernatant were retained and stored at -80°C until use.

2.9 Western blot analysis

To measure phospho-ERK 1/2 levels, a sample of cardiac myocytes were temperature preconditioned in the presence or absence of ERK1/2 inhibitors, either PD98059 (10µM) or U0126 (0.5µM), or the ROS scavenger MGP (300µM). The myocytes were then lysed and the collected protein (as described above) along with pre-stained all blue Precision Plus protein standards (BioRad) was subjected to SDS-PAGE mini gels (12.5% acrylamide gels) (BioRad mini Protean III gel system) at a constant voltage of 200 V for 40 minutes. The Bradford assay was used to determine the concentration of protein in cell lysates and 30µg of protein from each treatment group was loaded per well. The cell lysates were mixed with an equal volume of SDS-PAGE sample buffer (312.5 mM Tris-base, pH 6.8, 10% w/v SDS, 50% v/v glycerol, 250 mM DTT and 0.01% bromophenol blue) and heated to 100^{o}C for 5 minutes prior to SDS-PAGE. Running buffer contained (in mM) 25 Tris, 192 glycine, 0.1% SDS. Gels and nitrocellulose membranes were equilibrated in transfer buffer containing (in mM) 25 Tris, 192 glycine, 0.01% SDS, and 10% methanol for 20 minutes prior to electrophoretic transfer using a wet transfer cell (BioRad mini Protean II gel transblot system). Proteins were transferred to nitrocellulose membrane at 100V for 60 minutes. Following transfer of proteins to nitrocellulose membranes, non-specific binding sites were blocked by incubation of the membrane in 5% BSA in TBS-T for 1 hour at room temperature. TBS-T contained (in mM) 50 Tris, 150 NaCl and 0.1% Tween-20. The membranes were incubated with anti-phospho-Thr202/Tyr204-ERK antibody (Cell Signalling Technology) overnight while shaking at 4^{o}C. Primary antibodies were diluted in TBS-T containing 5% BSA. Membranes were washed four times for 5 minutes each in TBS-T. The membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit

secondary antibody in TBS-T containing 5% BSA for 1 hour at room temperature with agitation, then washed six times for 5 minutes each. Following this, blots were developed using ECL and exposure to HyperFilm. Relative densitometry was determined using Felix, a computerised software analysis package. To strip blots, membranes were incubated with stripping buffer containing 0.1M glycine pH 2.8 with 1% glycine for 45 minutes at room temperature. Membranes were then blocked with 5% non-fat dried milk and re-probed with anti-total ERK (p44/p42) antibody. Total ERK was used as a loading control for each well and phospho-ERK levels were normalised to the respective total ERK levels in each well. This was done to overcome any differences in protein loading. Both ERK1/2 antibodies were obtained from Cell Signalling Technology (Hitchin, UK) and were used in accordance with the manufacturer's instructions.

2.10 Materials

Fura-2-AM, Carboxy snarf-AM, MitoTracker deep red-AM and Image-IT ™ LIVE Mitochondrial Transition Pore Assay and TMRM were obtained from Molecular Probes (Invitrogen). Media 199, DMEM, penicillin and streptomycin were from Gibco (Invitrogen). The ERK1/2 inhibitors U0126 and PD98059 were from Tocris Bioscience. Anti-phospho (Thr202/Tyr204) ERK1/2 and anti-total ERK1/2 antibodies (Cell Signalling Technology) were obtained from New England Biolabs (NEB UK). Goat anti-rabbit IgG horseradish peroxidase conjugated antibody was from AB Frontier. Xanthine, xanthine oxidase, ITS+3, carnitine, creatine, taurine, T3, DNP, CsA and nitrocellulose membranes were all obtained from Sigma-Aldrich. All other laboratory compounds used in this study were obtained from Sigma-Aldrich or Fisher Scientific.

2.11 Statistics

Values are expressed as mean ± SEM. Data was analysed by an unpaired student's t-test, unless otherwise stated, in which case a 1-way analysis of variance (1-way ANOVA) followed by Turkey's post test for multiple comparisons was conducted. GraphPad Prism 5 was used for statistical analysis and $P < 0.05$ was considered significant. Where data is expressed as 'normalised to control', this indicates that all data values were divided by the mean control value for the dataset. Data are presented as mean ± S.E.M. Data concerning isolated myocytes is presented as n = cells, experiments, hearts. Data concerning cell lines is presented from a minimum of 40 cells over 6 separate experiments.

3 Temperature preconditioning of isolated ventricular myocytes

3.1 Introduction

The benefits of hypothermia to the myocardium have been known for several decades and its uses today vary from organ preservation to cold cardioplegia and localized cooling used in the clinical setting to preserve heart function (Hale & Kloner, 2011). It was first recognized in the late 1970s that hypothermia could be used to limit the damage caused by myocardial infarction. Abendschein *et al.* demonstrated in dogs that the ischaemic myocardium could be protected by whole-body hypothermia (Abendschein *et al.*, 1978). This pioneering study highlighted the potential of hypothermia, applied during ischaemia, to limit infarct size caused by ischaemia without reperfusion. Subsequently, Haendchen *et al.* demonstrated that the benefits of hypothermia on limiting infarct size also extended to reperfused dog myocardium's (Haendchen *et al.*, 1983). Since then the cardioprotective effects of hypothermia have been shown to be present in several animal species and hypothermia is now proposed to be one of the most potent cardioprotective strategies available (Darbera *et al.*, 2011), (Dixon *et al.*, 2002), (Miki *et al.*, 1998).

In 2007, Khaliulin *et al.* described the use of hypothermia to achieve cardioprotection in a novel manner using a protocol they termed 'temperature preconditioning' (TP). This involves exposing the heart to brief periods of hypothermic perfusion (26^{o}C) interspersed by periods of normothermic perfusion (37^{o}C) for three cycles prior to an index ischaemic episode. Khaliulin *et al.* compared the cardioprotective effects of TP against the gold standard of cardioprotection achieved by ischaemic preconditioning

(IPC) and claimed the protective effects of TP were greater. Following 25 minutes of global ischaemia and 60 minutes of reperfusion in the Langendorff perfused isolated rat heart, TP was found to significantly improve haemodynamic recover assessed at the end of reperfusion. TP resulted in a significantly greater recovery of left ventricular developed pressure (LVDP) and RPP (a work index calculated as the product of LVDP and heart rate) than both IPC and control hearts. Further TP was found to decrease the incidence of arrhythmias which was assessed during the first 20 minutes of reperfusion. IPC was found to decrease the number of episodes of ventricular premature beats (VPB), ventricular tachycardia (VT - defined as a run of four or more VPB) and ventricular fibrillation (VF – no discrete QRS deflections) compared to controls. In contrast, the anti-arrhythmic effects of TP were even greater than IPC, totally abolishing the occurrence of VF and reducing the incidence of VT to only 30% of hearts. Consequently TP achieved a significantly lower arrhythmia score (quantified using the scoring system of Curtis and Walker 1988 (Curtis & Walker, 1988)) than both IPC and control hearts. IPC and TP were also found to significantly reduce necrotic damage during reperfusion as assessed by lactate dehydrogenase (LDH) release. However, once again TP was found to be more protective than IPC as significantly less LDH was collected in the perfusate of TP hearts than IPC or control hearts when measured at 10 minutes of reperfusion. The protective signalling pathways of TP were found to involve a modest increase in reactive oxygen species (ROS), protein kinase Cε (PKC_{ϵ}) translocation to the particulate fraction and increased phosphorylation of AMP-activated protein kinase (AMPK) observed after 5 minutes of index ischaemia (Khaliulin *et al.*, 2007). A subsequent study conducted by the same group also demonstrated the involvement of cyclic AMP (cAMP)-dependent protein kinase A (PKA) activation, which

was shown to be upstream of PKC activation (Khaliulin *et al.*, 2010). More recently this group demonstrated that the protective effects of TP are not confined to using a hypothermic temperature of 26^{o}C. TP-induced protection was also observed when 32^{o}C and 17^{o}C were used as the hypothermic temperature in place of 26^{o}C. However it was found that using 7^{o}C as the hypothermic temperature to precondition the myocardium was associated with adverse effects, exacerbating the damage caused by ischaemia-reperfusion injury (Khaliulin *et al.*, 2011).

To date, all studies concerning TP have been conducted on the isolated perfused rat heart. In the present study I have investigated the effects of TP on isolated ventricular myocytes. Several advantages stem from isolated myocyte studies, two of which are the measurement of contractile function and intracellular Ca^{2+} during ischaemia and reperfusion which are parameters that cannot easily be determined in the whole heart. Furthermore, these are parameters which are yet to be investigated with regards to TP and were therefore investigated in the present study.

The results in this chapter describe the effects of exposing isolated ventricular myocytes to metabolic inhibition (MI) and reenergisation (simulated ischaemia and reperfusion) while assessing the consequences on contractile function and intracellular Ca^{2+}. These are end points used to assess cellular injury in isolated myocyte studies (Diaz & Wilson, 2006) and previous studies have demonstrated that these are adversely affected by MI and reenergisation (Rodrigo *et al.*, 2002; Rodrigo & Samani, 2008; Rodrigo & Standen, 2005). Rodrigo *et al.* have demonstrated that exposing isolated ventricular myocytes to MI and reenergisation causes myocyte hypercontracture and impairs contractile function when assessed following 10 minutes

of reenergisation. The mechanical damage induced by hypercontracture is proposed to occur upon reenergisation as a result of rapid resumption of oxidative phosphorylation which produces ATP in an intracellular background of elevated Ca^{2+}. Myocyte hypercontracture is shown to be dependent on oxidative ATP produced at reperfusion but not Ca^{2+} influx (Rodrigo & Standen, 2005). Ca^{2+} influx at the time of reperfusion is linked to a loss of Ca^{2+} homeostasis, resulting in a significant rise of intracellular Ca^{2+} concentration. Loss of Ca^{2+} homeostasis can be blocked by the mitochondrial permeability transition pore (mPTP) inhibitor cyclosporine A (CsA), suggesting that loss of Ca^{2+} homeostasis may be a consequence of mPTP opening in response to Ca^{2+} influx (Rodrigo & Standen, 2005). It was found that preconditioning myocytes with dinitrophenol (DNP) significantly increased the number of myocytes that recovered contractile function while reducing the rise in intracellular Ca^{2+} on reenergisation, compared to controls (Rodrigo *et al.*, 2002). Thus DNP preconditioning of isolated myocytes is considered cardioprotective as it reduces the damaging effects associated with ischaemia-reperfusion injury. Rodrigo *et al.* have also demonstrated that diazoxide preconditioning of isolated myocytes is similarly cardioprotective to DNP preconditioning and is associated with the same improvements of contractile function and augmentation in rise of intracellular Ca^{2+} (Rodrigo *et al.*, 2004). Furthermore, these investigators have demonstrated that myocytes isolated from ischaemic preconditioned hearts exhibit similar improvements in contractile function and Ca^{2+} handling following MI and reenergisation (Rodrigo & Samani, 2008). Thus it is clear that assessing contractile function and intracellular Ca^{2+} following MI and reenergisation of isolated myocytes is a reliable way to assess cardioprotective effects. Therefore, the initial focus of this study was to determine whether TP of isolated

myocytes could improve recovery of contractile function and Ca^{2+} homeostasis following MI and reenergisation compared to untreated control myocytes.

3.2 Results

3.2.1 Temperature preconditioning improves recovery of contractile function of isolated ventricular myocytes exposed to metabolic inhibition and reenergisation

TP, like IPC, has been reported to be cardioprotective in the intact heart (Khaliulin *et al.*, 2007). In the present study I have investigated the effects of TP on isolated ventricular myocytes. In order to investigate TP-induced cardioprotection, adult rat hearts were dissociated into single ventricular myocytes and were subjected to simulated ischaemia-reperfusion injury.

Isolated ventricular myocytes were perfused with substrate-free Tyrode buffer and stimulated to contract by electrical field stimulation at 1Hz. Prior to ischaemia, all viable, rod shaped ventricular myocytes that were synchronously contracting in response to electrical field stimulation were marked for analysis and were monitored throughout the experiment. A typical experiment usually contained between 10 and 40 contractile myocytes (figure 3.1Ai). Ischaemia was simulated by perfusion with metabolic inhibition (MI) Tyrode which lacked the metabolic substrates glucose and pyruvate. Additionally, MI Tyrode contained the metabolic poisons iodoacetic acid (1mM) (inhibits glyceraldehyde 3-phosphate dehydrogenase) and cyanide (2mM) (inhibits electron transfer from complex IV to molecular oxygen); the combined effects of which are a reversible inhibition of ATP generation. Within several minutes of

perfusion with MI Tyrode, myocytes ceased contracting and shortened into a state of rigor contracture (figure 3.1Aii). After 7 minutes of perfusion with MI Tyrode, myocytes were reenergised with Tyrode buffer. This was associated with wash out of the metabolic poisons and re-introduced myocytes to metabolic substrates which allowed ATP synthesis to re-commence. Concomitant with reenergisation of myocytes with Tyrode buffer was a strong hypercontracture. This caused a change of morphology from a rigor, shortened in length rod shaped myocyte to an irregular sphere with membrane blebbings (figure 3.1Aiii). Following 10 minutes of reenergisation the number of myocytes that had regained synchronous contractile function in response to field stimulation was assessed. Myocytes that were found to be asynchronously contracting were quantified as not having regained contractile function. The contractile response of control myocytes during superfusion with substrate free-Tyrode (pre-simulated ischaemia); MI Tyrode (simulated ischaemia); and Tyrode buffer (simulated reenergisation) are illustrated in figure 3.1B.

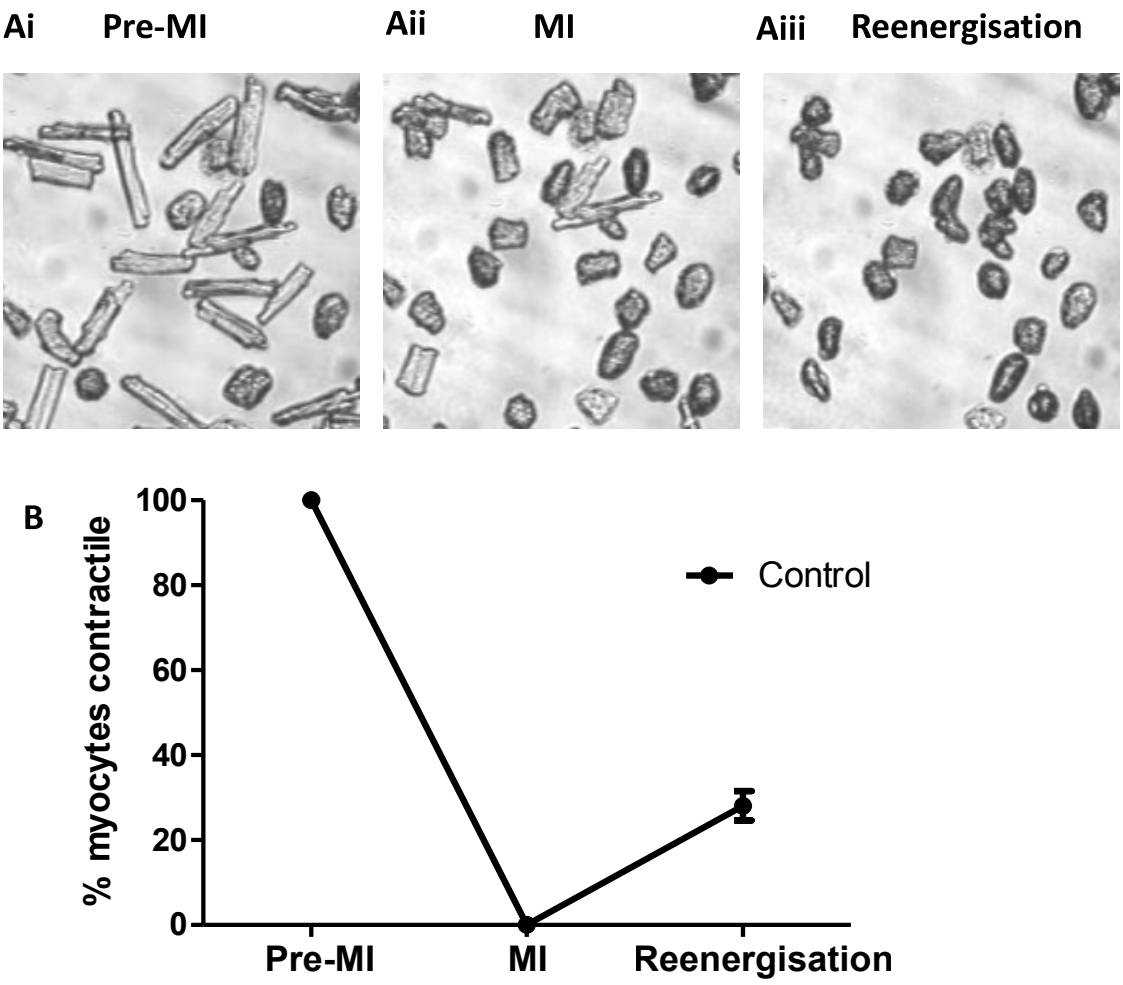

Figure 3.1: The effects of metabolic inhibition and reenergisation on myocytes morphology and ability to contract in response to electrical field stimulation.

Ai) Representative image of isolated cardiac myocytes prior to metabolic inhibition. Viable rod-shaped cardiac myocytes synchronously contract in response to electrical field stimulation (1 Hz).
Aii) During metabolic inhibition (simulated ischaemia) myocytes shorten into rigor contracture and lose their ability to contract in response to electrical field stimulation.
Aiii) Myocytes in hypercontracted state induced by reenergisation. The ability of myocytes to regain contractile function was assessed after 10 minutes of reenergisation.
B: The effects of MI and reenergisation on the ability of myocytes (control) to contract in response to electrical field stimulation (1Hz). Measurements were taken at three time points; immediately prior to MI, after 7 minutes of MI and following 10 minutes of reenergisation.
The majority of myocytes (control) fail to recover contractile function in response to electrical field stimulation following 7 minutes of metabolic inhibition and 10 minutes of reenergisation.

In control experiments, following exposure of isolated ventricular myocytes to 7 minutes of MI and 10 minutes of reenergisation, it was found that 31.5 ± 2.2% (n = 256, 8, 3) untreated control myocytes recovered contractile function. To determine the effects of TP, isolated ventricular myocytes where temperature preconditioned according to Khaliulin *et al.'s* guideline temperature of 26^{o}C prior to experimentation (see materials & methods for protocols). It was found that exposing temperature preconditioned (26^{o}C) myocytes to 7 minutes of MI and 10 minutes of reenergisation resulted in 43.1 ± 7.7% myocytes recovering contractile function (n = 206, 7, 3). This was found to be not significantly different from control (figure 3.2).

The effect of lowering the preconditioning temperature from 26^{o}C, as in Khaliulin's study, to 21^{o}C was investigated. Following MI and reenergisation it was found that 29.5 ± 2.8% control myocytes regained contractile function (n = 46, 2, 1) compared to, 39.2 ± 3.5% (n = 23, 2, 1) for temperature preconditioned (21^{o}C) myocytes. This too, was found not to be significantly different from control (figure 3.3).

Next, the effects of further lowering the preconditioning temperature to 16^{o}C was investigated as previous reports have demonstrated myocardial protection to be induced by similar temperatures (15^{o}C - 18^{o}C) (Chen *et al.*, 2002; Riess *et al.*, 2004). In this set of experiments it was found that 28.9 ± 5.3% (n = 229, 7, 3) control myocytes recovered contractile function following 7 minutes of MI and 10 minutes of reenergisation. In contrast, 51.6 ± 5.7% (n = 225, 7, 3) temperature preconditioned (16^{o}C) myocytes recovered contractile function which was significantly different compared to same day matched controls ($p < 0.05$) (figure 3.4).

TP using 16°C was the only temperature tested that showed a significant increase in contractile recovery. Moreover the cardioprotective effects induced by TP at 16°C were consistently reproducible. Thus, 16^{o}C was used as the preconditioning temperature for all subsequent experiments in this study.

In order to validate the protective effects of TP (16°C) on recovery of contractile function the effects of preconditioning with known cardioprotective agents following MI and reenergisation was investigated. Contractile recovery of dinitrophenol (DNP) (50 μM) preconditioned myocytes (56.5 ± 8.5%, n = 230, 6, 3) was found to be significantly higher than that of control myocytes (27.1 ± 4.7%, n = 199, 6, 3, $p < 0.05$) (figure 3.5). Similarly, cyclosporine A (CsA) (4 μM) (58.8 ± 6.8%, n = 187, 6, 3) treatment of myocytes was also found to significantly improve contractile recovery when measured following 10 minutes of reenergisation compared to same day matched controls (27.1 ± 4.7%, n = 199, 6, 3, $p < 0.005$) (figure 3.6).

Figure 3.7 summarises the protective effects of TP (16^{o}C), DNP (50 μM) preconditioning and CsA (4 μM) treatment on recovery of contractile function following 7 minutes of MI and 10 minutes of reenergisation. All three cardioprotective strategies produced similar improvements of contractile recovery relative to same day matched controls.

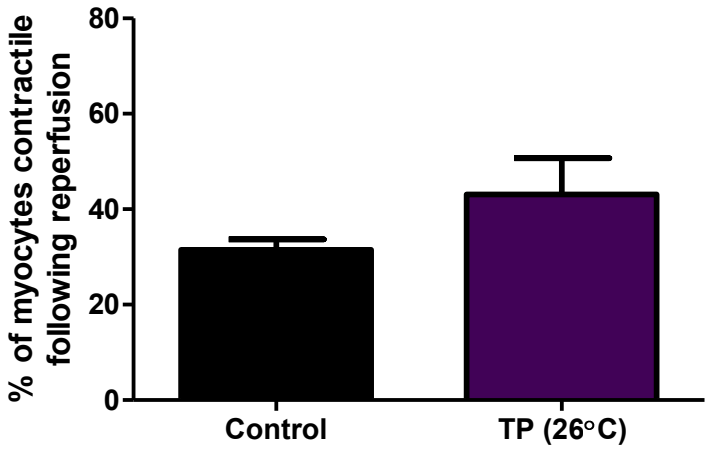

Figure 3.2: The effect of temperature preconditioning (26°C) on functional recovery of myocytes subjected to metabolic inhibition and reenergisation.

Myocytes were electrically field stimulated to contract at 1 Hz and exposed to 7 minutes of metabolic inhibition followed by 10 minutes of reenergisation. Recovery of contractile function was measured at 10 minutes of reenergisation. Percentage of myocytes that recovered contractile function for controls 31.5 ± 2.2 n =2 56, 8, 3; percentage recovery of temperature preconditioned (TP) myocytes 43.1 ± 7.7 n = 206, 7, 3.

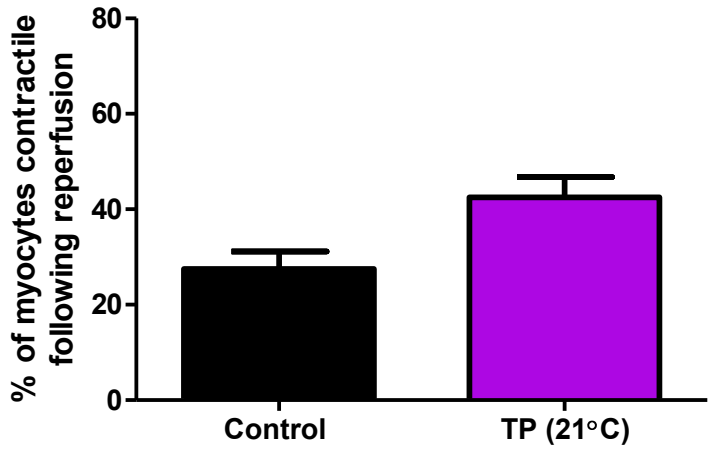

Figure 3.3: The effect of temperature preconditioning (21°C) on functional recovery of myocytes subjected to metabolic inhibition and reenergisation.

Myocytes were electrically field stimulated to contract at 1 Hz and exposed to 7 minutes of metabolic inhibition followed by 10 minutes of reenergisation. Recovery of contractile function was measured at 10 minutes of reenergisation. Percentage of myocytes that recovered contractile function for controls 29.5 ± 2.8 n = 46, 2, 1; percentage recovery of temperature preconditioned (TP) myocytes 39.2 ± 3.5 n = 23, 2, 1.

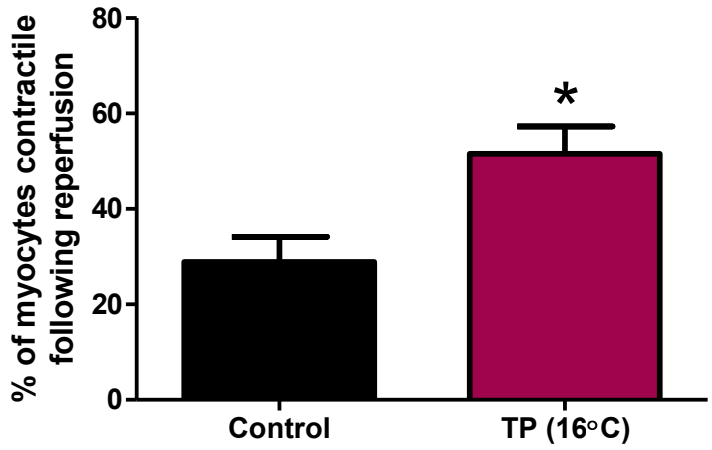

Figure 3.4: The effect of temperature preconditioning (16°C) on functional recovery of myocytes subjected to metabolic inhibition and reenergisation.

Myocytes were electrically field stimulated to contract at 1 Hz and exposed to 7 minutes of metabolic inhibition followed by 10 minutes of reenergisation. Recovery of contractile function was measured at 10 minutes of reenergisation. Percentage of myocytes that recovered contractile function for controls 28.9 ± 5.3 n = 229, 7, 3; percentage recovery of temperature preconditioned (TP) myocytes 51.6 ± 5.7 n = 225, 7, 3. * $p < 0.05$.

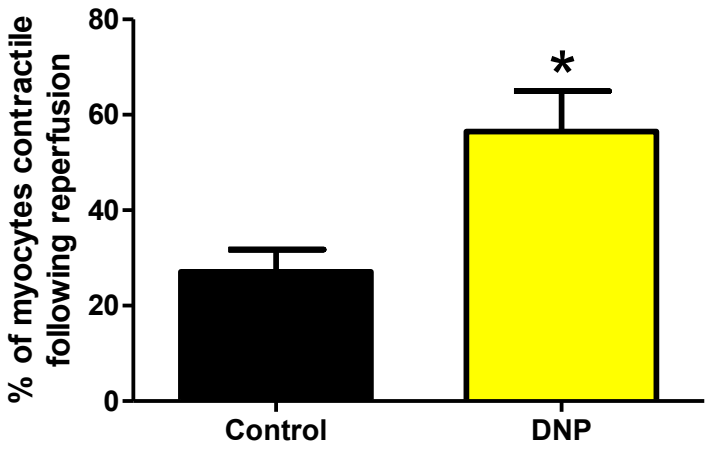

Figure 3.5: The effects of dinitrophenol (50µM) on functional recovery of myocytes subjected to metabolic inhibition and reenergisation.

Myocytes were electrically field stimulated to contract at 1 Hz and exposed to 7 minutes of metabolic inhibition followed by 10 minutes of reenergisation. Recovery of contractile function was measured at 10 minutes of reenergisation. Percentage of myocytes that recovered contractile function for controls 27.1 ± 4.7% n = 199, 6, 3; percentage recovery of dinitrophenol (DNP) preconditioned myocytes 56.5 ± 8.5% n = 230, 6, 3. * $p < 0.05$

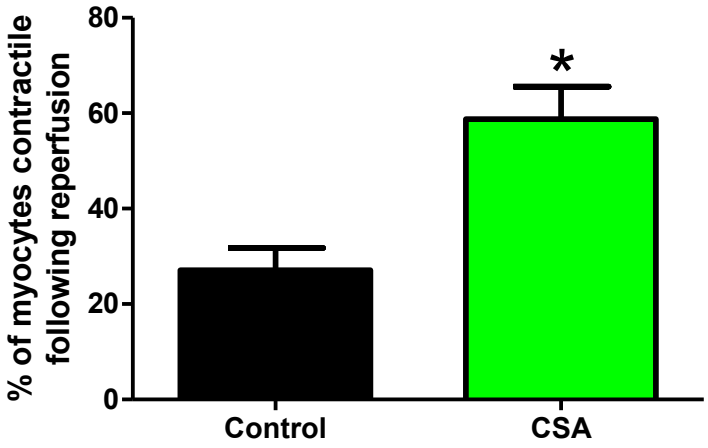

Figure 3.6: The effect of cyclosporine A (4µM) on functional recovery of myocytes subjected to metabolic inhibition and reenergisation.

Myocytes were electrically field stimulated to contract at 1 Hz and exposed to 7 minutes of metabolic inhibition followed by 10 minutes of reenergisation. Recovery of contractile function was measured at 10 minutes of reenergisation. Percentage of myocytes that recovered contractile function for controls 27.1 ± 4.7 n = 199, 6, 3; percentage recovery of cyclosporine A (CsA) preconditioned myocytes 58.8 ± 6.8 n = 187, 6, 3. * $p < 0.005$.

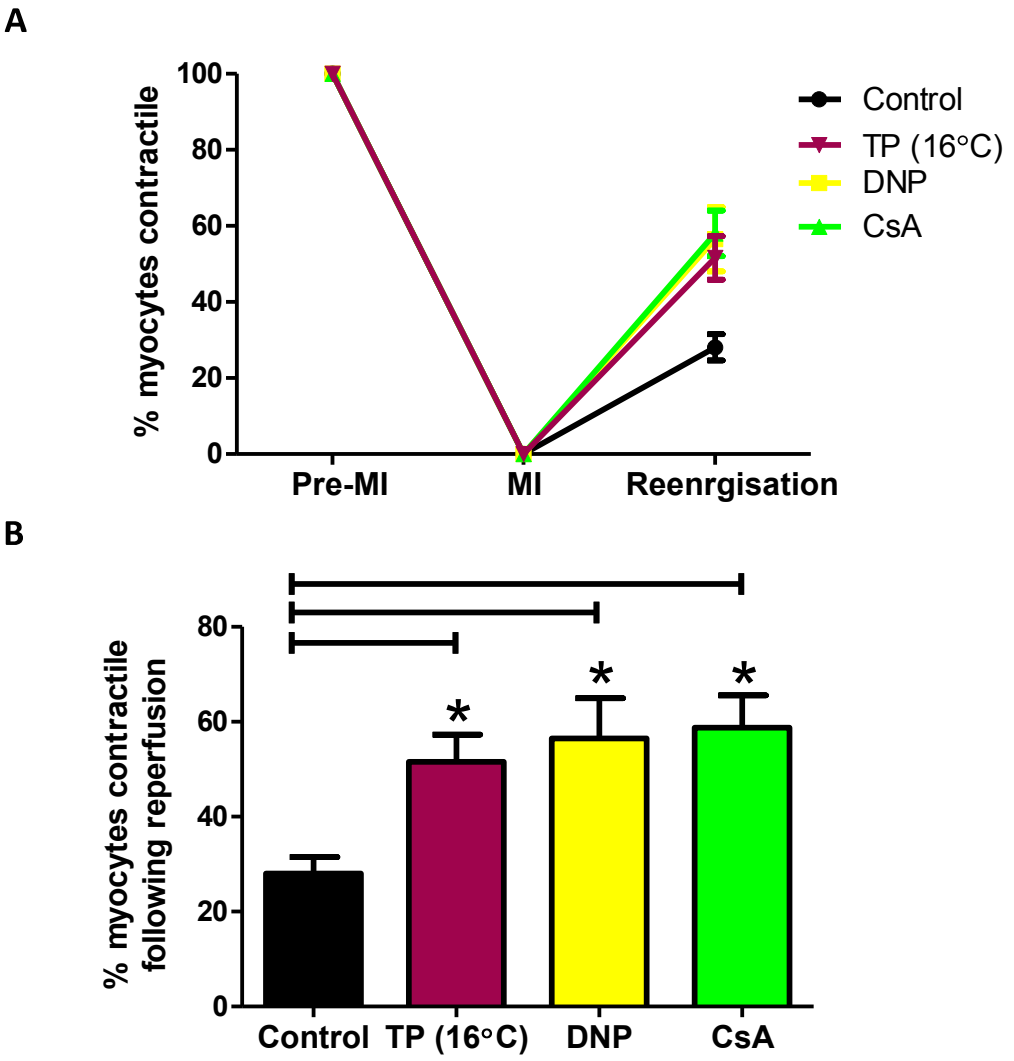

Figure 3.7: The effects of preconditioning on the ability of myocytes to recover contractile function following metabolic inhibition and reenergisation.

A: The effects of MI and reenergisation on the ability of myocytes (various treatments) to contract in response to electrical field stimulation (1Hz). Measurements were taken at three time points; immediately prior to MI, after 7 minutes of MI and following 10 minutes of reenergisation.
B: Mean data of percentage of myocytes that recovered contractile function following 7 minutes of MI and 10 minutes of reenergisation for control, n = 428; temperature preconditioned (TP 16^{0}C) n = 225; dinitrophenol (DNP 50µM) preconditioned, n = 230 and cyclosporine A (CsA, 4µM) treatment, n = 187,. At least three hearts were tested for each treatment. Data was analysed by a 1-way ANOVA; * $p < 0.05$ vs. control.

3.2.2 Temperature preconditioning improves Ca^{2+}homeostasis of isolated ventricular myocytes exposed to metabolic inhibition and reenergisation

Loss of Ca^{2+} homeostasis is routinely observed during prolonged ischaemia and reperfusion and improved Ca^{2+} homeostasis is a feature of cardioprotection (Piper *et al.*, 2004). IPC preconditioning and pharmacological preconditioning have been demonstrated to improve post ischaemia-reperfusion injury Ca^{2+} homeostasis and this has been suggested to contribute to the cardioprotection granted by these protective interventions (Rodrigo *et al.*, 2002; Ylitalo *et al.*, 2000; Liu *et al.*, 1998; Liu *et al.*, 2005). Loss of Ca^{2+} homeostasis is associated with a significant rise in intracellular Ca^{2+} which can consequently cause Ca^{2+}-activated enzymes such as proteases, nucleases and phospholipases to become activated, resulting in cellular injury. Additionally, uncontrolled rises in intracellular Ca^{2+} can cause Ca^{2+}overload-induced contracture (Piper *et al.*, 2004) and contractile dysfunction (Rodrigo & Samani, 2008; Bolli & Marban, 1999). Furthermore, elevated intracellular Ca^{2+} at the time of myocardial reperfusion is believed to be a critical trigger of irreversible mitochondrial transition pore opening which is associated with necrotic cell death (Halestrap, 2009). Hence, maintaining myocardial Ca^{2+} homeostasis during ischaemia and reperfusion is cardioprotective and favours myocardial survival.

Until present, the effects of TP on intracellular Ca^{2+} homeostasis during ischaemia and reperfusion have not been investigated. For this reason I set out to investigate this parameter in isolated ventricular myocytes that were subjected to MI and reenergisation. Isolated myocytes were loaded with the ratiometric Ca^{2+} sensitive dye

fura-2AM. Myocytes were then exposed to 7 minutes of MI followed by 10 minutes of reenergisation, while stimulated to contract at 1Hz throughout. Ca^{2+} measurements were taken throughout the experiment but sampling was increased, from 0.1Hz to 1Hz, at two phases during the experiment. This was conducted to allow more accurate determination of basal Ca^{2+} during these time points, at which, comparisons of basal Ca^{2+} levels was made between control and preconditioned myocytes. Figure 3.8 shows a representative 340/380 ratio fura-2 trace of a control and temperature preconditioned myocyte exposed to MI and reenergisation. The arrows indicate the time points at which the rate of sampling was increased (1Hz). For the remainder of the experiment the rate of sampling was 0.1Hz which resulted in an aliasing effect produced by the slow sampling of rapid Ca^{2+} transients. This resulted in the recording of field stimulation-dependent slowly oscillating Ca^{2+} levels, which can be used as an indicator of Ca^{2+} homeostasis and contractile function of the cells (Rodrigo & Samani, 2008). Prior to MI myocytes were superfused with Tyrode to allow a stable baseline of Ca^{2+} to be recorded. During this period synchronous Ca^{2+} oscillations were observed. Upon exposure of myocytes to MI Tyrode, Ca^{2+} oscillations progressively declined and this was followed by a rise in intracellular Ca^{2+} in both control and temperature preconditioned myocytes. In control myocytes, the rise in intracellular Ca^{2+} was augmented by reenergisation and few control myocytes recovered synchronous Ca^{2+} transients in response to field stimulation. In contrast, reenergisation of temperature preconditioned myocytes was typically accompanied by a smaller, transient, increase of intracellular Ca^{2+}. Further, this was followed by the reappearance of synchronous Ca^{2+} transients to field stimulation in the majority of myocytes.

To determine the effects of TP on Ca^{2+}homeostasis, measurements of Ca^{2+} transients were taken at the start (prior to MI and reenergisation) and end (following MI and reenergisation) of experiments and compared to controls. It was during these crucial time points that the rate of sampling was increased to 1Hz. Prior to MI, synchronous Ca^{2+} transients at a rate of 1Hz (as dictated by field stimulation at 1Hz) were observed in both control and temperature preconditioned myocytes (figure 3.9A). Basal Ca^{2+} was taken to be the trough of Ca^{2+} transients. The trough at 1 minute following the start of an experiment was recorded as the 'basal Ca^{2+} level prior to MI' and was compared between treatment groups. Following MI and reenergisation the majority of control myocytes had elevated intracellular Ca^{2+} and failed to recover synchronous Ca^{2+} transients. For a proportion of the myocytes Ca^{2+} levels spontaneously oscillated independent of field stimulation and continued to do so even after field stimulation was stopped, indicating loss of Ca^{2+} homeostasis (figure3.9B control trace).

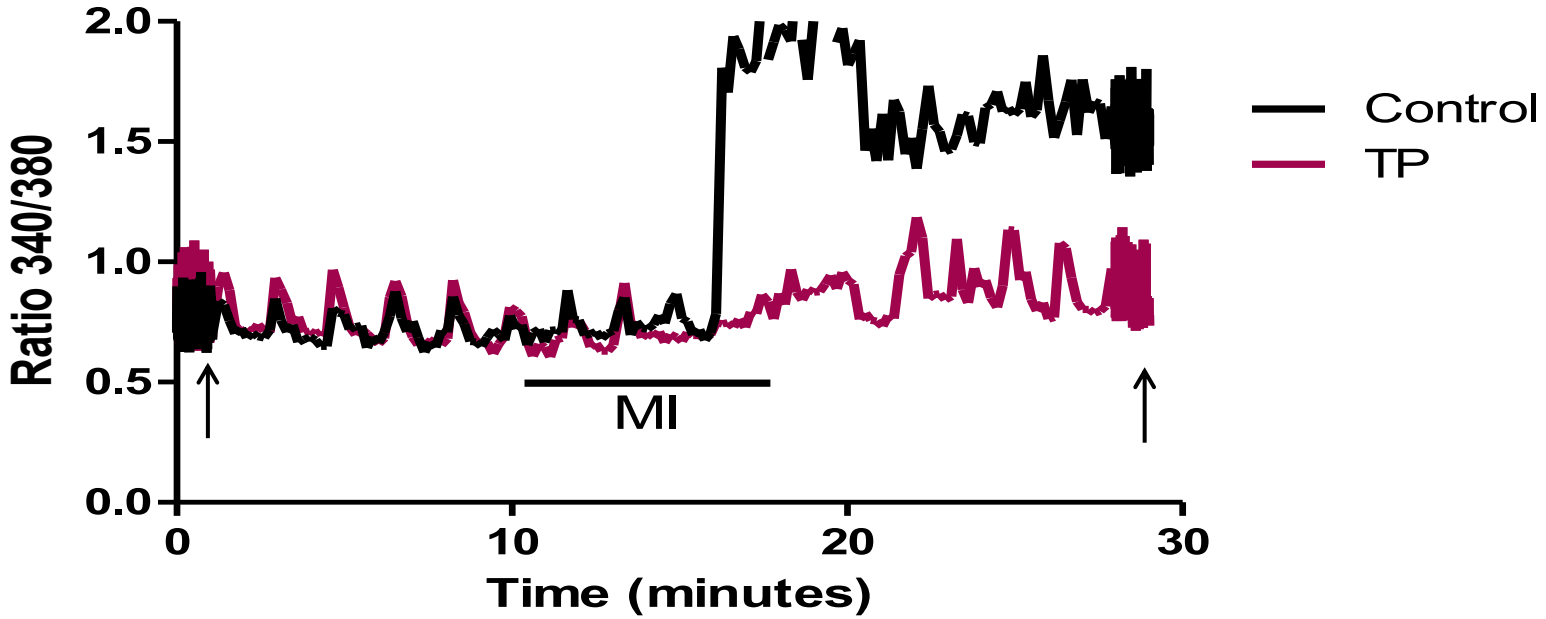

Figure 3.8: The effects of metabolic inhibition and reenergisation on calcium homeostasis of control and temperature preconditioned myocytes.

Typical 340/380 fura-2 trace from representative control and temperature preconditioned (TP) myocytes. Myocytes were exposed to 7 minutes of metabolic inhibition (MI) followed by 10 minutes of reenergisation with Tyrode solution. Electrical field stimulation (1Hz) was applied throughout the experiment. Measurements of intracellular Ca^{2+} were recorded at two time points; 10 minutes prior to MI and once again following 10 minutes of reenergisation (indicated by the arrows), at which time points the rate of sampling was increased from 0.1 Hz to 1 Hz to increase the accuracy of measurements.

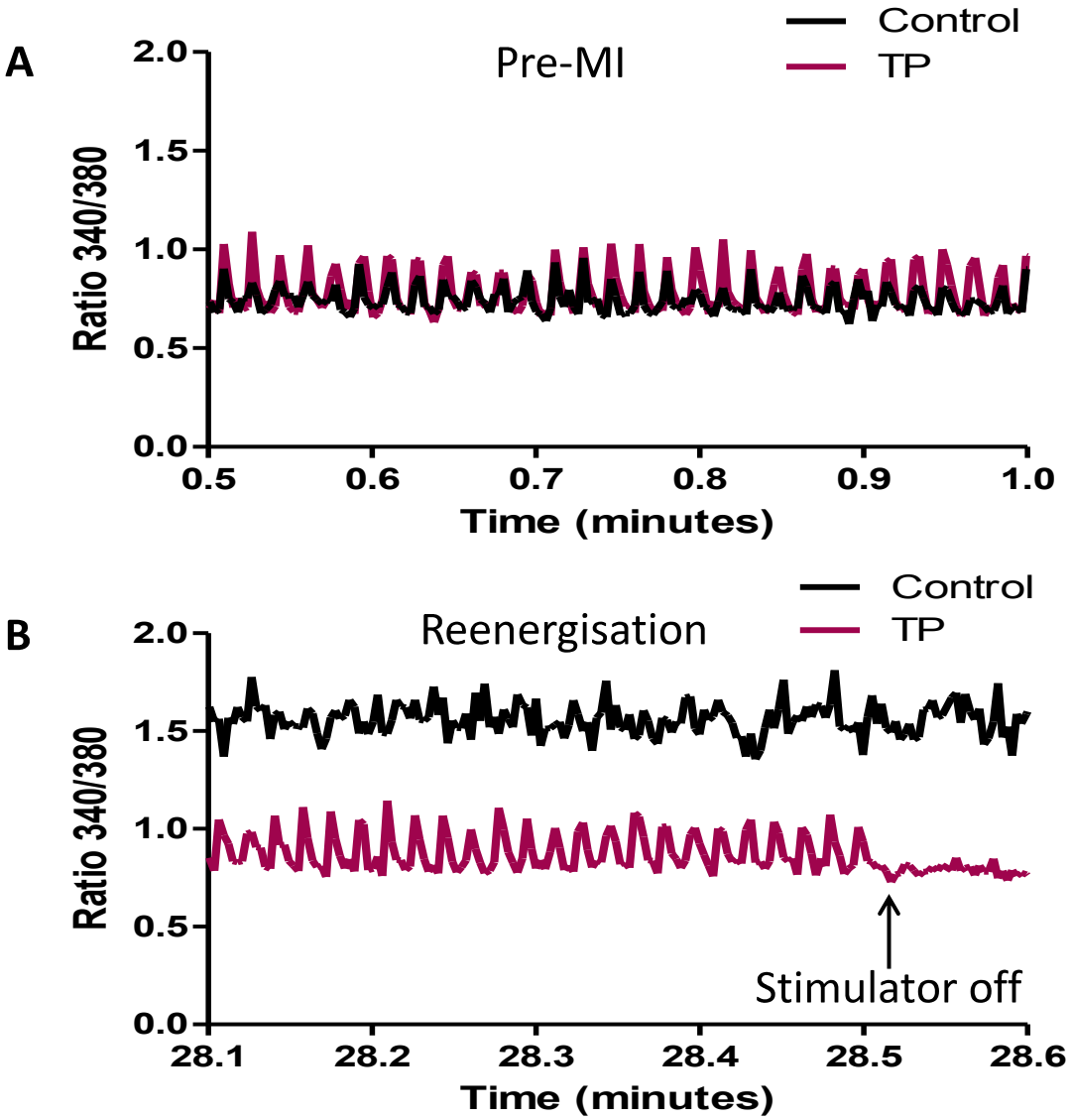
A
Pre-MI
Control
TP
Ratio 340/380
2.0
1.5
1.0
0.5
0.0
0.5
0.6
0.7
0.8
0.9
1.0
Time (minutes)
B
Reenergisation
Control
TP
Ratio 340/380
2.0
1.5
1.0
0.5
0.0
Stimulator off
28.1
28.2
28.3
28.4
28.5
28.6
Time (minutes)

Figure 3.9: Measurements of $[Ca^{2+}]_i$ from isolated ventricular myocytes exposed to metabolic inhibition and reenergisation.

Typical 340/380 fura-2 trace from representative control and temperature preconditioned (TP) myocytes during the two phases of high frequency sampling (1Hz) at which time points basal Ca^{2+} was recorded. A: Representative traces depicting real time changes in intracellular Ca^{2+} prior to metabolic inhibition. Ca^{2+} peaks (fura-2 340/380 peaks) were routinely observed in line with electrical field stimulation at 1 Hz and was a criterion for inclusion of analysis. Basal Ca^{2+} values prior to metabolic inhibition were measured at time = 1 minute. B: Following 10 minutes of reenergisation a second period of high frequency sampling was applied during which time it was found that the majority of control myocytes failed to regain synchronous Ca^{2+} oscillations in line with electrical field stimulation. After 30 seconds of high frequency sampling the electric field stimulator was turned off (indicated by arrow) to allow more accurate measurements of basal Ca^{2+} to be recorded. A calcium calibration was conducted to convert arbitrary fluorescence units into estimated free cellular calcium (nM) concentration. Ca^{2+} values recorded from the two time points were then analysed by a student's t-test (unpaired) and $p < 0.05$ was considered significantly different.

Basal Ca^{2+} was re-recorded at 10 minutes of reenergisation under conditions of no electric field stimulation (figure 3.9B). Of note, figure 3.9B shows that no significant change in basal fluorescence was observed when the stimulator was switched off. This indicated that the trough of Ca^{2+} transients (evoked by field stimulation) could be used to measure basal Ca^{2+} levels, as was conducted for pre-MI basal Ca^{2+} level measurements. A Ca^{2+} calibration was conducted to allow fura-2 fluorescence ratios to be converted into intracellular free Ca^{2+} concentrations (nM). Analysis revealed that mean basal Ca^{2+} levels were not significantly different between control and temperature preconditioned myocytes prior to MI. However, following MI and reenergisation the mean basal Ca^{2+} concentration was found to be significantly lower in temperature preconditioned (132.1 ± 17.6 n = 67, 6, 3) myocytes compared to controls (301.3 ± 52.7 n = 69, 6, 3; $p < 0.005$) (figure 3.10). TP (60.1 ± 5.1% n = 67, 6, 3) was also found to significantly increase the proportion of myocytes that recovered synchronous Ca^{2+} transients in response to field stimulation compared to controls (31.5 ± 3.0% n = 69, 6, 3; $p < 0.01$) (figure 3.11). It was found that basal Ca^{2+} levels following MI and reenergisation, in both control and temperature preconditioned myocytes that recovered Ca^{2+} transients was generally below 150nM. Therefore the number of myocytes that had an intracellular Ca^{2+} concentration below this value following MI and reenergisation was examined and was taken to represent the proportion myocytes with unimpaired Ca^{2+} homeostasis. A significantly higher percentage of temperature preconditioned myocytes (81.7 ± 11.0% n = 67, 6, 3) were found to have basal Ca^{2+} lower than 150nM compared to controls (41.1 ± 3.7% n = 69, 6, 3; $p < 0.05$) (figure 3.12).

The effects of DNP preconditioning (figure 3.13) were found to be similar to that of TP. No significant change in mean basal Ca^{2+} concentration was observed between control and DNP preconditioned myocytes prior to MI. However, DNP preconditioning (212.9 ± 10.0nM n = 42, 5, 3) of myocytes did cause a significant reduction in mean basal Ca^{2+} following MI and reenergisation compared to control (317.8 ± 38.5nM n = 44, 5, 3; $p < 0.05$). In contrast, CsA treatment (figure 3.14) was found to significantly lower mean basal Ca^{2+} levels both, prior to MI (83.76 ± 5.618 n = 93, 6, 3) and following MI and reenergisation (194.5 ± 23.5 n = 88, 6, 3) compared to control (104.7 ± 6.092 n = 75, 6, 3; $p < 0.05$; 430.9 ± 82.2 n = 70, 6, 3; $p < 0.005$, respectively).

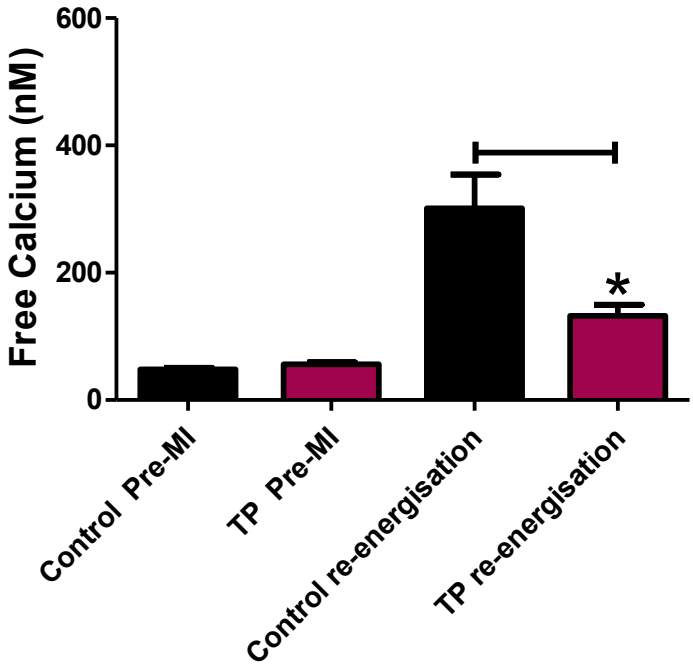

Figure 3.10: The effects of temperature preconditioning (16°C) on intracellular Ca^{2+} in myocytes subjected to 7 minutes of metabolic inhibition, followed by 10 minutes of reenergisation.

Fura-2 ratio was calibrated to give estimations of free Ca^{2+} concentrations (nM). Control reenergisation 301.3 ± 52.7nM n = 69, 6, 3; temperature preconditioned (TP) reenergisation 132.1 ± 17.6nM n = 67, 6, 3. * $p < 0.005$.

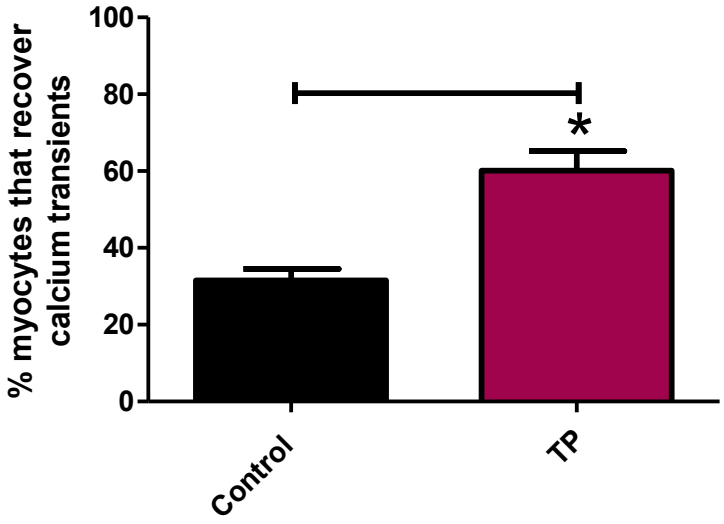

Figure 3.11: The proportion of myocytes that recovered synchronous calcium transients following 7 minutes of metabolic inhibition and 10 minutes of reenergisation.

At 10 minutes of reenergisation following metabolic inhibition, the number of myocytes that had recovered calcium transients in response to field stimulation (1Hz) was assessed. 31.5 ± 3.0% of control myocytes (n = 69, 6, 3) and 60.1 ± 5.1% of temperature preconditioned (TP) myocytes (n = 67, 6, 3) recovered synchronous calcium transients. * $p < 0.01$.

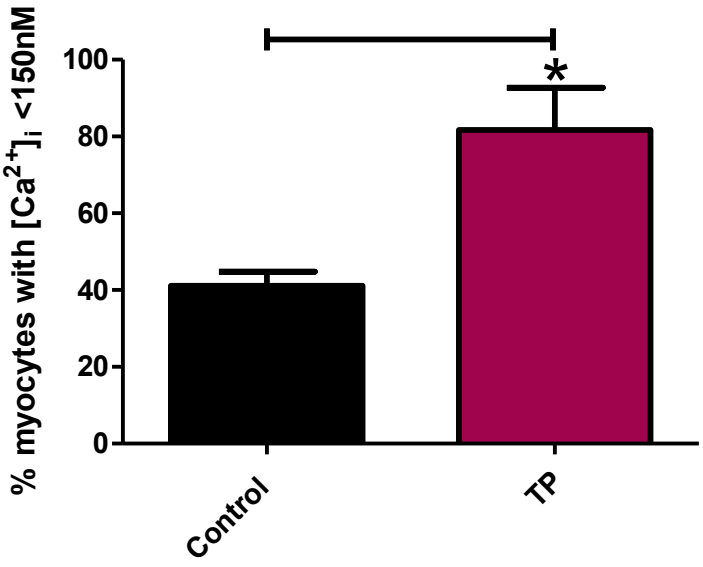

Figure 3.12: The proportion of myocytes that had intracellular calcium concentration less than 150nM following 7 minutes of metabolic inhibition and 10 minutes of reenergisation.

At 10 minutes of reenergisation following metabolic inhibition, the number of control myocytes that had $[Ca^{2+}]_i$ < 150nM was 41.1 ± 3.7% (n = 69, 6, 3) compared to 81.7 ± 11.0% for temperature preconditioned (TP) myocytes (n = 67, 6, 3).
* $p < 0.05$.

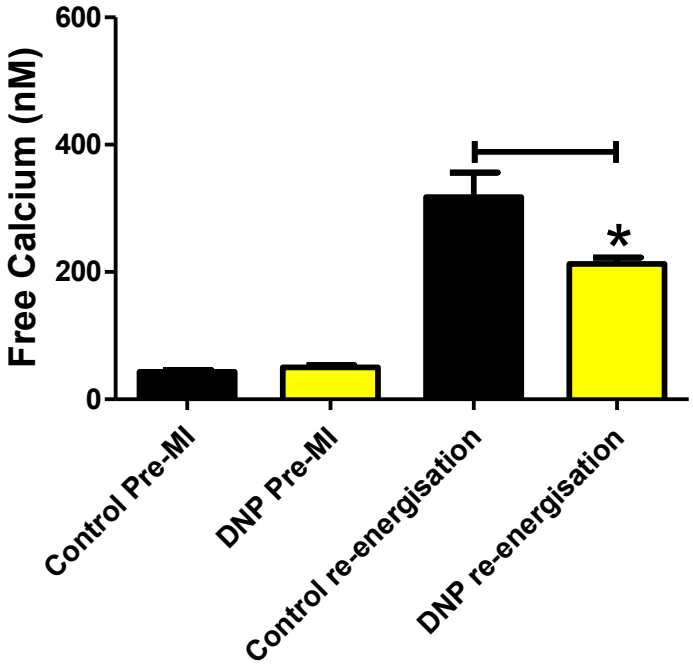

Figure 3.13: The effects of DNP (50µM) preconditioning on basal intracellular Ca^{2+} in myocytes subjected to 7 minutes of metabolic inhibition, followed by 10 minutes of reenergisation.

Fura-2 ratio was calibrated to give estimations of free Ca^{2+} concentrations (nM). At 10 minutes of reenergisation electrical field stimulation was stopped and basal free Ca^{2+} was measured. Control reenergisation 317.8 ± 38.5nM n = 44, 4, 3; DNP reenergisation 212.9 ± 10.0nM n = 42, 4, 3. * $p < 0.05$.

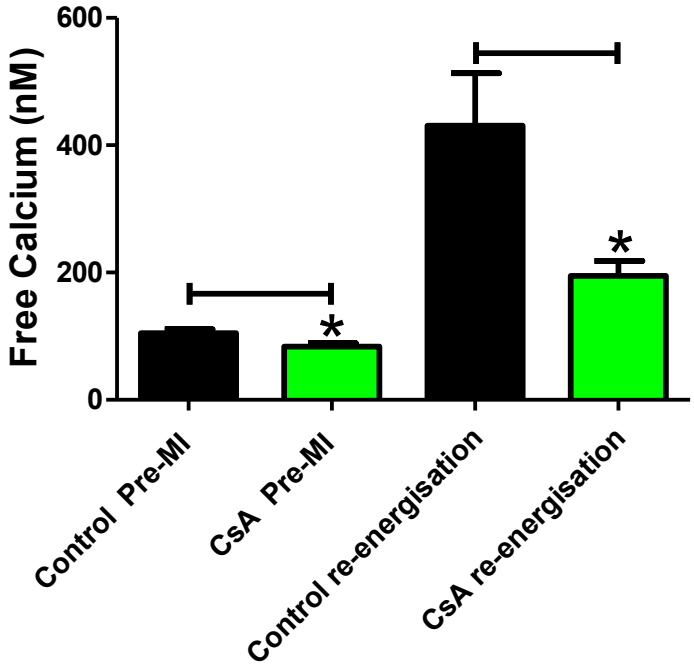

Figure 3.14: The effects of CsA (4µM) preconditioning on intracellular Ca^{2+} in myocytes subjected to 7 minutes of metabolic inhibition, followed by 10 minutes of reenergisation.

Fura-2 ratio was calibrated to give estimations of free Ca^{2+} concentrations (nM). Control pre-MI 104.7 ± 6.092 n = 75, 6, 3; CsA pre-MI 83.76 ± 5.618 n = 93, 6, 3. Control reenergisation 430.9 ± 82.2nM n = 70, 6, 3; CsA reenergisation 194.5 ± 23.5nM n = 88, 6, 3.* $p < 0.05$.

3.3 Summary of results

In this chapter it is demonstrated, that isolated ventricular myocytes are cardioprotected by TP. This finding implies that the molecular mechanisms responsible for TP-induced protection reside at the cellular level; independent of any nervous system influence or paracrine signalling from other cell types (such as endothelial cells and fibroblasts) present within the intact heart. Unlike the study in which TP was first reported (Khaliulin *et al.*, 2007), this study found that preconditioning myocytes using 26^{o}C did not induce a significant cardioprotective effect as assessed by recovery of contractile function following MI and reenergisation. On the contrary, this study found that a lower preconditioning temperature of 16^{o}C was required to induce cardioprotection. TP at 16^{o}C was found to represent a temperature which consistently induced robust cardioprotection and was therefore used for all subsequent experiments in this study.

TP (16^{o}C) significantly improved the proportion of myocytes that recovered contractile function following MI and reenergisation compared to controls. The improvement of contractile function induced by TP was found to be similar to that as induced by the known cardioprotective agents DNP and CsA.

TP (16^{o}C) was also found to significantly improve Ca^{2+} homeostasis following MI and reenergisation. Temperature preconditioned myocytes had significantly lower basal Ca^{2+} levels compared to controls after exposure to MI and reenergisation. Moreover, a higher percentage of temperature preconditioned myocytes regained synchronous Ca^{2+} transients in response to electric field stimulation following MI and reenergisation

compared to controls. This finding positively correlates with the improvement of contractile function as induced by TP and this may be causal.

Limitations

In this study, the end point used to assess a cardioprotective phenotype following MI and re-energisation was recovery of contractile function, which may be taken as a surrogate for increased viability. However, as myocardial stunning, which results in a reversible loss of contractile function following ischaemia-reperfusion injury, is a well documented occurrence known to effect cardiac myocytes, in essence this model may have only assessed recovery from stunning (Bolli, 1990). The more clinically relevant question to address is the effect of cardioprotective interventions on cell viability following ischaemia-reperfusion injury. Therefore, the question arises as to whether the model of MI and re-energisation used in this study was capable of inducing cardiac myocyte death in the time frame of the experiment. This could have been assessed by the addition of a hypotonic solution containing Trypan blue at the end of each experiment and would have improved experimental design. Trypan blue is impermeable to viable myocytes with intact membrane integrity and therefore only permeates dead myocytes. This allows determination of the percentage of viable myocytes in a mixed population of myocytes by light microscopy (Diaz & Wilson, 2006). In this regard, it was recently demonstrated by Turrell *et al,* using an almost identical MI and re-energisation model as used in this study, that this model is capable of inducing myocyte death (Turrell *et al.*, 2011). Thus, it is feasible that the improvement of functional recovery as induced by TP in this study may have been as a result of decreased viability. However, this should have been directly investigated by the addition of Trypan blue at the end of each experiment.

4 The effects of temperature preconditioning on opening of the mitochondrial permeability transition pore during simulated reperfusion injury

4.1 Introduction

In the previous chapter it was demonstrated that isolated ventricular myocytes are amenable to temperature preconditioning (TP). This resulted in improved recovery of contractile function and Ca^{2+} homeostasis following simulated ischaemia-reperfusion injury. In this chapter I investigate whether the protective effects induced by TP are due to inhibition of mitochondrial permeability transition pore (mPTP) opening; a critical factor underlying reperfusion injury (Yang *et al.*, 2010).

The most prominent roles of mitochondria are the regulation of cellular metabolism and energy production. However, in recent years it has emerged that mitochondria play a pivotal role in determining cardiomyocyte survival and death in the setting of ischaemia-reperfusion injury. Maintaining mitochondrial integrity is critical as this allows proper mitochondrial bioenergetics and functioning to take place. Mitochondrial dysfunction impairs energy production and can arise following ischaemia-reperfusion injury through opening of the mPTP. The mPTP is believed to span the mitochondrial outer and inner membranes and consequently its opening results in increased permeability of the mitochondrial inner membrane causing all small solutes to equilibrate across the inner membrane. However, only molecules less than 1500Da can permeate the mPTP and thus a high concentration of proteins is retained within the mitochondrial matrix. These exert a colloidal osmotic pressure that

draws in water and causes mitochondrial swelling. Additionally, mPTP opening causes dissipation of the mitochondrial membrane potential as protons are granted unrestricted passage across the, otherwise impermeable, mitochondrial inner membrane. As a result of this, mitochondrial depolarization occurs, oxidative phosphorylation is uncoupled and the action of the proton translocating ATPase is reversed. This ultimately leads to ATP depletion and myocyte death (Halestrap, 2010).

It is well established that elevated intramitochondrial Ca^{2+} is the primary trigger for opening of the mPTP, although it was recognized as early as the 1950's that several other factors can significantly sensitise pore opening to Ca^{2+} concentration (Crompton & Costi, 1988; Tapley, 1956; Hunter & Ford, 1955; Yang *et al.*, 2011; Crompton *et al.*, 1987; Chappell & Crofts, 1965). Of particular relevance to the setting of ischaemia-reperfusion injury are three factors; oxidative stress, depletion of mitochondrial matrix adenine nucleotides and high phosphate concentrations. The conditions which prevail in cardiomyocytes upon reperfusion following a long period of ischaemia are profoundly perturbed and are conducive to mPTP opening. The irreversible opening of the mPTP is responsible for the necrotic cell death associated with reperfusion injury.

During prolonged myocardial ischaemia cellular ATP concentrations are severely depleted owing to interrupted mitochondrial respiration and oxidative phosphorylation. Correspondingly, cellular ADP, AMP and Pi concentrations are increased which stimulate glycolysis through activation of phosphofructokinase. The increased rate of glycolysis acts to counteract the ATP deficit, but the amount of ATP produced is insufficient to maintain the high energy demands of the heart. Lactic acid accumulates as a byproduct of glycolysis causing intracellular pH to fall. This

progressively inhibits glycolysis through inhibition of phosphofructokinase and also activates the Na^+/H^+ antiporter which acts to rectify the changes in cellular pH. The action of the antiporter results in the inward movement of Na^+ coupled with the extrusion of H^+. Under normal conditions Na^+ balance would be regulated through the actions of the Na^+/K^+-ATPase, however, greatly reduced ATP concentrations impair the function of the Na^+/K^+-ATPase resulting in a progressive rise in intracellular Na^+ concentration. As a secondary mechanism to regulate intracellular Na^+ concentration, the cell utilizes the Na^+/Ca^{2+} antiporter, which under these conditions is stimulated to work in reverse mode, favoring Ca^{2+} accumulation (Ca^{2+} overload) in return for Na^+ extrusion. Increased intracellular Ca^{2+} concentration is characteristic of prolonged ischaemia and this coupled with severe ATP depletion, lead to a gradual decline in cellular integrity as degradative enzymes such as phospholipases (PLA_2) and Ca^{2+}-sensitive enzymes, including nucleases and proteases are activated. To exacerbate the situation further, hydrolysis of ATP leads to the formation of AMP mediated through the actions of adenylate kinase. AMP in turn is then broken down by the actions of 5'-nucleotidase producing adenosine. This is then further processed by the purine degradation pathway resulting in the formation of the nucleosides ionosine and xanthine with a consequent depletion of total nucleotides. The situation can be further compounded if sufficient residual oxygen is available to catalyze the breakdown of xanthine by xanthine oxidase producing superoxide as a byproduct (Halestrap, 2010; Halestrap, 2009; Halestrap *et al.*, 1998; Halestrap *et al.*, 2004; Silverman & Stern, 1994). This can be further metabolized to more damaging reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radical (Zweier & Talukder, 2006). In all, prolonged ischaemia results in an energy compromised myocardium in which Ca^{2+}-

activated enzymes attack cellular components resulting in necrotic cell death. Thus in order to prevent myocardial death and limit the damage caused by ischaemia, timely restoration of myocardial blood flow (reperfusion) is essential.

It is well documented that reperfusion following a short period of ischaemia results in very little, if any, damage to the myocardium. However, reperfusion following a prolonged period of ischaemia causes additional damage to that caused by ischaemia alone and this is referred to as reperfusion injury. Reperfusion of the ischaemic myocardium delivers oxygen and substrates which permit the restoration of mitochondrial bioenergetics and the reestablishment of a mitochondrial membrane potential. However, restarting of the respiratory chain which is permitted by the sudden availability of oxygen is associated with a burst of mitochondrial oxygen free radical production. This occurs in a background of elevated mitochondrial matrix Ca^{2+} concentration, low adenine nucleotides and elevated phosphate – consequences of prolonged ischaemia. As discussed above, these factors readily promote mPTP opening but this is delayed at the onset of reperfusion through the inhibitory effects of low pH. Within the first few minutes of reperfusion the mPTP remains closed until cellular pH is restored through the actions of pH regulatory pumps. Upon return to normal pH the inhibitory effects are withdrawn and mPTP opening occurs as confirmed by experimental data (Griffiths & Halestrap, 1995; Halestrap *et al.*, 1997; Kerr *et al.*, 1999), leading to cell death.

The central role of mPTP opening in reperfusion injury has been confirmed by the demonstration that blocking mPTP opening reduces myocardial damage. This was first demonstrated in isolated cardiac myocytes using the immunosuppressant cyclosporine A (CsA), a drug which inhibits cyclophilin-D (CyP-D) - a core component of the mPTP. CsA treatment of myocytes was found to reduce injury following substrate-free anoxia and reoxygenation (Nazareth *et al.*, 1991). Subsequently, the protective effect of CsA treatment of the Langendorff perfused heart against ischaemia-reperfusion injury was demonstrated (Griffiths & Halestrap, 1993). More recently, the protective effects of CsA were confirmed in a, proof of concept clinical trial, involving a small cohort of patients undergoing percutaneous coronary intervention. It was found that patients treated with CsA developed smaller infarcts compared to patients treated with normal saline (control) (Piot *et al.*, 2008). Furthermore, studies have also demonstrated that CyP-D-knockout mice develop significantly smaller infarcts following ischaemia and reperfusion. Further evidence implicating mPTP opening as a critical mediator of reperfusion injury comes from the demonstration that a number of cardioprotective interventions, including ischaemic preconditioning, ischaemic postconditioning and pharmacological preconditioning all inhibit mPTP opening (Lim *et al.*, 2007; Halestrap *et al.*, 2007). Given the wide array of cardioprotective protocols that have been shown to reduce mPTP opening on reperfusion, this chapter investigated the effects of TP on mPTP opening using a cellular model of reperfusion injury.

TP has previously been shown to inhibit Ca^{2+}-induced mPTP opening in isolated de-energised mitochondria at 25^{o}C, using a method which measures a decrease in light scattering that accompanies mitochondrial swelling following Ca^{2+} addition and mPTP opening (Khaliulin *et al.*, 2007). In this study I sought to replicate this in intact

cardiomyocytes using a well established model of mPTP opening (Hausenloy *et al.*, 2004; Huser *et al.*, 1998; Huser & Blatter, 1999; De Giorgi *et al.*, 2002; Duchen *et al.*, 1998). Studying mitochondrial functions in intact cardiomyocytes, as opposed to preparations of pure mitochondria, allows more faithful representation of the events associated with ischaemia-reperfusion injury in the intact heart (Diaz & Wilson, 2006). The photodamage model used to investigate mPTP opening in this study relies on the selective accumulation of tetramethyl rhodamine methyl ester (TMRM) into mitochondria. This coupled with illumination of TMRM results in mitochondria specific generation of reactive oxygen species (ROS) and therefore, this model represents a most appropriate means of simulating the mitochondrial oxidative stress observed in reperfusion injury (Jacobson & Duchen, 2002). Furthermore, based on the literature (Wang *et al.*, 2008), I have developed a model of xanthine and xanthine oxidase-induced mPTP opening which also relies on the production of ROS within mitochondria to induce mPTP opening. Thus pathological mPTP opening was assessed using two independent models which simulate the events of reperfusion injury by producing excessive amounts of mitochondrial ROS.

4.2 Results

4.2.1 Characterizing the effects of photodamage-induced mPTP opening in HeLa cells

Initial characterization of photodamage-induced mPTP opening was conducted in non-contractile HeLa cells. HeLa cells were grown on glass cover slips in Dulbecco's minimum essential medium (DMEM) containing GlutaMAX-1 and used for experimentation when approximately 70% confluent. When ready for use cover slips

were transferred to petri dishes containing 1 ml of media and the required concentration of tetramethyl rhodamine methyl ester (TMRM). HeLa cells were left to load in the dark at 37^{o}C for 15 minutes and then re-suspended in extracellular solution (135mM NaCl, 6mM KCl, 10mM HEPES, 1mM $MgCl_2$, 2mM $CaCl_2$ pH 7.4) ready for experimentation. Experiments were conducted at 34^{o}C and cells were perfused with extracellular solution throughout. TMRM-loaded HeLa cells were continuously excited with light at 546nm and data was sampled at 0.5Hz. All HeLa cells in the field of view were marked for analysis and the time to global mitochondrial depolarization (a surrogate for mPTP opening), as indicated by peak fluorescence increase, was recorded. Figure 4.1 depicts the fluorescent changes observed over time in representative TMRM-loaded HeLa cells exposed to photodamage. Initially TMRM-loaded cells displayed relatively low level fluorescence and this was due to autoquenching of the dye which was spatially localized to the mitochondria (figure 4.1A). Continuous illumination of TMRM caused the generation of free radicals which induced mPTP opening (Hausenloy *et al.*, 2004). Consequently mitochondrial depolarization occurred which resulted in the redistribution of TMRM to the cytosol where it dequenched producing an increase in fluorescence intensity (figure 4.1B). The time to peak fluorescence intensity was recorded and it was assumed that this represented global mPTP opening; all mitochondria were depolarized (figure 4.1C). Subsequent to global mPTP opening fluorescence intensity fell and this was believed to occur as a result of cell membrane rupture and dye loss (figure 4.1D).

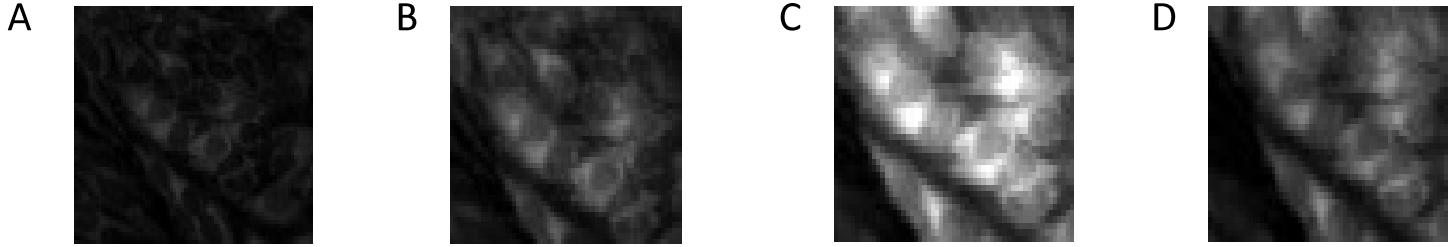

Figure 4.1: Representative time series showing fluorescent images of representative HeLa cells loaded with TMRM and subjected to photodamage over time.

HeLa cells were loaded with TMRM in the dark, at 37°C for 15 minutes. The cells were then re-suspended in fresh Tyrode solution prior to experimentation A: time = 0 minutes, TMRM fluorescence is relatively low due to TMRM localisation being confined to mitochondria, resulting in autoquenching of dye fluorescence. B: time = 6 minutes, TMRM fluorescence increases as mPTP begin to open and TMRM begins to diffuse into the cytosol. C: time = 12 minutes, HeLa cells exhibiting peak fluorescence indicating global mPTP openings. D: time = 17 minutes, following peak fluorescence TMRM fluorescence begins to decrease possibly due to cell membrane rupture and dye release. For analysis of time to mPTP opening, the time to state C was recorded.

The effects of photodamage-induced mPTP opening in TMRM-loaded HeLa cells was characterized using a range of concentrations from 100nM to 4µM TMRM and a concentration-dependent effect was observed. During the first three minutes of all experiments, non-photodamage (illumination at 0.1Hz as opposed to continuous illumination) of TMRM-loaded HeLa cells was instituted resulting in stable baseline fluorescence (figure 4.2). After three minutes, continuous illumination (photodamage) was started and it was found that the lowest concentration of TMRM (100nM) was insufficient to induce mPTP opening within the maximum time-frame of the experiment (30 minutes). 500nM was found to be the lowest concentration of TMRM required to induce mPTP opening and higher concentrations of TMRM correlated with shorter times to global mPTP opening (figure 4.3). A positive correlation was also observed between the concentration of TMRM and the peak fluorescence observed as a result of global mPTP opening. The higher the concentration of TMRM the greater was the fluorescence observed upon mPTP opening (figure 4.4).

In order to demonstrate that the mitochondrial depolarisation measured in this assay was a dependable reflection of mPTP opening, it was necessary to demonstrate that the time to mPTP opening could be influenced using the mPTP inhibitor cyclosporine A (CsA). To proceed with testing this hypothesis required choosing a concentration of TMRM for use in control experiments (untreated HeLa cells). The concentration chosen was 2µM as this was found to produce robust mPTP opening within a reasonable time. Furthermore, HeLa cells loaded with 2µM TMRM were found to have a stable baseline during the period of non-photodamage (first three minutes) and the fluorescent changes induced by mPTP opening were clearly identifiable (figure 4.2 & 4.5). CsA is believed to inhibit mPTP opening thorough inhibition of the activity of cyclophilin-D; a

core component of the mPTP. Thus treatment of HeLa cells with CsA would be expected to delay the time of photodamage-induced mPTP opening. To test this, HeLa cells were pre-treated with CsA (4μM) for 20 minutes prior to experimentation. Furthermore, CsA (4μM) was present throughout the experiment. Treatment of HeLa cells with CsA was found to delay the time to mPTP opening (figure 4.5). Under control conditions the time to photodamage-induced mPTP opening was found to be 13.2 ± 0.2 minutes compared to 14.2 ±0.2 minutes for CsA treated HeLa cells and this was found to be significantly different (* $p < 0.05$) (figure 4.6).

Having characterised this assay in HeLa cells, the effect of photodamage-induced mPTP opening was next investigated in TMRM-loaded isolated myocytes.

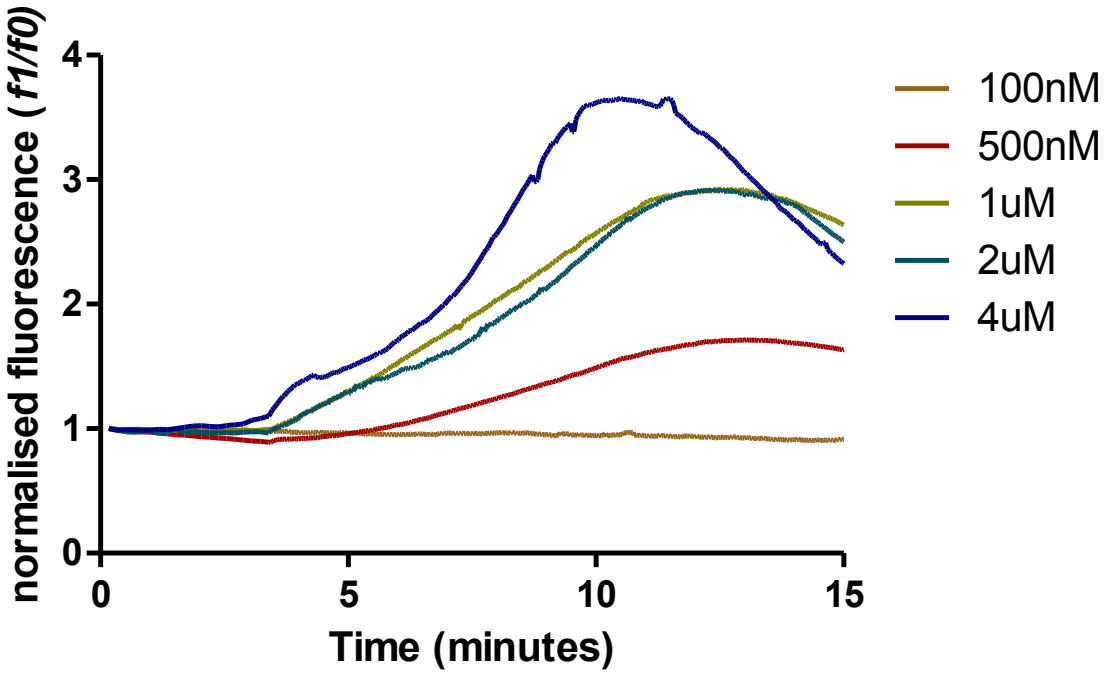

Figure 4.2: The effects of loading HeLa cells with increasing concentrations of TMRM on time to photodamage-induced mPTP opening.

TMRM-loaded HeLa cells were exposed to photodamage. During the first 3 minutes of the assay cells were not exposed to photodamage resulting in stable baseline fluorescence. Subsequently cells were subjected to photodamage (continuous illumination at 546nm) to induce mPTP opening, reflected by an increase in fluorescence intensity. The time to global mPTP opening was recorded.

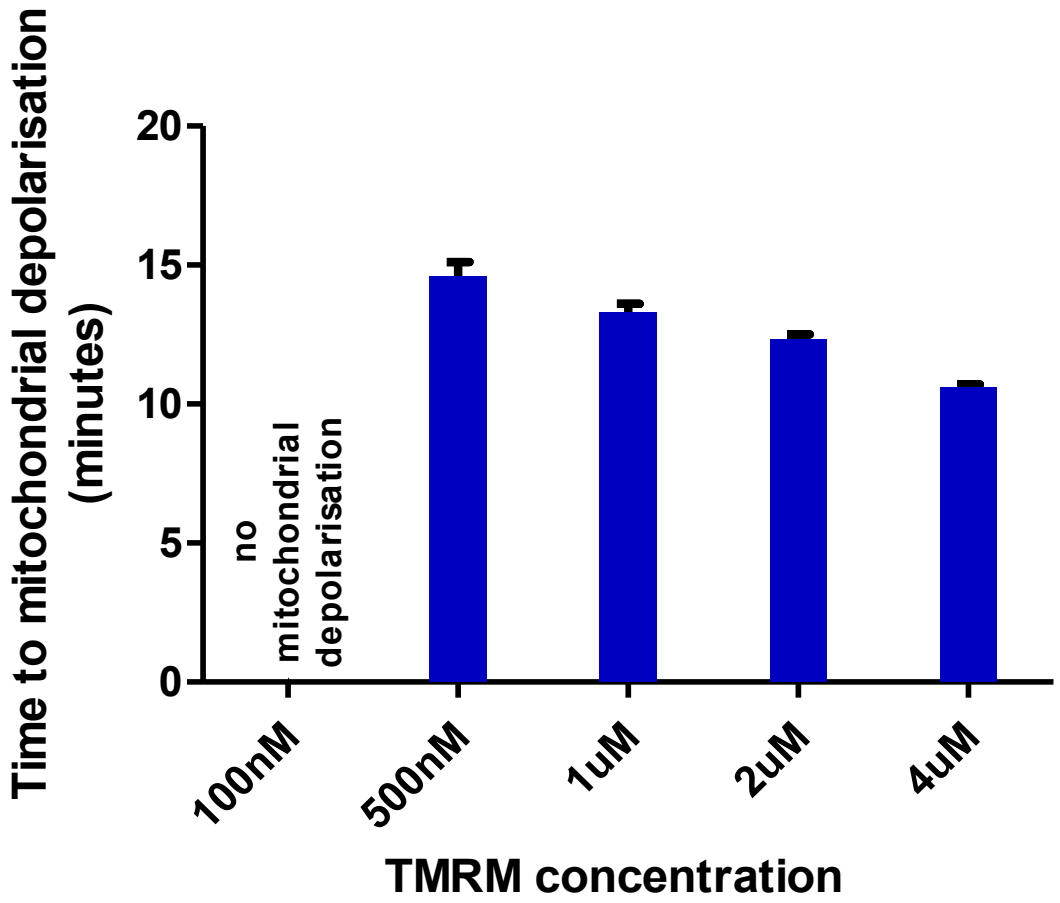

Figure 4.3: The effects of varying TMRM concentration on time to mean mPTP opening in HeLa cells subjected to photodamage.

The mean times to global mPTP opening in HeLa cells loaded with increasing concentrations of TMRM and subjected to photodamage-induced mPTP opening. At the lowest concentration of TMRM (100nM) no mPTP opening was observed within the maximum experimental period (30 minutes).

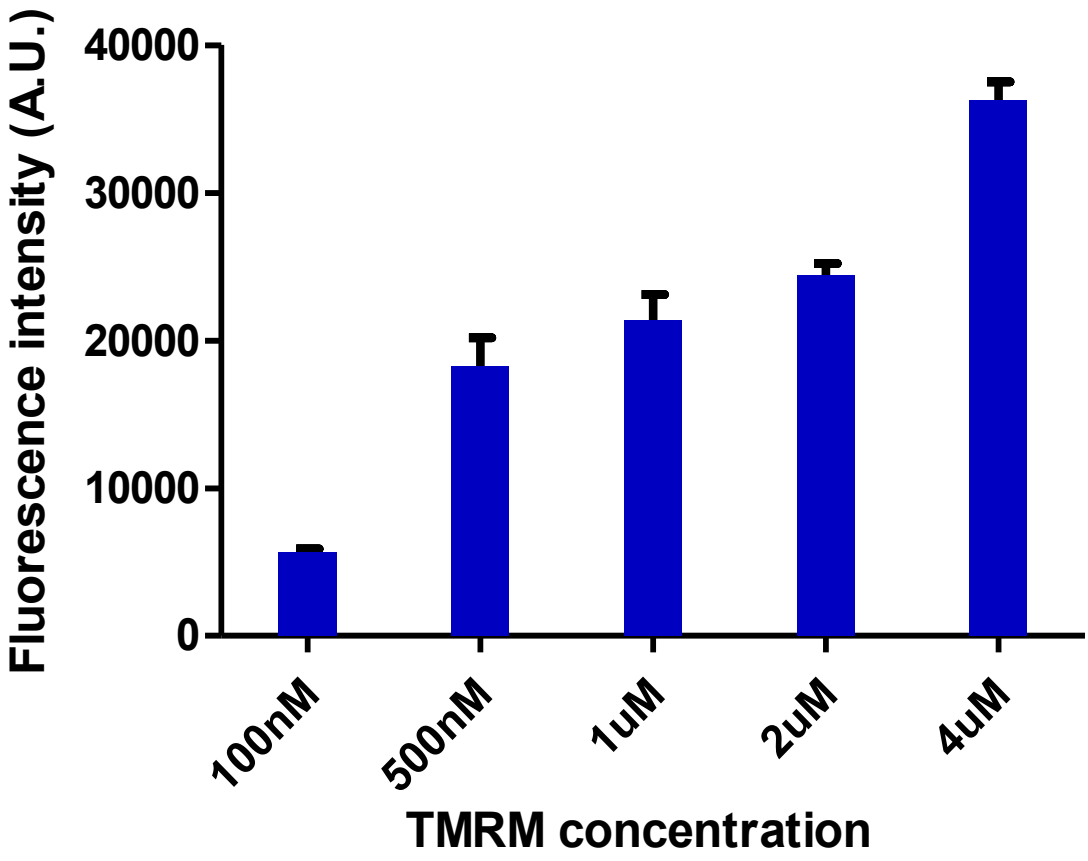

Figure 4.4: The effects of varying TMRM concentration on average peak fluorescence exhibited at global mPTP opening in HeLa cells subjected to photodamage.

A positive correlation was observed between the concentration of TMRM loaded and the peak fluorescence observed during global mPTP opening. The higher the concentration of TMRM, the greater the fluorescence intensity at global mPTP opening.

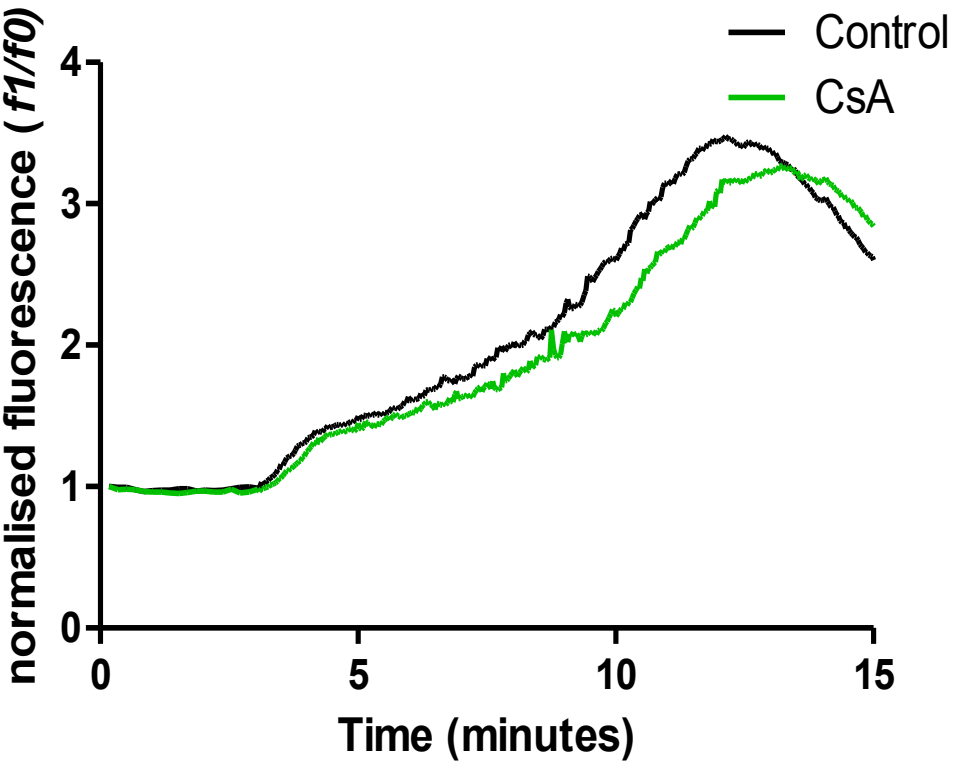

Figure 4.5: The effects of CsA (4µM) treatment of TMRM-loaded HeLa cells on time to photodamage-induced global mPTP opening.

Representative traces of control and CsA (4µM) treated TMRM-loaded (2µM) HeLa cells on time to global mPTP opening. CsA treated HeLa cells were pre-treated for 20 minutes before experimentation followed by experiments being conducted in the presence of CsA.

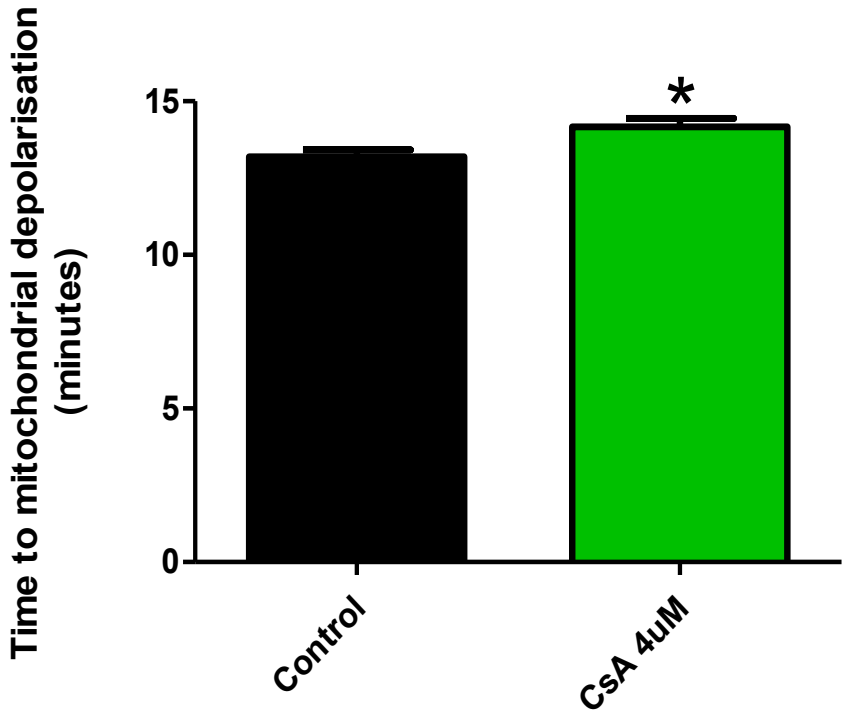

Figure 4.6: The effect of CsA (4µM) treatment of HeLa cells on time to mean photodamage-induced global mPTP opening.

TMRM-loaded (2µM) HeLa cells were continuously illuminated to induce global mPTP opening. Control 13.2 ± 0.2 minutes; CsA 14.2 ±0.2; minutes; * $p < 0.05$.

4.2.2 Characterizing the effects of photodamage-induced mPTP opening in isolated myocytes

To investigate the effects of photodamage-induced mPTP opening, freshly isolated ventricular myocytes in Tyrode solution were loaded with TMRM, in the dark at 37^{o}C for 15 minutes, and then re-suspended in fresh Tyrode solution. Figure 4.7 depicts the typical fluorescent changes exhibited by TMRM-loaded myocytes that are exposed to photodamage-induced mPTP opening. Initially cellular fluorescence is relatively low as the fluorescence of TMRM is autoquenched in the mitochondria (figure 4.7A). Continuous illumination (546nm) of TMRM-loaded myocytes caused the generation of free radicals which induced mPTP opening. This was reflected by the movement of TMRM from the mitochondria into the cytosol where the fluorescence exhibited was increased. mPTP opening was responsible for causing mitochondrial depolarization which forced the cationic TMRM molecules into the cytosol. Often this occurred firstly in a subsection of a myocyte and then gradually spread throughout the myocyte (figure 4.7B & C). Shortly after myocytes had undergone global mitochondrial depolarization (global mPTP opening) myocytes were found to hypercontract indicating ATP depletion and impaired cell health (figure 4.7D).

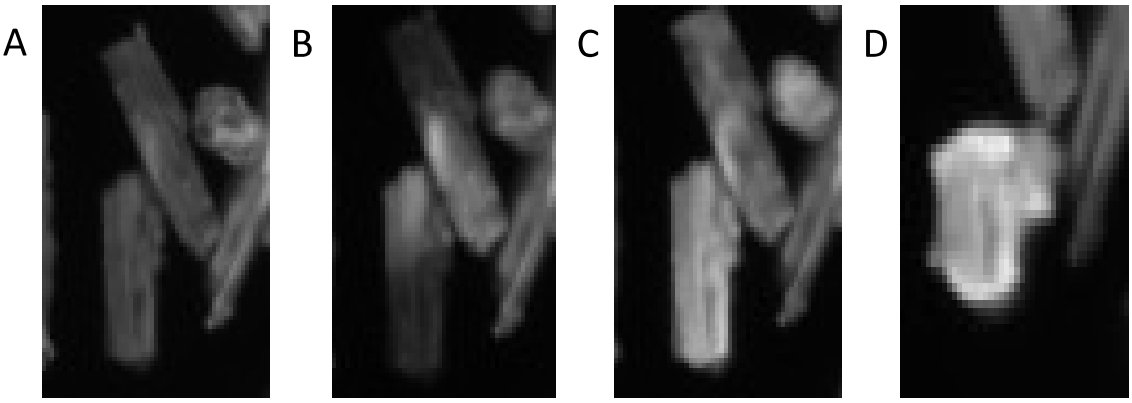

Figure 4.7: Time series showing fluorescent images of representative myocytes loaded with TMRM and subjected to photodamage.

A: time = 0 minutes, myocytes exhibiting relatively low level TMRM fluorescence due to localisation in mitochondria causing auto quenching of dye. B: time = 4.39 minutes, increased fluorescence in a subsection of myocytes indicating mitochondrial depolarisation which is caused by mPTP opening. C: time = 5.29 minutes, myocytes displaying peak fluorescence indicating global mPTP opening. D: time= 6.48 minutes, myocytes hypercontract implying ATP depletion and poor cell health.

The effects of loading myocytes with increasing concentrations of TMRM ranging from 125nM to 20µM were investigated on the time to photodamage-induced mPTP opening. As observed in HeLa cells a positive correlation existed between the concentration of TMRM loaded into myocytes and the time to mPTP opening. Higher concentrations of TMRM were found to shorten the time to photodamage-induced mPTP opening (figure 4.8). A concentration of 250nM TMRM was identified as the minimum concentration required to induce mPTP opening within a 30 minute experimental period. Using a concentration below this threshold resulted in no measurable changes in TMRM fluorescence, implying that no photodamage-induced mPTP opening had occurred. Increasing the concentration of TMRM appeared to linearly shorten the time to photodamage induced mPTP opening, although at the highest concentrations of TMRM used the effects appeared to reach a plateau (figure 4.9). The same was true for the peak fluorescence observed in TMRM loaded myocytes that had undergone mPTP opening. At the lower concentration of TMRM from 125nM to 2µM the peak fluorescence observed following mPTP opening was found to rise linearly with increasing concentrations of TMRM. However above 2µm TMRM the response was found to plateau (figure 4.10).

Having characterised the effects of loading myocytes with increasing concentrations of TMRM on time to photodamage-induced mPTP opening, it was necessary to demonstrate that the time to mPTP opening, for a given concentration of TMRM, could be manipulated using the mPTP inhibitor CsA (4µM). The concentration of TMRM used to investigate this was 5µM as this concentration resulted in robust mPTP opening within a reasonable time. Figure 4.11 depicts the typical fluorescent changes exhibited in representative control and CsA (4µM) treated myocytes that were exposed to

photodamage-induced mPTP opening. The peak of the fluorescence increase was taken to be global, irreversible mPTP opening (figure 4.11 see arrows). Shortly after this event had occurred the myocytes shortened into a hypercontracted state, followed by a loss of fluorescence intensity.

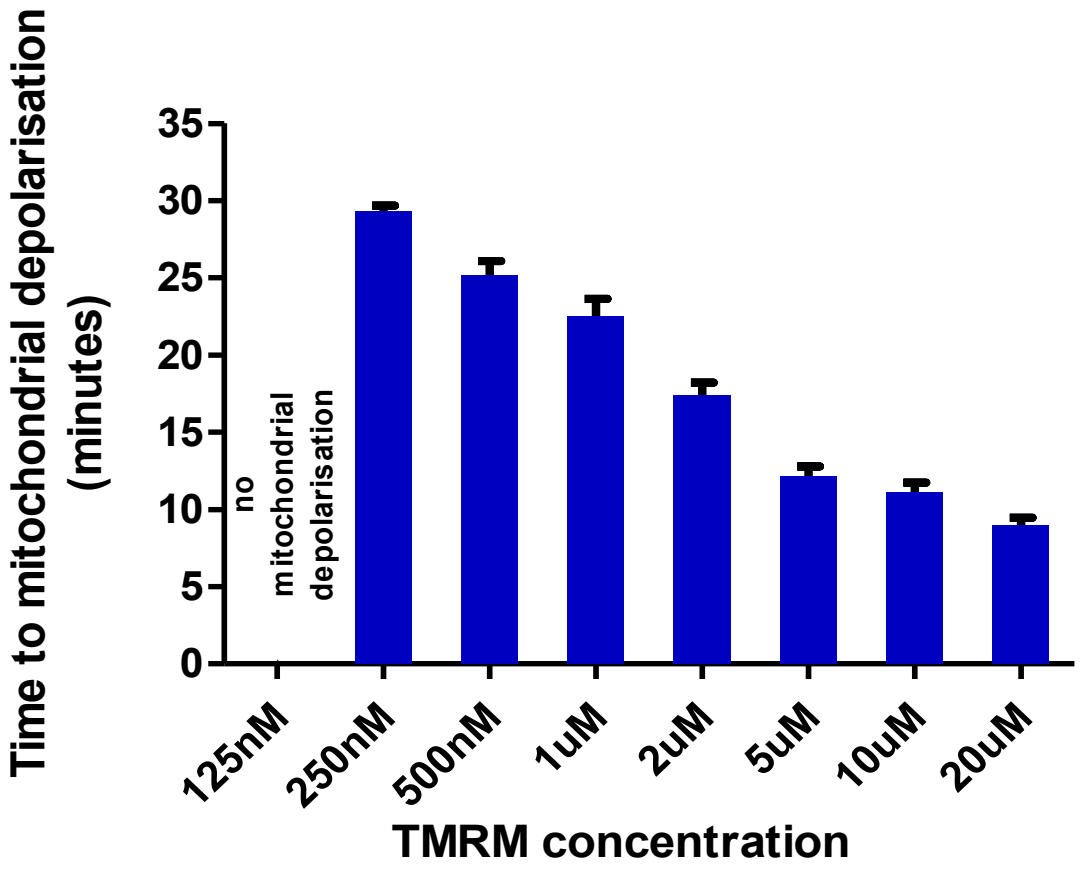

Figure 4.8: The effects of loading isolated myocytes with increasing concentrations of TMRM on the time to average mPTP opening.

Myocytes were loaded with TMRM in the dark at 37°C for 15 minutes. The myocytes were then re-suspended in fresh Tyrode and subjected to photodamage (continuous illumination at 546nm) and the time to global mPTP opening was recorded.

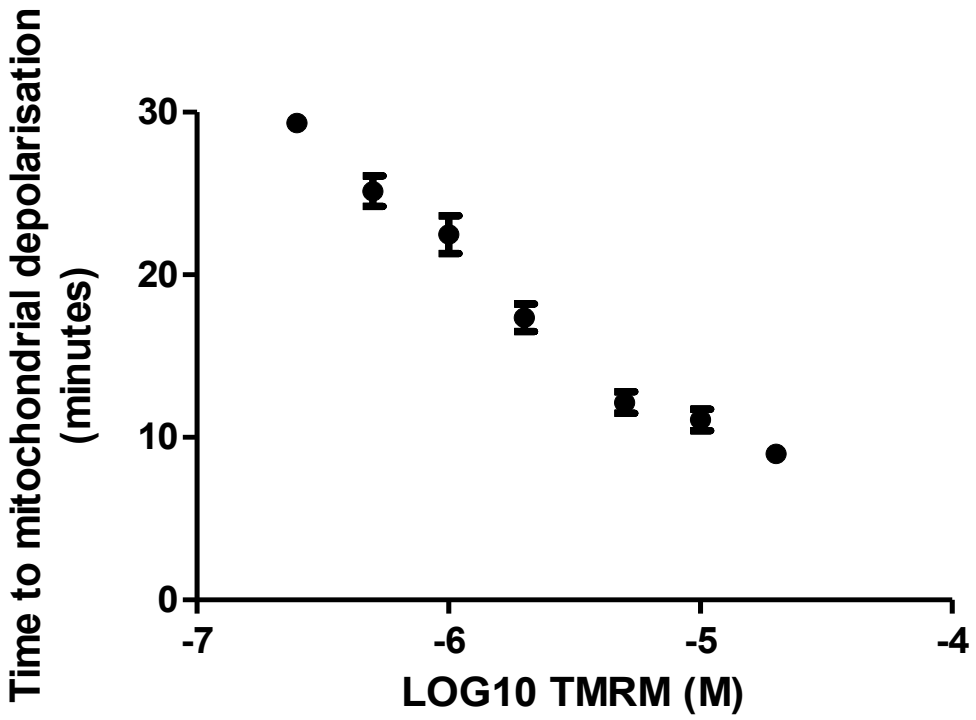

Figure 4.9: TMRM concentration response curve.

Graph showing the relationship between TMRM concentration and time to global mPTP opening (mitochondrial depolarisation). The concentration of TMRM chosen for mPTP experiments was 5µM.

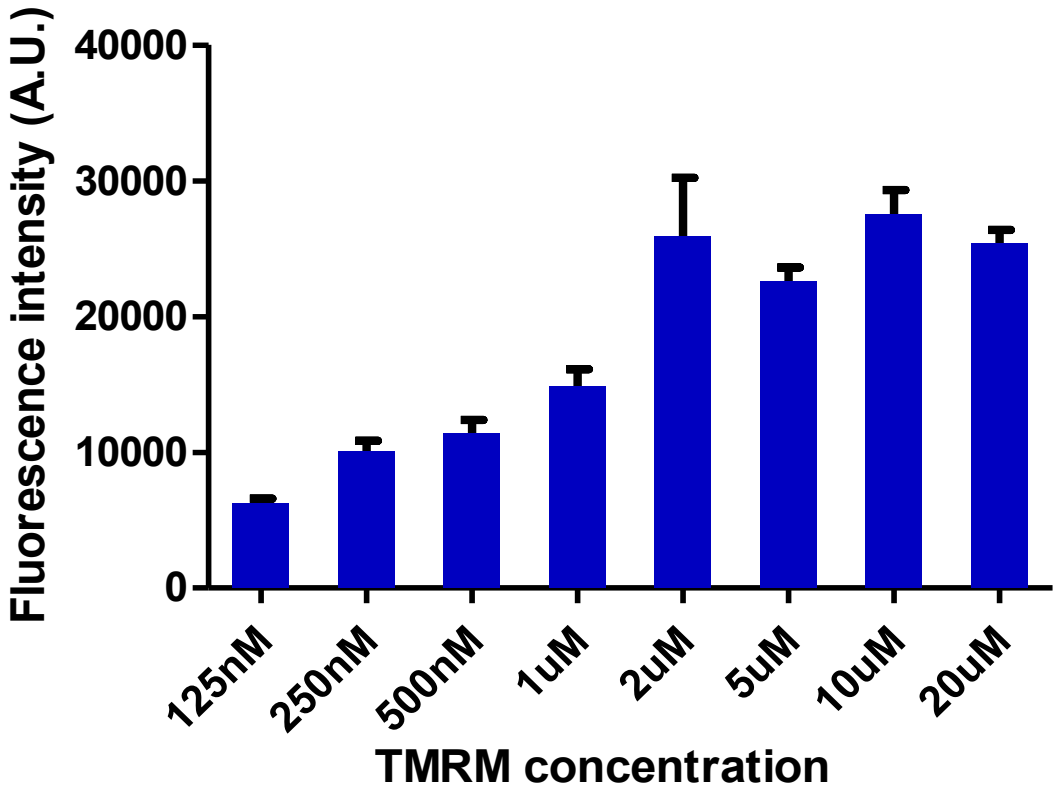

Figure 4.10: The effect of varying TMRM concentration on peak fluorescence exhibited upon global mPTP opening.

Myocytes were loaded with TMRM in the dark at 37°C for 15 minutes. The myocytes were then re-suspended in Tyrode solution and subjected to photodamage. The peak fluorescence exhibited upon global mPTP opening was recorded. As global mPTP opening did not occur using 125nM the peak fluorescence exhibited during the 30 minute experimental period was recorded.

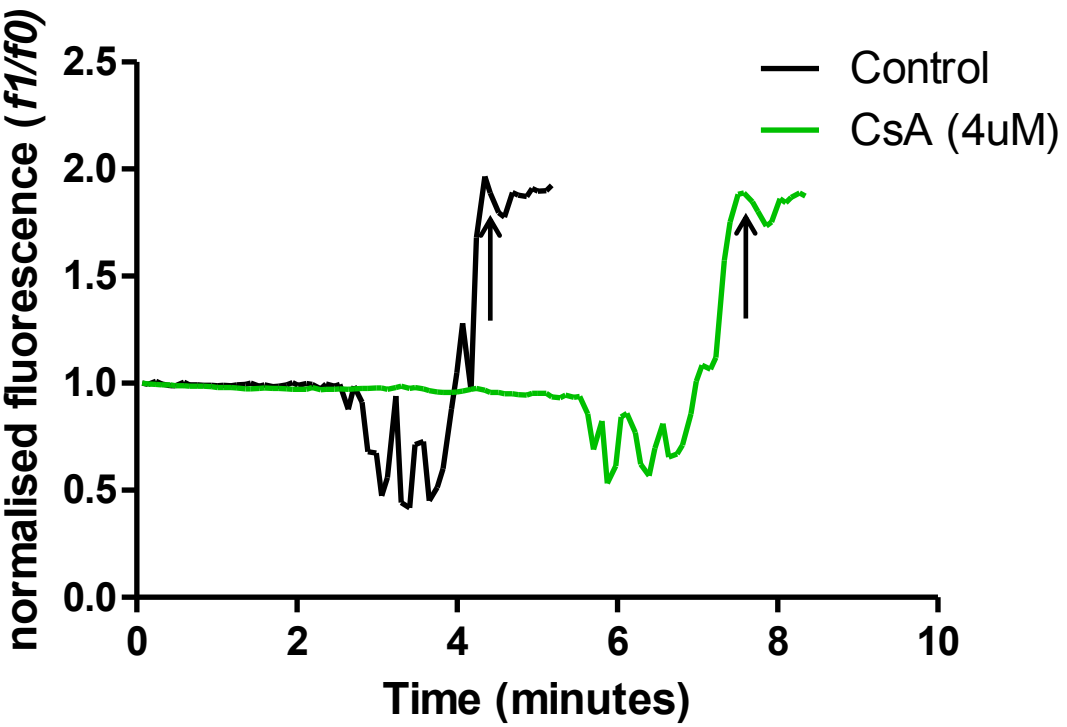

Figure 4.11: Representative traces showing the effects of CsA (4µM) treatment on time to mPTP opening in myocytes loaded with TMRM (5µM).

Pre-treatment of myocytes (15 minutes) with CsA (4µM) followed by experimenting in the presence of CsA significantly delayed the time to photodamage-induced mPTP opening. The times to peak (see arrows) were recorded and compared between groups.

Interestingly, immediately prior to global mPTP opening, both control and CsA treated myocytes often displayed a transient decrease in fluorescence followed by a large increase in fluorescence which exceeded baseline fluorescence. The cause of this transient dip in fluorescence is unknown but may be due to mitochondrial hyperpolarization prior to mPTP opening. The mean time to global mPTP opening for control myocytes was found to be 4.9 ± 0.2 minutes (n = 152, 16, 3) compared to 6.5 ± 0.3 minutes (n = 162, 18, 3) for CsA treated myocytes (figure 4.12). This was found to be significantly different ($p < 0.0001$). CsA is believed to inhibit mPTP opening through inhibiting the activity of cyclophilin-D (CyP-D), a pore forming component of the mPTP. However, CsA is also known to inhibit calcineurin at higher concentrations. Thus in order to investigate that the observed effect of CsA on delaying mPTP opening was not due to an off target effect on calcineurin, the effects of treating myocytes with FK-506 (1µM) a calcineurin inhibitor were investigated. TMRM-loaded myocytes were pre-treated with FK-506 for 10 minutes prior to experimentation followed by continuous perfusion of FK-506 (1µM in Tyrode) throughout the experiment. In these experiments the mean time to global mPTP opening for control myocytes was 1.88 ± 0.17 minutes (n = 42, 4, 3) compared to 1.87 ± 0.16 (n = 44, 4, 3) for FK-506 treated myocytes (figure 4.13). These times were found to be not significantly different.

To further increase confidence that this assay measured mPTP opening in response to photodamage, the effects of treating myocytes with carboxyatractyloside (7.5µM) an mPTP opener were investigated. TMRM-loaded myocytes were pre-treated with carboxyatractyloside for 10 minutes prior to experimentation followed by continuous perfusion of carboxyatractyloside (7.5µM in Tyrode) throughout the experiment. In control experiments (no treatment of TMRM-loaded (5µM) myocytes) the mean time

to global mPTP opening was found to be 3.24 ± 0.22 minutes (n = 63, 6, 3) compared to 2.26 ± 0.17 minutes (n = 51, 6, 3) for carboxyatractyloside treated myocytes (figure 4.14). These times were found to be significantly different ($p < 0.001$).

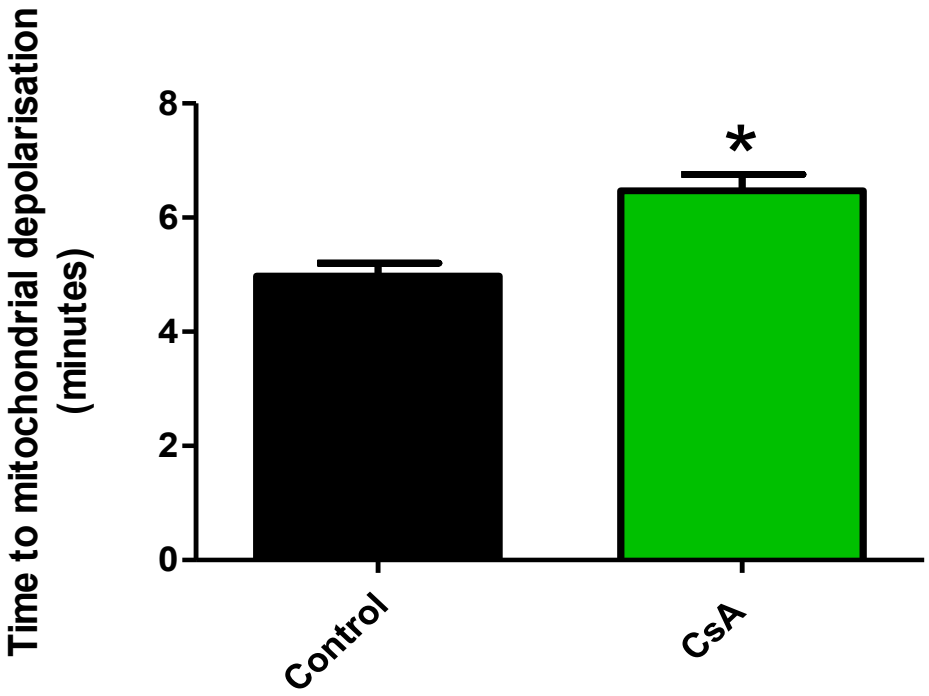

Figure 4.12: The effects of CsA (4µM) treatment on time to mean mPTP opening in myocytes loaded with TMRM (5µM).

Pre-treatment of myocytes (15 minutes) with CsA (4µM) followed by experimenting in the presence of CsA significantly delayed the time to photodamage-induced mPTP opening. Control 4.9 ± 0.2 minutes n = 152, 16, 3; CsA 6.5 ± 0.3 minutes n = 162, 18, 3; * $p < 0.0001$.

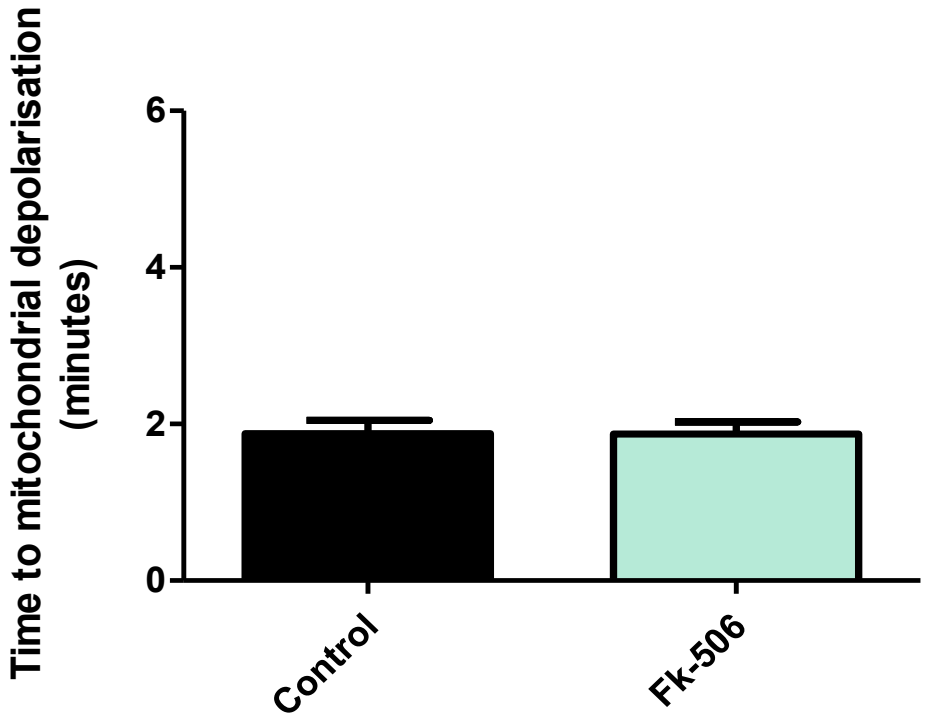

Figure 4.13: The effect of FK-506 (1µM) treatment on time to mean mPTP opening in myocytes loaded with TMRM (5µM).

FK-506, a calcineurin inhibitor, was used as a control to confirm that the effect of CsA on delay of mPTP opening were not as a result of calcineurin inhibition; a known secondary effect of CsA at higher concentrations. Control 1.88 ± 0.17 minutes n = 42, 4, 3; FK-506 1.87 ± 0.16 minutes n = 44, 4, 3.

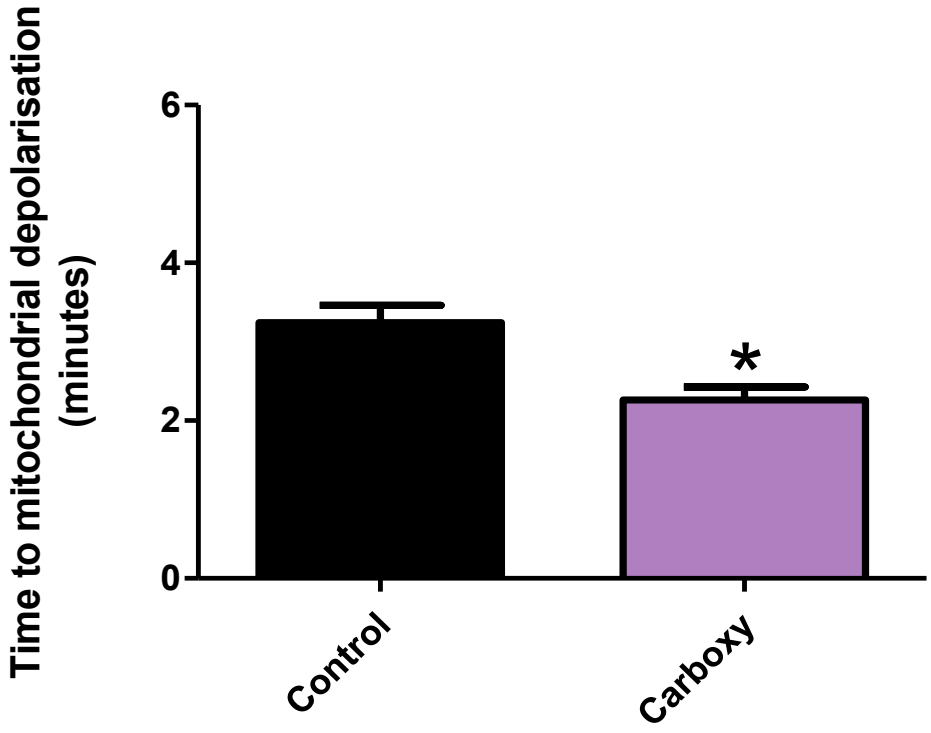

Figure 4.14: The effect of carboxyatractyloside (7.5µM) treatment on time to mean mPTP opening in myocytes loaded with TMRM (5µM).

Carboxyatractyloside (Carboxy) is a known mPTP opener; hence this compound was used to confirm that mitochondrial depolarisation was a true reflection of mPTP opening. Control 3.24 ± 0.22 minutes n = 63, 6, 3; Carboxy 2.26 ± 0.17 minutes n = 51, 6, 3; * $p < 0.001$.

4.2.3 Temperature preconditioning of isolated myocytes significantly delays photodamage-induced pathological mPTP opening

Following characterisation of the photodamage-induced mPTP opening assay in isolated myocytes, I was confident that mitochondrial depolarisation as measured in this assay was a reliable reflection of mPTP opening. I therefore went on to investigate the effects of TP on time to mPTP opening.

TMRM (5µM) loaded myocytes were temperature preconditioned (16^{o}C) prior to experimentation (see material and methods fro protocol). TP of myocytes (5.07 ± 0.53 minutes n = 44, 5, 3) was found to significantly ($p < 0.0005$) delay the time to photodamage-induced mPTP opening compared to controls (3.02 ± 0.25 minutes n = 49, 5, 3) (figure 4.15). The effects of DNP (50µM) preconditioning of myocytes on photodamage-induced mPTP opening were also investigated. TMRM loaded myocytes were treated with DNP (50µM) for 10 minutes followed by wash in Tyrode solution. DNP preconditioning of myocytes (8.3 ± 0.8 minutes n = 61, 6, 3) was also found to significantly ($p < 0.0001$) delay the mean time to mPTP opening compared to controls (3.5 ± 0.2 n = 89, 7, 3) (figure 4.16).

Figure 4.17 summarises the results of the characterisation experiments conducted using pharmacological agents to manipulate mPTP opening, along with the effects of TP and DNP preconditioning on mPTP opening. Interestingly, both TP (1.7 fold increase) and DNP preconditioning (2.5 fold increase) resulted in greater delays to mPTP opening compared to the known mPTP inhibitor CsA (1.4 fold increase), relative to same day controls.

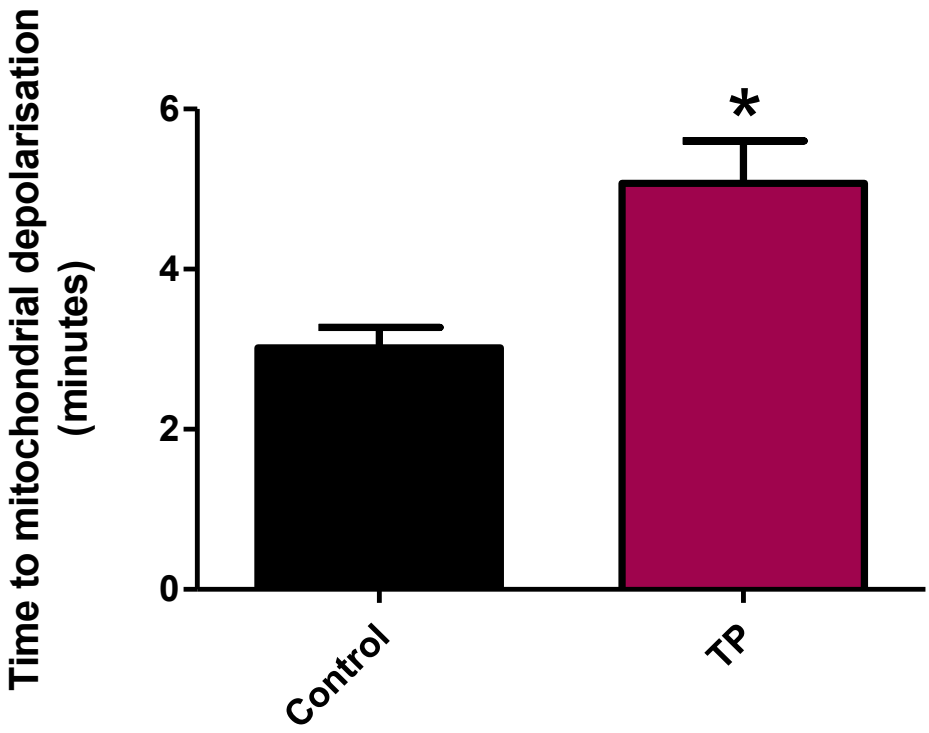

Figure 4.15: The effect of temperature preconditioning 16°C on time to mean mPTP opening in myocytes loaded with TMRM (5µM).

Temperature preconditioning (TP) myocytes prior to experimentation significantly delayed the time to photodamage-induced irreversible mPTP opening. Control 3.02 ± 0.25 minutes n = 49, 5, 3; TP 5.07 ± 0.53 minutes n = 44, 5, 3; * $p < 0.0005$.

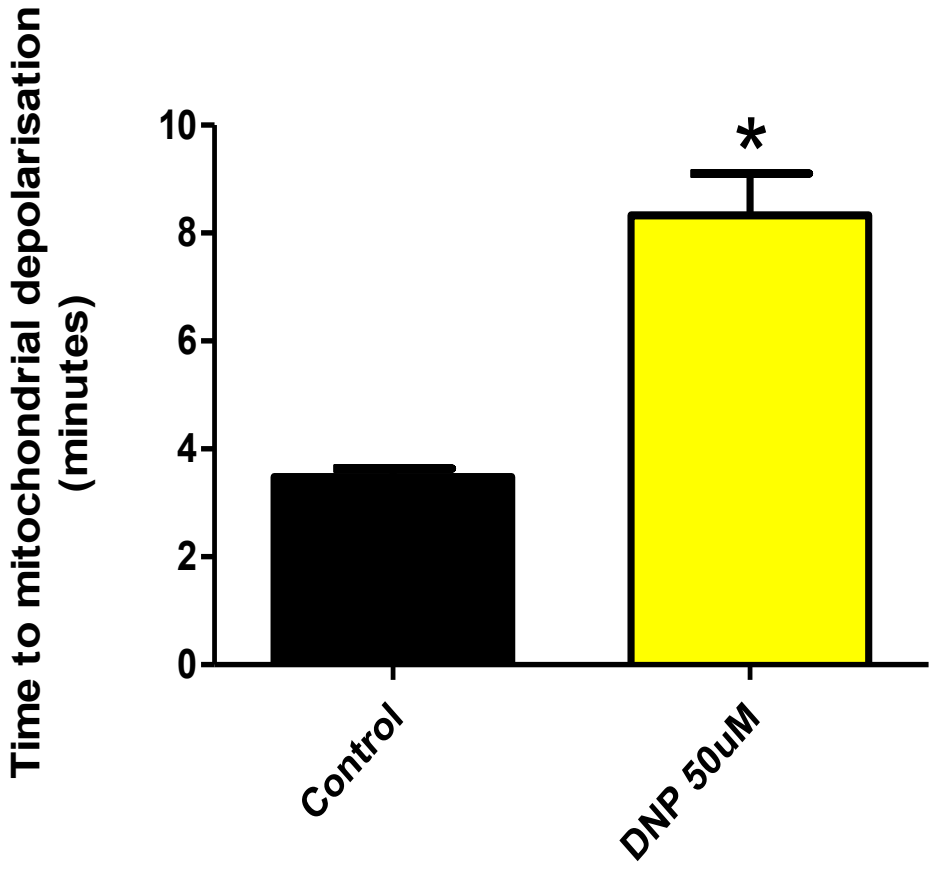

Figure 4.16: The effect of DNP (50µM) preconditioning on time to mean mPTP opening in myocytes loaded with TMRM (5µM).

Prior to experimentation myocytes were DNP preconditioned for 5 minutes followed by a wash in Tyrode solution. DNP preconditioning significantly delayed the time to irreversible mPTP opening Control 3.5 ± 0.2 minutes n = 89, 7, 3; DNP 8.3 ± 0.8 minutes n = 61, 6, 3; * $p < 0.0001$.

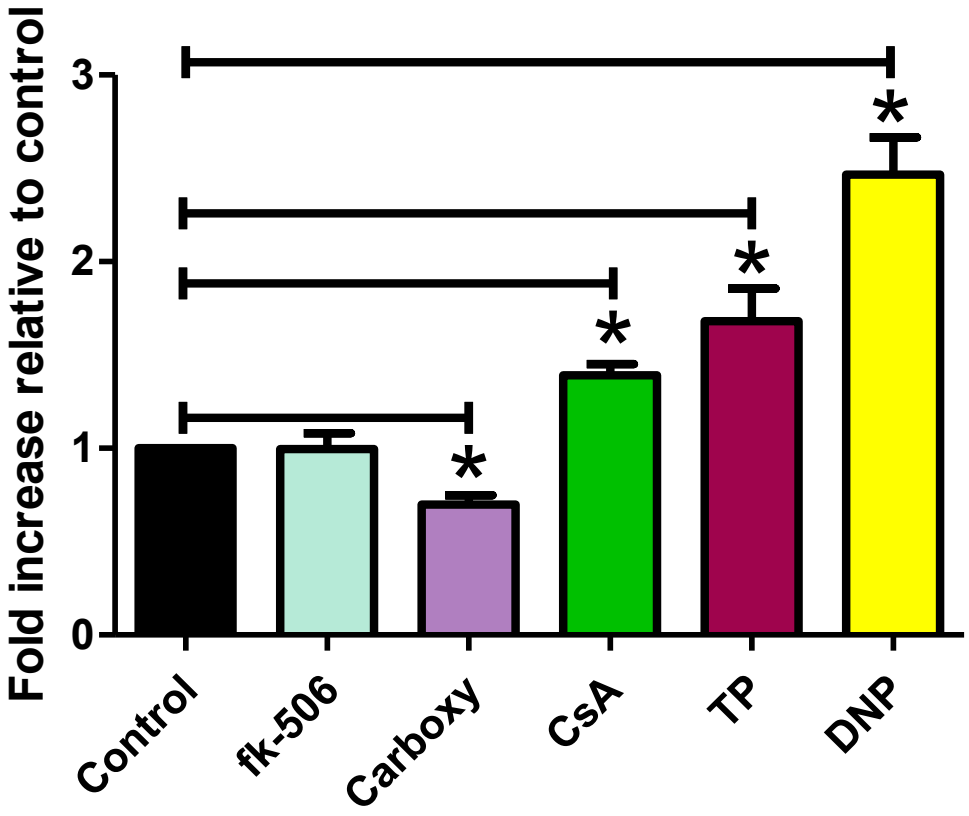

Figure 4.17: Summary of the effects of, FK-506 (1µM), Carboxyatractyloside (7µM), CsA (4µM); temperature preconditioning (16°C) and DNP (50µM) on time to mean mPTP opening normalised to same day matched controls.

The time to irreversible TMRM fluorescence increase was measured in control myocytes (n = 353) and was compared to the time taken in myocytes that were treated with fk-506 (1µM) n = 44; carboxyatractyloside an mPTP opener, (Carboxy, 7.5 µM) n = 51; cyclosporine A, an mPTP blocker (CsA, 4µM) n = 162; temperature preconditioning (TP) n = 126 and dinitrophenol preconditioning (DNP, 50µM) n = 90. At least three hearts were tested for each treatment. Data was analysed by a 1-way ANOVA; * $p < 0.005$ vs. control.

4.2.4 Temperature preconditioning of isolated myocytes significantly delays xanthine and xanthine oxidase-induced pathological mPTP opening

In the above section the effects of photodamage-induced mPTP opening were thoroughly characterised in isolated myocytes and the assay was proven to be one in which, mitochondrial depolarization is a dependable reflection of mPTP opening. However, an accepted limitation of the above mentioned assay is the use of TMRM to both provoke, by way of photo-induced ROS production and detect mPTP opening. Thus a second model of mPTP opening was developed in which a relatively low concentration of TMRM (50nM) was used solely to measure mPTP opening. In this assay the ROS required to induce mPTP opening was produced by the reaction of xanthine with xanthine oxidase. TMRM (50nM) loaded myocytes were sampled at a rate of 0.2Hz while being superfused with Tyrode solution containing xanthine (2mM) and xanthine oxidase (20mU) to induce mPTP opening. The low rate of sampling (0.2Hz) was intended to minimize the production of ROS by photo-excited TMRM molecules (photodamage). Furthermore, a relatively low concentration of TMRM (50nM) was used in this assay compared to the photodamage-induced mPTP opening assay (5µM) and this served as a second way to reduce photodamage-induced ROS production.

TMRM selectively accumulates in mitochondria due to the mitochondrial membrane potential. This results in punctate fluorescence of TMRM (50nM) loaded myocytes with bright bands running longitudinally throughout myocytes (figure 4.18Ai). This differs from the photodamage-induced mPTP opening assay described above, in which the

high concentration of TMRM (5µM) used causes fluorescence to be quenched within the mitochondria resulting in a diffuse fluorescent signal. In the absence of xanthine and xanthine oxidase, no photodamage-induced mPTP opening was observed in TMRM (50nM) loaded myocytes that were sampled at a frequency of 0.2Hz (figure 4.18B). This is reflected by the maintenance of a punctate fluorescent pattern after a 35 minute sampling period (figure 4.18Aii), during which time no decrease in the fluorescent signal was observed (figure 4.18B).

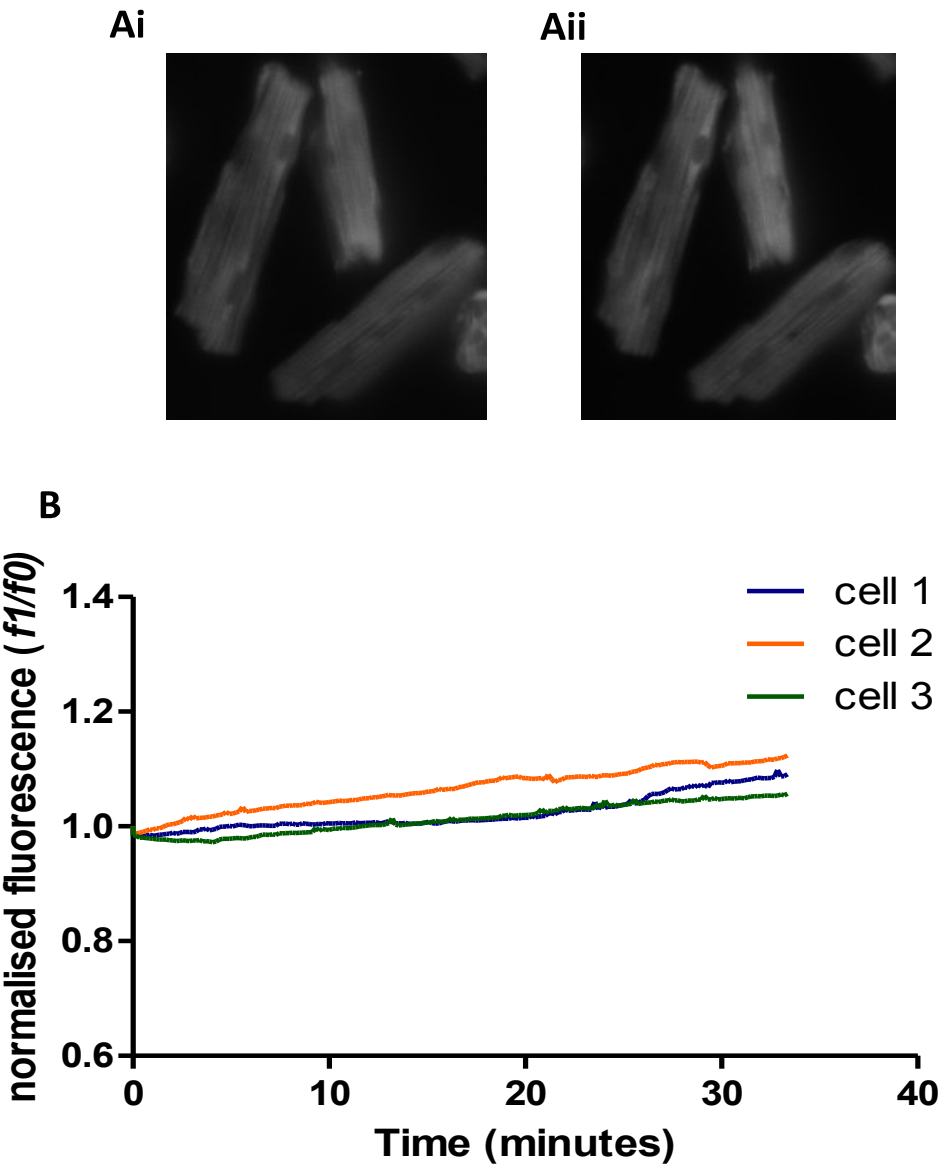

Figure 4.18: Low dose TMRM (50nM) does not cause photodamage-induced mPTP opening.

Myocytes were loaded with TMRM (50nM) and illuminated for 25ms every 5 seconds to observe the effects on mPTP opening. Ai: Time = 0 minutes, TMRM (50nM) loaded control myocytes displaying relatively high, mitochondrially localised fluorescence. Aii: There was no significant change in TMRM fluorescence after 35 minute of data acquisition; indicating that irreversible mPTP opening had not occurred. B: Representative traces from three TMRM-loaded (50nM) control myocytes. Under these experimental conditions TMRM fluorescence remains relatively stable over a 30 minute period.

When TMRM (50nM) loaded myocytes were challenged with xanthine and xanthine oxidase a significant decrease in myocyte fluorescence was observed which indicated mPTP opening (figure 4.19A & B). Xanthine and xanthine oxidase-induced mPTP opening caused a sudden decrease in the fluorescent signal which was caused by the movement of TMRM from the mitochondria into the cytosol. This movement was driven by mitochondrial depolarization which is a consequence of mPTP opening. Subsequent to mPTP opening cellular TMRM fluorescence rapidly declined and this followed shortly after by myocyte hypercontracture. mPTP opening was recorded and taken to be the time at which TMRM fluorescence initially began to decline (figure 4.19B see arrows). For control myocytes (TMRM loaded myocytes challenged with xanthine and xanthine oxidase) the mean time to xanthine and xanthine oxidase-induced mPTP opening was found to be 10.3 ± 0.9 minutes (n = 17, 4, 3). To confirm this assay as one which measures mPTP opening, experiments were conducted in the presence of CsA (mPTP inhibitor) and this was found to significantly ($p < 0.05$) delay the mean time to mPTP opening (15.5 ± 1.1 minutes, n = 14, 4, 3). Next, the effects of TP (15.1 ± 1.3 minutes, n = 18, 4, 3) was investigated and this too was found to significantly ($p < 0.05$) delay the time to xanthine and xanthine oxidase-induced mPTP opening (figure 4.20).

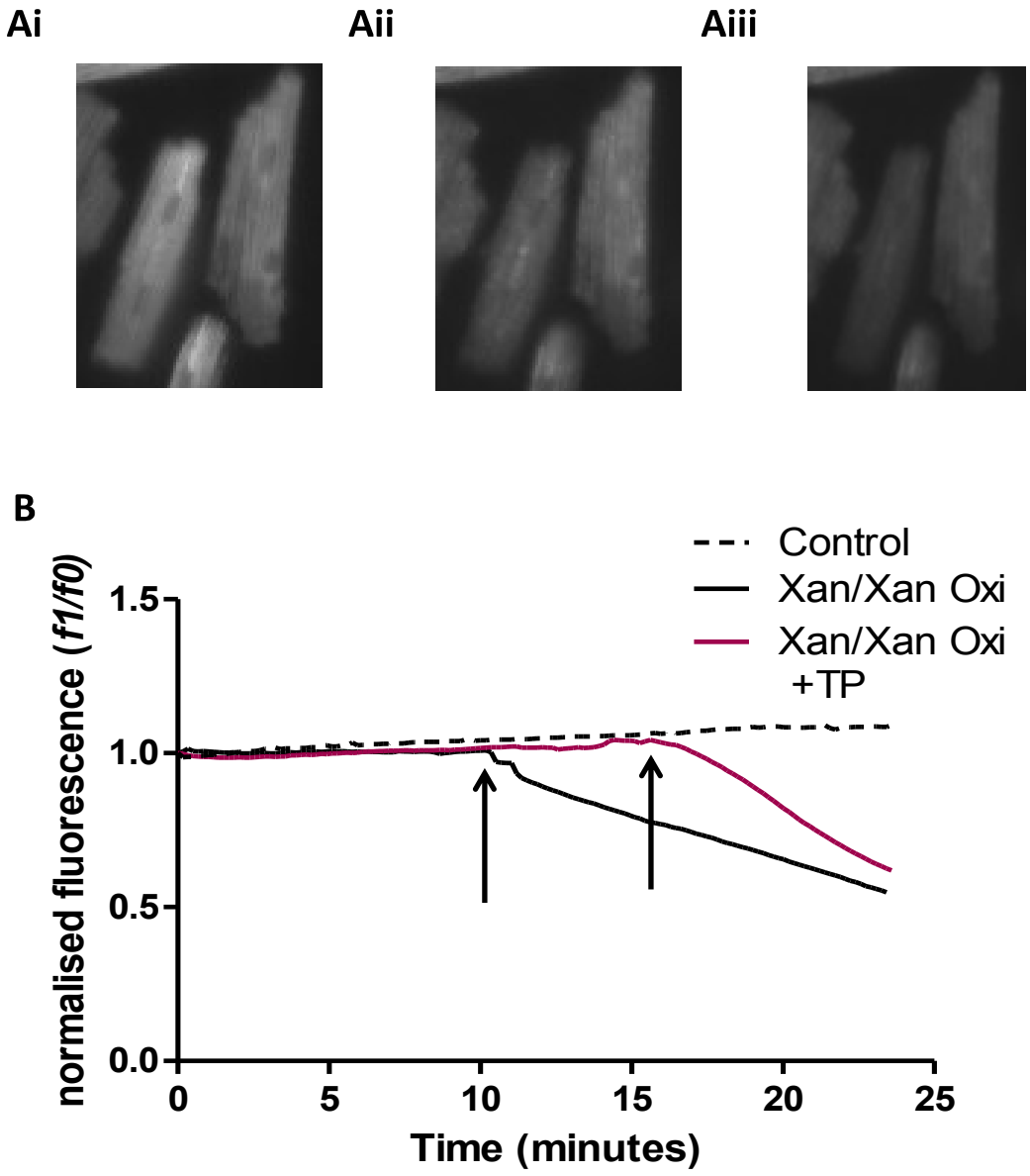

Figure 4.19: Temperature preconditioning delays xanthine/xanthine oxidase-induced mPTP opening.

TMRM (50nM) loaded myocytes were challenged with xanthine/xanthine oxidase (exogenous ROS donor) to induce mPTP opening. Ai: Time = 0 minutes, myocytes displaying relatively high, mitochondrially localized fluorescence. Aii: Time = 10 minutes, the onset of mPTP opening (mitochondrial depolarisation) which causes redistribution of TMRM from the mitochondria to the cytosol, resulting in a decrease in cellular fluorescence. The time to onset of mPTP opening was recorded and analysed between treatment groups. Aiii: Time = 15 minutes, global mPTP opening causes a loss of mitochondrial TMRM fluorescence. B: Representative traces from control (unchallenged myocytes), control myocytes challenged with xanthine (Xan) and xanthine oxidase (Xan Oxi) and temperature preconditioned (TP) myocytes challenged with xanthine (Xan) and xanthine oxidase (Xan Oxi).

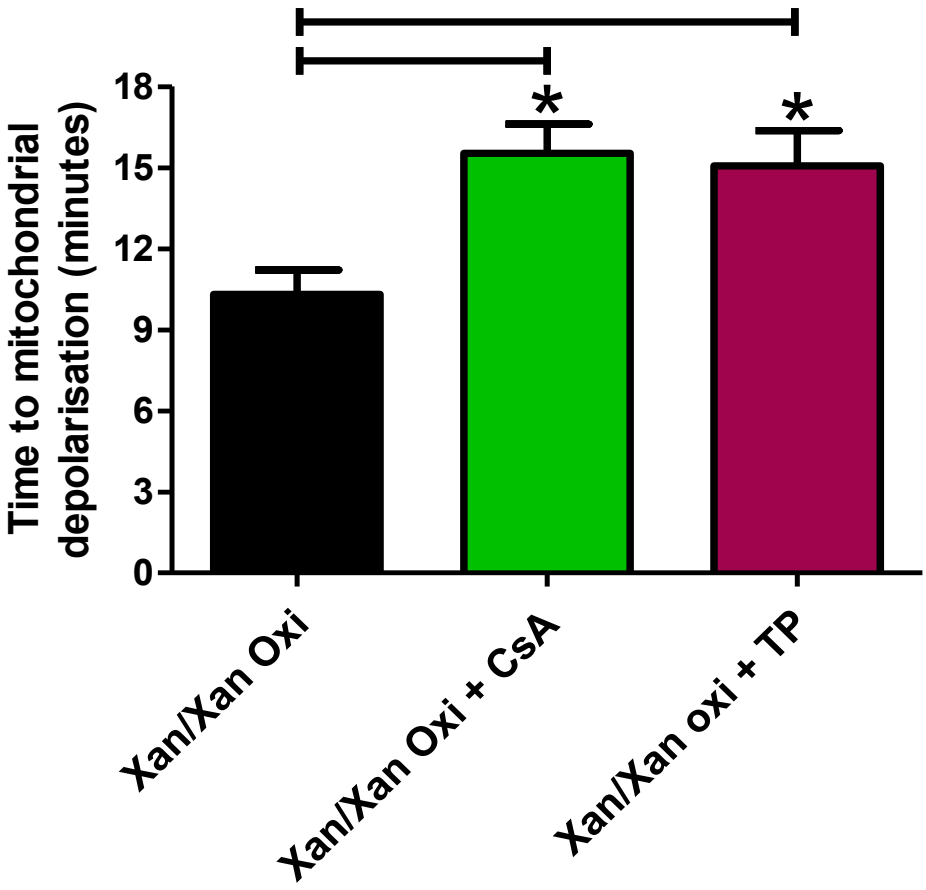

Figure 20: CsA (4µM) and temperature preconditioning (16^{o}C) delay the mean time to xanthine/xanthine oxidase-induced mPTP opening.

Mean times to onset of mPTP opening for control myocytes n = 17, 4, 3; cyclosporine A (CsA, 4µM) treated myocytes n = 14, 4, 3; and temperature preconditioned (TP) myocytes n = 18, 4, 3. Data was analysed by a 1-way ANOVA; * = $p < 0.05$.

4.2.5 Temperature preconditioning of isolated myocytes does not alter non-pathological mPTP flickering

It has been demonstrated that non pathological, transient mPTP flickering occurs under basal conditions and it has been suggested that this may serve as a way to regulate mitochondrial ROS and Ca^{2+} concentration within myocytes (Huser & Blatter, 1999). The effects of TP on non-pathological mPTP flickering was investigated using a well established calcein-cobalt quench assay (Hausenloy *et al.*, 2010; Petronilli *et al.*, 1999; Saotome *et al.*, 2009). Isolated ventricular myocytes were co-loaded with calcein-AM (1μM) and cobalt chloride (1mM). Calcein-AM readily crosses cell and mitochondrial membranes whilst cobalt chloride is spatially restricted to the cytosol. Cobalt chloride binds to and quenches cytosolic calcein fluorescence to reveal a punctuate pattern of mitochondrial calcein fluorescence. Over time, due to basal mPTP flickering, calcein is released from mitochondria into the cytosol where its fluorescence is quenched by cobalt chloride. This is observed as a change in the fluorescence pattern from a mitochondrial punctuate pattern (figure 4.21Ai) to a decreased and diffuse fluorescence pattern (figure 4.21Aii). Over the course of a 30 minute experiment three images of cellular fluorescence where captured at times 0, 10 and 30 minutes (figure 4.21B). The frequency of sampling was intentionally low in order to minimize photodamage-induced oxidative stress on the cells. For each time-point, the mean calcein fluorescence was expressed as a percentage of the initial value.

In control myocytes loaded with calcein and cobalt chloride, it was found that mean calcein fluorescence fell to 80.2 ± 7.2% (n = 13, 3, 3) of initial fluorescence after a 30 minute experimental period. To show that the loss of calcein fluorescence was mPTP flickering-dependent, experiments were conducted in the presence of the mPTP

inhibitor CsA (4μM). Under these conditions mean calcein fluorescence was found to be 101.2 ± 2.9% (n = 11, 3, 3) of initial fluorescence at the end of a 30 minute experimental period. This indicated that mPTP flickering was responsible for the loss of calcein fluorescence and proved the efficacy of this assay as one which measured non-pathological mPTP flickering. The effects of TP on basal mPTP flickering were next investigated. Myocytes were temperature preconditioned (16^{o}C) and then subsequently used for experimentation to assess if TP blocked basal mPTP flickering. It was found that mean calcein fluorescence fell to 78.8 ± 4.1% (n= 12, 3, 3) of initial fluorescence in temperature preconditioned myocytes which was not dissimilar to the fluorescent changes observed in control myocytes (figure 4.21B). The mean fluorescent changes recorded at 30 minutes for CsA and temperature preconditioned myocytes were compared to controls (figure 4.22). CsA, but not TP, was found to significantly ($p < 0.05$) retain calcein fluorescence over a 30 minute period compared to controls.

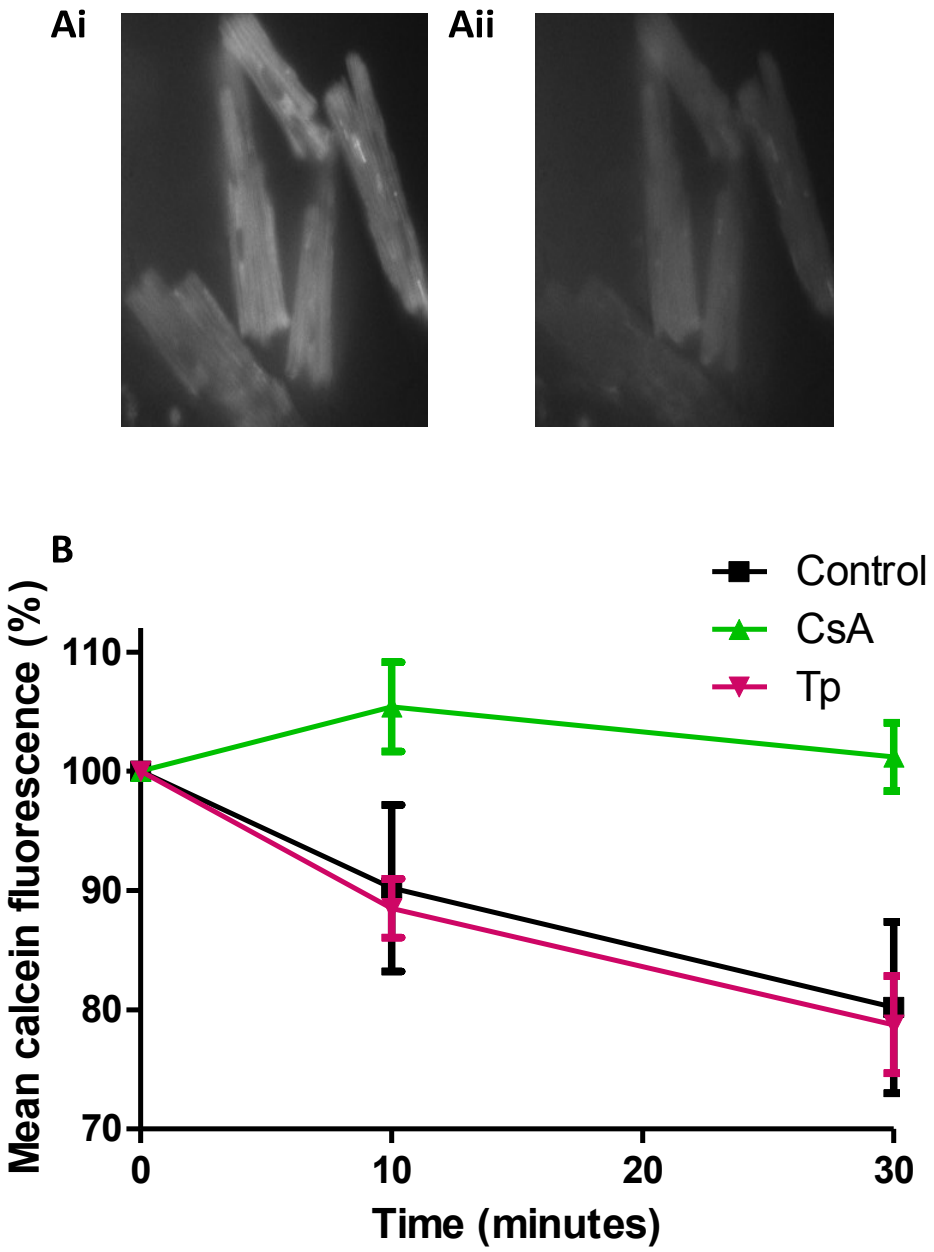

Figure 4.21: Unlike CsA (4µM) treatment, temperature preconditioning does not alter basal rate mPTP flickering.

The calcein-cobalt quench assay was used to assess basal rate of mPTP flickering. Ai: Time = 0 minutes, control myocytes loaded with calcein-AM and cobalt chloride. Cytoplasmic calcein fluorescence is quenched resulting in a punctuate pattern of fluorescence emitting from mitochondria. Aii: Time = 30 minutes, mPTP flickering causes redistribution of calcein fluorescence from mitochondria to the cytosol where the signal is lost. B: Calcein fluorescence was measured at times 0, 10 and 30 minutes and was expressed as a percentage of initial fluorescence (time = 0 minutes).

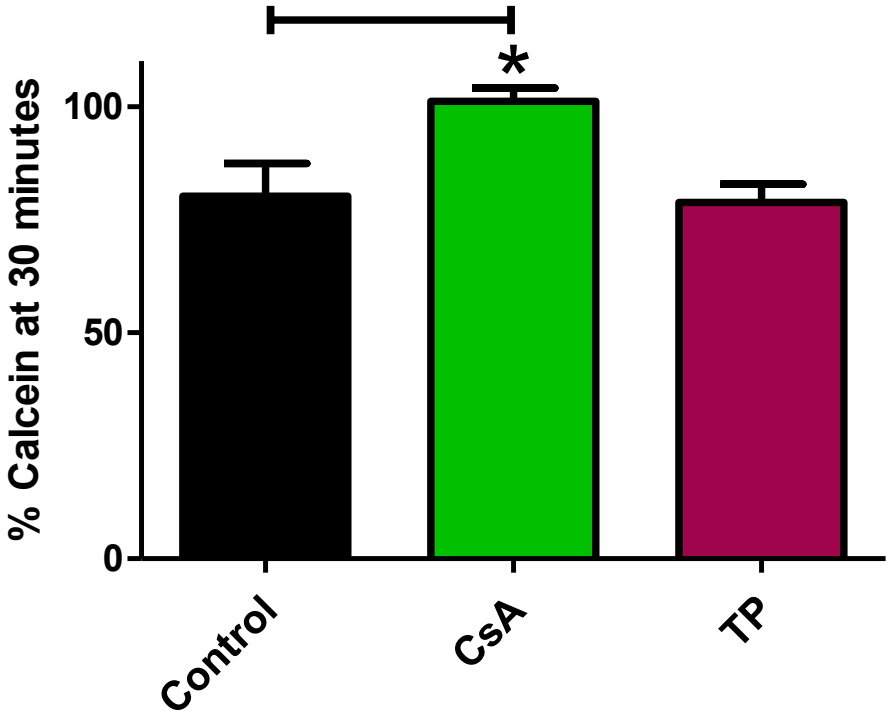

Figure 4.22: Temperature preconditioning does not alter basal rate mPTP flickering.

Fluorescence values (expressed as a percentage of fluorescence at time = 0 minutes) at time = 30 minutes were compared between control n = 13, 3, 3; cyclosporine A (CsA) n = 11, 3, 3; and temperature preconditioned (TP) n = 12, 3, 3 myocytes. Data was analysed by a 1-way ANOVA; * $p < 0.05$.

4.3 Summary of results

In this chapter a model of photodamage-induced mPTP opening is characterised in TMRM loaded HeLa cell and acutely isolated ventricular myocytes. A concentration dependent effect of TMRM on time to mPTP opening, indicated by mitochondrial depolarization, is demonstrated. Pharmacological manipulators of the mPTP were utilized to demonstrate that mitochondrial depolarization is a dependable reflection of mPTP opening. Subsequently, TP was demonstrated to significantly delay photodamage-induced mPTP opening relative to controls.

This chapter also describes a novel model of xanthine and xanthine oxidase-induced mPTP opening in isolated myocytes. A by product of the reaction of xanthine with xanthine oxidase is ROS which is a key trigger of mPTP opening. The efficacy of the model as one which measure mPTP opening was confirmed using the mPTP inhibitor CsA which was found to significantly delay mPTP opening relative to controls. Subsequently TP was also found to significantly delay xanthine and xanthine oxidase-induced mPTP opening relative to controls. Thus this chapter demonstrates the protective effects of TP on delaying mPTP opening using two different models.

Finally in this chapter the effects of TP on basal, non-pathological mPTP flickering were investigated using an established calcein-cobalt quench assay. Using this assay it was demonstrated that control myocytes display basal mPTP flickering which can be completely blocked by the presence of CsA. However, it was found that TP did not block basal mPTP flickering and temperature preconditioned myocytes exhibited mPTP flickering almost identically to control myocytes. This finding, taken together with other results discussed in this chapter indicate that TP only inhibits pathological mPTP

opening in the presence of noxious stimuli (photodamage or xanthine and xanthine oxidase produced ROS), while having no effect on non-pathological mPTP flickering. This differs from the actions of CsA which was found to inhibit both pathological and non-pathological mPTP opening.

Limitations

A significant limitation of the photodamage and xanthine/xanthine oxidase assays used in this investigation to assess pathological mPTP opening in isolated myocytes, is that they only focus on one aspect of reperfusion injury while completely ignoring ischaemia. In the intact heart, myocardial ischaemia precedes reperfusion and is responsible for causing cellular ionic and pH changes which are critically involved in increasing the likelihood of mPTP opening at reperfusion. Moreover, the assays used primarily focus on generating ROS to induce mPTP opening, although several other factors, such as calcium and phosphate concentration, are to known to play pivotal roles on regulating mPTP opening at reperfusion (Halestrap, 2010).

The photodamage and xanthine/xanthine oxidase induced mPTP opening assays used in this study utilised mitochondrial depolarisation as a surrogate marker for mPTP opening. This was confirmed to be a dependable reflection of mPTP opening using pharamacological agents that are known to modulate mPTP opening. However the efficacy of these control experiments is only as good as the selectivity of the pharmacological agents used to manipulate mPTP opening. Unfortunately selective mPTP inhibitors which directly prevent pore opening do not exist, since the pore forming component of the mPTP remains to be defined. However, in support of using mitochondrial depolarisation as a surrogate for mPTP opening, Hausenloy *et al*

demonstrated that photodamage induced mitochondrial depolarisation closely coincided with the release of mitochondrially entrapped calcein. Further, it was demonstrated that both events could be inhibited by the presence of the known mPTP inhibitor CsA (Hausenloy *et al.*, 2004).

The experiments conducted in this chapter concerning pathological mPTP opening could have been better investigated by directly investigating mPTP opening. This could have been investigated using the calcein-cobalt quench assay. However, it should be noted that this assay too is not exempt from criticism, since it requires cells to be loaded with cobalt chloride to quench cytosolic calcein fluorescence and evidence exist implicating cobalt as having toxic effects in myocytes (Liu *et al.*, 2010).

5 Investigating the roles of mitochondrial ROS and phospho-ERK in the process of temperature preconditioning of isolated ventricular myocytes

5.1 Introduction

The seminal finding of ischaemic preconditioning (IPC) by Murry *et al.* in 1986 was the first demonstration that the heart could be preconditioned to increase its tolerance to a subsequent period of prolonged ischaemia followed by reperfusion. IPC involves subjecting the heart to brief periods of sub-lethal ischaemia with intermittent reperfusion prior to a prolonged episode of index ischaemia (Murry *et al.*, 1986). Murry *et al.* used canine hearts to demonstrate that IPC greatly reduced the infarct size resulting from a 40 minute ischaemic insult to only 25% of that observed in the control group; an amount of protection previously unparalleled. Moreover, unlike previously described interventions, IPC could be consistently reproduced by all who investigated it and was subsequently demonstrated to be effective in several species including mouse, rabbit, rat, feline, sheep and monkey. However to the present day, despite almost three decades of research, the exact mechanisms underlying IPC-induced protection remains to be elucidated.

The protective effects of IPC were first realised to be a receptor triggered mechanism through the work of Liu *et al*. in 1991. This group reported that IPC-induced protection could be mimicked by the A_1 receptor-selective agonist R(-)-N^6-(2-phenylisopropyl) adenosine (PIA) and that IPC-induced protection could be blocked by an adenosine receptor antagonist. It was proposed that endogenous adenosine released by the IPC

protocol activated A_1 adenosine receptors resulting in a preconditioned phenotype (Liu *et al.*, 1991). In subsequent years, two other autacoids bradykinin (Wall *et al.*, 1994) and opioids (Schultz *et al.*, 1995), which like adenosine are agonists of G_i protein coupled receptors, were also demonstrated to be released by the IPC protocol and were shown to be crucial for protection. It is believed that receptors of these three agonists work in parallel to trigger signalling pathways which underlie IPC-induced protection. The pathways activated by these receptors are appreciated to be very divergent but are thought to ultimately converge on a single target; PKC whose inhibition eliminates IPC-induced protection, while having no effect on non-preconditioned hearts. Further, pharmacological activation of PKC by phorbol esters mimics the protection afforded to the myocardium by IPC (Mitchell *et al.*, 1995; Ytrehus *et al.*, 1994).

The role of ROS in IPC-induced protection has also been established beyond doubt and has been shown to act upstream of PKC. IPC-induced protection can be abolished by the presence of a ROS scavenger and conversely, protection can be mimicked by transient exposure to an oxygen radical generating system (Baines *et al.*, 1997; Tritto *et al.*, 1997). In 2005, Hausenloy *et al.* described a pro-survival protein kinase pathway termed the reperfusion injury salvage kinase (RISK) pathway which confers powerful cardioprotection when activated at the time of myocardial reperfusion. The RISK pathway includes PI3 kinase/Akt and MEK1/2/ERK1/2 and is known to be recruited by IPC (Hausenloy *et al.*, 2005b). Furthermore, research conducted in the past several years have revealed that numerous reagents can protect the myocardium from ischaemia-reperfusion injury when administered at the time of myocardial reperfusion. Such reagents, to mention a few are, insulin (Baines *et al.*, 1999), adenosine A_1/A_2

agonist AMP 579 (Xu *et al.*, 2000), transforming growth factor-β1 (Baxter *et al.*, 2001), urocortin (Schulman *et al.*, 2002), bradykinin (Yang *et al.*, 2004), natriuretic peptides (Yang *et al.*, 2006) and cyclosporine A (Hausenloy *et al.*, 2009). With the exception of cyclosporine A, all of the above mentioned reagents, like IPC, depend on the activation of the RISK pathway to occur for protection to be achieved. A proposed downstream target of the RISK pathway is GSK-3β which may represent an integration point for several preconditioning pathways (IPC, pharmacological preconditioning, ischaemic postconditioning). Evidence suggests that GSK-3β inhibition strongly inhibits mitochondrial permeability transition pore (mPTP) formation; the ultimate end effector of preconditioning signalling cascades (Juhaszova *et al.*, 2004; Juhaszova *et al.*, 2009).

In 2007, a novel preconditioning intervention was described by Khaliulin *et al.* in which brief periods of hypothermia interspersed by periods of normothermia was found to protect the isolated heart against a subsequent bout of prolonged ischaemia followed by reperfusion This intervention was termed temperature preconditioning (TP) and its cardioprotective effects were claimed to be greater than IPC (Khaliulin *et al.*, 2007). Following 25 minutes of global ischaemia and 60 minutes of reperfusion, TP was found to increase haemodynamic recovery, decrease arrhythmias and reduce necrotic damage (as assessed by lactate dehydrogenase release) more than IPC. The underlying mechanisms of protection of TP were investigated and were found to involve inhibition of mPTP opening at reperfusion. This was proposed to be as a result of less oxidative stress on reperfusion in TP hearts, which was again less than that observed in IPC hearts. Like IPC, TP was found to recruit the activation of PKC_{ϵ} and its inhibition blocked the protective effects of TP. A partial block of the protective effects of TP was

also observed using an AMP-activated protein kinase (AMPK) inhibitor and cardioprotection was abolished using a free radical scavenger. These findings, implicated PKC_{ϵ}, AMPK and ROS as mediators of the protective effects of TP. In a second study of TP conducted by the same group in 2010, it was proposed that switching between hypothermia and normothermia during the TP protocol may cause β-adrenergic stimulation, which is accompanied by cyclic AMP (cAMP) dependent PKA activation (Khaliulin *et al.*, 2010). As hypothesised it was found that TP significantly increased tissue concentrations of cAMP and PKA activity. Thus this group further studied the temporal relationship between PKA and PKC activation in TP hearts and demonstrated that PKA stimulation prior to PKC stimulation can mimic the protective effects of TP. Block of β-adrenergic stimulation during the TP protocol only partially abolished the protective effects of TP, whilst blocking PKA during the TP protocol completely abolished the TP-induced protection. This finding implied that β-adrenergic stimulation was only partially responsible for PKA activation during the TP protocol and the authors suggested a ROS-mediated pathway my represent a second mechanism of PKA activation, but this was not investigated.

In the present chapter I expand upon the finding that ROS is involved in TP and seek to characterise exactly when and how during the TP protocol that ROS is produced. Further, I explore the role of ERK in TP and investigate if a functional relationship exists between ROS and ERK. Such a relationship in the context of TP is yet to be defined.

5.2 Results

5.2.1 Characterising the effects of the mitochondrial matrix-targeted superoxide biosensor in CHO cells

To investigate the possible role and dynamics of mitochondria derived ROS in the process of TP, a recently described novel mitochondrial matrix-targeted superoxide biosensor was used. The biosensor is a circularly permuted yellow fluorescent protein (cp-YFP), which was previously used as the core structure for the Ca^{2+} indicator pericam and was discovered as a novel biosensor for superoxide by serendipity. The probe was engineered to be expressed in the mitochondrial matrix of adult cultured cardiomyocytes using the cytochrome C oxidase subunit IV (COX IV) targeting sequence (mt-cpYFP). The protein is purported to be a highly sensitive and reversible superoxide-selective probe, bearing Cys171 and Cys193 residues that form a reactive superoxide sensor (Wang *et al.*, 2008). However, other groups that have characterised the properties of cp-YFP have disputed its sensitivity to superoxide but on the contrary suggest the probe to be highly responsive to pH (Schwarzlander *et al.*, 2011). Thus, given the somewhat conflicting reports with regards to cp-YFP I set out to characterise its properties in CHO cells and isolated ventricular myocytes.

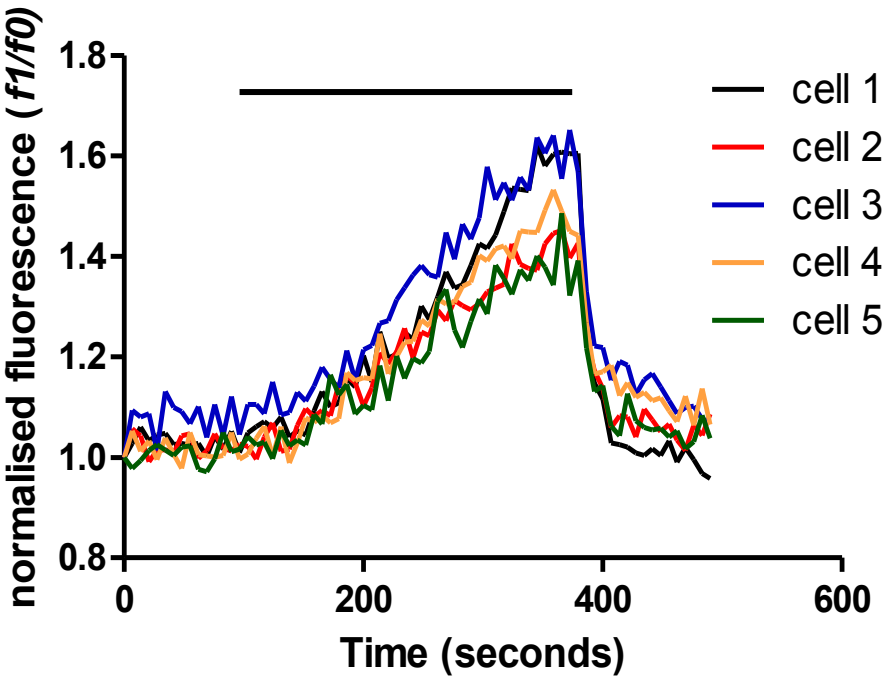

Figure 5.1: The effects of xanthine (2mM) and xanthine oxidase (2mU) on CHO cells infected with mitochondrially targeted superoxide biosensor.

CHO cells were infected with superoxide biosensor virus for 48 hours. Representative traces showing a xanthine/xanthine oxidase-induced increase in cpYFP fluorescence; indicating an increase in mitochondrial superoxide levels. The black bar indicates the duration of exposure of CHO cells to xanthine (2mM) and xanthine oxidase (20mU).

CHO cells were grown on glass cover slips in minimum essential medium (MEM) α containing ribonucleosides, deoxyribonuleosides and L-glutamine. The cells were left in culture for 48 hours with adenovirus encoding the mt-cpYFP probe after which time approximately 60-80% of cells were infected and expressed the mt-cpYFP ROS biosensor. Glass cover slips that were approximately 70% confluent were used for experimentation. Experiments were conducted at 34^{o}C and CHO cells were initially perfused with Tyrode solution to establish a stable fluorescent baseline. The fluorescence emission collected at 515nm of mt-cpYFP expressing CHO cells excited at 488nm increased by approximately 60% with the addition of xanthine (2mM) and xanthine oxidase (20mU) to the perfusing solution (figure 5.1). The superoxide (generated by xanthine and xanthine oxidase under aerobic conditions) induced increase in fluorescence was synchronous in almost all mt-cpYFP expressing cells. Further, the superoxide-induced increase in fluorescence was rapidly reversed when xanthine and xanthine oxidase was withdrawn from the perfusing solution and fluorescence returned to baseline levels.

These initial characterization experiments conducted in CHO cells demonstrated the sensitivity of the mt-cpYFP biosensor to ROS. Therefore, further characterization experiments of the probe were conducted in isolated myocytes.

5.2.2 Characterising the effects of the mitochondrial matrix-targeted superoxide biosensor in isolated myocytes

Characterisation of the mt-cpYFP probe was conducted in ventricular myocytes that were infected with adenovirus encoding mt-cpYFP for 48 hours. Ventricular myocytes were cultured in media 199 supplemented with ITS+3 (2mg/ml), carnitine (2mM),

creatine (5mM), taurine (5mM), T3 (1.5pM) and penicillin and streptomycin (100 units) at 37°C and 5%CO_2. After 48 hours approximately 70% of myocytes were infected (figure 5.2A) and when challenged with xanthine (2mM) and xanthine oxidase (20mU) a sharp increase in fluorescence was observed (figure 5.2Bii & figure 5.3).

A

superoxide biosensor infection	fields of view counted	total cells	% infection efficiency
1	2	8	88
2	6	23	61
3	5	12	50
4	4	15	66
Total/Average	17	58	66

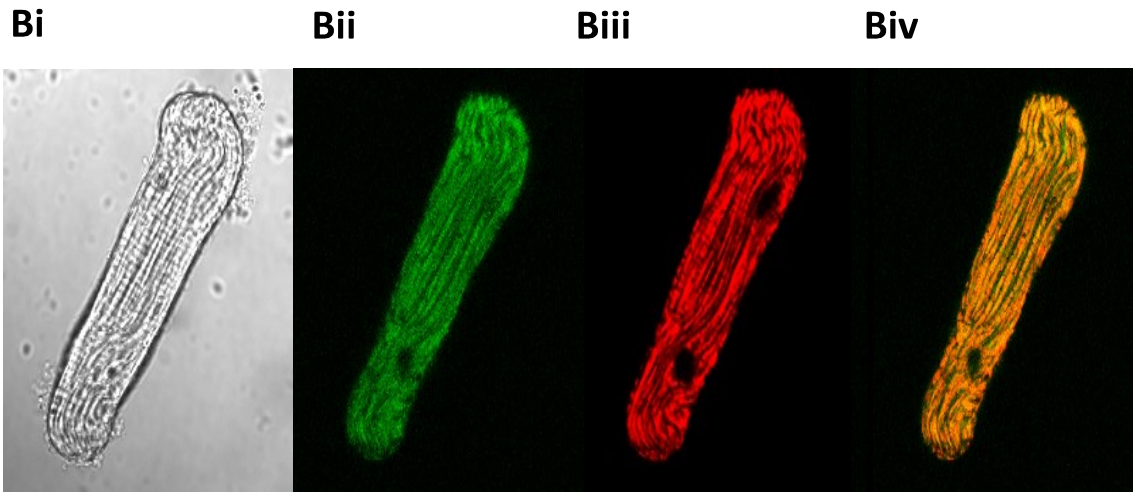

Figure 5.2: Superoxide biosensor infection efficiency and co-localisation with MitoTracker deep red.

A: Table showing the infection efficiency of the ROS biosensor adenovirus. Infected myocytes were counted from a selection of fields of view from four separate infections and the average percentage of infected myocytes was calculated.
B: Representative image of an infected isolated ventricular myocyte following 48 hour in culture with the superoxide biosensor virus. Bi: Bright field image of an mt-cpYFP expressing myocyte. Bii: ROS biosensor fluorescence when excited at 488nm and challenged with xanthine and xanthine oxidase. Biii: MitoTracker deep red (selective mitochondrial marker) fluorescence when excited at 640nm and Biv: Merged image of the fluorescence from the ROS biosensor and mitoTracker deep red. The orange/yellow fluorescence indicates co-localisation of the ROS biosensor and mitochondria.

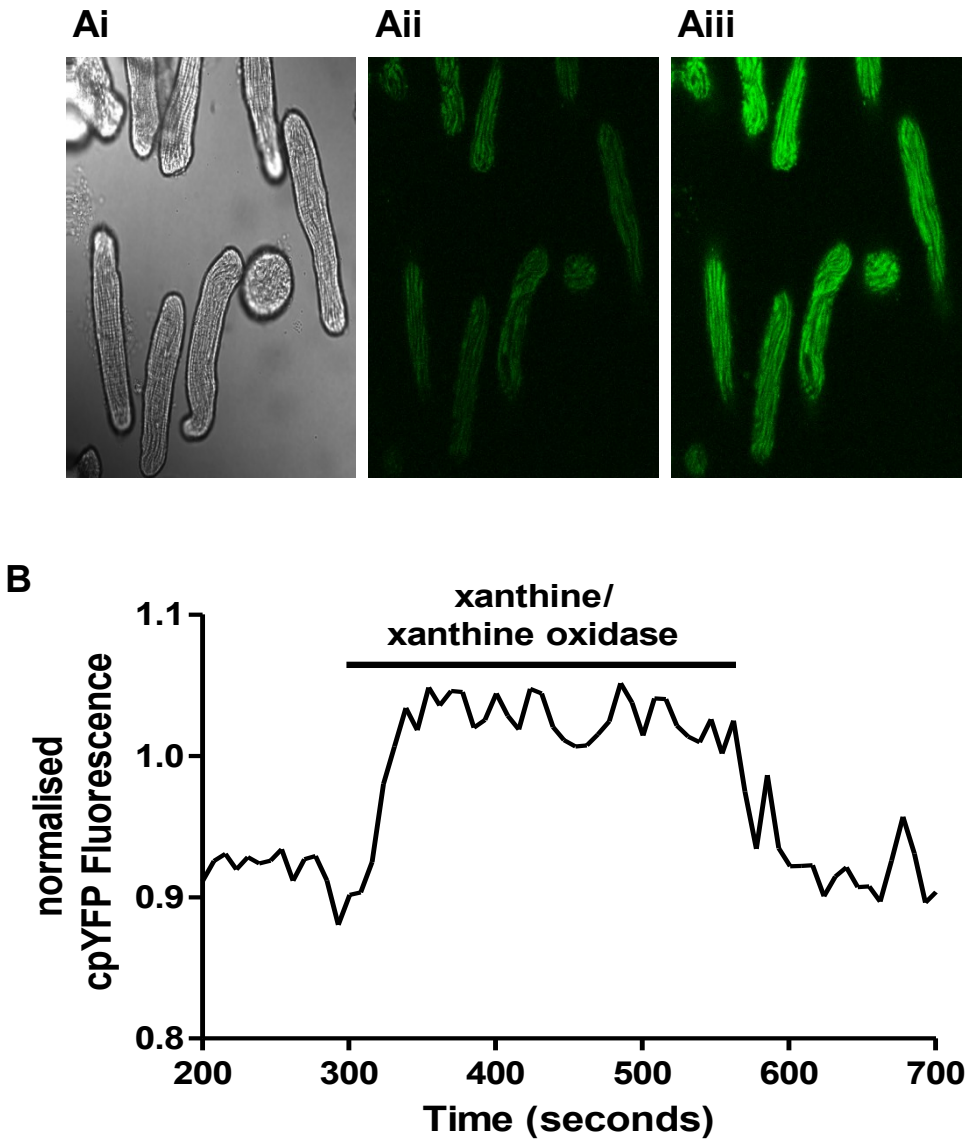

Figure 5.3: Superoxide biosensor infected myocytes are sensitive to exogenous ROS.

Ai: A bright field image of a representative field of view of adenovirally infected mt-cpYFP expressing myocytes. Aii: A fluorescent image of the same myocytes exhibiting low level basal fluorescence. Aii: After exposure to xanthine/xanthine oxidase the fluorescence exhibited is significantly stronger.
B: Representative trace showing the effect of xanthine and xanthine oxidase on the fluorescence of an mt-cpYFP-expressing myocyte.

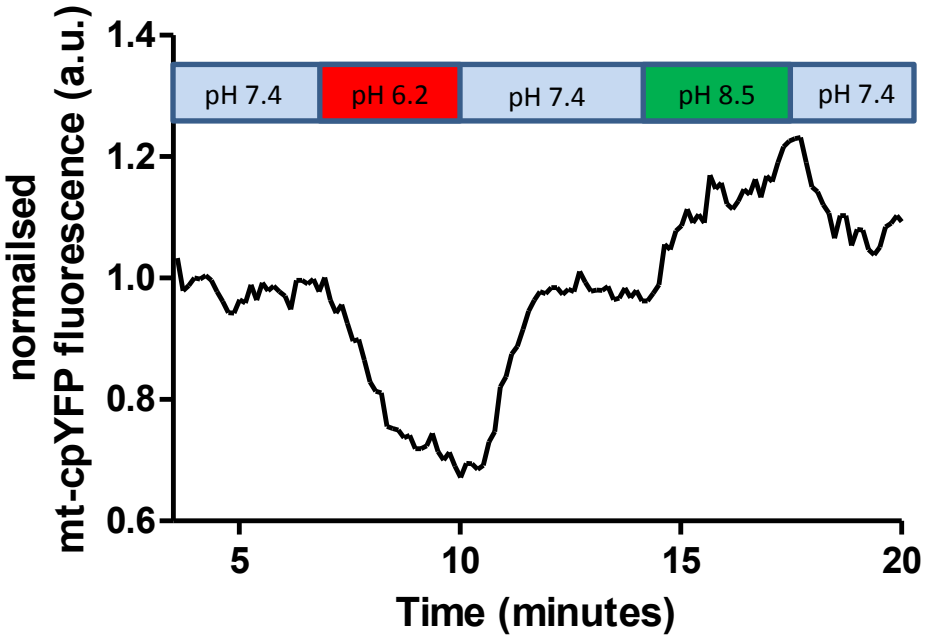

Figure 5.4: superoxide biosensor fluorescence is sensitive to pH changes.

Representative trace showing the effect of pH changes on the fluorescence of an mt-cpYFP-expressing myocyte.

To confirm that the changes in fluorescence emanated from mitochondria, and to therefore confirm the targeting of the probe to the mitochondrial matrix, co-localisation analysis was conducted. 48 hour infected myocytes expressing mt-cpYFP were loaded with the mitochondria specific fluorescent dye mitoTracker red (figure 5.2Biii) and the fluorescence of mt-cpYFP and mitoTracker red were found to significantly overlap (figure 5.2Biv). The response of mt-cpYFP expressing myocytes to xanthine (2mM) and xanthine oxidase (20mU) is illustrated in figure 5.3. As observed in CHO cells, the basal fluorescence of mt-cpYFP expressing myocytes perfused with Tyrode solution is low (figures 5.3Aii & 5.3B). The addition of xanthine and xanthine oxidase to the perfusing solution causes a rapid and robust increase in fluorescence (figures 5.3Aiii and 5.3B) indicating an increase in mitochondrial matrix superoxide concentration. A decrease in fluorescence accompanied the removal of xanthine and xanthine oxidase demonstrating that the binding of the probe to superoxide was reversible (figure 5.3B).

The sensitivity of the mt-cpYFP probe to changes in pH was next investigated by exposing 48 hour infected myocytes expressing mt-cpYFP to Tyrode solutions of pH 7.4, pH 6.2 and pH 8.5. As can be seen from figure 5.4 the mt-cpYFP probe was found to be sensitive to changes in pH. Acidic pH caused a decrease in mt-cpYFP fluorescence while alkaline conditions caused an increase in fluorescence above that observed under basal conditions (pH 7.4). The fluorescence changes induced by alterations of pH could not be inhibited by the addition of the ROS scavenger MPG (300µM), indicating that the changes in fluorescence did not arise from a secondary effect of pH on superoxide production.

The characterisation experiments conducted in this study reveal that mt-cpYFP is responsive to both superoxide and pH. This finding indicated that mt-cpYFP could be used to determine the effects of TP on mitochondrial superoxide production. However, it was realised that any fluorescent changes seen to be induced by TP would require control experiments to be conducted to determine if the changes were due to effects on superoxide levels or changes to pH.

5.2.3 Temperature preconditioning of isolated myocytes stimulates mitochondrial ROS production which is essential for protection

The importance of ROS in TP has previously been demonstrated (Khaliulin *et al.*, 2007), but exactly how, or when, ROS is involved during the TP protocol remains to be elucidated. The effects of TP were therefore investigated on mt-cpYFP expressing myocytes to observe when, if any, changes to superoxide production would be induced by the protective protocol.

48 hour infected myocytes expressing mt-cpYFP were perfused with Tyrode at 37^{o}C for the duration of 20 minutes and no significant change in fluorescence was observed (figure 5.5A). However, when 48 hour infected myocytes expressing mt-cpYFP were temperature preconditioned, fluorescence was seen to significantly increase at two stages during the protocol. The increase in fluorescence routinely coincided with perfusion of myocytes at 16^{o}C resulting in two peaks of increased fluorescence during the TP protocol (figure 5.5B). It was further observed that if TP was conducted in the presence of the ROS scavenger MPG (300µM), the effects of cold perfusion (16^{o}C) on increases of fluorescence was significantly dampened (figure 5.5B). This demonstrated

that the TP-induced increase in fluorescence was, at least in part, as a result of increased superoxide production. Figure 5.5 outlines the increases in fluorescence induced during the two stages of cold perfusion both, in the presence and absence of MPG (300μM). In the absence of MPG, the mean fluorescence increases from baseline observed during cold perfusion were 1020 (a.u.) and 1190 (a.u.) for peak 1 and peak 2 respectively. In contrast, when TP was conducted in the presence of MPG the mean fluorescence increases from baseline were significantly reduced to 510 (a.u.) and 217 (a.u.) for peak 1 and peak 2 respectively (figure 5.6).

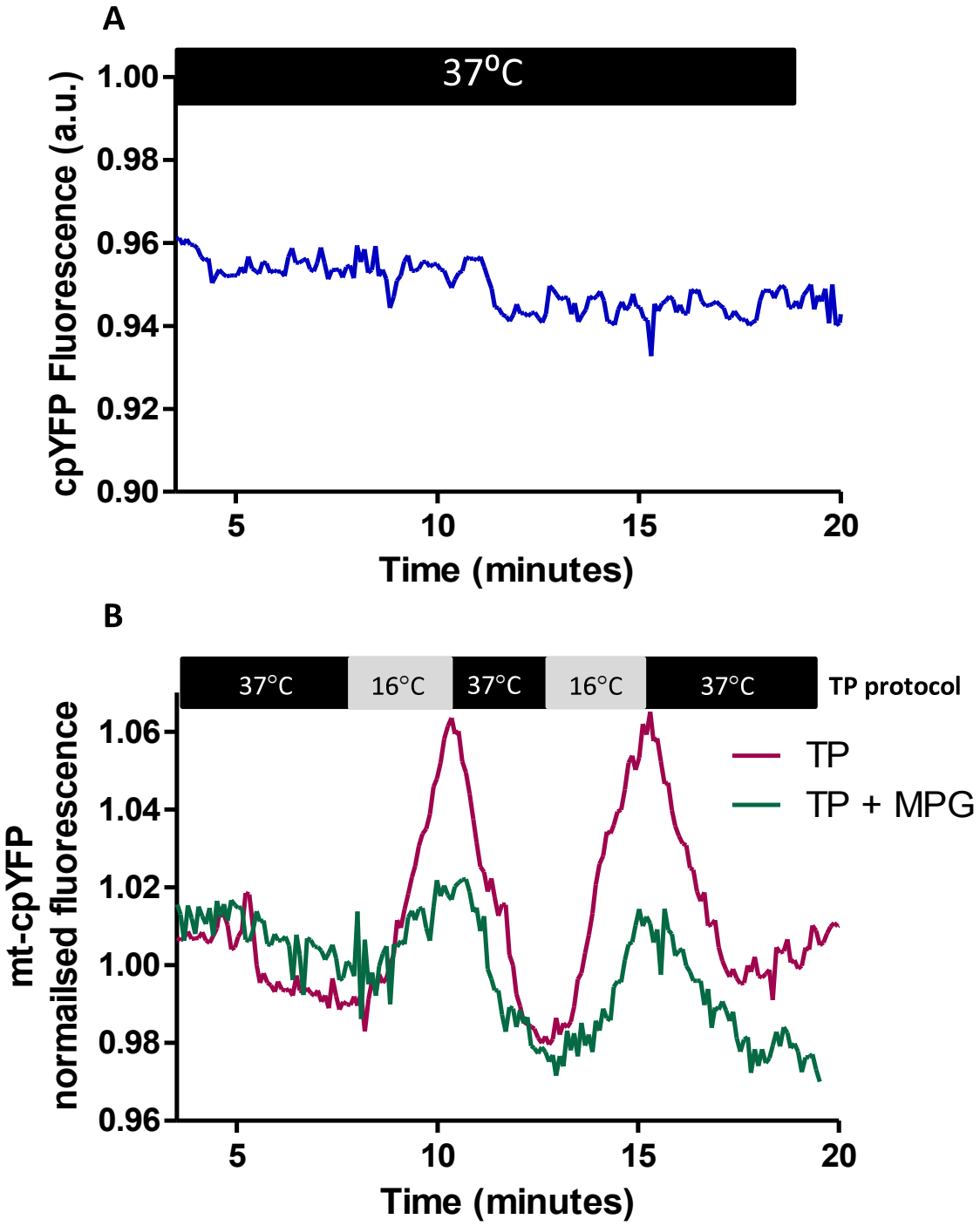

Figure 5.5: Cold perfusion during temperature preconditioning increases mitochondrial superoxide concentration.

A: Representative trace of an mt-cpYFP-expressing myocyte perfused at 37°C for 20 minutes. B: Representative traces of mt-cpYFP-expressing myocytes exposed to temperature preconditioning (TP) in the absence (red trace) and presence (green trace) of a ROS scavenger MPG (300 µM).

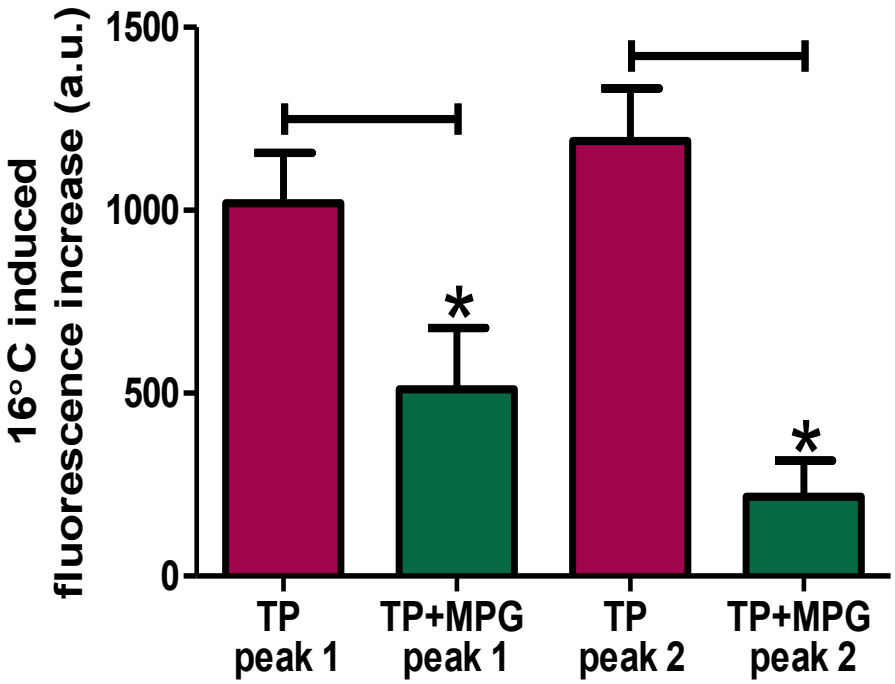

Figure 5.6: Cold perfusion during temperature preconditioning causes two peaks of superoxide production.

The mean fluorescence observed during temperature preconditioning (TP) at peak 1 and peak 2 for control (red bar) n = 19 and MPG treated (green bar), n = 18 myocytes. Data was analysed by a 1-way ANOVA; * $p < 0.05$.

Control experiments were conducted in order to assure that TP-induced increases in fluorescence were due to increased superoxide production and not changes to pH. Uninfected 48 hours cultured myocytes were loaded with the pH sensitive fluorescent dye, carboxy snarf (10µM), using a protocol which favoured mitochondrial loading (see materials and methods). To confirm this, myocytes were co-loaded with mitoTracker red (500nM) and mitochondrial loading of carboxy snarf was observed (figure 5.7). When carboxy snarf loaded myocytes were exposed to Tyrode solutions of different pH, clear changes of fluorescence were observed. Acidic Tyrode (pH 6) solution caused decreases in emitted fluorescence whilst alkaline Tyrode solution (pH 9) caused increases in fluorescence (figure 5.8). This indicated that carboxy snarf loaded myocytes were capable of detecting mitochondrial pH changes. Carboxy snarf loaded myocytes were subsequently exposed to TP but no change in emitted fluorescence was observed (figure 5.9). This indicated that the fluorescence change induced by TP in mt-cpYFP expressing myocytes was as a result of increased superoxide concentration and not changes in cellular pH. The consequence of scavenging ROS during the TP protocol was next investigated by assessing if TP-induced protection against mPTP opening was affected. In the previous chapter TP was demonstrated to significantly delay the time to photodamage-induced mPTP opening.

Uninfected 48 hours cultured isolated myocytes were temperature preconditioned and assayed for photodamage-induced mPTP opening (chapter 4 / figure 4.15). The ability of TP to delay pathological photodamage-induced mPTP opening was not lost after 48 hours in culture. TP (1.5 fold increase compared to control) significantly delayed mPTP opening compared to untreated control myocytes, but protection was lost if TP was performed in the presence of the ROS scavenger MPG (300µM) (figure 5.10). This

suggests that the TP-induced increases in superoxide levels (figure 5.5) are crucial for TP-induced protection against pathological mPTP opening.

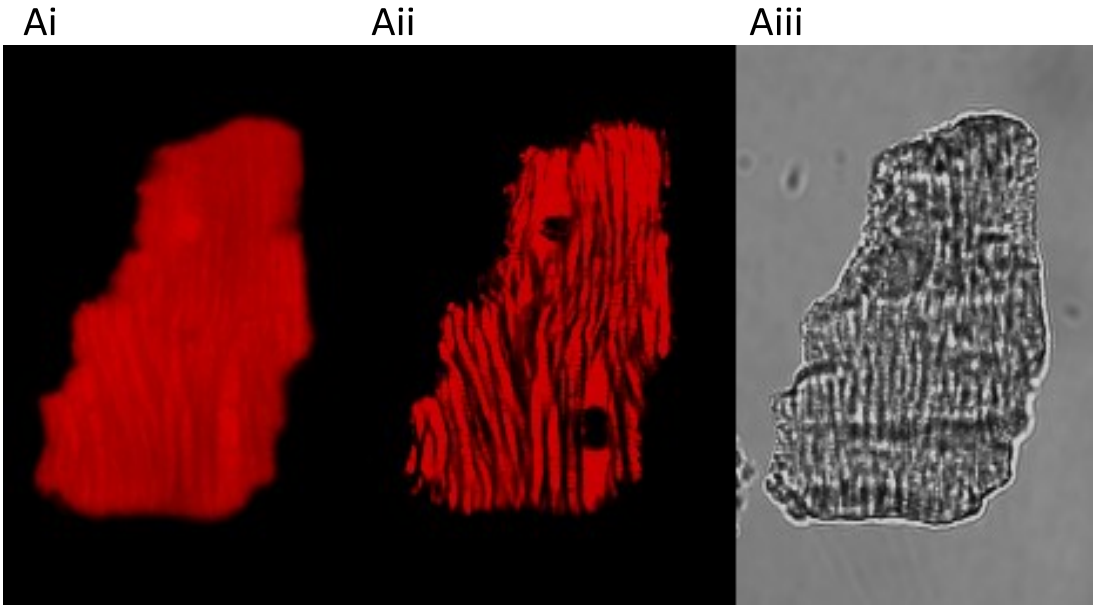

Figure 5.7: Localisation of carboxy snarf and MitoTracker deep red.

Representative image of an uninfected 48 hours cultured myocyte loaded with carboxy snarf using a protocol which favours mitochondrial localisation. Ai: Carboxy snarf fluorescence. Aii: MitoTracker deep red fluorescence. Aiii: Bright field image of the myocyte.

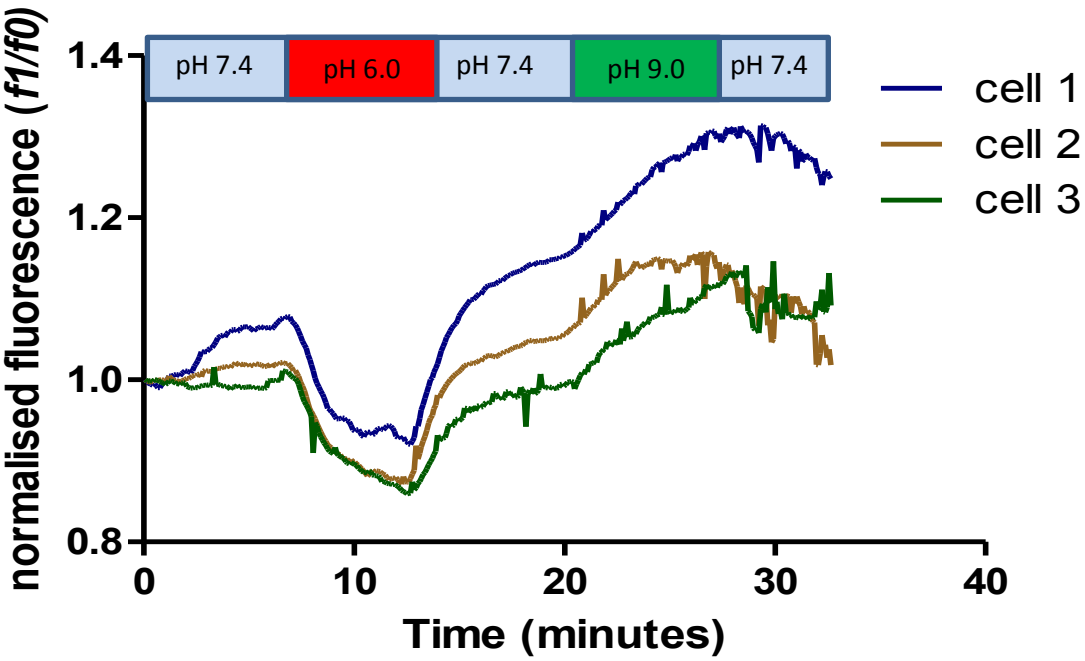

Figure 5.8: Myocytes loaded with carboxy snarf are sensitive to pH changes.

Representative traces of 48 hours cultured uninfected myocytes loaded with the pH sensitive fluorescent dye carboxy snarf. Myocytes were perfused with Tyrode of differing pH and the fluorescent changes induced were recorded.

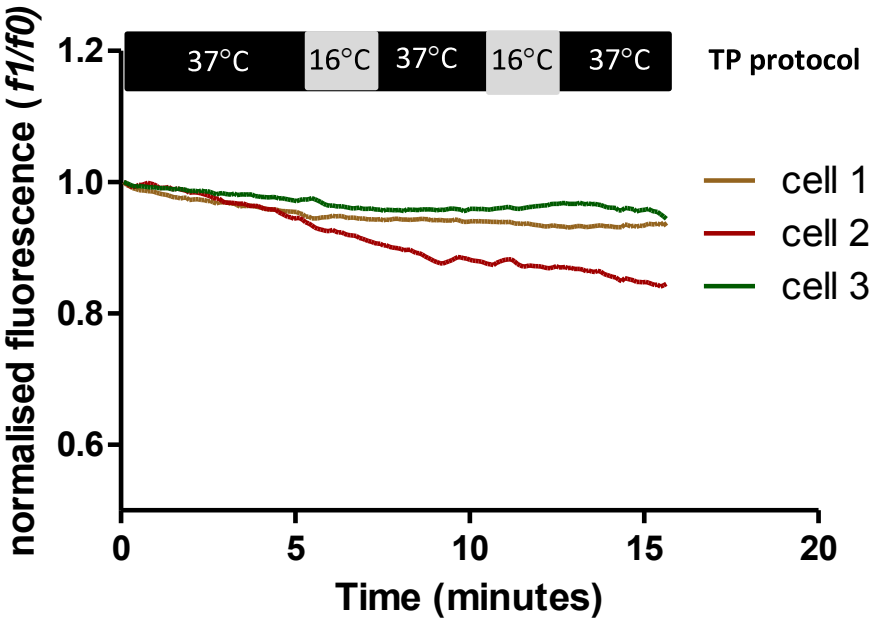

Figure 5.9: Cold perfusion during temperature preconditioning does not alter mitochondrial pH.

Representative traces of 48 hours cultured uninfected myocytes loaded with the pH sensitive fluorescent dye carboxy snarf. A mitochondrial specific loading protocol was followed to ensure mitochondrial dye loading. Mitochondrial pH was unaltered during temperature preconditioning (TP).

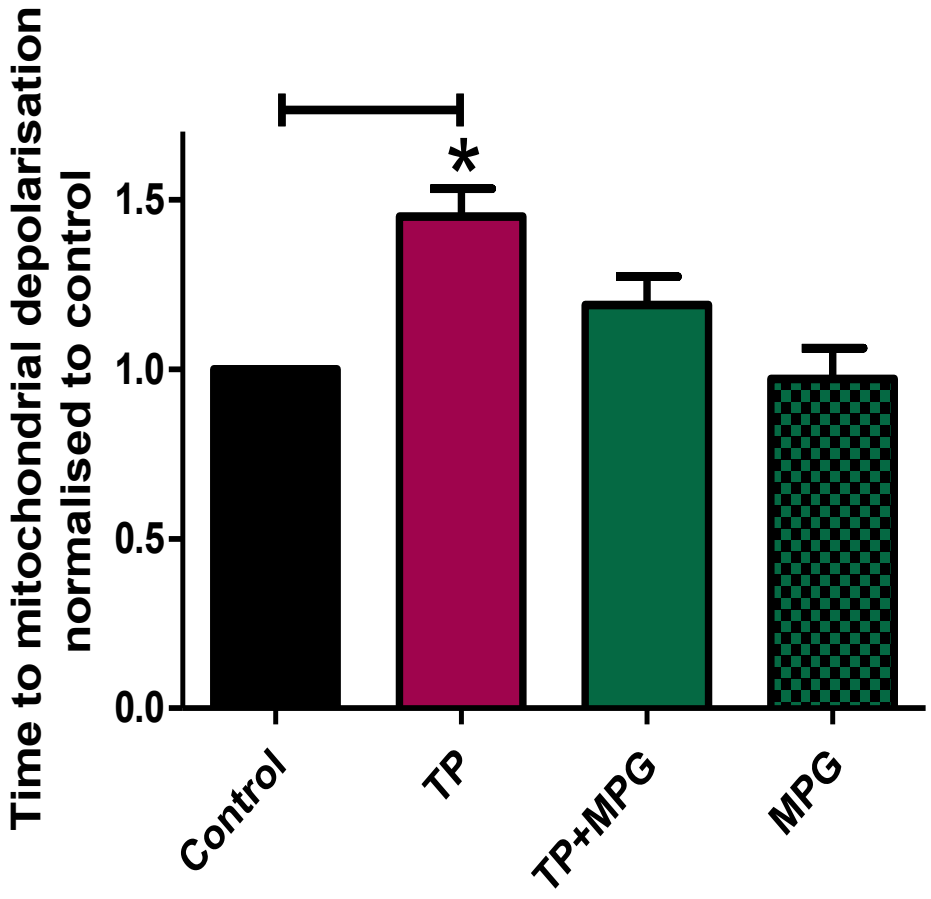

Figure 5.10: Scavenging ROS during temperature preconditioning abolishes temperature preconditioning-induced delay of mPTP opening.

TMRM loaded myocytes were continuously illuminated to induce mPTP opening. Temperature preconditioning (TP) (red bar) significantly delayed mPTP opening, but this effect was lost when TP was conducted in the presence of MPG (green bar). MPG alone had no significant effect compared to untreated control myocytes. Data were normalised to the mean of same day controls. Control, n = 41, TP, n = 42 or MPG (300 µM) treated TP myocytes, n= 45, MPG alone n = 26. Data was analysed by a 1-way ANOVA; * $p < 0.0001$. .

5.2.4 Temperature preconditioning of isolated myocytes induces phosphorylation of ERK which is essential for protection

Extracellular signal-regulated kinases (ERK) are associated with cell survival and proliferation. ERK along with PI3K and Akt are members of the reperfusion injury salvage kinase (RISK) pathway which is shown to be activated by IPC and confers protection against reperfusion injury (Hausenloy *et al.*, 2005b). The involvement of ERK in TP is yet to be investigated and is therefore the subject of investigation in this chapter.

To investigate the involvement of ERK in TP, myocytes were either temperature preconditioned in the presence or absence of ERK inhibitors and then subsequently assayed for tolerance against photodamage-induced mPTP opening. TP (1.4 fold increase compared to control) in the absence of ERK inhibitors significantly delayed mPTP opening relative to untreated control myocytes. On the other hand, TP in the presence of either PD98059 (10µM) or U0126 (0.5µM) abolished TP-induced delay of mPTP opening compared to control. Treating myocytes with PD98059 or U0126 alone did not significantly alter the time to mPTP opening compared to controls (figure 5.11).

To confirm the role of ERK in TP, western blot analysis was conducted. Temperature preconditioned myocytes demonstrated significantly greater phosphorylation of ERK compared to control myocytes (figure 5.12). However, myocytes that were temperature preconditioned in the presence of either PD98059 (10µM) or U0126 (0.5µM) failed to demonstrate a significant increase in ERK phosphorylation compared to control. This indicated that TP-induced phosphorylation of ERK. Further, it was found that TP in the presence of the ROS scavenger MPG (300µM) also abolished TP

induced phosphorylation of ERK. This result indicates that a functional relationship exists between TP-induced superoxide production and ERK phosphorylation. It further implies that TP-induced superoxide production is upstream of ERK phosphorylation.

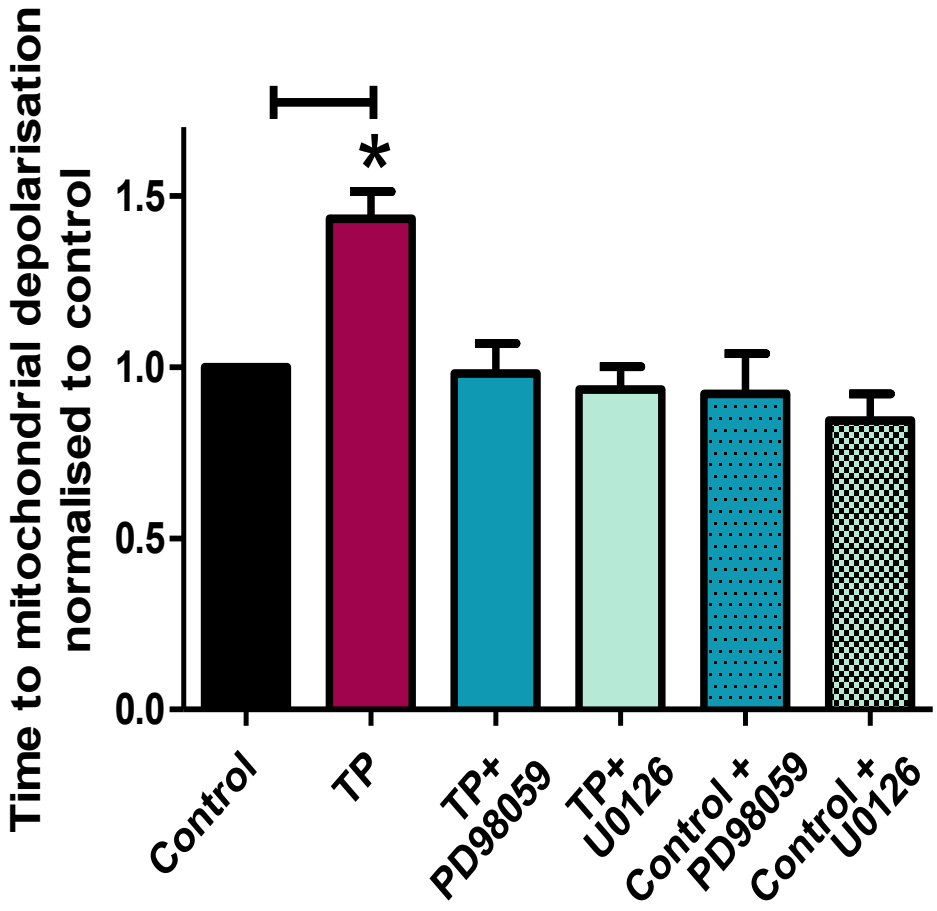

Figure 5.11: Inhibiting phosphorylation of ERK during temperature preconditioning abolishes temperature preconditioning-induced delay of mPTP opening.

TMRM loaded myocytes were continuously illuminated to induce mPTP opening. Temperature preconditioning (TP) (red bar) significantly delayed mPTP opening, but this effect was lost when TP was conducted in the presence of either PD98059 (turquoise bar) or U0126 (pale blue bar). PD98059 or U0126 alone had no significant effect compared to untreated control myocytes. Data were normalised to the mean of same day controls. Control, n = 38; TP, n = 47; TP with PD98059, n= 18; TP with U0126 n = 31; PD98059 alone n = 21; U0126 alone n = 31. Data was analysed by a 1-way ANOVA; * $p < 0.0005$.

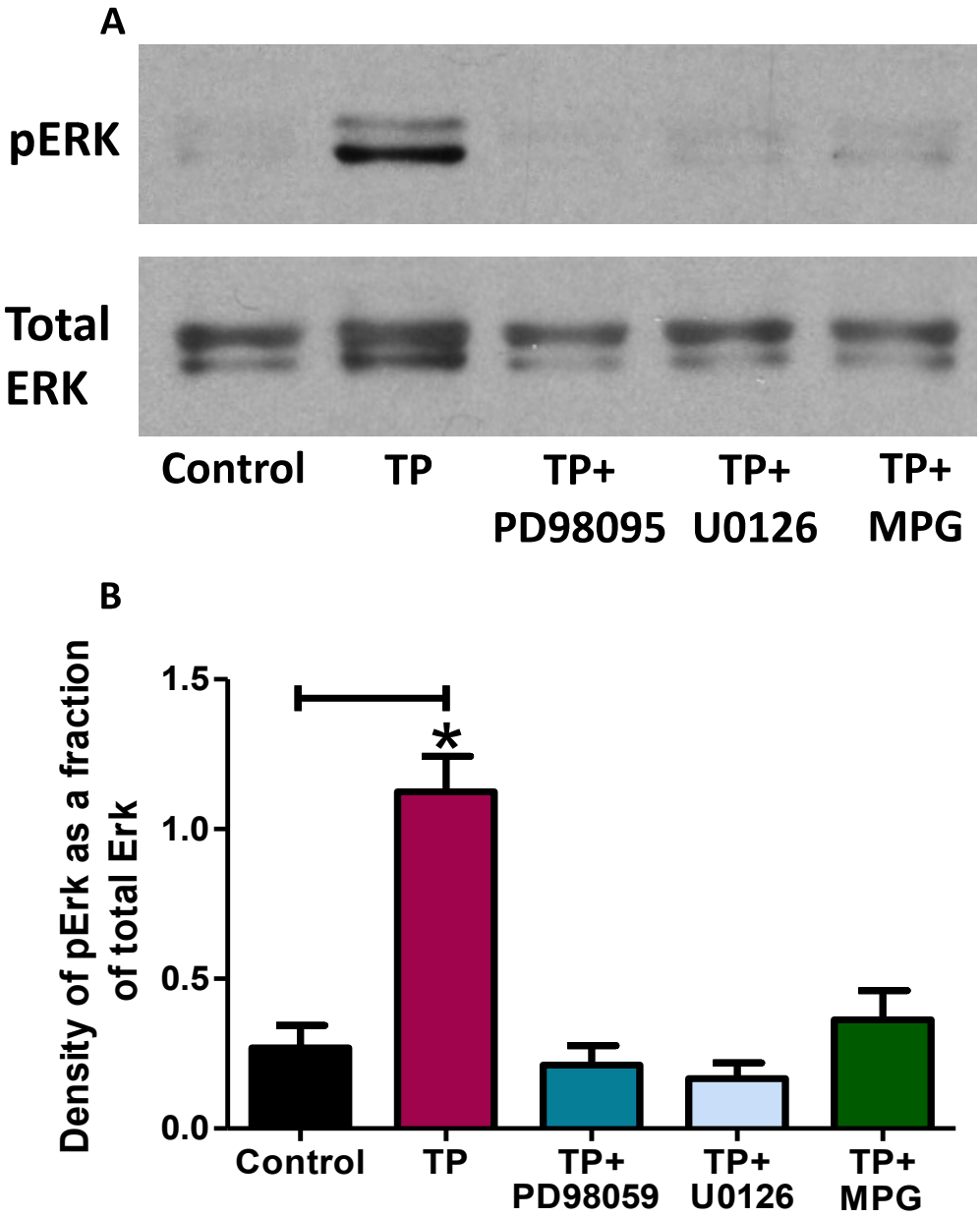

Figure 5.12: Temperature preconditioning significantly increases phosphorylation of ERK.

A: Representative Western blot showing total ERK and phosphorylated ERK in response to temperature preconditioning (TP) with and without ERK inhibitors PD98059 (10µM) or U0126 (0.5µM) and ROS scavenger MPG (300µM). B: Average densitometry showing the effects of TP; TP in the presence of PD98059 or U0126 (ERK inhibitors); or MPG (ROS scavenger) on ERK phosphorylation. n = 3 Western blots form 3 hearts. Data was analysed by a 1-way ANOVA; * $p < 0.005$.

5.3 Summary of results

In this chapter the properties of mt-cpYFP, a purported novel mitochondrial matrix-targeted superoxide biosensor are characterised. Characterisation of the probe was conducted initially in CHO cells and then more thoroughly in isolated ventricular myocytes. The mt-cpYFP probe was demonstrated to be highly sensitive to superoxide (generated by xanthine and xanthine oxidase) in both CHO cells and isolated myocytes. It was further illustrated in isolated myocytes that the mt-cpYFP probe preferentially localises to mitochondria and that its binding to superoxide is reversible. However, characterisation experiments in isolated myocytes also revealed that mt-cpYFP is responsive to pH in addition to superoxide and therefore requires care when interpreting results. For this reason, necessary control experiments were conducted using pH sensitive fluorescent dyes to allow confirmation of the cause of changes in mt-cpYFP fluorescence, when observed.

Using the mt-cpYFP probe it was demonstrated that TP is associated with two burst of increased superoxide production that is induced exclusively during the hypothermic (16^{o}C) phase of the protocol. It was subsequently demonstrated that scavenging ROS during the TP protocol significantly dampened the measured increase in mt-cpYFP fluorescence and resulted in a consequential loss of cardioprotection. This finding indicated that the TP-induced changes in mt-cpYFP fluorescence were most likely as a result of an increase in mitochondrial superoxide concentration rather than TP-induced pH changes. However, it was still possible that the TP-induced fluorescence changes were evoked as a result of matrix alkalinisation which could have secondarily stimulated superoxide production; thereby explaining the loss of cardioprotection

when TP was conducted in the presence of a ROS scavenger. Therefore to confirm that the TP-induced changes in mt-cpYFP fluorescence were not due to pH changes, TP was conducted in carboxy snarf (pH sensitive dye) loaded myocytes and no pH changes were observed during the TP protocol. Hence, the fluorescence changes observed in mt-cpYFP expressing myocytes subjected to TP were confirmed to be as a result of hypothermia-induced increases in mitochondrial superoxide concentration and not pH changes.

In this chapter the role of ERK in the process of TP was also investigated and it was demonstrated that TP-induced delay of mPTP opening was lost when TP was conducted in the presence of ERK inhibitors. It was further illustrated by means of western blots, that phosphorylation of ERK is significantly increased by TP. The increase in ERK phosphorylation was found to be abolished when TP was conducted in the presence of ERK inhibitors and also when TP was conducted in the presence of MPG, a ROS scavenger. The latter result implies a TP-induced functional coupling between ROS and ERK phosphorylation, where ROS is upstream of and is required to be activated for phosphorylation of ERK to occur.

In summary, this chapter demonstrates that TP-induced protection of isolated myocytes is dependent on increased mitochondrial ROS production and subsequent phosphorylation of ERK.

Limitations

In this investigation myocytes were infected with adenovirus encoding the mt-cpYFP superoxide biosensor for 48 hours. Successful infection was determined by the appearance of low level basal fluorescence in mt-cpYFP expressing myocytes, when

excited at 488nm and emission collected at 515nm. Conversely, following 48 hours in culture, uninfected myocytes displayed no basal fluorescence. The fluorescence of mt-cpYFP expressing myocytes was demonstrated to significantly increase by the addition of xanthine and xanthine oxidase, indicating an increase in mitochondrial superoxide concentration. The xanthine/xanthine oxidase induced fluorescence changes rapidly returned to basal levels upon removal of xanthine/xanthine oxidase from the perfusing solution. In order to confirm that the superoxide sensitivity of mt-cpYFP expressing myocytes was not merely due to the presence of virus DNA, control experiments should have been conducted in which myocytes were infected for 48 hours with scrambled DNA.

The pH sensitive dye carboy snarf was used to determine whether TP resulted in pH changes in mitochondria of intact myocytes. It was demonstrated that perfusion of carboxy snarf loaded myocytes with Tyrode titrated to either pH 6 or pH 9 induced robust fluorescence decreases and increases, respectively. Carboxy snarf is a single excitation to dual emission dye, owing to the protonated and deprotonated forms of the molecule, which allows for ratiometric imaging. This corrects for differences in dye loading or movement artefacts and therefore provides a more accurate means to monitor changes in intracellular pH. A limitation of this study is that carboxy snarf imaging was conducted using single excitation to single emission. However, this still produced clearly identifiable, reversible changes in intracellular pH. Moreover, the experiments in which carboy snarf was used were conducted on non-contracting, quiescent myocytes greatly reducing the chances of false fluorescent changes due to movement artefacts.

6 Discussion

The aims of this study were to examine the cellular changes induced by temperature preconditioning (TP) of adult rat myocytes. Isolated ventricular myocytes were temperature preconditioned by two brief episodes of profound hypothermia (16°C), resulting in protection against simulated ischaemia-reperfusion injury. TP induced protection was found to be associated with a delay of pathological mitochondrial permeability transition pore (mPTP) opening; believed to be the central determinant of myocyte survival in the setting of ischaemia-reperfusion injury (Halestrap, 2010). However, it was found that non-pathological transient mPTP opening was unaltered by TP. A crucial part of the signaling pathway underlying protection by TP was found to involve brief periods of hypothermia-induced mitochondrial ROS production which was measured using a superoxide biosensor. The TP-induced mitochondrial ROS production was found to be an obligatory signaling event required for TP. It was found that the presence of the ROS scavenger MPG during TP significantly reduced mitochondrial ROS and resulted in a loss of TP-induced delay of mPTP opening. TP was also found to significantly increase ERK1/2 phosphorylation. Further, it was found that TP-induced mitochondrial ROS production was upstream of ERK1/2 and was essential for ERK1/2 phosphorylation. The presence of ERK 1/2 inhibitors, U0126 or PD98059, during TP abolished ERK 1/2 phosphorylation. Moreover, preventing ERK 1/2 phosphorylation was found to abrogate the protective effect of TP on delaying mPTP opening. This is the first study to investigate the effects of TP on isolated ventricular myocytes. Further, this study is the first to characterize the timing of ROS involvement in TP and also to demonstrate the involvement of the reperfusion injury salvage kinase (RISK) pathway protein ERK1/2 in the cardioprotective signaling cascade of TP.

6.1 Temperature preconditioning protects isolated myocytes against simulated ischaemia-reperfusion injury

In 2007, TP was found to confer cardioprotection in the isolated heart subjected to 25 minutes of global ischaemia followed by 60 minutes of reperfusion. The protective effects of TP manifested in the form of improved haemodynamic recovery, decreased arrhythmias and reduced necrotic damage (assessed by lactate dehydrogenase release) when assessed during reperfusion (Khaliulin *et al.*, 2007).

In the current study, the effects of TP on isolated ventricular myocytes were investigated and it is demonstrated that the protective effects of TP can be induced at the single cell level. Protection was determined by observing that TP of myocytes resulted in improved recovery of contractile function following exposure to 7 minutes of metabolic inhibition (MI) and 10 minutes of reenergisation. A key feature of this finding is the implication that the signalling pathways involved in TP exist at the level of individual myocytes and are independent of any signalling influence from other cell types present within the heart, such as endothelial cells and fibroblasts. The finding in this study that TP of isolated myocytes improves recovery of contractile function following MI and reenergisation is in agreement with previously published findings (Khaliulin *et al.*, 2007). Khaliulin *et al.* reported that TP of the whole heart improved haemodynamic recovery, resulting in significantly higher LVDP during reperfusion. Since LVDP is a measure of cardiac contractility, this correlates positively with the finding in this study that TP improves recovery of contractile function of isolated myocytes. Further, Khaliulin *et al.* also reported decreased arrhythmias and reduced necrotic damage as a result of TP and these findings are also in agreement with the

results of this study. This study reports TP-induced improved recovery of contractile function following MI and reenergisation and this can be taken as a surrogate of improved viability, since the ability to contract is directly dependent on viability. Therefore the TP-induced improvement of contractile function may represent increased viability, which in turn would reduce necrotic damage as observed in the whole heart by Khaliulin *et al*. Additionally, it was found in this study the TP significantly increased the percentage of myocytes that recovered synchronous Ca^{2+} transients following MI and reenergisation compared to control, indicating improved Ca^{2+} homeostasis (chapter 3 / figure 3.11). This is consistent with Khaliulin's finding of decreased arrhythmias which are typically caused by Ca^{2+} mismanagement. Thus the TP-induced improvement of contractile function may be the result of improved viability and improved Ca^{2+} homeostasis.

Positive control experiments were conducted using established cardioprotective agents in order to confirm and compare the degree of cardioprotection induced by TP of isolated myocytes. The mitochondrial uncoupler 2, 4-dinitrophenol (DNP) is documented to have cardioprotective properties which have been demonstrated in both whole heart (Minners *et al.*, 2000) and isolated myocyte studies (Rodrigo *et al.*, 2002). DNP preconditioning of isolated myocytes was found to improve contractile function in a similar manner to TP. Likewise, it was found that conditioning of isolated myocytes with the mPTP inhibitor cyclosporine A (CsA), a widely reported cardioprotective agent (Nazareth *et al.*, 1991; Piot *et al.*, 2008), also resulted in a significant improvement in contractile recovery to a degree which was similar to that observed for TP. These findings validate the use of the MI and reenergisation protocol used in this study to simulate ischaemia-reperfusion injury and further serves to

confirm the protective efficacy of the results obtained with regards to TP of isolated myocytes.

In this study the end point used to determine cardioprotection was recovery of contractile function following MI and reenergisation and this was significantly improved by TP, DNP preconditioning and CsA conditioning. This may have been the result of decreased cell death following MI and reenergisation as suggested above, but in reality is uncertain since viability was not directly measured and loss of contractile function can also arise as a result of myocardial stunning. This is a post-ischaemic dysfunction which can impair contractile function for prolonged periods but is entirely reversible. Therefore the improved recovery of contractile function of temperature preconditioned myocytes observed in this study may have been the result of a TP-induced acceleration of recovery of stunned myocytes. For this reason, the use of contractile function as an end point of preconditioning studies is undesirable as discerning myocytes that are dead as opposed to stunned based on morphology is undetectable. Notwithstanding these limitations, the use of contractile recovery as an end point is advantageous as it gives an output of functional myocytes. Although stunning is a reversible process it is not without detrimental health implications as loss of contractile function in significant portions of the heart would adversely impact cardiac output. Consequently, functional regions of the myocardium will be subject to increased burden thereby increasing susceptibility to arrhythmias. Hence, interventions such as TP which improve recovery of contractile function, either by increasing cell viability or accelerating recovery from stunning, are considered beneficial.

In this study, initial investigations of TP of isolated myocytes were conducted following the protocol of Khaliulin *et al.* using a hypothermic temperature of 26^{o}C interspersed with normothermic incubation at 37^{o}C, for three cycles. However, it was found that TP in this manner produced inconsistent results. TP using 26^{o}C was found to significantly reduce Ca^{2+} overload following MI and reenergisation compared to control (data not shown), but did not significantly improve contractile recovery (chapter 3 / figure 3.22). For this reason, the hypothermic temperature was progressively reduced to 16^{o}C at which temperature robust TP-induced cardioprotection was consistently observed (chapter 3 / figures 3.4 & 3.10). The reasons for the discrepancy in the preconditioning temperatures between this study and Khaliulin's are unclear and may relate to the fact that the myocytes in this study are isolated. Nonetheless, using profound hypothermic temperatures to precondition the myocardium is not uncommon and previous reports have documented protection of isolated hearts using hypothermic perfusion at 17^{o}C (Chen *et al.*, 2002; Riess *et al.*, 2004). A recent study conducted by Khaliulin *et al.* investigated the most effective hypothermic temperature to temperature precondition the myocardium. A range of temperatures including 32^{o}C, 26^{o}C, 17^{o}C and 7^{o}C were investigated in the *ex vivo* Langendorff perfused rat heart and it was reported that 26^{o}C granted optimal protection. Nevertheless 32^{o}C and 17^{o}C were also found to confer protection to the myocardium, whereas preconditioning with 7^{o}C actually exacerbated reperfusion injury (Khaliulin *et al.*, 2011). TP using 7^{o}C was associated with substantially greater Ca^{2+} accumulation and this is proposed to be a contributing factor to the greater damage caused by ischaemia-reperfusion injury. Hypothermia is known to inhibit ATP-dependent ion channels which results in cellular Ca^{2+} accumulation. The severity of inhibition is greater at lower temperatures and therefore

excessive Ca^{2+} accumulation is observed at 7^{o}C. This finding is supported by an independent study which found that rapid cooling of the myocardium to 1^{o}C caused contracture as a result of Ca^{2+} release from sarcoplasmic reticulum (Bers *et al.*, 1989). Ca^{2+} accumulation was also observed at preconditioning temperatures of 32^{o}C, 26^{o}C and 17^{o}C in Khaliulin's study, although to a much lesser extent. However, evidence suggests that moderate intracellular Ca^{2+} accumulation prior to prolonged ischaemia exerts a cardioprotective effect and may represent a crucial part of the signaling cascades involved in TP (Przyklenk *et al.*, 1999; Sanada & Kitakaze, 2004). This will be discussed in more detail under section 6.5.

6.2 Temperature preconditioning improves calcium handling following simulated ischaemia-reperfusion injury

Elevated Ca^{2+} in myocytes following ischaemia-reperfusion injury has pathophysiological implications associated with cellular injury (Rodrigo & Standen, 2005). High intracellular Ca^{2+} underlies cardiomyocyte hypercontracture, the development of areas of cell death by necrosis (Piper *et al.*, 2004; Altschuld *et al.*, 1985)(), the activation of pro-apoptotic proteins and Ca^{2+}-dependent proteases (Dong *et al.*, 2006) and pathological mPTP opening (Halestrap *et al.*, 2007). Ischaemic preconditioning (IPC) along with other cardioprotective interventions have been shown to significantly reduce post ischaemia-reperfusion Ca^{2+} loading and augment contractile responsiveness to Ca^{2+} (Rodrigo & Samani, 2008; An *et al.*, 2001). The effects of TP on intracellular Ca^{2+} following ischaemia-reperfusion injury are yet to be investigated and was therefore examined in the present study.

In addition to improving recovery of contractile function following simulated ischaemia-reperfusion injury, TP was also found to significantly decrease basal Ca^{2+} and improve Ca^{2+} homeostasis compared to control (chapter 3 / figures 3.10, 3.11 & 3.12). The majority of control myocytes were found to incur significant elevation in basal calcium upon reenergisation and typically associated with this was an impairment of the myocytes ability to regain synchronous calcium transients in response to electrical field stimulation. Conversely, temperature preconditioned myocytes were found to have significantly lower basal calcium levels (typically < 150nM) at the end of reenergisation compared to controls. Moreover, an increased percentage of temperature preconditioned myocytes regained synchronous calcium transients in response to field stimulation which is intuitive, since TP was also found to improve recovery of contractile function (see previous section), which is directly dependent on proper calcium homeostasis. However, whether the improvement of basal Ca^{2+} as induced by TP is the cause of improved recovery of Ca^{2+} transients is unclear. It was occasionally observed that myocytes exhibiting elevated basal Ca^{2+} following MI and reenergisation managed to re-establish synchronous Ca^{2+} transients within 10 minutes of reenergisation. In contrast, it was also observed that some myocytes with relatively low basal Ca^{2+} following MI and reenergisation were unable to recover synchronous Ca^{2+} transients (data not shown). Thus it is obvious that basal Ca^{2+} level alone is not the sole determinant for restoration of synchronous Ca^{2+} transient, although the finding that a higher percentage of myocytes with low basal Ca^{2+} recovered synchronous Ca^{2+} transients indicates a correlation does exist (chapter 3 / figures 3.11 & 3.12).

The improvement of Ca^{2+} homeostasis induced by TP would certainly favor myocyte survival since high intracellular calcium is known to contribute to the damage caused

by ischaemia-reperfusion injury. High mitochondrial matrix Ca^{2+} concentration is a critical trigger evoking the irreversible opening of the mPTP (Griffiths & Halestrap, 1995). Therefore, maintenance of Ca^{2+} homeostasis of temperature preconditioned myocytes may underlie many of the therapeutic benefits of TP already reported including decreased infarct size and reduced arrhythmias (Khaliulin *et al.*, 2007).

In this study the observed reduction in basal Ca^{2+} of temperature preconditioned myocytes following MI and reenergisation was also mimicked by DNP preconditioning and CsA conditioning of myocytes. This suggests that preventing Ca^{2+} overload may be a common outcome of all cardioprotective interventions. In agreement with the results of this study, previous studies have also reported DNP preconditioning to reduce Ca^{2+} overload following MI and reenergisation (Rodrigo *et al.*, 2002). Similarly, several other groups have demonstrated that pharmacological preconditioning, using a number of different agents, can prevent Ca^{2+} overload upon reperfusion and thereby prevent cardiomyocyte death (Gao *et al.*, 2007; Baczko *et al.*, 2004; Light *et al.*, 2001; Chen *et al.*, 2009). The literature suggests that the mechanism by which intracellular Ca^{2+} in preconditioned myocytes is maintained low upon reperfusion is not universal, but rather varies depending on the preconditioning agent used. For example, sodium ferulate preconditioning is believed to prevent Ca^{2+} overload via a NO/cGMP/PKG pathway (Chen *et al.*, 2009), whilst PMA preconditioning is believed to act through activation of sarcolemmal K_{ATP} channels (Light *et al.*, 2001). Further, DNP preconditioning is believed to mediate its protection through mitochondrial depolarisation and depletion of mitochondrial NADH (Rodrigo *et al.*, 2002), whereas CsA conditioning is proposed to prevent Ca^{2+} overload through inhibition of pathological mPTP opening (Rodrigo & Standen, 2005). This study investigates the

effects of TP on intracellular Ca^{2+} following simulated ischaemia-reperfusion injury and demonstrates that TP acts to reduce reenergisation-induced Ca^{2+} overload. The mechanism by which TP reduces Ca^{2+} overload was not directly investigated, but evidence from other experiments conducted in this study concerning the mitochondrial permeability transition pore (mPTP) indicate this may involve inhibition of pathological mPTP opening. In the next section, the finding that TP increases the tolerance of isolated myocytes to pathological mPTP opening is discussed. The mPTP is known to open during the first few minutes of reperfusion and is responsible for causing necrotic cell death. Preventing mPTP opening with agents such as CsA and sanglifehrin A has previously been shown to be sufficient to prevent Ca^{2+} overload in isolated myocytes (Rodrigo & Standen, 2005). Therefore, as TP also inhibits mPTP opening, it is plausible to postulate that TP may also reduce Ca^{2+} overload through an mPTP-dependent mechanism upon reperfusion.

TP of isolated myocytes significantly increased the percentage of myocytes with intracellular Ca^{2+} <150nM following MI and reenergisation to 81.7 ± 11.0% (n = 67, 3) from 41.1 ± 3.7% (n = 69, 3) for control myocytes. Additionally, TP also significantly increased the proportion of myocytes that recovered synchronous calcium transients following MI and reenergisation to 60.1 ± 5.1% (n = 67, 3) from 31.5 ± 3.0% (n = 69, 3) for control myocytes. This correlated well with the proportion of myocytes that were found to recover contractile function following MI and reenergisation which was 51.6 ± 5.7% (n = 225, 3) for temperature preconditioned myocytes compared to 28.9 ± 5.3% (n = 229, 3) for control myocytes. These results taken together illustrate that not all myocytes that recover Ca^{2+} transients will necessarily recover contractile function. This is consistent with the findings of Rodrigo and Standen (Rodrigo & Standen, 2005) who

demonstrated a similar trend when investigating the effects of IPC on Ca^{2+} overload and contractile recovery following MI and reenergisation, in isolated myocytes. Intact Ca^{2+} homeostasis is undoubtedly a prerequisite for recovery of contractile function since proper calcium cycling underlies excitation-contraction coupling. However the results of this study and those of Rodrigo and Standen highlight that secondary mechanisms may also be impaired following MI and reenergisation which impair contractile function independent of Ca^{2+} homeostasis. Such mechanisms may cause damage to the excitation-contraction machinery and in doing so, impair contractile function. Alternatively, the lower recovery of contractile function compared to the proportion of myocytes that recovered Ca^{2+} transients may be as a result of myocardial stunning which would be expected to recover given sufficient time. Therefore, it is possible that had experiments conducted in this study been extended for a longer period of time following MI and reenergisation, the proportion of myocytes that recovered contractile function would more closely match the figures for the proportion of myocytes that recovered Ca^{2+} transients.

6.3 Temperature preconditioning of isolated myocytes increases tolerance against oxidative stress-induced pathological mPTP opening

The opening of the mPTP was first proposed to be a mediator of cell death in the setting of acute ischaemia-reperfusion injury by Crompton's group in 1986 (Al-Nasser & Crompton, 1986). Since then the opening of the mPTP at reperfusion has become well established as the key determinant of cell fate by causing mitochondrial dysfunction, ATP depletion and subsequent necrotic cell death. Although how mPTP

opening is modulated is not fully understood (Halestrap, 2010). Cardioprotective interventions such as IPC and ischaemic post conditioning (IPost) have been shown to inhibit mPTP opening, with several of the mediatory intracellular signal transduction pathways reported to terminate at the mPTP (Halestrap *et al.*, 2007; Hausenloy & Yellon, 2007a). Likewise, inhibiting mPTP opening at the time of myocardial reperfusion with agents such as CsA, or genetic treatments designed to prevent mPTP opening result in cardioprotection and are capable of reducing myocardial infarct size by 30-50%. Moreover, genetic ablation of cyclophilin-D, a core component of the mPTP, was found to confer protection against ischaemia-reperfusion injury which could not be further enhanced by CsA or IPost. (Griffiths & Halestrap, 1993),(Gomez *et al.*, 2007; Hausenloy & Yellon, 2003; Argaud *et al.*, 2005; Baines *et al.*, 2005; Hausenloy *et al.*, 2004). Thus the current paradigm suggests that an intervention which inhibits mPTP opening would likely be cardioprotective. In 2007, Khaliulin *et al.* demonstrated that TP could inhibit Ca^{2+}-induced mitochondrial swelling (a reflection of mPTP opening) in de-energised isolated mitochondria (Khaliulin *et al.*, 2007). In this study I sought to confirm this in intact cardiomyocytes using a well established model of mPTP opening (Hausenloy *et al.*, 2004) and a second novel model developed by myself.

This study utilised a well established oxidative stress-induced mPTP opening model to directly examine the effects of TP on mPTP opening in the intact myocyte. As expected, it was found that mPTP opening was significantly delayed by treatment of myocytes with the mPTP inhibitor CsA, whereas mPTP opening was augmented by treatment with the mPTP opener carboxyatractyloside. Importantly, treatment of myocytes with the calcineurin inhibitor FK-506 was found not to alter the time to mPTP opening compared to control myocytes. This indicated that CsA was acting to inhibit mPTP

opening via a mechanism that was independent of calcineurin inhibition. This model of mPTP opening examines the induction of the mPTP in response to oxidative stress. The relationship between oxidative stress and mPTP opening is relevant to investigate since both factors have been shown to contribute to myocyte death following ischaemia-reperfusion injury. Temperature preconditioned myocytes were found to have increased tolerance to oxidative stress-induced mPTP opening and the time to mPTP opening was significantly delayed compared to control. Likewise, pharmacological preconditioning with DNP was also found to increase tolerance to mPTP opening. In fact both TP and DNP preconditioning were found to delay oxidative stress-induced mPTP opening to a greater extent than with treatment with the known mPTP inhibitor CsA. It is believed that CsA inhibits mPTP opening by inhibiting the activity of cyclophilin-D, a core component of the mPTP (Halestrap *et al.*, 1997). The increased tolerance to oxidative stress induced by TP and DNP preconditioning suggest they may inhibit mPTP opening by an alternative mechanism to CsA, possibly involving several signalling mediators which act to directly and indirectly, ultimately inhibit mPTP opening. Direct inhibition of mPTP opening would involve signalling cascades converging on a regulatory component of the mPTP, modulating its action to decrease the likelihood of mPTP opening. In contrast an indirect mechanism would serve to alter factors which influence mPTP opening such as calcium regulation, oxidative stress, ATP concentrations and pH regulation. How TP may affect these factors to increase tolerance of myocytes to mPTP opening is the subject of section 6.8 and will be discussed in more detail.

In this study CsA was consistently used at a concentration of 4µM to inhibit mPTP opening. It is widely reported that CsA has a relatively narrow therapeutic range of

efficacy and becomes toxic at higher concentrations. Studies in which CsA is used to inhibit mPTP opening typically use CsA at concentrations between 0.1μM - 2μM. In this study, I too began by using concentrations of CsA within this range, but found greatest inhibition of mPTP opening using CsA at 4μM. Lemaster's group found that CsA used in excess of 5μM concentration in isolated myocytes was cytotoxic and proposed that the loss of CsA induced cytoprotection at higher concentrations may be due to inhibition of calcineurin (Kim *et al.*, 2006). Although the concentration of CsA used in this study was atypical and above that of the reported therapeutic range, the results of this study clearly demonstrate that CsA at 4μM produced a cytoprotective rather than cytotoxic effect on isolated myocytes exposed to simulated ischaemia-reperfusion injury.

The above mentioned results relate to an established model used to quantify mPTP opening from intact myocytes in real-time. This model relies on photoactivated TMRM molecules to produce oxidative stress which induce mPTP opening. Within the same experiment, the fluorescence of TMRM is used to report changes to mitochondrial membrane potential, a surrogate marker for mPTP opening. Using this model, TP was clearly shown to increase tolerance of isolated myocytes to mPTP opening. To confirm this result, I developed a second assay in which TMRM was used at a significantly lower concentration (5μM vs. 50nM) only to measure changes to mitochondrial membrane potential (mPTP opening), coupled with xanthine and xanthine oxidase used to produce the ROS necessary for inducing mPTP opening. The rationale for using xanthine and xanthine oxidase to induce mPTP opening was based on a recent finding by Wang *et al.* (Wang *et al.*, 2008). This group used a superoxide biosensor to demonstrate that perfusion of cells with xanthine and xanthine oxidase caused a marked increase in mitochondrial ROS concentration. Therefore using xanthine and

xanthine oxidase in this model is most appropriate, as it closely reflects the events of reperfusion injury in which a burst of mitochondrial ROS production is observed and is believed to be a key factor evoking mPTP opening. The efficacy of this model as one which measures mPTP opening was confirmed using CsA. Treatment of myocytes with CsA significantly delayed the time to xanthine and xanthine oxidase induced mPTP opening compared to untreated control myocytes. This model of xanthine and xanthine oxidase-induced mPTP opening was then used to confirm that TP delays pathological mPTP opening.

Thus, TP was found to delay mPTP opening in two sets of experiments using different models. This finding is consistent with reports that de-energised, isolated mitochondria harvested from temperature preconditioned hearts also exhibit increased tolerance to Ca^{2+}-induced mitochondrial swelling, which is also considered to be a reflection of mPTP opening (Khaliulin *et al.*, 2007).

6.4 Temperature preconditioning of isolated myocytes does not block non-pathological transient mPTP flickering

Irreversible mPTP opening is pathological and is characterised by deregulation of mitochondrial matrix Ca^{2+} release, termination of oxidative phosphorylation, mitochondrial matrix swelling with concomitant inner membrane unfolding and eventual outer membrane rupture and cell death (Rasola & Bernardi, 2011). Irreversible opening of the mPTP represents a point of no return in committing a cell to death. In contrast, evidence exists supporting a transient form of mPTP opening, or 'flickering', found in both isolated mitochondria (Huser & Blatter, 1999) and in intact cells (Petronilli *et al.*, 1999). This low conductance-state of the mPTP is believed to

release accumulated Ca^{2+} from mitochondria when a concentration gradient forms between the matrix and the external medium (Huser & Blatter, 1999; Jacobson & Duchen, 2002; Ichas & Mazat, 1998). In this way, transient mPTP flickering may serve to contribute to organelle Ca^{2+} homeostasis acting as a fast Ca^{2+}-induced Ca^{2+} release mechanism by which mitochondria avoid matrix Ca^{2+} overload and in turn, irreversible pathological mPTP opening. Thus the notion of the mPTP has shifted from one which solely implicates it as a regulator of cell death in various pathologic states, including ischaemia-reperfusion injury; to one in which transient mPTP flickering has an important physiological function in regulating Ca^{2+}. In support of this notion, recent evidence has demonstrated that cyclophilin-D (a core component of the mPTP) knock out mice, which are chronically protected against ischaemia-reperfusion injury (Baines *et al.*, 2005), have an increased propensity to develop heart failure after transaortic constriction, overexpression of Ca^{2+}/calmodulin dependent protein kinase IIδc or swimming exercise (Elrod *et al.*, 2010). Moreover, cyclophilin-D knock out was associated with heart mitochondria possessing higher basal Ca^{2+}, suggesting a defect in mitochondrial Ca^{2+} handling related to loss of mPTP function. Furthermore, CsA treatment of cultured cardiomyocytes was found to acutely reduce mitochondrial Ca^{2+} efflux and leak (Elrod *et al.*, 2010). In the previous section, the protective effects of TP on inhibiting irreversible pathological mPTP opening were discussed in light of evidence found in the current study and also that which was found by Khaliulin *et al.* (Khaliulin *et al.*, 2007). However, to date, the effect of TP on transient mPTP flickering are yet to be investigated and was therefore investigated in this study.

Given the knowledge that TP inhibited irreversible pathological mPTP opening, I set out to investigate the effects on non-pathological basal mPTP flickering after a

standard TP protocol. Transient mPTP flickering was measured using an established calcein-cobalt quench assay (Hausenloy *et al.*, 2010; Petronilli *et al.*, 1999; Saotome *et al.*, 2009) and the specificity of the assay, as one that measures mPTP flickering, was confirmed using CsA which completely blocked transient mPTP flickering (chapter 4 / figures 4.21 & 4.22). This was reflected by retention of mitochondrial calcein fluorescence which can only leave mitochondria via the open mPTP. Hence in the presence of CsA which blocks transient mPTP opening mitochondrial calcein fluorescence is retained. In contrast, mitochondrial calcein fluorescence in control and temperature preconditioned myocytes fell by a similar percentage over the course of an experiment, indicating transient mPTP flickering had occurred. This indicated that transient mPTP flickering rates were unaltered following TP of isolated myocytes when measured during basal (non-stressed) conditions. This result suggests that the mechanism of mPTP inhibition differs between CsA treatment and TP of isolated myocytes. While CsA treatment of myocytes causes a total block of both transient mPTP flickering and irreversible mPTP opening, TP was only found to effect irreversible mPTP opening. The total block of mPTP opening as induced by CsA treatment is supported by previous studies and the mechanism is strongly believed to be cyclophilin-D dependent. While much evidence exits surrounding the properties of the mPTP, its molecular structure remains largely unresolved. Cyclophilin-D is a matrix peptidylprolyl *cis-trans* isomerase and represents the only protein with a confirmed regulatory role of the mPTP as confirmed by genetic studies (Bernardi & Rasola, 2007). CsA inhibits mPTP opening by binding to cyclophilin-D which evidently results in complete block of mPTP opening. By comparison, TP only appears to effect irreversible mPTP opening, while permitting transient mPTP flickering to ensue. How TP permits

transient mPTP flickering to occur while inhibiting irreversible mPTP opening was not directly investigated in this study, but will be considered below.

In addition to a postulated physiological role in regulating Ca^{2+}, it has recently emerged that transient mPTP flickering plays a protective signalling role in hypoxic preconditioning (Hausenloy *et al.*, 2010). Hausenloy *et al.* demonstrated that the rate of mPTP flickering was augmented during a hypoxic preconditioning protocol and that this was necessary for cardioprotection. Previous work conducted by the same group demonstrated that cardioprotection induced by IPC or pharmacological preconditioning, using various agents, could be abolished by the presence of CsA during the preconditioning phase (Hausenloy *et al.*, 2004). This strongly implicates transient mPTP opening as a mediator of cardioprotection. Thus, an emerging picture presents the mPTP as a double-edged sword, with both protective and deleterious actions. In the present study, transient mPTP opening rates were measured following the TP phase. However, based on the literature, I hypothesise that had transient mPTP flickering been measured during the preconditioning protocol, an increased rate of mPTP flickering compared to control myocytes would have been observed. It is proposed that preconditioning protocols which impose innocuous stress upon myocytes, such as IPC and TP, are likely to expose mitochondria to modest Ca^{2+} overload conditionings which may be sufficient to trigger transient mPTP flickering (Korge *et al.*, 2011). This is corroborated for TP by an earlier study which demonstrated that moderate hypothermia decreases the activity of membrane bound Ca^{2+}-pumps resulting in increased cellular Ca^{2+} concentration (Liu *et al.*, 1991). Subsequent to increased mPTP flickering may be the increased incidence of superoxide flashes (Wang *et al.*, 2008) that would increase mitochondrial ROS concentration. An increase in

mitochondrial ROS concentration is demonstrated in the present study to be essential for TP-induced protection and will be discussed in more detail in the next section. Previous studies have also shown ROS to be a crucial mediator of several other preconditioning protocols including IPC, and treatment with a ROS scavenger during the preconditioning phase is sufficient to abolish protection (Hausenloy *et al.*, 2004). Thus an increased rate of mPTP flickering induced during TP is a highly probable occurrence that likely represents a key signalling event, linked to the release of mitochondria derived ROS required to induce cardioprotection.

In addition to increasing mPTP flickering rates during the phase of TP, I hypothesise that TP would result in myocytes in which the mPTP is primed to increase low conductance flickering in response to noxious stimuli, such as that which exists during reperfusion following prolonged ischaemia. This would serve to limit the build up of Ca^{2+} (a key trigger of irreversible mPTP opening) within the mitochondrial matrix thereby increasing the threshold for irreversible pathological mPTP opening. Transient mPTP opening would cause mitochondrial depolarisation long enough to extrude accumulated matrix calcium while retaining essential matrix metabolites such as NADH, after which mitochondria rapidly repolarise. The rate of reuptake via the Ca^{2+} uniporter is expected to be significantly slower then the Ca^{2+} flushing rate and therefore only a tiny fraction of mitochondria (less than 1%) are depolarised at any given point in time. Since mPTP openings are stochastic and asynchronous, it would be predicted that transient mPTP flickering in this manner would not impair cellular energy generation and would increase tolerance to irreversible mPTP opening (Korge *et al.*, 2011). This hypothesis offers an explanation for the finding in intact cardiac myocytes that irreversible mPTP opening is difficult to induce with Ca^{2+} loading alone,

but requires the accompaniment of high ROS levels. In fact, emerging evidence suggests that ROS rather than Ca^{2+} is largely responsible for irreversible mPTP opening during reperfusion injury (Kim *et al.*, 2006; Juhaszova *et al.*, 2008).

In conclusion, the results of this study, taken together with previously published work, highlights the mPTP as a highly dynamic structure. The mPTP serves a physiological role in mitochondrial Ca^{2+} homeostasis, along with a central role in cell protection and cell death.

6.5 Superoxide biosensor reveals mitochondrial ROS production during temperature preconditioning of isolated ventricular myocytes

As with the mPTP, the role of ROS in the setting of ischaemia-reperfusion injury is considered to be both protective and deleterious. It is well established that excessive ROS production upon reperfusion of the ischaemic myocardium is a key trigger evoking irreversible mPTP opening which results in necrotic cell death. However, increasing evidence supports a role for ROS in cardioprotective signaling cascades during preconditioning. This has previously been reported with regards to TP and it was demonstrated that cardioprotection induced by TP was abrogated by the presence of MPG (ROS scavenger) during the preconditioning phase (Khaliulin *et al.*, 2007). In the current study, this finding was expanded upon and I sought to characterise the timing of ROS involvement during the TP phase. This was achieved with the aid of a mitochondrially targeted reversible superoxide biosensor which allowed real-time measurements of dynamic changes in superoxide levels to be detected in living cells.

This study reports that mitochondrial ROS is produced during the hypothermic phase of TP and that this ROS production is essential for cardioprotection. The TP protocol consisted of two cycles of 2 minutes of hypothermia followed by 3 minutes of normothermia. Bursts of mitochondrial ROS production were detected exclusively during the two hypothermic (16^{o}C) periods of the TP protocol. Hypothermia-induced mitochondrial ROS production was found to be an obligatory signalling event required for TP and it was found that the presence of a ROS scavenger during the TP phase significantly dampened ROS production. Further, the presence of ROS scavengers during the preconditioning phase ablated the TP-induced delay in irreversible mPTP opening. Excess ROS generation is a well documented cardiac occurrence associated with reperfusion following ischaemia. It is also known that ischaemia and acute hypothermia cause cardiorespiratory depression. (Brookes *et al.*, 1971; Dufour *et al.*, 1996; Deveci & Egginton, 2007; Ali *et al.*, 2010). Taking this information together I hypothesised that increasing the temperature from 16^{o}C to 37^{o}C during the TP protocol would be associated with an increase in mitochondrial ROS production, as the rate of mitochondrial respiration recovers from depression. However, results were to the contrary and mitochondrial ROS production was detected during the hypothermic phase of the preconditioning protocol. This finding is consistent with a previous report in which it was demonstrated that hypothermia (17°C) by cold perfusion of the isolated whole-heart resulted in dihydroethidium (DHE) fluorescence changes across the intact myocardial wall, suggesting an increase in ROS production (Riess *et al.*, 2004). Further, it was recently demonstrated in isolated mouse brain mitochondria, that hypothermia caused respiratory slowdown which was coupled to increased ROS production, hydrogen peroxide release and elevation of mitochondrial membrane potential (Ali *et*

al., 2010). In the present study the mechanism and source of increased mitochondrial ROS production was not directly investigated. However, given the relationship that has been described between transient mPTP opening and ROS production, it is possible that hypothermia-induced increases in the rate of mPTP flickering could account for the increase in ROS production. A point of potential conflict here is that Ali *et al.* observed in an isolated mitochondria study, that hyperpolarisation of the mitochondrial membrane at lower temperatures was associated with an increase in ROS production. This therefore implies that mitochondrial depolarisation, as occurs with mPTP opening, should decrease ROS production. However, recent evidence in intact cells indicate that brief openings of the mPTP causes transient mitochondrial depolarisation that stimulates ROS production (Huser *et al.*, 1998; Romashko *et al.*, 1998; Duchen, 2000; Zorov *et al.*, 2000). The mechanism by which transient mPTP opening stimulates ROS production in intact myocytes is not entirely known, but it was demonstrated that transient mPTP opening-triggered superoxide flashes were produced in an electron transport chain (ETC)and ATP dependent manner. Blockade of the electron transport chain at any site, or inhibition of the ATP synthase was found to be sufficient to abolish superoxide flash activity (Wang *et al.*, 2008), but the exact source of mPTP-triggered superoxide flashes remains undefined. Since complexes I and III of the respiratory chain are sources of electron leak and sites of ROS formation in the presence of O_2, these complexes have been suggested to be sites of hypothermia-induced ROS production (Riess *et al.*, 2004). The link between metabolic depression, as induced by hypothermia, and ROS production may be explained by decreased activity of the F1/F0 ATPase and reduced O_2 consumption by the ETC. This would have the consequence of increasing mitochondrial O_2 levels that can promote ROS production

(Ali *et al.*, 2010). In support of the notion that metabolic depression promotes ROS production, Hausenloy *et al.* recently reported that hypoxic preconditioning also stimulated mitochondrial ROS production (Hausenloy *et al.,* 2010). Moreover, they reported that ROS production was dependent on cyclophilin-D; implicating the mPTP as a critical trigger/generator of ROS production. In light of this, it may be proposed that any form of cellular stress which causes a partial inhibition of the respiratory chain, such as that caused by TP and IPC, would be sufficient to induce mitochondrial ROS production.

To explain the link between transient mPTP flickering and ROS production, a model of ROS induced-ROS release has been proposed. In this model, a preconditioning stimulus which confers mitochondrial stress would be expected to generate ROS due to a partial inhibition of the ETC. Consequently, ROS production would trigger transient mPTP flickering (ROS is a key trigger of mPTP opening), which in turn would trigger ETC-mediated and ATP-dependent bursts in superoxide production. The produced superoxide may then be converted to more stable freely diffusible ROS derivatives which could gather to create high ROS concentration signalling microdomains (Wang *et al.*, 2008). It is proposed that under basal conditions, the naturally occurring ROS-scavenging system prevents excessive superoxide production. However, when ROS production is stimulated by a preconditioning stimulus, the amount of ROS generated overwhelms the ROS scavenging system, resulting in the accumulation of ROS which may enter the cytosol and activate downstream mediatory kinases involved in cardioprotection (Hausenloy *et al.*, 2010). Alternatively, hypothermia-induced increases in mitochondrial Ca^{2+} concentration during the TP protocol (Przyklenk *et al.*, 1999; Sanada & Kitakaze, 2004) may be directly responsible for increased rates of

transient mPTP flickering which are associated with superoxide flashes. This is supported by the knowledge that Ca^{2+} is a key trigger of mPTP opening (Halestrap & Davidson, 1990). Ca^{2+} is also known for its roles as a second messenger and its ability to regulate mitochondrial metabolism. Mitochondrial Ca^{2+} controls critical elements of the Krebs cycle and the activity of Ca^{2+}-sensitive dehydrogenases, thereby allowing Ca^{2+}-mediated modulation of oxidative phosphorylation (Riess *et al.*, 2004). Thus, elevated mitochondrial Ca^{2+} can result in the coordinated upregulation of respiratory chain activity, resulting in increased ATP synthesis. This would be expected to be coupled to increased O_2 consumption. However the increased rate of respiration would also increase the probability of electron slippage from the respiratory chain to O_2, producing superoxide. This may represent an alternative mechanism by which hypothermia induces superoxide production.

To summaries, I have demonstrated that hypothermia-induced mitochondrial ROS production is an obligatory signaling event required for the observed protection granted by TP. I propose that changes in the rate of mitochondrial respiration is the initial trigger firing the activation of cardioprotective signaling cascades irrespective of the preconditioning stimulus (e.g. IPC, TP). Alterations in the rate of mitochondrial respiration may signal to the cell the need to modulate function in order to cope with an impending cellular or organismal challenge. One possibility is that this signal is carried away from the mitochondria by ROS, which may differ in source and type from the detrimental ROS associated with reperfusion injury. It is possible that the signaling ROS accumulates to form discrete pockets of high concentration ROS, within which, secondary downstream effector(s) are activated. These signaling microdomains may be spatially removed from mPTP's and thus would not trigger pathological irreversible

mPTP opening. Alternatively the signaling ROS, produced as a result of mPTP flickering, may enter the cytosol and directly interact with downstream effectors.

6.6 Specificity and implications of using the mt-cpYFP superoxide biosensor to measure mitochondrial ROS production

mt-cpYFP was first described as a mitochondrially targeted superoxide biosensor by Wang *et al* in 2008 (Wang *et al.*, 2008). Extensive *in vitro* research revealed the superoxide selectivity of the biosensor over physiologically relevant oxidants and metabolites. Moreover, superoxide-induced (generated by 2mM xanthine and 20mU xanthine oxidase under aerobic conditions) increases in mt-cpYFP fluorescence were reversed by the subsequent addition of Cu/Zn-superoxide dismutase (SOD), or prevented by prior addition of SOD. However the selectivity of the biosensor for superoxide was questioned in 2011 as experiments conducted in *Arabidopsis* mitochondria failed to detect superoxide flashes. Instead these experiments revealed the biosensor to be highly sensitive to pH (Schwarzlander *et al.*, 2011). In light of these conflicting results I characterised the sensitivity of the mt-cpYFP probe to both pH and superoxide radicals in CHO cells and isolated myocytes.

The results of this study support the claim of Wang *et al.* that the biosensor is sensitive to superoxide. mt-cpYFP fluorescence changes were detected in response to xanthine and xanthine oxidase in both CHO cells and adenovirally infected myocytes expressing the biosensor. However, as expected and acknowledged by Wang *et al*., I found that the protein-based fluorescent probe is also sensitive to pH. TP of isolated myocytes was found to be associated with two bursts of superoxide production observed during the hypothermic phase of the TP protocol. The detected superoxide produced was

found to be highly sensitive to ROS scavengers. In contrast, TP was not found to alter mitochondrial pH as detected with a mitochondrially loaded pH sensitive probe (carboxy snarf). This finding implied that the fluorescence changes of mt-cpYFP in response to TP were caused by increased detection of superoxide anions and not mitochondrial alkalinisation. Thus, I report that the mt-cpYFP probe can be used as a biosensor for superoxide anions so long as the correct controls are conducted.

In its initial description, the mt-cpYFP probe was thoroughly characterised and subsequently used to detect superoxide flashes in a range of living cells including adult cardiac myocytes. Real-time imaging using mt-cpYFP revealed intermittent, quantal bursts of superoxide production within the mitochondrial matrix of all cell types tested (Wang *et al.*, 2008). Investigation into the molecular mechanism of superoxide genesis revealed a functional coupling between stochastic openings of the mPTP and superoxide flashes. Each superoxide flash was found to coincide with transient mitochondrial depolarisation and release of mitochondrial-entrapped rhod-2, suggesting that each flash permitted a fraction of rhod-2 to escape the mitochondrial matrix via the transiently open mPTP. Moreover, pharmacological and genetic manipulation of cyclophilin-D, a critical component of the mPTP, significantly attenuated the incidence of superoxide flashes. This led the investigators to propose that monitoring superoxide flash activity provides a means for detecting transient mPTP flickering in living cells (Wang *et al.*, 2008). The implications of this with regards to this study is that TP-induced ROS production, as measured using mt-cpYFP, was triggered by transient mPTP opening. Future experiments could confirm this by TP myocytes in the presence of CsA and based on the reports of Wang *et al*., this would be expected to abrogate both hypothermia-induced ROS production and TP-induced

protection. This hypothesis is supported by previous experiments that demonstrated that IPC and pharmacological preconditioning induced protection was abrogated by the presence of CsA during the preconditioning phase (Hausenloy *et al.*, 2004). Furthermore, a recent study found that hydrogen peroxide preconditioning was also abolished by CsA, implicating transient mPTP flickering as a crucial mediator of cardioprotection (Saotome *et al.*, 2009). Further evidence supporting the notion of transient mPTP triggered superoxide production comes from the demonstration that hypoxic preconditioning generates mitochondrial ROS in wild type but not cyclophilin-D deficient mice (Hausenloy *et al.*, 2010). Moreover, hypoxic preconditioning was found to protect wild type cardiomyocytes against simulated ischaemia-reperfusion injury but not cyclophilin-D deficient myocytes. This strongly implicates both transient mPTP opening and the downstream ROS produced, as a consequence of transient opening, as crucial mediators in the cardioprotective signalling cascades of hypoxic preconditioning. Taken together, these findings illustrate the principal role of transient mPTP opening in cardioprotective signalling cascades and suggest ROS to be a downstream consequence of transient opening, which is essential for cardioprotection to be achieved. Thus, it is likely that superoxide production detected using mt-cpYFP in this study in response to TP was triggered by transient mPTP opening, as previously illustrated by Wang *et al*. This therefore implies that transient mPTP opening plays a pivotal role in the signaling cascades of TP. Furthermore, transient mPTP opening is responsible for downstream ROS production which was demonstrated in the present study to be essential for cardioprotection to be achieved.

6.7 ERK1/2 phosphorylation is downstream of temperature preconditioning induced ROS production and is essential for cardioprotection

The role of the pro-survival kinase ERK1/2 is well established in cardioprotective signaling pathways (Hausenloy *et al.*, 2010; Solenkova *et al.*, 2006)(Solenkova *et al.*, 2006)

. Evidence shows that the protective effects of several cardioprotective strategies (Samavati *et al.*, 2002) including IPC are, in part, due to activation of ERK1/2 (Hausenloy *et al.*, 2005a). The response of ERK to a preconditioning stimulus is believed to be bi-phasic; firstly activated shortly after the preconditioning stimulus and again during reperfusion. It is reported that ERK activation upon reperfusion is critical to protection, since blocking this second activation is sufficient to render loss of cardioprotection (Hausenloy *et al.*, 2005a). In addition to mediating cardioprotection induced by preconditioning (e.g. IPC), ERK1/2 activation is also demonstrated to be involved in the protective signaling pathways of hypothermia-induced protection. It was recently demonstrated that when isolated whole hearts were maintained in mild hypothermia (35°C) during ischaemia, ERK1/2 phosphorylation was preserved for the duration of the ischaemic period instead of steadily declining which occurs in normothermia (Yang *et al.*, 2011). The preservation of ERK1/2 during the hypothermic ischaemic period conferred a significant cardioprotection. The majority of hypothermia related investigations have focused on targeting the ischaemic period to protect the myocardium against ischaemia-reperfusion injury (Gotberg *et al.*, 2008; Miki *et al.*, 1998; Hale *et al.*, 1997; Maeng *et al.*, 2003; Hale & Kloner, 1997; Dae *et al.*, 2002; Tissier *et al.*, 2010). These differ from classical preconditioning studies in which

signaling pathways are activated prior to an ischaemic insult. In this regard, TP is somewhat different to the majority of hypothermia related studies. TP utilizes hypothermia to precondition the myocardium and therefore is likely to share aspects of the signaling events associated with classical preconditioning such as IPC. The role of ERK in TP has not previously been investigated and was therefore examined in this study.

The present study reports that TP results in a robust phosphorylation of ERK1/2 when measured ten minutes after the TP stimulus, but prior to the ischaemic insult. Further, cardioprotection by TP was blocked by pharmacological inhibition of the upstream protein kinase MEK1/2. This shows that ERK1/2 activation is a key step in the cardioprotective pathway of TP. Furthermore, it was found that ROS produced during TP is responsible for the downstream activation of ERK1/2. The effect of scavenging ROS during the TP protocol was found to be sufficient to abolish ERK1/2 activation and any subsequent cardioprotection. This indicates that mitochondrial ROS production is upstream and necessary for ERK1/2 activation in the cardioprotective signaling cascades of TP in cardiac myocytes. This finding is supported by the recent demonstration that cyclophilin-D deficient murine hearts are unable to generate mitochondrial ROS or activate ERK1/2 in response to an IPC stimulus. Whereas mitochondrial ROS and ERK1/2 were robustly activated in response to IPC in wild type hearts (Hausenloy *et al.*, 2010). This finding not only implicates ROS in the activation of ERK1/2, but also implicates transient mPTP flickering as a mediator of ERK1/2 activation, since cyclophilin-D knockout was sufficient to abolish ERK1/2 activation. This is likely true for TP too and could be investigated by examining whether the presence of CsA during TP abolishes ERK1/2 activation. Further evidence supporting

the notion of ROS induced ERK1/2 activation was demonstrated by Xu *et al*. These investigators found that administration of *S*-nitroso-*N*-acetyl penicillamine (SNAP) induced ROS generation in both isolated myocytes and whole hearts and had protective effects. Protection was abolished by the ROS scavenger MPG or the ERK inhibitor PD98059. Western blot analysis revealed that SNAP significantly enhanced phosphorylation of ERK and that this was blocked by MPG (Xu *et al.*, 2004). A ROS-ERK1/2 relationship was also found to underlie the protective effects of sevofluorane postconditioning in the Langendorff perfused rat heart subjected to ischaemia-reperfusion injury. Sevofluorane postconditioning was found to decrease infarct size and inhibit mPTP opening following ischaemia-reperfusion injury in a ROS-ERK1/2 dependent manner. Western blot analysis demonstrated ROS to be upstream of ERK1/2 (Yao *et al.*, 2010).

The above mentioned studies serve to demonstrate that a functional relationship between ROS and ERK1/2 is clearly established in the protective signalling cascades of several disparate pre- and postconditioning interventions. Thus, it may be that TP and other protective interventions share a common cardioprotective signalling pathway. This common pathway may stem from a preconditioning stimulus driven alteration in mitochondrial respiration that results in the production of mitochondrial ROS (Samavati *et al.*, 2002). This subtoxic level signalling ROS may then enter the cytosol to modulate signal transduction pathways, one of which certainly involves ERK1/2. In this study it was demonstrated that the presence of the ROS scavenger MPG or ERK inhibitors during TP eliminated TP-induced inhibition of pathological mPTP opening. This suggests that ROS-ERK1/2 signalling may ultimately converge on the mPTP to prevent irreversible opening following ischaemia-reperfusion injury.

6.8 How does temperature preconditioning increase tolerance of myocytes to simulated ischaemia-reperfusion injury?

Previous studies have demonstrated that TP involves a modest increase in ROS, PKA activation followed by downstream PKC_{ϵ} activation and a potential role for AMPK (Khaliulin *et al.*, 2007; Khaliulin *et al.*, 2010). The present study confirms a role of ROS in TP. Moreover, it demonstrates that ROS is produced exclusively during the hypothermic phase of the TP protocol and may be produced via a transient mPTP flickering-dependent manner. Furthermore, this study highlights a critical role for ERK1/2 activation in TP and implicates ROS as a mandatory mediator of ERK1/2 activation. A common observation between this study and that of previous TP studies is that TP induces an inhibitory effect on irreversible pathological mPTP opening, which underlies necrotic cell death in the setting of ischaemia-reperfusion injury. Thus, inhibition of irreversible mPTP opening may represent the ultimate end point of cardioprotective signaling cascades and through this means increase cell viability following ischaemia-reperfusion injury. Below I discuss what is known of the signaling modules involved in TP and how these may interact to inhibit irreversible mPTP opening, thereby granting protection against simulated ischaemia-reperfusion injury.

The superoxide biosensor mt-cpYFP was used in this study to demonstrate the essential role of ROS in TP. Brief periods of hypothermia during TP were shown to stimulate ROS production. This in turn may have stimulated transient mPTP flickering. Alternatively, hypothermia-induced mitochondrial Ca^{2+} loading may be responsible for increased mPTP flickering. Regardless of the exact mechanism, increased mPTP flickering would be expected to cause flashes of ETC and ATP-dependent superoxide

production (Wang *et al.*, 2008). These superoxide anions would then be rapidly converted to more stable ROS derivatives that modulate cardioprotective signaling pathways. As proposed above, ROS may enter the cytosol and cause the direct activation of ERK1/2, which was demonstrated in the present study to be essential for protection to be achieved. Evidence also exists for a direct mechanism of PKA activation by ROS (Brennan *et al.*, 2006) and this may account for mediating PKA involvement in TP as demonstrated by Khaliulin *et al.* (Khaliulin *et al.*, 2010). These investigators found that cAMP and PKA concentration was increased following TP and that block of PKA activation with either, the non-selective β-adrenergic blocker sotalol or the PKA inhibitor H-89, attenuated the cardioprotective effects of TP. H-89 was found to totally abolish the cardioprotection granted by TP whilst sotalol only partially abolished the protective effects. This finding suggests that β-adrenergic stimulation is only partially responsible for PKA activation in response to TP. In contrast, repeated β-adrenergic stimulation with norepinephrine or isoproterenol has been show to mimic the protective effects of IPC (Asimakis *et al.*, 1994) and attenuation of calpain-mediated degradation pathways of structural proteins has been proposed as the underlying mechanism (Inserte *et al.*, 2004). PKA is also known to mediate phosphorylation of the β2-adrenergic receptor, consequently increasing its affinity for G_i protein and contributing to its anti-apoptotic effect (Zhu *et al.*, 2001). PKA activation could also contribute to the cardioprotective effects of TP by phospholamban phosphorylation, causing increased SR Ca^{2+} uptake and a reduction in cytosolic Ca^{2+} (Sichelschmidt *et al.*, 2003). This may lead to decreased mitochondrial Ca^{2+} loading thereby decreasing the likelihood of irreversible mPTP opening upon reperfusion. Khaliulin *et al.* demonstrated that isoproterenol mediated stimulation of β-adrenergic

receptors increased PKC activity in the heart (Khaliulin *et al.*, 2010). This supports a role for PKA activation of PKC as proposed by Khaliulin *et al.* and this is supported by similar findings in independent studies (Nagasaka *et al.*, 2007; Wallukat, 2002).

The role of PKC in cardioprotection is well documented and it appears that PKC_{ϵ} in particular is both required and sufficient alone to induce cardioprotection (Dorn *et al.*, 1999). Block of PKC activation was found to abolish IPC-induced cardioprotection as reported by several groups (Hausenloy *et al.*, 2007; Ytrehus *et al.*, 1994). With regards to TP, in addition to activation mediated by PKA, PKC may also be directly activated by ROS as found to occur in anesthetic preconditioning signaling cascades (Novalija *et al.*, 2003). The mechanism by which PKC activation is protective is uncertain, but Costa and Garlid have proposed a PKG mediated activation of two pools of mitochondrial PKC_{ϵ} in response to a preconditioning stimulus. Cytosolic PKG activates one pool of PKC_{ϵ} which is proposed to be located in close proximity to the mitochondrial inner membrane. When activated this pool of PKC_{ϵ} is believed to phosphorylate and open mK_{ATP} channels. Consequently mitochondrial matrix K^+ uptake occurs resulting in matrix alkalinisation, and the generation of superoxide and its product, hydrogen peroxide, from complex I (Andrukhiv *et al.*, 2006). The produced ROS then further activates PKC_{ϵ}, maintaining ROS in a positive feedback loop. Additionally, the produced ROS also activates a second mitochondrial pool of PKC_{ϵ} which is functionally coupled to the mPTP. This pool of PKC_{ϵ} is suggested to be the end-effector that determines cell fate by directly inhibiting irreversible pathological mPTP opening (Costa & Garlid, 2008). In support of this, it was found that direct activation of this pool of PKC_{ϵ} using hydrogen peroxide resulted in mPTP inhibition, independent of mK_{ATP} opening (Costa *et al.*, 2006). The mechanism by which PKC_{ϵ} inhibits mPTP opening is unclear, although it had

previously been suggested that phosphorylation of the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane or disruption of the interaction between cyclophilin-D and the adenine nucleotide translocate (ANT) may underlie this protective effect (Baines *et al.*, 2003). However, neither VDAC nor ANT is now considered to be an essential component of the mPTP, leaving the mechanism by which PKC_{ϵ} inhibits mPTP opening undefined.

In addition to directly inhibiting mPTP opening PKC_{ϵ} is also proposed to activate ERK1/2 and form signaling PKC_{ϵ}-ERK signaling modules which phosphorylate and inactive the pro-apoptotic protein Bad (Baines *et al.*, 2002). This, in part, is suggested to contribute to PKC_{ϵ}-mediated cardioprotection. In light of this, I propose that PKC_{ϵ} may represent a mediator of the interaction between ROS and ERK1/2 found in this study in response to a TP stimulus, which is essential for cardioprotection. However evidence also exists for direct activation of ERK1/2 by H_2O_2 in rat cardiomyocytes (Aikawa *et al.*, 1997). Therefore it is possible that both mechanisms are responsible for ERK1/2 activation in response to TP. This study is the first to demonstrate ERK1/2 activation in response to TP and this finding prompts the suggestion that the PI3K-Akt component of the reperfusion injury salvage kinase (RISK) pathway may also be involved. The RISK pathway was discovered by Hausenloy *et al.* as a major signal transduction pathway responsible for mediating cardioprotection at the time of myocardial reperfusion. The recruitment of RISK has been shown to be involved in IPC, IPost and in preconditioning associated with several pharmacological agents (Hausenloy *et al.*, 2005a; Hausenloy & Yellon, 2007b). The recruitment of the PI3K-Akt component of the RISK pathway in TP was investigated by Khaliulin *et al.* but these investigators failed to detect phosphorylation of Akt at 15 minutes of reperfusion

following prolonged ischaemia (Khaliulin *et al.*, 2010). However the involvement of Akt in TP can not be ruled out since Akt phosphorylation could be transient and therefore explain why it was not observed in Khaliulin's study. It would be interesting to investigate Akt phosphorylation immediately following the TP protocol as this was the time at which ERK1/2 activation was found in this study. Based on the literature I would expect the activation of ERK1/2 in temperature preconditioned myocytes to be bi-phasic (Fryer *et al.*, 2001); with a second activation at the time of myocardial reperfusion, as found for IPC, which is demonstrated to inhibit mPTP opening (Juhaszova *et al.*, 2004). The mechanism by which ERK1/2 inhibits mPTP opening is not known but this study demonstrates that block of ERK1/2 activation in response to TP with either U0126 or PD98059 is sufficient to render loss of protection against mPTP opening. A possible mediator of ERK1/2-induced mPTP inhibition is GSK-3β which is a protein that regulates a wide variety of cellular processes including metabolism, growth and apoptosis. The importance of GSK-3β in IPC-induced protection was first demonstrated by Tong *et al.* in 2002. These investigators demonstrated that phosphorylation and inactivation of GSK-3β was essential for cardioprotection and that pretreatment of rat hearts with GSK3-β inhibitors prior to ischaemia could mimic the protective effects of IPC (Tong *et al.*, 2002). Subsequently Juhasova *et al.* confirmed the role of GSK3-β inactivation in cardioprotection by demonstrating that cardiomyocytes containing a constitutively activated form of GSK3-β were not amenable to cardioprotection (Juhaszova *et al.*, 2004). It is proposed that GSK3-β inactivation mediates mPTP inhibition through dephosphorylation of VDAC which prevents mitochondrial entry of adenine nucleotides. This would be expected to facilitate mitochondrial depolarisation which would secondarily reduce mitochondrial

Ca^{2+} accumulation and ROS production during myocardial ischaemia. Consequently, mPTP opening would be less likely to occur at the time of myocardial reperfusion (Das *et al.*, 2008). However, this mechanism of protection is debatable since it is unclear how GSK3-β would inhibit mPTP opening at the time of myocardial reperfusion given the necessity of adenine nucleotides to enter mitochondria to allow mitochondrial re-energisation and recovery of cardiomyocytes (Hausenloy *et al.*, 2009). Moreover, the role of GSK3-β in cardioprotection is even more contentious as it was recently demonstrated that mice containing a mutated form of GSK3-β that was insensitive to phosphorylation and inhibition, were still amenable to the infarct-limiting effects of both ischaemic pre- and postconditioning (Nishino *et al.*, 2008). A second mechanism by which ERK1/2 may mediate the protective effects of TP could involve the generation of nitric oxide (NO) via eNOS which is a known downstream target of ERK1/2. The produced NO may then go on to activate cGMP-PKG (Costa *et al.*, 2005) or it may directly inhibit mPTP opening through PKC_{ϵ} (Costa & Garlid, 2008). Lemasters' group have demonstrated that administration of NO at the time of myocardial reperfusion results in mPTP inhibition and experimental evidence also implicates NO in protection induced by IPost (Yang *et al.*, 2004). Therefore, ERK1/2 mediated activation of NO may play a role in the protection afforded by TP.

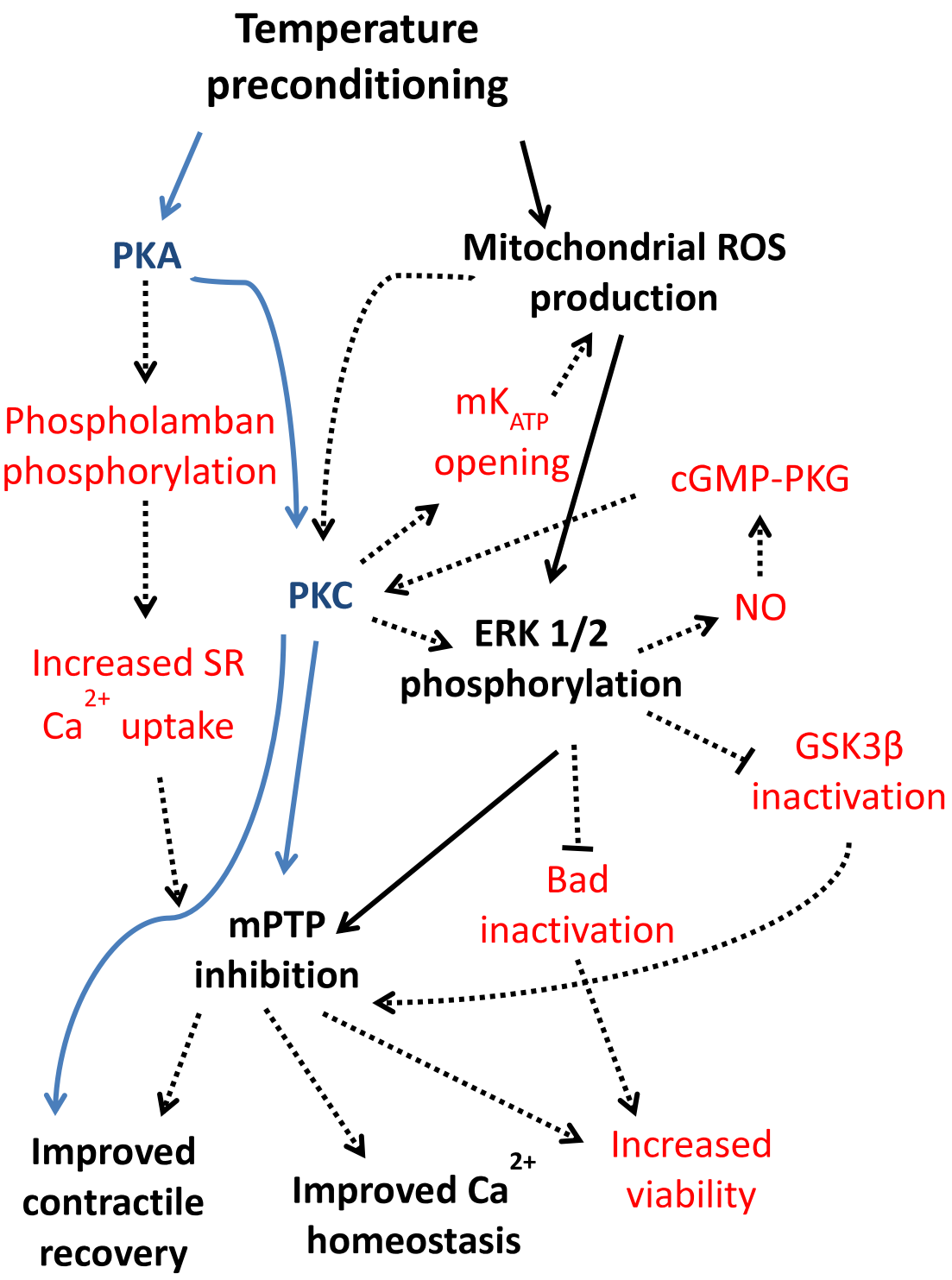

Figure 6.1: Signalling pathways of temperature preconditioning.

Schematic diagram illustrating the signaling pathways activated in response to a temperature preconditioning stimulus in cardiac myocytes. Processes shown in black are demonstrated in the present study. Processes in blue were demonstrated in previous temperature preconditioning studies. Processes in red are likely to be involved in temperature preconditioning based on the literature. Solid arrows indicate certain events in temperature preconditioning while dashed arrows indicate proposed events.

Thus far, considerations of the mechanisms by which TP may ultimately result in cardioprotection have focused on direct mechanisms of mPTP inhibition. By this I refer to the activation of signal transduction pathways which converge directly onto the mPTP to inhibit opening and this is summerised in figure 6.1. In contrast, indirect mechanisms of mPTP inhibition refer to modifications of factors which regulate mPTP opening such as oxidative stress, Ca^{2+} regulation, pH regulation and ATP levels. TP may act by modulating these factors at the time of reperfusion to create a cellular milieu which does not favor mPTP opening. This study has demonstrated that TP is associated with lower basal Ca^{2+} levels following simulated ischaemia-reperfusion injury compared to controls. Given that Ca^{2+} is a regulator of mPTP opening this may lead to the suggestion that reduced Ca^{2+} results in less mPTP opening and therefore underlies the protection granted by TP. However since the effect of intracellular Ca^{2+} on mPTP opening was not investigated directly this can not be stated for certain. Moreover, the question arises, is lower Ca^{2+} observed in temperature preconditioned myocytes the cause of less mPTP opening, or the consequence? Preventing Ca^{2+} overload using antagonists of plasma membrane or mitochondrial Ca^{2+} channels such as ruthenium red or verapamil has been shown to protect hearts against reperfusion injury (Massoudy *et al.*, 1995; Garcia-Rivas Gde *et al.*, 2006). However a direct demonstration that this involved inhibition of the mPTP remains to be reported. Furthermore, whether Ca^{2+} is actually a factor which regulates mPTP opening is uncertain and Lemasters' work suggests that it is not. On the contrary Lemasters group propose that Ca^{2+} overload is the consequence of bioenergetic failure subsequent to irreversible mPTP opening (Kim *et al.*, 2006).

Unlike Ca^{2+}, low pH is firmly established as a factor which regulates/inhibits mPTP opening (Halestrap, 2010). It was demonstrated that the mPTP remains closed during ischaemia due to intracellular acidosis, but opens at reperfusion when pH has returned to normal. In support of this, it is reported that inhibition of sodium/hydrogen exchanger 1 (NHE1) in the setting of ischaemia-reperfusion injury reduced mPTP opening *in situ* (Javadov *et al.*, 2008) and also protected heart against reperfusion injury (Mentzer *et al.*, 2003). Furthermore, post conditioning has been shown to reduce mPTP opening (Ferrera *et al.*, 2007) and oxidative stress (Correa *et al.*, 2008). It has been suggested that the protective effects of post conditioning may involve the maintenance of low pH during reperfusion (Halestrap, 2010). The effects of TP on cellular pH at the time of reperfusion have not been investigated but may well represent an indirect mechanism of mPTP inhibition, contributing to TP-induced cardioprotection.

Preservation of myocardial ATP levels is also suggested to be a factor which prevents mPTP opening at reperfusion. Interestingly, in its initial description, the protective effects of IPC were reported to be dependent on the preservation of ATP levels (Murry *et al.*, 1986). This is supported by later studies which demonstrated reduced ATP consumption during myocardial ischaemia (Murry *et al.*, 1990; Kobara *et al.*, 1996). The inhibitory effects of adenine nucleotides on the mPTP were demonstrated to increase mitochondrial sensitivity to Ca^{2+}-induced mPTP opening (Halestrap *et al.*, 1997). The mechanism by which preserved ATP levels are thought to inhibit mPTP opening is by increased binding to the adenine nucleotide translocase (ANT) which is proposed to be either a pore forming component of the mPTP, or at the least, a regulatory component (Halestrap, 2010). TP was previously shown to increase

myocardial preservation of ATP, creatine phosphate, β-NAD+ and total adenine nucleotides (ATP+ADP+AMP) in the reperfused heart (Khaliulin *et al.*, 2007). This suggests that TP enhances preservation of energy metabolism which may serve to inhibit mPTP opening at reperfusion.

TP was also demonstrated to be associated with decreased oxidative stress at reperfusion as detected by decreased protein carbonylation (Khaliulin *et al.*, 2007). Oxidative stress generated at the time of myocardial reperfusion as a result of reoxygenation of the ETC is a major factor which regulates mPTP opening. Since TP reduces reperfusion induced oxidative stress, this may largely account for the deceased mPTP opening in temperature preconditioned myocytes. IPC has also been shown to have an anti-oxidant effect, resulting in decreased ROS production on reperfusion of the ischaemic myocardium (Khaliulin *et al.*, 2007; Clarke *et al.*, 2008). Likewise, IPost is also demonstrated to reduce reperfusion induced oxidative stress (Sun *et al.*, 2005), but the exact mechanism by which this occurs for either ischaemic pre- or postconditioning remains unclear. Thus, attenuation of oxidative stress at the time of myocardial reperfusion may represent a universal outcome of all protective interventions and account for decreased mPTP opening. However, direct demonstration that IPC, IPost, or TP-induced reduced oxidative stress actually mediates mPTP inhibition is yet to be shown.

In summary, I propose that the protective effects of TP against simulated ischaemia-reperfusion injury ultimately arise through inhibition of irreversible mPTP opening at reperfusion. This may occur as a combination of factors and signaling cascades act to directly and indirectly inhibit mPTP opening. Consequently, the selective permeability

of the mitochondrial inner membrane remains intact allowing the generation of ATP and sparing the myocyte necrotic death.

6.9 Conclusions

This study demonstrates that TP of isolated myocytes improves recovery of contractile function and Ca^{2+} homeostasis following MI and reenergisation. Further it demonstrates that TP inhibits irreversible mPTP opening but does not affect non-pathological transient mPTP flickering. Also demonstrated in the current study is that the hypothermic phase of the TP protocol is associated with bursts of mitochondrial ROS production. Further the activation and critical role of ERK1/2 in the signaling cascades of TP is demonstrated. I have shown that hypothermia-induced mitochondrial ROS production is upstream and essential for ERK1/2 activation. Blocking either ROS production or ERK1/2 activation was sufficient to render loss of protection against irreversible mPTP opening.

Based on the findings of the current study and that which is previously reported about TP, I find the similarities between the signaling modules involved in TP and IPC to be striking. This leads me to suggest that TP, like IPC, may activate a universal stress pathway which results in cardioprotection. I propose that a change in the rate of mitochondrial respiration, as induced by brief periods of sub lethal ischaemia or hypothermia, is sufficient to trigger protective signaling cascades which ultimately converge on the mPTP to inhibit its opening. The signaling cascades of the two protective interventions may not be entirely identical, and this may account for the reported additional protection of TP over IPC (Khaliulin *et al.*, 2007). However, until present the key players shown to be involved in IPC are the same ones illustrated to be

involved in TP. Therefore, I propose that pharmacological manipulation of these key mediators of cardioprotection can be targeted for use in the clinical stetting. Further, I suggest that activating this potentially universal protective stress pathway at multiple levels using several activators and inhibitors, may prove more beneficial than targeting a single identified trigger, such as mitochondrial ROS, alone. This is because cardioprotection is most likely achieved by the synergistic actions of several signaling modules and cascades all acting in parallel to decrease susceptibility to ischaemia-reperfusion injury.

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