The role of Protein Kinase B and Mitochondrial Permeability Transition Pore in ischaemic preconditioning of myocardium

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Dedication

This thesis is dedicated to my father Dr Zakir Husain Husainy and my mother Mrs Shirin Husainy. I am what I am today because of their vision, love and support.

I also dedicate this thesis to my friend and wife, Dr Farhina Husainy and daughter Zahra for their help and unconditional love.

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Abstract:

Background:

Protein kinase B (PKB) plays a critical role in cell survival, growth and proliferation. The investigation of its involvement has been limited by the lack of specific pharmacological agents. Similarly mitochondrial permeability transition pore (MPTP) plays a critical role in necrotic and apoptotic cell death. Opposed role of MPTP (transient versus sustained opening) in cardioprotection has been proposed. Therefore the primary aims of this thesis were to (i) to investigate the influence of PKB in the tolerance to ischaemia/reoxygenation (I/R), (ii) to define the relationship of PKB with the mitoK_{ATP} channel and with p38 MAPK in the signal transduction mechanism of cardioprotection by IP, (iii) to determine how two structurally different MPTP inhibitors (Cyclosporine A and Bongkrekic Acid) administered for varying time regimes influenced ischemia/reperfusion (I/R)-induced injury and (iv) to explore how pharmacologic MPTP opening at different time points during I/R modulated myocardial injury.

Methods & Results:

Myocardial slices from rat left ventricle and from the right atrial appendage of patients undergoing elective cardiac surgery were subjected to 90 min ischaemia/120 min reoxygenation at 37◦C. Tissue injury was assessed by creatine kinase (CK) released and determination of cell necrosis and apoptosis. Myocardial caspase 3 was also assessed.

Muscles were randomized to receive various treatments. The results showed that blockade of PKB activity caused significant reduction of CK release and cell death, a benefit that was as potent as ischaemic preconditioning and could be reproduced by blockade of phosphatidylinositol 3-kinase (PI-3K) with wortmannin and LY 294002. The protection was time dependent with maximal benefit seen when PKB and PI-3K were inhibited before ischaemia or during both ischaemia and reoxygenation. PKB inhibition induced a similar degree of protection in the human and rat myocardium and, importantly, it reversed the unresponsiveness to protection of the diabetic myocardium.

The status of MPTP can dramatically influence ischemic/reoxygenation-induced injury and protection of the rat left ventricular myocardium. Importantly, the status of the MPTP during first 10 min of reoxygenation is of critical importance with both opening and closing of the pore being as protective as ischemic preconditioning.

Conclusion:

Inhibition of PKB plays a critical role in protection of the mammalian myocardium and may represent a clinical target for the reduction of ischaemic injury.

Both formation and inhibition of the MPTP can be exploited for therapeutic purposes and that there is a defined therapeutic window, with the first few minutes of reoxygenation being a crucial period to achieve cardioprotection.

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Publications

- Linares-Palomino J, **Husainy MA**, Lai VK, Dickenson JM, Galiñanes M.

  Selective blockade of protein kinase B protects the rat and human myocardium against ischaemic injury.

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- **Husainy MA**, Dickenson JM, Galiñanes M.

  The MPTP status during early reoxygenation is critical for cardioprotection.

  J Surg Res. 2012 May 1; 174(1): 62-72.

Abstract

- Josè Linares-Palomino, **Mohammad Ali Husainy**, John Dickenson, Vien Khach Lai, Nicholas Standen, Manuel Galiñanes.

  Specific inhibition of protein kinase B protects against ischemic myocardial injury in rat and man.

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CHAPTER 1

INTRODUCTION

ISCHAEMIA

Definition: When a tissue is subjected to oxygen deprivation, it results in hypoxia. In ischaemia the blood flow is also disrupted which prevents the wash out of lactic acid and other waste products from the cells. If oxygen delivery to cells is insufficient for the demand (hypoxia), hydrogen will be shifted to pyruvic acid converting it to lactic acid (Figure 1). This temporary measure (anaerobic metabolism) allows small amounts of energy to be produced. Lactic acid build up in tissues and blood is a sign of inadequate mitochondrial oxygenation, which may be due to hypoxemia, poor blood flow (e.g., shock) or a combination of both (1). If severe or prolonged it could lead to cell death.

Pathophysiology: Myocardial ischaemia is characterized by ischaemia to the heart muscle, usually due to disease. It can occur as a result of increased myocardial oxygen demand, reduced myocardial oxygen supply or both. Clinically it manifests as angina with

ST-segment deviation on ECG, reduced scintigraphy using Thallium 201 or Technetium 99 or cardiac arrhythmia.

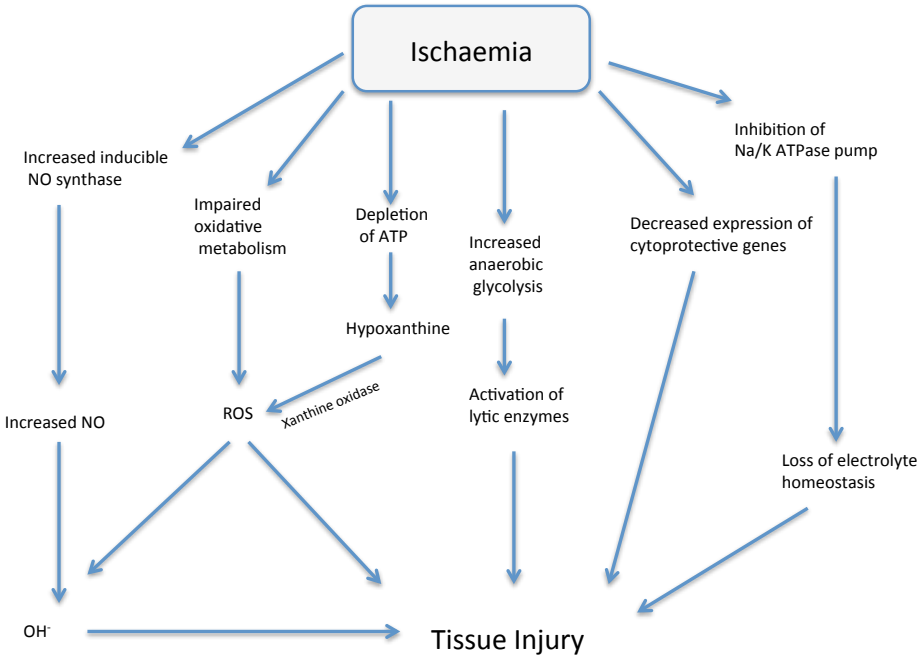

Figure 1. Schematic diagram of pathways of ischaemic injury

Atherosclerosis in the coronary vessels disrupts the normal coronary physiology and introduces resistance to coronary flow. This acquired resistance is in series with the physiological resistors and thus has its impact on coronary flow reserve reducing this on occasions to levels not allowing sufficient oxygen delivery to the myocardium(2).

The human heart receives about 1/20th of cardiac output under basal conditions. Myocardial oxygen consumption ($M\dot{V}O_2$) and oxygen delivery forms the core of understanding of

myocardial ischaemia. The human heart receives about 1/20th of cardiac output under basal conditions. With exercise, coronary blood flow may rise three- to four fold to accommodate the increase in $M\dot{V}O_2$. Indeed, there is an almost perfect linear relation between coronary blood flow and $M\dot{V}O_2$. The mandatory increase in coronary blood flow is needed by the myocardium because of near complete oxygen extraction under basal conditions (coronary sinus oxygen saturation at rest can be as low as 25–30%). This contrasts with other muscle groups where increased oxygen requirements can be met, in part, by increasing oxygen extraction and thus widening of the atrio-ventricular (AV) difference in oxygen saturation. One of the consequences of this balance between coronary blood flow and $M\dot{V}O_2$ is that it forms the basis of the practical utility of exercise testing.

Coronary blood flow, at least to the left ventricle, occurs predominantly in diastole since the resistance vessels within the heart are occluded during systolic contraction. The flow within the coronary vessel is determined by the driving pressure (diastolic blood pressure), the resistance (the tone within the walls of the resistance arterials and the compressive forces of the myocardium), and to a lesser extent the elastic recoil found within the conduit and capacitance vessels. The highly mutable component of these three parameters is the tone within the resistance vessels, and this accounts for a significant amount of the variability in angina symptoms and other atypical angina. It is of less therapeutic potential, however, because of the absence of selective coronary microvascular vasodilator drugs (2).

Clinical Consequences: Coronary Artery Disease (CAD) is the leading cause of death worldwide. While the symptoms and signs of coronary artery disease are noted in the advanced state of disease, most individuals with coronary artery disease show no evidence of

disease for decades as the disease progresses before the first onset of symptoms, often a "sudden" heart attack, finally arises. After decades of progression, some of these atheromatous plaques may rupture and (along with the activation of the blood clotting system) start limiting blood flow to the heart muscle. The disease is the most common cause of sudden death(3), and is also the most common reason for death of men and women over 20 years of age. According to present trends in the United States, half of healthy 40-year-old males will develop CAD in the future, and one in three healthy 40-year-old women (4).

REPERFUSION INJURY

Definition: Reperfusion injury refers to damage to tissue caused when blood supply returns to the tissue after a period of ischemia. The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation can result in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function

Causes of Reperfusion injury: The damage of reperfusion injury is due in part to the inflammatory response of damaged tissues. White blood cells, carried to the area by the newly returning blood, release a host of inflammatory factors such as interleukins as well as free radicals in response to tissue damage(5). The restored blood flow reintroduces free oxygen within cells that damages cellular proteins, DNA, and the plasma membrane. Damage to the cell's membrane may in turn cause the release of more free radicals (Figure 2). Such reactive species may also act indirectly in redox signalling to turn on apoptosis. Leukocytes may also build up in small capillaries, obstructing them and leading to more ischemia.

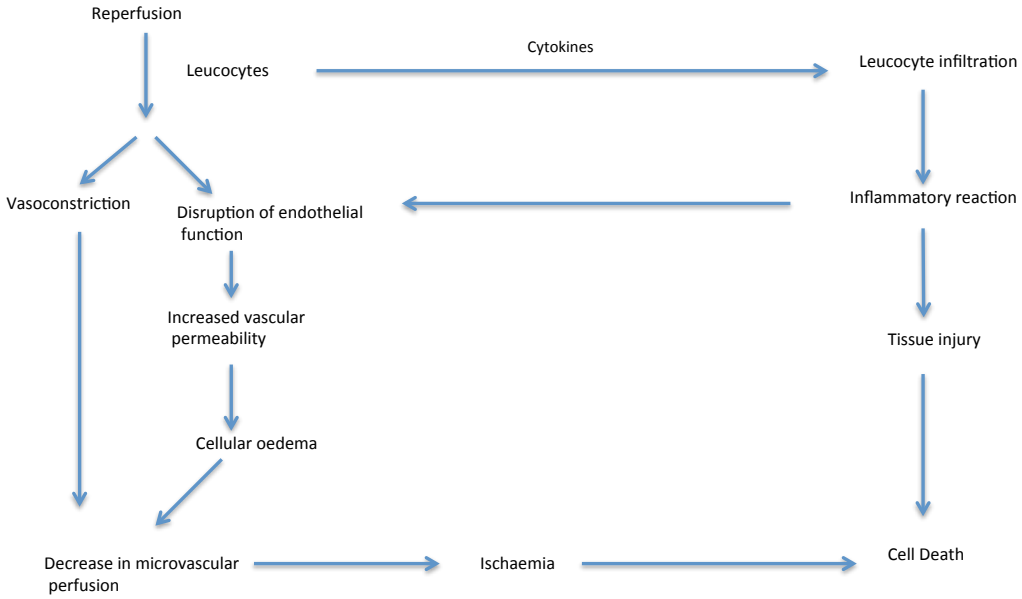

Figure 2. Schematic diagram of pathways in reperfusion injury.

During ischaemia, the drop in pH that occurs due to the build up of lactic acid leads to activation of the Na^+/H^+ antiporter to try and restore intracellular pH(pHi). Since ATP concentrations are greatly reduced, the Na^+/K^+ATPase is inhibited and the Na^+ that enters the cell cannot be pumped out again, which results in increased intracellular Na^+. This in turn causes Ca^{2+} to rise because the Na^+/Ca^{2+} antiporter, which usually pumps Ca^{2+} out of the cell, is inhibited or reversed. The conversion of ATP to ADP and AMP is rapid and reversible. AMP is slowly converted into adenosine and then inosine and xanthine through a purine degradation pathway. These nucleosides leak out of the cell and lead to a gradual depletion of adenine nucleotide, which may contribute to the complications of ischaemia. If sufficient oxygen is available, xanthine may be further oxidised by xanthine oxidase, which produces oxygen free radicals that are very damaging to the tissue. The depletion of ATP and elevated

Ca^{2+} that occurs in ischaemia leads to a gradual decline in cellular integrity as degradative enzymes are activated and ATP-dependent repair processes are unable to operate. If the tissue remains ischaemic for prolonged periods, this deterioration leads to necrotic cell death. However, shorter periods of ischaemia are accompanied by less damage and ATP-dependent processes can reverse this provided the mitochondria remain sufficiently intact to generate the ATP upon reoxygenation.

Calcium Overload

The intracellular accumulation of Na^+ and Ca^{2+} plays a key role in ischemia-induced myocardial injury. The accumulation of intracellular H^+ and the activation of Na^+-dependent pH regulatory mechanisms, including the Na^+-H^+ exchanger (NHE-1) and the Na^+-HCO_3^- cotransporter, contribute to Na^+ accumulation. Intracellular Na^+ accumulation, coupled with the NHE-1, then causes Ca^{2+} overload. As glycolysis uncoupled from glucose oxidation is an important determinant of the rate of H^+ production, factors that affect glucose metabolism, including degree of ischemia, and competition from other energy substrates, are expected to influence Na^+ and Ca^{2+} accumulation(6).

Calcium overload causes unique phenomena in cardiac myocytes called contraction band necrosis. It occurs during reperfusion from hypercontraction secondary to excessive influx of calcium causing sarcolemmal rupture (7). Two mechanism have been proposed for contraction band necrosis:

a. Calcium dependent mechanism: Calcium overload secondary to ischaemia activates contractile machinery

b. Calcium independent mechanism: Low ATP levels causing activation of contractile machinery(7).

Oxygen free radical generation

Readmission of oxygen might paradoxically contribute to ischaemic damage by the formation of oxygen free radicals (8 -15).

Normally, the anti-oxidant defence systems in the aerobic myocardium are able to get rid of the oxygen free radicals. However, ischaemia reduces this ability, especially in the mitochondrial compartment, making the tissue more susceptible to injury (16,17).

Modes of myocardial death

Necrosis: When heart muscles are subjected to repeated ischaemia and reperfusion, it suffers from characteristic fluid and electrolyte imbalance leading to accumulation of water, which results in rupture of the cell and organelles. It also causes margination and clumping of nuclear chromatin that eventually leads to severe membrane permeability defect and unregulated influx of calcium causes irreversible cardiomyocyte injury. Ultimately, the cell develops physical defects in the cell membrane and rupture (18).

Apoptosis: It is defined as programmed cell death. It is an energy dependent biochemical event characterised by blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation leading to cells changes and death(18- 19).

Cardiomyocyte apoptosis has been implicated in diseases of the human heart like heart failure (20), cardiomyopathies (21) and arrhythmias (22). It has been shown to occur in animal models of ischaemia and reperfusion (23-25) and in isolated cell lines exposed to oxidative stress (26).

Mechanism of Apoptosis:

A cascade of cell signals are involved in the process of apoptosis, which has got extracellular (extrinsic) and intracellular (intrinsic) inducers. Toxins, hormones, growth factors, cytokines, nitric oxide form the extrinsic inducers. They can either positively or negatively affect apoptosis (27,28).

Intrinsic inducers initiate apoptosis through stress. This can be in the form of heat, hypoxia, radiation, nutrient deprivation, and glucocorticoids(28).

Before enzymes precipitate the actual process of cell death, apoptotic signals must cause regulatory proteins to initiate the apoptosis pathway. This step allows apoptotic signals to

cause cell death, or the process to be stopped, should the cell no longer need to die. Several proteins are involved, but two main methods of regulation have been identified: targeting mitochondria functionality, or directly transducing the signal via adaptor proteins to the apoptotic mechanisms. Another extrinsic pathway for initiation identified in several toxin studies is an increase in calcium concentration within a cell caused by drug activity, which also can cause apoptosis via calcium binding protease calpain.

Mitochondrial regulation

Mitochondria are essential for aerobic metabolic cycle. Apoptotic proteins target mitochondria resulting in swelling by formation of pore or by increasing the mitochondrial membrane. This causes leakage of apoptotic effectors such as nitric oxide.

Mitochondria-derived activators of caspases (SMACs) are released into cytosol following the increased permeability (28). SMAC binds to inhibitor of apoptosis proteins (IAPs) causing their deactivation and preventing them from arresting the process of apoptosis.

There is also release of cytochrome *c* due to the formation of mitochondrial outer permeability pore (MAC), which is regulatory in role (29). Cytochrome *c*, once released, binds with Apoptotic protease activating factor-1 (Apaf-1) and ATP. This new complex then binds to procaspase-9 to create another protein complex known as an apoptosome. It cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector caspase-3 (Figure 3).

The Bcl-2 family of anti-apoptotic genes regulates MAC. They are homologs of the *ced-9* gene found in *C.elegans* (30). They exert direct control on apoptosis by exploiting MAC. Bax and Bak form the pore, while Bcl-2, Bcl-xL or Mcl-1 inhibits its formation.

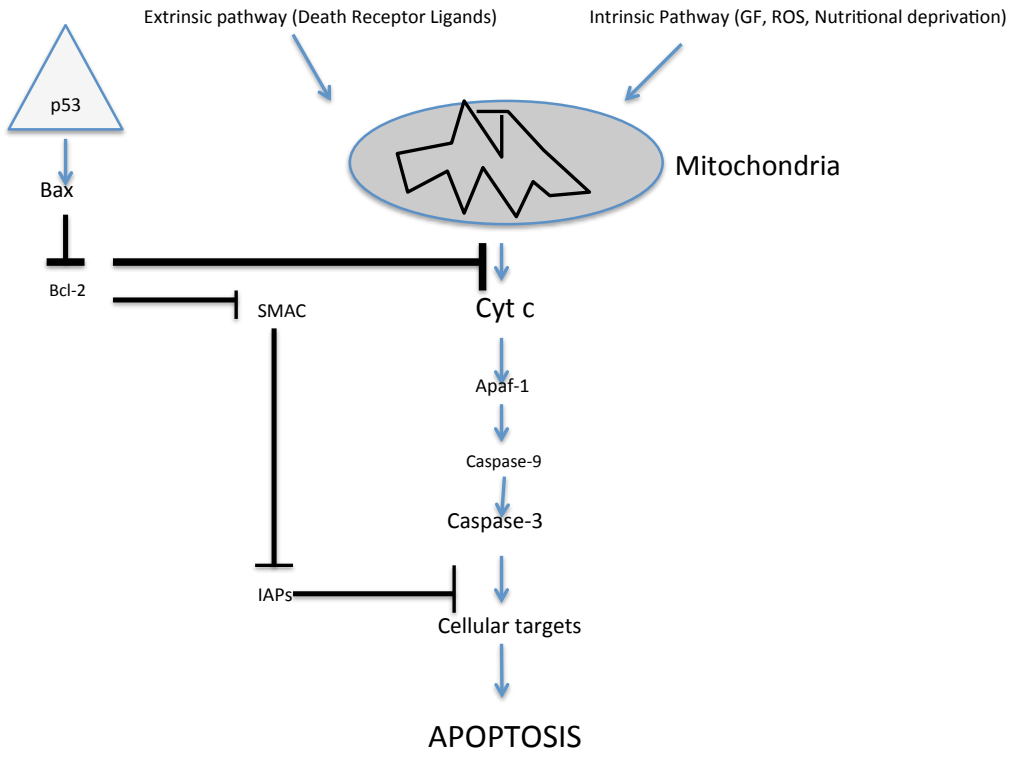

Figure 3: Schematic diagram of apoptotic signalling cascade.

Direct Signal Transduction

There are two widely accepted models for this mechanism. They are

a) TNF-induced model
b) Fas-Fas ligand mediated model

TNF-induced (tumour necrosis factor) model: TNF is a cytokine produced by activated macrophages. The human body cells have two types of receptors for TNF: TNF-R1 and TNF-R2. When TNF binds with TNF-R1, it initiates the caspase activation through the *TNF receptor- associated death domain* (TRADD) and *Fas-associated death domain protein* (FADD) (31). It can also lead indirectly to the activation of inflammatory response through activation of transcription factors.

Fas receptor ligand mediated model: (also known as Apo-1 or CD95) binds the Fas ligand (FasL), which is a trans membrane part of the TNF family (17). It results in formation of the death-inducing sin galling complex (DISC), which contains the FADD, caspase-8 and caspase-10. Processed caspase-8 by activating other members of the caspase family triggers apoptosis of the type I cells. In type II cells this complex starts a feedback loop that spirals into increasing release of pro-apoptotic factors from mitochondria and the amplified activation of caspase-8.

There exists a balance between pro-apoptotic (BAX, BID, BAK or BAD) and anti-apoptotic (Bcl-xL and Bcl-2) members of the Bcl-2 family. It is the proportion of pro-apoptotic homodimers that form in the outer-membrane of the mitochondria. These homodimers are

essentially required for the permeability of mitochondrial membrane to release caspase activators *viz.* Cytochrome *c* and SMAC.

Apoptosis in heart disease

Cell death via apoptosis has been implicated in many disease of the heart. Cardiovascular remodelling may involve cell proliferation, apoptosis and fibrosis. Tieger et al. (32) demonstrated in a rat model of cardiac hypertrophy that highest levels of apoptosis were observed just before the development of cardiac hypertrophy and the levels decreased as the hypertrophy progressed. Kajstura et al. (33) demonstrated apoptosis is increased in comparison to necrosis after myocardial infarction. Apoptotic cell death may contribute to the progression from good (>50%) to moderate (30-50%) or poor (<30%) left ventricular ejection fraction after an acute myocardial infarction.

Apoptosis in ischaemia/reperfusion injury of the heart

Apoptosis can be a result when cardiomyocytes are subjected to oxidative stress leading to production of reactive oxygen species (ROS) (17) (34). During cardiac surgery, the myocardium is subjected to a period of global ischaemia while on cardiopulmonary bypass. It will by itself, lead to activation of neutrophils leading to the release of ROS like superoxide anion (O^{-2}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (13,35) and the activation of cytokines and complement (36). Reperfusion of the ischaemic myocardium may lead to damage to the endothelium leading to inhibition of nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS) (37). The damage is further exacerbated by the

relatively low anti-oxidant status of the myocardium as a result of preceding ischaemia (14). In ischaemia/ reperfusion injury of myocardium, apoptosis has been prevented by NO synthesis through inhibition of caspase-3 activity (38). It has also been shown that adhesion of neutrophils to the endothelium during reperfusion releases ROS(13,35). This severe oxidative stress environment targets lipids and lipid-soluble anti-oxidants in patients resulting in formation of lipid hydroxyperoxidase (39). These free radicals can also bind to sulphydryl groups on membrane proteins. Our laboratory has already demonstrated that in diabetic patients there is a rapid increase in nitrotyrosine, an index of peroxynitrite generation before the initiation of cardiopulmonary bypass (39). There is also a steady increase in protein carbonyls in the plasma before and during cardiopulmonary bypass further supporting the concept that oxidative stress is exacerbated in diabetics as compared to non-diabetics during cardiac surgery. In the same study(39), glyceryl trinitrate (GTN), a nitric oxide donor, decreased the formation of carbonyls and lipid hydroperoxides in patients with diabetes and the production of nitrotyrosines in both diabetic and non-diabetic patients.

Chen et al. (40) have demonstrated that over expression of Bcl-2 renders the heart more resistant to apoptosis and I/R injury. A mitochondrial pathway that is resistant to oxidative stress induced apoptosis involves activation of mitochondrial ATP-sensitive potassium (mito K_{ATP}) channels in cultured neonatal rat myocytes(41). Furthermore, over expression of heat shock proteins (HSP), HSP60 and HSP10, individually and in combination result in resistance to apoptosis induced by simulated ischaemia and reoxygenation (42). There is evidence that the inhibition of p38 mitogen-activated protein (MAP) kinase results in decrease in apoptotic cell death and improved cardiac function in isolated rabbit hearts(43).

In a nutshell, there is plethora of evidence that indicate that oxidative stress induces apoptosis in animal models and that this injury can be ameliorated by anti-oxidant therapy.

AUTOPHAGY

It is a self cannibalization process in which cell undergoes degradation of its own component, recycling amino acids and other building blocks that eventually can be reused through lysosomal machinery(44). Three types of autophagy have been identified, namely macro-authopagy, micro-autophagy and chaperone-mediated authopagy. Macroautophagy involves sequestration of cytoplasm into cytosolic vesicle called as autotophagosome. On the other hand, microauthopagy involves direct engulfment of cytoplasm by protrusion, septation or invagination.

Cardiomyocytes are poorly replaced and under constant attack of ROS and therefore autophagy plays an important role in turnover of damaged organelles (45). As a response to stress and to meet increased requirement for repair, autophagy is activated. It eventually leads into production of energy or delivering building blocks for anabolic processes. If the damage is irreversible to cardiac myocytes, the injury takes the classical apoptosis pathway.

CARDIOPROTECTION

The feasibility and practicability of establishing the perfusion to an infarcting heart has led to the usage of the term 'cardioprotection'. It is an umbrella term used for variety of interventions to slow down the rate of progression of ischaemic injury to an irreversible injury before reperfusion can be provided. There are some known understood mechanisms through which the cardioprotection can be achieved. These include

a) Promotion of aerobic metabolism with exogenous glucose
b) The reduction of intracellular Ca2+ overload with calcium channel blockers
c) Limiting cell swelling with osmotic agents.

The abovementioned methods have led to success in achieving improvement in cardiac function and therefore been implemented from laboratory to the clinical settings (46).

Methods of cardio-protection during cardiac surgery

Three different approaches have been shown to reduce cardiac injury, namely;

a) Using chemical cardioplegic agents such as potassium (47,48), acetylcholine (49) or tetrodotoxin (50) or the depletion of extracellular calcium(46).
b) Using hypothermia to decrease cardiac metabolism and function.
c) The use of 'intermittent ischaemia', which uses short periods of ischaemia with adequate periods of reperfusion (51).

Cardioplegia

This is achieved by infusing cold crystalloid cardioplegic solution into the coronary vessels. It achieves asystole and protects myocardium from damage. However, the effect brought about may not be homogenous and in severe coronary artery disease a large area is vulnerable to ischaemic changes.

Hypothermia

It works by reducing myocardial oxygen consumption. It is further classified into

i) Mild (34^{o} C)

ii) Moderate (26^{o} C) or

iii) Deep ($15\text{-}22^{o}$ C).

The last method is a well-recognised method of achieving neuroprotection during total circulatory arrest and thus associated with serious implications to the patient. Also by applying this method, a reduction of only 7% in myocardial oxygen consumption is reported. Therefore, the extra benefit obtained from hypothermia is low.

Moderate hypothermia (26^{o} C) has been recently shown to be most cardioprotective in Langendorff-perfused rat hearts (52).

The adverse effects of hypothermia include metabolic acidosis, left shift of oxyhaemoglobin dissociation curve and increase in plasma viscosity. Due to the abovementioned side effects, hypothermia is at present less frequently used.

Intermittent ischaemia

Intermittent cross clamping is well-recognised method of avoiding ischaemic injury during cardiac surgery. It has been reported though that cardiac function immediately after cessation of cardiopulmonary bypass is better with cardioplegia than with intermittent cross-clamping (53) and that cardioplegia results in a better preservation of high-energy phosphates, glycogen, and ultrastructure than intermittent cross-clamping (53).

ISCHAEMIC PRECONDITIONING

It was observed that brief episodes of ischaemia with intermittent reperfusion did not lead to progressive depletion of high-energy phosphates in the canine myocardium (54). The onset of infarction was delayed in pre-treated hearts with significant reduction in ultimate myocardial infarct size, resulted in recognition of the concept of ischaemic preconditioning. Myocardial ATP concentrations fell during the first brief coronary artery occlusion, these concentrations were preserved during further identical occlusions and no necrosis occurred. Since then this phenomenon has been demonstrated in every animal species studied (55). It was further demonstrated that this process was able to offer protection against myocardial stunning and reperfusion arrhythmias (56-58).

There is a biphasic time frame protection characteristic of ischaemic preconditioning. The 'classical' or 'early' phase last 1 to 2 hours after the preconditioining stimulus and then a second window 12 to 72 hours later(59,60). This protection was limited to the ischaemic insults lasting for ~30 to 90 minutes and ineffective when this period was extended to 3 hours (55).

This phenomenon has opened a great interest in identifying the underlying cell signalling pathways with the aim to develop pharmacological tools in order to enhance myocardial tolerance to ischaemic insults. A number of membrane receptor-linked cellular triggers, intracellular signalling cascades and potential cytoprotective end-effector proteins involved have been identified (55).

Due to ethical considerations, the evidence for ischaemic preconditioning in human heart is indirect. In vitro models using human ventricular myocytes (61,62) and isolated atrial trabeculae suggests that protection can be induced using metabolic and functional end points. A close resemblance has been observed between the mechanism of protection in human tissue and that of many animal species, namely, the involvement of adenosine as an important trigger, protein kinase B and C as intermediate intracellular messenger, and the ATP-dependent K^+ (K_{ATP}) channel as a potential end-effector protein (63-66).

There is also evidence that preconditioning suppress apoptosis in addition to necrosis in intact hearts (67). This may be a result of opening of channels in the mitochondrion following activation of intra-mitochondrial signalling pathways.

The opening of mitochondrial ATP sensitive potassium (mito-KATP) channels can lead to either the enhancement or attenuation of cardioprotection with the preconditioning mechanism highly dependent on the phasing of channel opening (68). Thus mitoK_{ATP} channels may signal protection through:

i) inhibition of cytochrome *c* release,
ii) the optimisation of energy production through depolarisation of the mitochondrial membrane potential with alterations in mitochondrial Ca^{2+} handling, and/or
iii) the modulation of reactive oxygen species (ROS) production, including oxygen free radicals (69,70) and nitric oxide (71) , during ischaemia or reperfusion.

Our group has shown that opening of mitoK_{ATP} channels signals the activation of both PKC and p38MAPK. Both downstream elements have shown to be inhibited by the antagonists 5-hydroxydecanoate (5-HD: a presumed specific mitoK_{ATP} channel inhibitor) or chelerythrine

and SB203580, respectively (72). By inducing a sub lethal production of ROS, the short ischaemia stimulus associated with preconditioning by ischaemia may confer cardioprotection through opening of mitoK_{ATP} channels (73) or via activation of protein kinase C isozymes leading to attenuation of ROS (36) and the inhibition of caspase-8 induced apoptosis (74).

The cell signalling pathways of reperfusion injury is still a topic of debate. Yellon et al. have coined a term reperfusion injury salvage kinases (RISK) for various intracellular protein kinase activation (75). The signalling cascade includes activation of PI3-Kinase /PKB and ERK1/2 through G protein couple ligands located in sarcolemma (Figure 4). It subsequently activates downstream kinases like p70S6 and GSK3β before finally converging on mitochondrion.

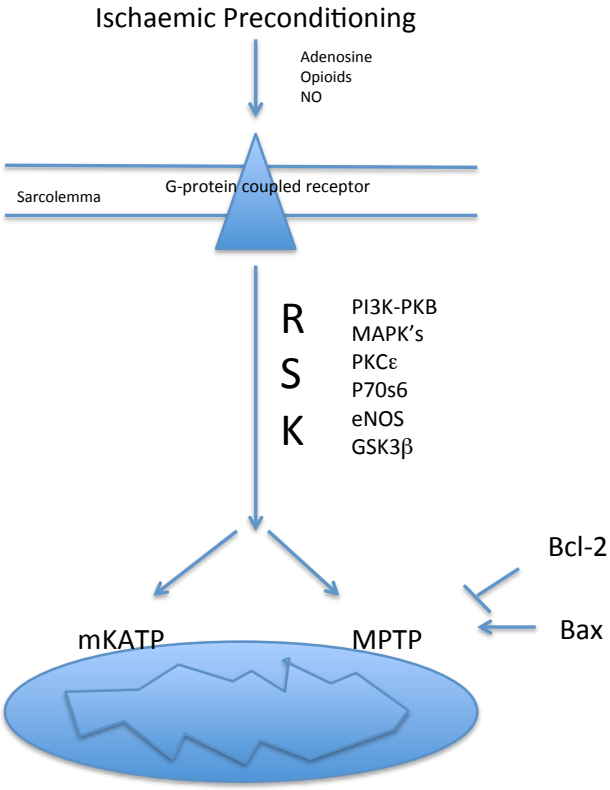

Figure 4: Schematic diagram of signalling pathways used in ischaemic preconditioning.

POSTCONDITIONING

Vinten-Johansen's group first described post conditioning (POC) in canine hearts (76). After prolonged ischaemia, controlled repetitive cycle (3 episodes) of intermittent reperfusion (30 seconds) and ischaemia (30 seconds) results in myocardial protection, which is termed as POC (77). An early moment in ischaemia/reperfusion period is critical because cardioprotection could not be attained 1-3 minutes after the onset of reperfusion (77). POC has been shown to not only reduce infarct size ~20-70% but also reduces apoptosis, necrosis, endothelial dysfunction, ROS production and inflammatory response depending on species and models (77-79).

POC can be excellent tool in a variety of clinical situations. Due to practical and ethical limitations, IP is not feasible in clinical settings POC can be applied to coronary artery bypass graft surgery, aortic cross clamp and percutaneous coronary interventions (PCI). Various groups have demonstrated that POC during PCI for acute myocardial infarction has reduced infarct size and improved heart function and decreased production of ROS (80-82).

PROTEIN KINASE B

Protein Kinase B (PKB; also known as Akt) is a serine/threonine kinase, belonging to the AGC superfamily of protein kinases, which plays a prominent role in regulating cell survival, growth, proliferation, angiogenesis, metabolism and migration (83).

Rowe et al. originally identified Akt1 as the oncogene in the transforming retrovirus, AKT(84). It was isolated from an AKR mouse spontaneous thymoma cell line by co-cultivation with an indicator mink cell line. PKBγ were cloned from a transformed mink cell clone and these sequences were used to identify PKBα and PKBβ in human clone library(84).

There are three known isoforms of PKB namely PKBα/Akt1, PKBβ/Akt2 and PKBγ/ Akt3 and all are expressed in the myocardium, with PKBα and PKBβ most abundant (85).

All three PKB isoforms are activated in a phosphatidylinositol 3-kinase (PI-3K)-dependant manner involving either Class IA or Class IB PI-3Ks, which in turn are activated by tyrosine kinase and G-protein-coupled receptors, respectively.

PKB possess a protein domain known as a PH domain, or Pleckstrin Homology domain. It binds either PIP_3 (phosphatidylinositol (3,4,5)-triphosphate, phosphatidylinositol (3,4,5) P3) or PIP_2 (phosphatidylinositol (3,4)-biphosphate, PtdIns (3,4) P2 (86). PIP_2 is phosphorylated

by PI3-kinases and upon receipt of chemical messengers, which tell the cell to begin the growth process (Figure 5). Once activated, PI3-kinase phosphorylates PIP_2 to form PIP_3.

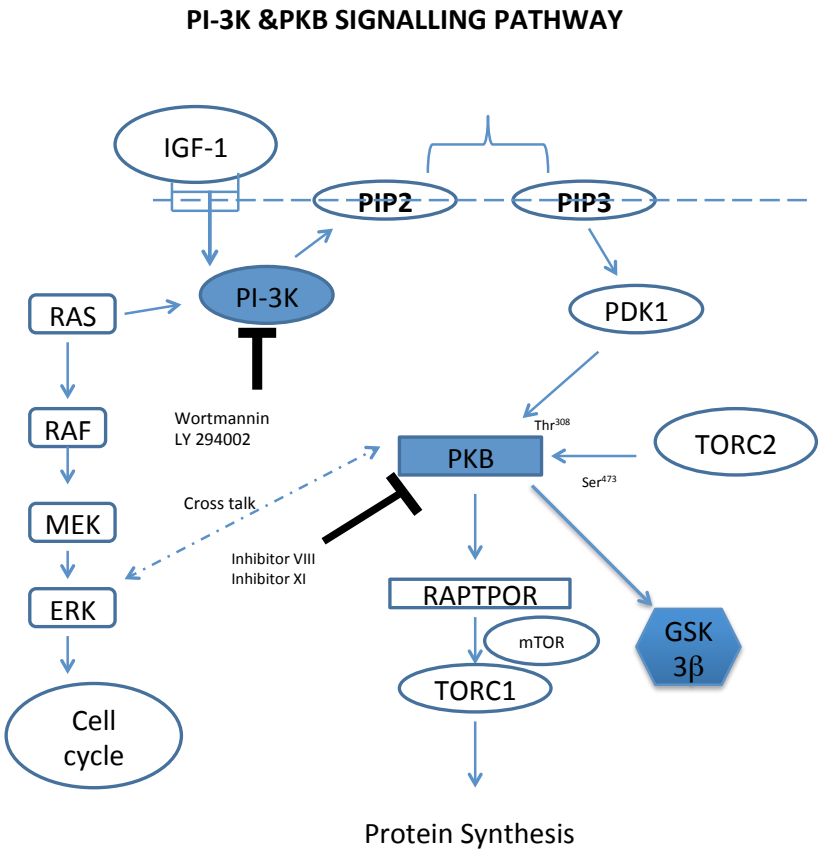

Figure 5: PI-3K signalling pathway

After PKB has been positioned at the membrane through binding of PIP_3, it phosphorylates by phosphoinositide dependent kinase 1 (PDPK1 at threonine 308) and mTORC2 (at serine 473). mTORC2 along with Integrin-linked kinase (ILK) and Mitogen-Activated Protein Kinase Activated Protein Kinase-2 can functionally acts as PDK2 molecule. Phosphorylation by mTORC2 stimulates the subsequent phosphorylation of PKB by PDK1.

Once activated PKB then goes on to activate or deactivate its myriad substrate (eg mTOR) via its kinase activity.

Genetics

C.elegans and *Drosophila* genetics have been used for describing signalling pathways for kinase substrates. Using genetic approach in *C.elegans*, it was shown that Fokhead box P (FOXO) transcription factor Daf-16 is downstream of the PI3K and PKB (87).

Deletion alleles of PKB have also been important in confirming the kinase specificity. A range of iso-form specific PKB knockout mice has been used to demonstrate the phosphorylation reactions. Knocking of a PDK1-PH domain mutant (PKD-1-RRR472-474LLL or PDK1-PH_{K1}) results in defective phosphorylation and activation of PKB isoform. It however does not effect in phosphorylation with other AGC kinases (88).

Validation of this result has also been obtained by embryonic stem cells expressing PDK1-PDF pocket mutant (PDK-1L155E) (89).

Cell Survival

PKB plays an important role in promoting cell survival by blocking the function of proapoptotic proteins and processes. The proapoptotic effects of Bcl-2 homology domain 3 (BH3)- only proteins are negatively regulated by PKB. It inhibits the BH3-only protein BAD by direct phosphorylation (90,91). The phosphorylation of BAD on S136 is done through PKB, which in turn creates a binding site for 14-3-3 proteins. This process results in release

of BAD from its target protein (92). It has been shown that this phosphorylation of S136 on BAD is critical for cell survival effects of PKB on neurons and other cells (90,93).

BH3-only proteins are inhibited by PKB through FOXO – mediated transcription of target genes (94). Another target is MDM2 through which PKB can inhibit BH3-only proteins and causes degradation of p53(95-96).

There remains a considerable gap in the understanding of other targets of PKB towards cell survival. Cross et al. (97) have shown that PKB phosphorylates GSK3 isoforms causing inactivation of the kinase. GSK3 directly inhibits the prosurvival Bcl-2 family member MCL-1 (98). The PKB activation inhibits the processing of procaspase-9 through Bcl-2 family members and it results in decrease in protease activity of caspase-9 in vitro (99).

Crosstalk with other pathways remains a possibility for PKB to bring cell survival effects. For example, PKB signalling can activate the NF-kB transcription factor through the stimuli by tumour necrosis factor (TNFa) and platelet-derived growth factor (PGDF) (100,101). PKB through phosphorylating c-Raf causes inhibition of ERK pathway (102). PKB signalling has also been shown to inhibit MAPKinase, JNK and p38.

Cell Growth

PKB through activation of mTOR complex 1(MTORC1) plays a vital role in promoting cell growth. Nutrients and growth factor signalling regulate this step. MTORC1 is a critical regulator of translation initiation and ribosome biogenesis and plays an evolutionary conserved role in cell growth control (103). Tuberous sclerosis complex 2 (TCS2) and

proline-rich PKB substrate of 40kDA (PRAS40) has been shown to act as PKB substrate for MTORC1 activation (104,105). PKB has also been suggested in to contribute new lipids synthesis through ATP citrate lyase (ACL) (106). The conversion of citrate to cytosolic acetyl CoA results providing scaffolding for lipid biosynthesis.

Angiogenesis

Vascular endothelial growth factor (VEGF) results in activation of PI3K-PKB pathway in endothelial cells (107). It subsequently contributes to endothelial cell survival, growth and proliferation. PKB activates endothelial nitric oxide (NO) synthase (eNOS) releasing NO causing vasodilation, vascular remodelling and angiogenesis (108).

Cellular Metabolism

PKB signalling plays important part in cellular metabolism. It can also induce protein synthesis pathways and therefore is a key signalling protein for skeletal muscle hypertrophy and general tissue growth.

PKBβ is an important component of insulin signalling pathway and induce glucose transport. Garofalo et al. (109) demonstrated separate roles of PKBα and PKBβ in 'knocked out' mice model. In null PKBα mice, glucose haemostasis was unperturbed. It is consistent with a role for PKBα in growth. In null PKBβ mice, there was a mild growth deficiency and displayed insulin resistance therefore postulating the idea that PKBβ is more specific for insulin

receptor signalling pathway The role of PKBγ is less clear, though it appears to be predominantly expressed in brain. It has been reported that mice lacking PKBγ have small brains(86).

PKBβ regulates the uptake of glucose in response to insulin. On insulin stimulation, PKBβ combines with glucose transporter 4(Glut4)-containing vesicles, it leads to Glut4 translocation to the plasma membrane (110,111). Rab-GAP AS160 is an important direct target of PKB involved in this process. S588 and T642 are amongst the five other PKB sites that are phosphorylated on AS160 in response to insulin. PKB has also been suggested to regulate the cell surface expression of transporters of amino acids through an unclear mechanism (111).

It can also cause alteration in cellular glucose and lipid metabolism. Glucose in converted to glucose 6-phosphate through the action of hexokinases. PKB enhances the association of hexokinase with mitochondria and increases phosphorylation of glucose. PKB signalling can regulate both storage and catabolization of glucose. When PKB is activated, it increases the rate of glycolysis through insulin signalling pathway (112).

Novel Inhibitors of PKB

As discussed earlier, PKB plays a crucial role in cell survival, growth and angiogenesis. The classical studies to explore the role of PKB have used PI-3K inhibitors such as wortmannin and LY 294002. Mocanu et al. used these two inhibitors to show that in vivo blocking of PI-

3K in rabbit reversed the decrease in infarct size offered by IP (113). Also, using wortmannin and LY294002 decreased the PKB phosphorylation, thereby confirming its indirect negative effect on it.

Until recently, specific role of PKB and other protein kinases have been very difficult to study because of lack of specific inhibitors. Zhao et al. reported a novel series of potent and selective allosteric PKB inhibitors.(114). These inhibitors belong to 2,3-diphenylquinoxaline core 1 family. It was shown to inhibit the phosphorylation of both PKB1 and PKB2 *in vitro.* Furthermore, it was shown that these inhibitors have selectivity to AGC family kinases such as PKA, PKC, SGK as well as selectivity for isoforms of PKB. Due to its non-competition with ATP and allosteric mode of binding, this inhibitor was able to show this selectivity.

Barve et al. developed copper based complex that was able to interact with amino acid in the Pleckstrin Homology (PH) and the kinase domain of the PKB protein(115). This compound showed a very potent inhibition of PKB on hormone-independent and metastatic breast (BT20), prostate (PC-3) and K-ras mutant (COLO357) and K-ras wild type (BxPC-3) pancreatic cancer cells.

Calleja et al. explained the molecular mechanism of PKB inhibition by inhibitor VIII(116). It has been shown that interaction of the PH domain and PKB's inactive state prevents phosphorylation by PDK1, which is its upstream activator. They have also postulated that PKB inhibition through inhibitor VIII may involve the C-terminus also.

These developments have surely made a significant contribution in not only designing experiments for cancer researches but also cell signalling pathways involving myocardial injuries.

MITOCHONDRIAL PERMEABILITY TRANSITION PORE (MPTP)

Mitochondrion is a key player in events leading to apoptosis and necrosis in myocardial ischaemia and reperfusion. Mitochondria forms a pivot around which multiple signal transduction pathways revolves and bring about cardioprotection through the opening of mitochondrial ATP-sensitive K^+channels (mitoK_{ATP}).

Crompton et al. (117), Howarth and Hunter et al. (118,119) described the existence of a non-specific channel with a molecular cut off of 1.5 kDa which was termed as mitochondrial permeability transition pore (MPTP). Amongst the major activators of MPTP are oxidative stress, phosphates and adenine nucleotide depletion, which are normally encountered during the ischemic/reperfusion phase of heart (120-122).

The opening of MPTP causes loss of barrier to protons thereby resulting in uncoupling of oxidative phosphorylation. This prevents ATP production inside mitochondria. Also, it leads to disruption of metabolic gradients between the mitochondria and cytosol, resulting in loss of mitochondrial membrane potential and mitochondrial swelling.

Crompton et al. (117,123) showed for the first time that opening of this pore can account for the reperfusion injuries after ischaemia. Since then many experiments have targeted the MPTP as a potential pharmacological modulator for cardiac protection mechanism.

Sanglifehrin A (SfA), cyclosporine A (CsA), N-methyl-4-isoleucine-CsA (NIM811) and D-3MeAla-4EtVal-CsA (Debio-25) were identified as direct inhibitors of MPTP (124-128).

CsA and SfA initially promised to be of great value in cardiac protection but because of interactions with other cyclophilins and counter mechanism of MPTP opening (Ca^{2+}) proved them to be not clinically feasible.

Ischaemic preconditioning is triggered by G-protein coupled receptors (GPCR) agonist like opioid peptides, adenosine and bradykinin. This activation converges ultimately to mitoK_{ATP} opening and production of ROS. PKC inhibitor can block this protection and PKC epsilon has been proposed as a common target of cardioprotective signalling (129).

Non-GPCR pathways like digitalis have been also shown to offer protection from ischaemic reperfusion injury through $Na^{+}K^{+}$ATPase activation of SRC kinase resulting in the formation of 'binary receptor'. This in turn phosphorylates transmit signals to the intracellular compartments (130,131). This has been termed as Ouabain signalling. It is dependent on mitoKATP opening and mitochondrial ROS production.

Signalling enzymes are transformed into vesicular, multimolecular signalling complexes termed as signalosomes. They are assembled in caveolae and relay the signal to the mitochondrial outer membrane (MOM) (132).

Once it is relayed to MOM, a cascade of intra mitochondrial signalling starts. The primary function of it is to inhibit opening of MPTP. It can be largely grouped into these identifiable pathways:

1. Opening mitoK_{ATP} by activation of PKCepsilon: PKC epsilon is embedded in the mitochondrial inner membrane. Upon activation by IP, ischaemic post conditioning and pharmacological preconditioning through mitoKATP results in PKC epsilon activation(133).

2. Mitochondrial K^+ uptake: Opening of mitoKATP channels causes influx of K^+ that is counterbalanced by efflux of H^+ to maintain the pH of matrix. This results in matrix alkalisation. This process results in Complex I to produce increased amount ROS and thereby activating PKC epsilon (134).

3. Activation of PKC epsilon by endogenous ROS: A second mitochondrial PKC epsilon 'PKC epsilon 2' is activated through increased ROS production. This acts as an inhibitor to MPTP thereby reducing cell death (135).

The cellular mechanisms associated with mitoK_{ATP} induced cell survival are still not fully understood but probably involve modulation of mitochondrial Ca^{2+} overload and reactive oxygen species formation during ischemia and reperfusion (136,137).

AIMS OF MY STUDY

My study has the following specific research aims:

1) To investigate the influence of PKB in the tolerance to ischaemia/reoxygenation (I/R) –induced injury of the mammalian (rat and human) myocardium using novel specific PKB inhibitors binding to the PH domain.
2) To define the relationship of PKB with the mitoK_{ATP} channel and with p38 MAPK, two identified essential steps in the signal transduction mechanism of cardioprotection by IP
3) Given the opposed roles of the MPTP in cardioprotection (transient *versus* sustained opening) the primary aim of this study was to determine how two structurally different MPTP inhibitors (cyclosporine A and bongkrekic acid) administered for varying time regimes influenced ischemia/reperfusion (I/R)-induced injury in myocardial slices from rat left ventricle.
4) To explore how pharmacologic MPTP opening (using atractyloside) at different time points during I/R modulated myocardial injury.

CHAPTER 2

MATERIALS AND METHODS

Procurement of myocardial tissue

Study animals

Male Wistar rats, weighing 250–350 g, were purchased from Charles River Labs (Margate, UK). Strict adherence to the *Guide for the Care and Use of Laboratory Animals* (US National Institutes of Health –Ref No. 85-23, revised 1996) was observed. Animals received care in accordance with the *Guidance on the Operation of the Animals* (Scientific Procedures Act 1986 (Her Majesty's Stationary Office, London, UK). Rats were culled by cervical dislocation and heart was retrieved immediately by thoracotomy.

Human study subjects

At the time of right atrial cannulation during elective coronary artery bypass graft or aortic valve surgery, right atrial appendage from diabetic and non-diabetic patients were retrieved. Poor ejection fraction (30%), atrial fibrillation and those patients who had opioids, catecholamines or potassium channel openers (nicorandil/ diazoxide) were excluded from this study. Local ethical approval was obtained and strict adherence to *Declaration of Helsinki*

was maintained. Written informed consents were obtained from patients to obtain myocardial samples.

Experimental preparation

Our laboratory has previously validated and characterised the *in vitro* experimental model used for this study (138)) and extensively used by our laboratory. The rats were killed by cervical dislocation followed by immediate thoracotomy to retrieve the heart. The heart was placed in ice-cooled pre-oxygenated Krebs- Henseleit-Hepes (KHH) buffer at 4^{o} C for 3 minutes. The left ventricle was then mounted on ice-cooled ground glass plate and using surgical skin graft blades (Swann-Morton Ltd, Sheffield, UK), they were sliced to a thickness of between 300-500 μm, weighing between 30 and 50 mg each. These slices were then transferred to Erlenmeyer flasks (25mls, Duran, Astell Scientific, Sideup, UK), which contained 10ml of KHH-buffered solution in a shaking water bath at 37^{o}C. It was left in it for equilibration for 40 minutes where the tissues were oxygenated by 95% O_2-5%CO_2 gas mixture to obtain a pO_2 between 25-30kPa and CO_2 between 6.0 and 6.5 kPa at 37^{o}C.

To induce ischaemic preconditioning, these slices were subjected to one cycle of 5 minutes of ischaemia (pH 6.6, no glucose, 95% N_2 & 5%CO_2) followed by one cycle of 5 minutes of reoxygenation before inducing 90 minutes of ischaemia. This regime has been known to provide maximal protection in this experimental model (139).

Similar protocol was used for the human right atrial appendage. This method has also been validated through other studies using identical protocols of simulate ischaemia and reoxygenation (138).

Solutions and chemicals

The KHH-buffered solution (pH 7.4 at 37°C) with following concentration was prepared (in mM): NaCl (118), KCl (4.8), $NaHCO_3$ (27.2), MgCl2 (1.2), KH_2PO_4 (1.0), $CaCl_2$ (1.20), Glucose.H2O (10) and Hepes (20).

Wortmannin (0.1 μM), LY 294002 (10 μM), chelerythrine (10 μM), 5-hydroxydecanoate (5-HD, 1 mM), SB203580 (10 μM) and diazoxide (100 μM) were purchased from Sigma and their doses were selected following preliminary dose–response studies for each of them (72).

Dose response studies were performed for novel inhibitors of PKB, inhibitors XI and VIII (Calbiochem) at the concentrations of 0.1, 1, 10μM for PKB inhibitor XI and 0.05, 0.5 and 5 μM for PKB inhibitor VIII. The most effective concentration was therefore identified.

DMSO was used as solvent for wortmannin, PKB inhibitor VIII, SB203580 and diazoxide. It was eventually added into the KHH buffer.

For MPTP experiments, agent cyclosporine A (CsA, 20 μM), bongkrekic acid (BKA, 100 μM), and atractyloside (ATR, 50 μM) were purchased from Sigma (Gillingham, Dorset, UK) and their doses were selected following preliminary dose-response studies to CsA and BKA. CsA, BKA, and ATR were dissolved in DMSO before being added into the KHH buffer.

The final concentration of DMSO was <0.1%, a concentration that has been shown to have no effects on the degree of ischemia/reoxygenation-induced injury (140).

Assessment of tissue injury

CK release into the media was assayed by a kinetic ultraviolet curve to measure tissue injury after 120 minutes of reoxygenation. The principle of this assay is based on detecting the formation of NADPH. The enzymatic reactions involved in the assay are as follows:

Creatine Phosphate +ADP <-------CK-------→Creatine + ATP

ATP+ Glucose ←----------HK----------→ ADP + G-6-P

Glucose-6-Phosphate + NADP ←-------G-6-PDH---→6-PG + NADPH

CK is the catalyst for conversion of creatine phosphate and adenosine diphosphate (ADP) to creatine and adenosine triphosphate (ATP) with the consumption of ATP in phosphorylating glucose to glucose 6-phosphate (G6P). The latter step of the reaction is catalysed by hexokinase (HK).

Glucose 6-phosphatase dehydrogenase (G-6PDH) catalyses the oxidation of G6P into 6-phosphogluconate (6PG). This oxidation requires the presence of nicotinamide adenine dinucleotide (NADP) resulting in its reduction to NADPH and increasing absorbance at 340nm. The rate of transformation from NADP to NADPH is directly proportional to CK levels in the media.

To measure the enzyme activity, we used commercial assay kit (30-3060/R2: Abbott Laboratories, Diagnostic Division, Kent, UK), plate reader (BioTek Instruments, model ELx800uv), Winooski, VT, USA). The results were expressed after subtraction of the aerobic control value in International Units as mg wet weight^{-1}.

Assessment of cell death

At the end of the experimental protocols, the myocardial slices were incubated for 15 min with 30 μg ml^{-1} propidium iodide in PBS at pH 7.4 followed by three washes in PBS.

Using 4% paraformaldehyde in PBS, these myocardial tissues were fixed and stored in darkness at 4°C before sectioning. The tissues were then embedded with optical cutting temperature embedding matrix (Tissue-Tek, Agar Scientific Ltd, Stansted, UK). 7μm thick frozen sections were made in a Bright Cryotome (OTF model) at *ca* – 22° C and the sections were collected on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany).

Tissues underwent permeabilisation in a humidity chamber in 0.02mg ml^{-1} proteinase-K for 10 minutes at 37°C. They were also pre-sensitised for 1 min in a microwave oven at 800 W in 0.1% Triton X-100 and 0.1 M tri-sodium citrate at pH 6.0.

Minimum of 100 nuclei per section were counted for assessment of cell death. For assessment of apoptosis, we used a commercial kit (Roche Diagnostics GmbH, Penzberg, Germany). It has deoxynucleotidyl transferase to incorporate fluorescein (FITC)-labelled dUTP oligonucleotides to DNA strand breaks at the 3'-OH termini in a template-dependent manner (TUNEL technique). A fluorescence excitation of 515 nm and FITC fluorescence emission range of 600–630 nm were used.

For necrosis, we used helium-neon LASER light at 543nm to excite propidium iodide-labelled nuclei. The resulting fluorescence was detected using 600nm emission.

Sections were mounted using Antifade-mounting medium (Prolong Antifade kit, Invitrogen, Paisley, UK). It was stained with 4'6-diamidino-3-phenylindole (DAPI). For fluorescence, excitation at 340nm and detection at 456nm was done.

Using a fluorescence microscope (Axiovert 200M, Zeiss fluorescence microscope, Göttingen, Germany) at ×40 magnification we assessed cell necrosis and apoptosis. OpenLab v.5 program (Improvision, Coventry, UK) was used to acquire images (Figures 6,7 &8).

As described by Hassouna et al. (140), using analytical digital photomicroscopy, six to ten randomised fields from each slide of the triple-labelled high resolution imaging samples were analysed. Colorimetric methodology was employed to study fluorescence signals (141). Without changing the camera and microscope settings, at least three photographs were taken from each field to obtain an average pixel value through Adobe Photoshop 6.0 (Adobe Systems Incorporated, San Jose, CA, USA). Necrosis and apoptosis were expressed as a percentage of pixels representing the total nuclei. A pilot study (authors' unpublished data) performed in our laboratory showed no differences between the colorimetric and manual counting of apoptotic, necrotic or total nuclei.

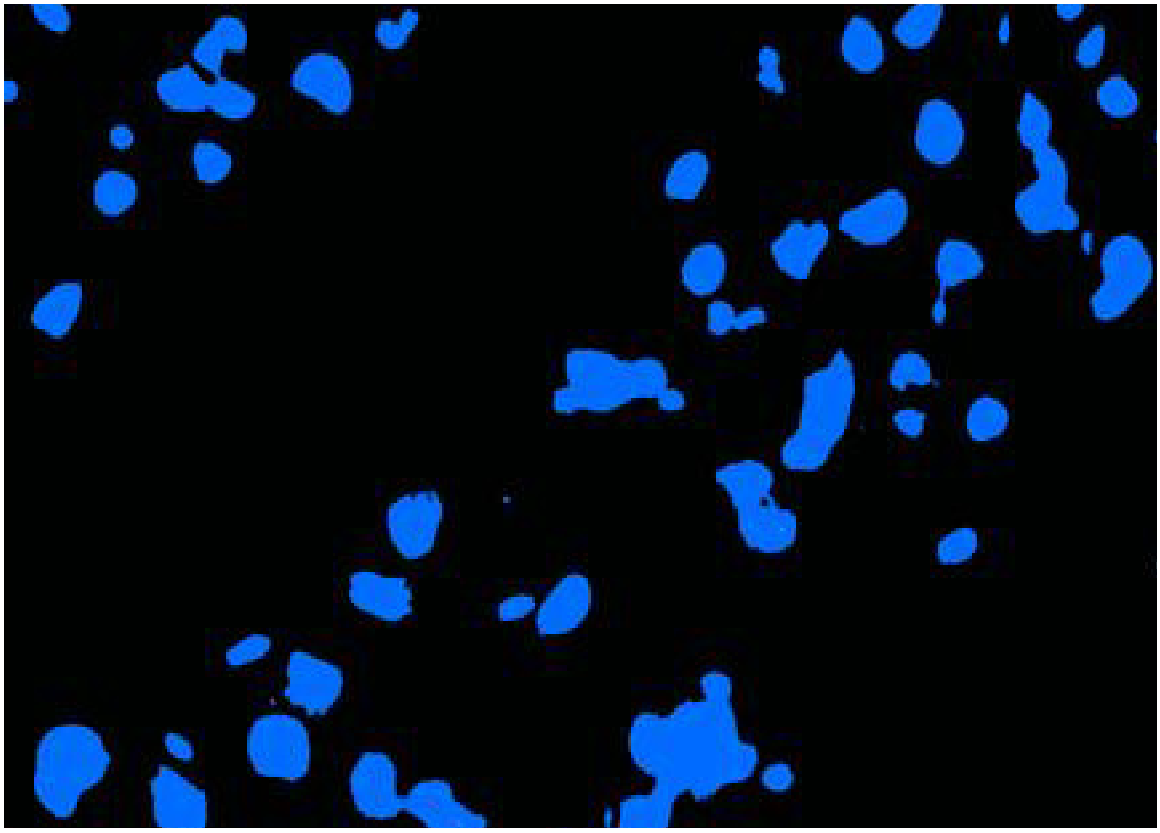

Figure 6: Representative image of DAPI (blue- normal nuclei)

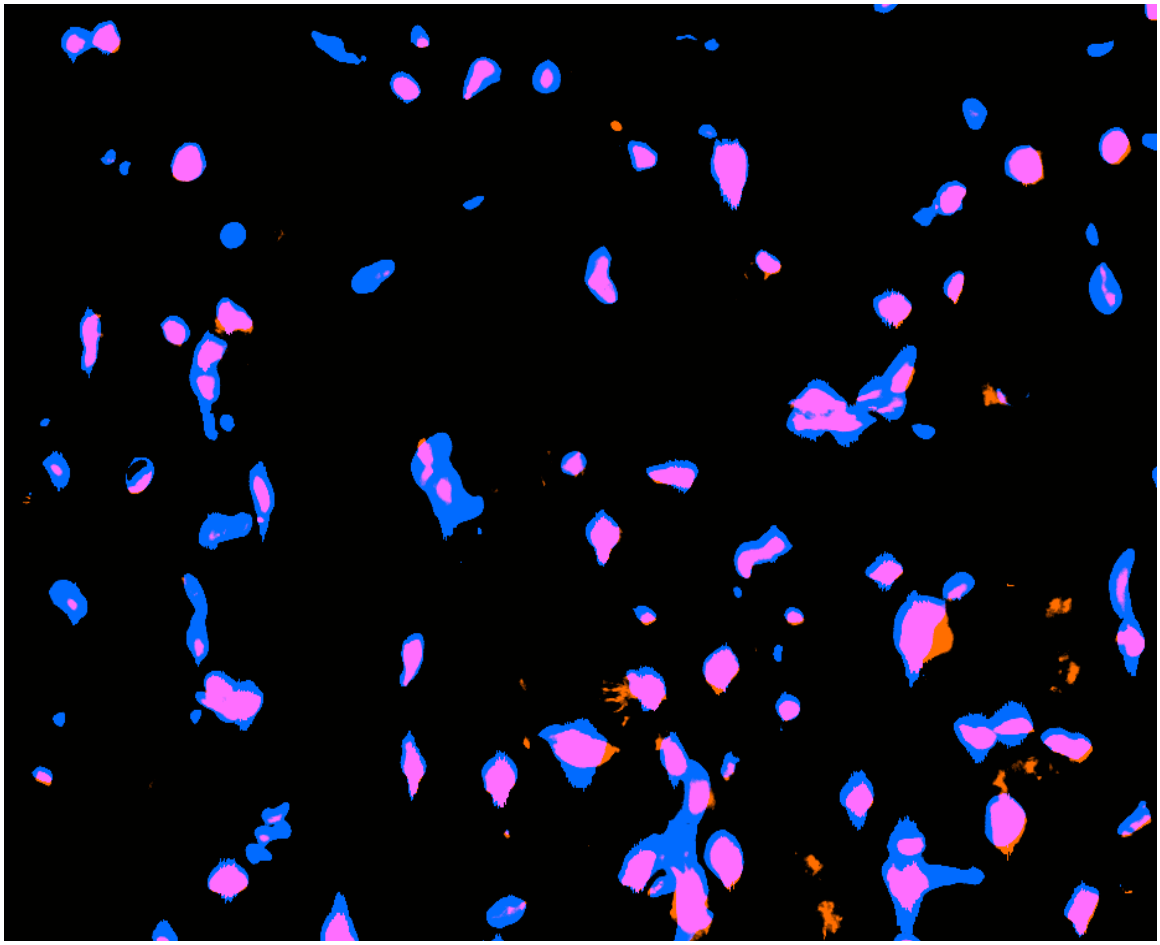

Figure 7: Representative image of DAPI & PI staining (pink- necrotic nuclei, blue-normal nuclei)

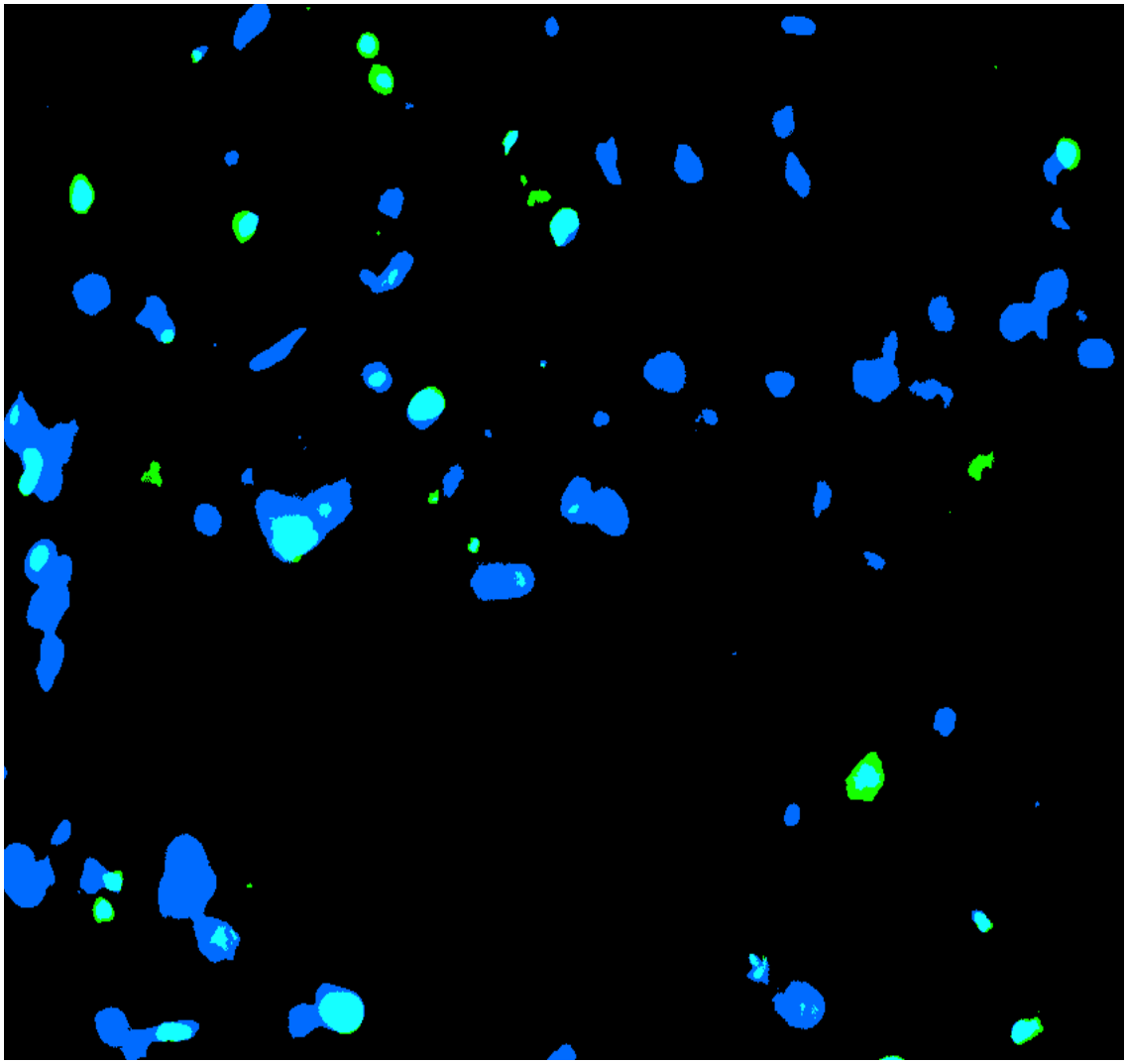

Figure 8: Representative image of TUNEL staining (green- apoptotic nuclei, blue-normal nuclei)

Proteome Profiler Array

For parallel determination of the relative levels of MAPKs, ERKs, JNKs and p38 Kinases in ischaemic-reperfusion injuries in rat and human myocardium, we attempted to use the novel Proteome Profiler Arrays (Human Phospho-MAPK Array Kit, Catalog Number ARY002B, R&D Systems Inc., USA). This array can detect 26 kinases in one single nitrocellulose membrane and therefore is a promising alternative for western blot analysis. Antibodies of these protein kinases have been selected using lysate samples prepared from cell lines known to express the target proteins and is spotted on nitrocellulose membrane.

Cell lysates were diluted, mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with the Proteome Profiler Human Phospho-MAPK Array. The membrane was then washed to remove unbound material. Streptavidin-HRP and chemiluminescent detection reagents were applied, and a signal was produced at each capture spot corresponding to the amount of phosphorylated protein bound.

The positive signals seen on developed film can be identified by placing a transparency overlay on the array image and aligning it with the pairs of reference spots in three corners of each array.

Pixel densities on developed x-ray film were collected and analyzed using transmission-mode scanner and image analysis software. The average signal of different arrays was compared to determine the relative change in protein phosphorylation between samples.

Drawbacks:

The Human Phospho-MAPK array kit claims to be suitable for rat and human cells. Also,

there are inherent negative and positive controls in the nitrocellulose membrane for validating the results. However, in our experiments, we observed that the negative controls were showing significant signals as compared to positive controls. Therefore it raised serious possibilities of error in the analysis and interpretation of the status of protein kinases. After thoughtful consideration, this method was given up and standard western blot technique for adopted.

Western blot analysis

To determine the phosphorylation of PKB (Ser^{473}), tissue samples were homogenised in RIPA buffer containing protease inhibitor, PMSF (1 mM), DDT (0.5 mM), glycerophosphate (25 μM) and sodium orthovanadate (1 mM). The homogenate was centrifuged at 10 000 *g* for 30 min and the supernatant obtained was analysed for protein concentration using the Bio-Rad *DC* protein assay kit. Tissue supernatant (20 μg total protein) was electrophoresed on 10% SDS-PAGE and blotted onto nitrocellulose membrane. Following transfer the membranes were washed with Tris-buffered saline (TBS) and blocked for 1 h at room temperature (20–24°C) in blocking buffer (TBS, 5% (w/v) skimmed milk powder, 0.1% (v/v) Tween-20). Blots were then incubated overnight at 4°C with primary rabbit monoclonal antibody against phosphorylated (Ser^{473}) PKB (Cell Signalling Technology) at 1:1000 dilution in blocking buffer. The primary antibody was removed, blots extensively washed three times for 5 min in TBS/0.1% Tween 20 (v/v) and incubated for 1 h at room temperature with goat anti-rabbit secondary antibody coupled to horseradish peroxidase at 1:5000 dilution in blocking buffer. Following removal of the secondary antibody, blots were extensively washed as above and developed using the enhanced chemiluminescence detection system

(Amersham, Little Chalfont, UK) and quantified by densitometry using Scion image (Scion, Frederick, MD, USA). The uniform transfer of proteins to the nitrocellulose membrane was routinely monitored by transiently staining the membranes with Ponceau S stain (Sigma Chemical Co.) prior to application of the primary antibody. In addition, replicate samples from each experiment were analysed on separate blots using a rabbit antibody (1:1000) that recognises unphosphorylated (total) PKB (Cell Signalling Technology).

Assessment of Caspase 3 Activity

Myocardial tissue was homogenized in lysis buffer containing 50 mM HEPES, 0.15 M NaCl, 2 mM EDTA, and pH of 7.40. This colorimetric assay was performed according to the commercial kit guidelines (GENios, Hayward, CA). Briefly, the homogenate was incubated with 1% CHAPS (non-ionic detergent, Sigma) and 1 mM DTT for 3 h. Then absorbance was measured using a wavelength of 405 nm using a Tecan GENios plate reader set up (MTX Lab Systems, Vienna, VA) and the results were expressed as optical density units.

Statistical analysis

Data are expressed as mean ±S.E.M. Each reported value was obtained after subtracting the value from the corresponding time-matched aerobic control. One-way ANOVA was used to compare the significance between groups. All the analyses were performed using the SPSS program and differences were considered to be statistically significant if $P < 0.05$.

Chapter 3

Role of Protein Kinase B in ischaemic preconditioning of rat and human myocardium

Introduction

Protein Kinase B (Akt) belongs to the AGC superfamily of protein kinases. It is a serine/threonine kinase. They play an important role in regulating cell survival, growth proliferation, angiogenesis, metabolism and migration (85).Duronio et al. have demonstrated that the activation of these PKB isoforms is through phosphatidylinositol 3-kinase (PI-3K) involving Class 1A or Class 1B PI-3Ks. It is subsequently activated by tyrosine kinase and G-protein-couple receptors (142).

PKB possess a protein domain known as a PH domain, or Pleckstrin Homology domain. It binds either PIP_3 (phosphatidylinositol (3,4,5)-triphosphate, phosphatidylinositol (3,4,5) P3) or PIP_2 (phosphatidylinositol (3,4)-biphosphate, phosphatidylinositol (3,4) P2 (86). PIP_2 is

phosphorylated by PI3-kinases and upon receipt of chemical messengers that tell the cell to begin the growth process. Once activated, PI3-kinase phosphorylates PIP_2 to form PIP_3.

After PKB has been positioned at the membrane through binding of PIP_3, it phosphorylates by phosphoinositide dependent kinase 1 (PDPK1 at threonine 308) and mTORC2 (at serine 473). mTORC2 along with Integrin-linked kinase (ILK) and Mitogen-Activated Protein Kinase Activated Protein Kinase-2 can functionally acts as PDK2 molecule. Phosphorylation by mTORC2 stimulates the subsequent phosphorylation of PKB by PDK1.

Activated PKB phosphorylates a number of downstream targets which have prominent roles in regulating apoptosis including the pro-apoptotic Bcl-2 family member BAD, caspase 9, glycogen synthase kinase 3β (GSK-3β) and the Forkhead family of transcription factors (83,143).

Various investigators have shown that PI-3K/PKB plays a central role in cardioprotection induced by ischaemic preconditioning (IP), ischaemic postconditioning (POC) and pharmacological preconditioning (85,144,145). Due to the unavailability of selective PKB inhibitors, most of these investigators had limited choice of using PI-3K inhibitors wortmannin and LY294002 only. Also, in vitro and in vivo expression of dominant negative or constitutively active PKB mutants were used to study the specific role of PKB (146-148).

There are more than 500 protein kinases known in the human genome. Designing specific inhibitors for these protein kinases has been a major challenge due to their molecular alignment. Most of these inhibitors are ATP competitive. The ATP binding domain of these kinases is highly conserved and therefore targeting them has been problematic.

The solution to this problem came with the advent of non-ATP competitive inhibitors. These are allosteric inhibitors ie. regulatory sites of these kinases are distinct from its active site. These inhibitors show selectivity towards AGC protein kinase family and towards PKB isoforms.(114-116,149).

Two important PKB inhibitors, inhibitors VIII and XI work on allosteric inhibition principle. They bind to the PH domain of PKB and make them inactive and prevent subsequent phosphorylation of Thr^{308} and Ser^{473} (115,116).

The primary aim of this study was to investigate the influence of PKB in the tolerance to ischaemia/reoxygenation (I/R)-induced injury of the mammalian (rat and human) myocardium using novel specific PKB inhibitors binding to the PH domain. A second objective was to define the relationship of PKB with the mitoK_{ATP} channel and with p38 MAPK; two identified essential steps in the signal transduction mechanism of cardioprotection by IP.

Following were the objectives of this study:

1. To investigate the effect of PKB inhibitors of I/R- induced injury and IP
2. To elucidate whether PI-3K inhibitors can reproduce the results obtained with PKB inhibitors on I/R-induced injury and IP.
3. To determine the optimal time of administration of PKB and PI-3K inhibitor induced cardioprotection.
4. To determine the relationship between PKB inhibition and mitoK_{ATP} channels.
5. To investigate whether PKB inhibition also protects the human myocardium.
6. To determine the effect of PKB inhibition with the inhibitor XI on PKB Ser^{473} phosphorylation.

Methods

Study animals

This has been described in Chapter 2.

Human study subjects

This has been described in Chapter 2.

At the time of right atrial cannulation during elective coronary artery bypass graft or aortic valve surgery, right atrial appendage from diabetic and non-diabetic patients were retrieved. Table 1 illustrates the pre operative medical treatment and patient's characteristic. This has been described in Chapter 2.

Table 1. Characteristics and medical treatment prior to surgery of right atrial appendage donor patients.

A. Non diabetics

	Pat #1	*Pat #2*	*Pat #3*	*Pat #4*	*Pat #5*	*Pat #6*
Gender	Male	Male	Female	Male	Female	Male
Age (years)	60	37	71	51	85	63
Diagnosis	CAD	CAD	AVS	AVS	AVS	AVS
Type of Surgery	CABG	CABG	AVR	AVR	AVR	AVR
Medication						
Aspirin	Yes	Yes	Yes	No	No	Yes
Clopidogrel	No	No	No	No	No	No
Omeprazole	Yes	Yes	No	No	Yes	Yes
β-blocker	Yes	Yes	Yes	No	Yes	No
ACE inhibitor	Yes	Yes	Yes	No	Yes	Yes
Statin	Yes	Yes	Yes	No	Yes	Yes

B. Diabetics

	Pat #1	*Pat #2*	*Pat #3*	*Pat #4*	*Pat #5*	*Pat #6*
Gender	Male	Male	Male	Female	Female	Male
Age (years)	38	75	68	79	62	55
Type of Diabetes	I	II	II	II	II	II
Diagnosis	CAD	CAD	CAD	AVS	CAD	CAD
Type of Surgery	CABG	CABG	CABG	AVR	CABG	CABG
Medication						
Insulin	Yes	No	No	No	No	No
Glicazide	No	Yes	Yes	No	No	Yes
Metformin	No	No	Yes	Yes	Yes	Yes
Aspirin	Yes	Yes	Yes	Yes	Yes	Yes
Clopidogrel	Yes	Yes	No	No	Yes	No
Omeprazole	Yes	No	No	Yes	No	No
β-blocker	Yes	Yes	No	No	Yes	Yes
ACE inhibitor	No	Yes	Yes	No	No	No
Statin	Yes	Yes	Yes	No	Yes	Yes

ACE, angiotensin-converting enzyme; AVR, aortic valve replacement; AVS, aortic valve stenosis; CABG, coronary artery bypass graft; CAD coronary artery disease; DM, diabetes mellitus.

Experimental preparation

The study was carried using established model of simulated ischaemia and reperfusion as described in Chapter 2.

Assessment of tissue injury

The criteria of tissue injury, CK leakage and cell death by apoptosis and necrosis have been described in Chapter 2 under assessment of tissue injury section.

Western blot analysis

This has been described in chapter 2.

Study protocols

Myocardial slices (*n*= 6/group, unless otherwise indicated) were randomly allocated to different groups so that the tissues from each animal or patient donor were not utilised more than once for the same group. Time-matched aerobic controls, values of which are shown in Table 2, were used in each experiment and the rest of the tissues were subjected to 90 min ischaemia/120 min reoxygenation.

Table 2. Mean±SEM values of myocardial slices subjected to time-matched aerobic control for all the studies.

	CK leakage (U/mg wet wt)	*Necrosis (% of nuclei)*	*Apoptosis (% of nuclei)*
Study 1			
PKB inhibitor VIII	0.22±0.02	10.6±0.9	8.6±0.7
PKB inhibitor XI	0.27±0.07	9.3±0.4	8.4±1.1
Study 2	0.25±0.03	16.6±1.5	12.8±1.0
Study 3	0.24±0.03	9.5±1.6	10.1±1.5
Study 4	0.52±0.07	10.5±1.0	10.9±0.6
Study 5	0.31±0.04	8.8±0.6	9.9±0.4
Study 6	0.33±0.03	17.0±3.2	14.5±3.2
Study 7			
Non diabetics	0.54±0.08	9.1±0.7	9.9±0.8
Diabetics	0.49±0.08	9.3±0.8	9.0±0.7

The following studies were sequentially carried out:

1. Study 1. To investigate the effect of PKB inhibitors on I/R-induced injury and IP, rat ventricular muscles were exposed to different concentrations of the PKB inhibitors VIII (0.05, 0.5 and 5 μM) and XI (0.1, 1 and 10 μM) for 20 min prior to I/R. (Figure 9 a1 and a2).

2. Study 2. To elucidate whether PI-3K inhibitors can reproduce the results obtained with PKB inhibitors on I/R-induced injury and IP, rat ventricular muscles were subjected to 20 min exposure with LY 294002 (10 μM) and wortmannin (0.1 μM) before I/R as shown in (Figure 10a1 and a2).

3. Study 3 &4. To determine the optimal time of administration of PKB and PI-3K inhibitor-induced cardioprotection, slices from rat ventricular myocardium were exposed to the PKB inhibitor XI (1 μM) (Figure 11a) and wortmannin (0.1 μM); see Fig. 12a) for 20 min before ischaemia; during ischaemia; during reoxygenation; during ischaemia and reoxygenation; and before ischaemia and throughout ischaemia and reoxygenation.

4. Study 5 &6. To determine the relationship between PKB inhibition and mitoK_{ATP} channels (see Fig. 13a), rat ventricular slices were exposed to the PKB inhibitor XI (1 μM) in the presence of the mitoK_{ATP} channel blocker 5-HD (10 μM). Similarly, to determine the relationship of PKB with p38 MAPK (see Fig.6a), rat ventricular slices

were exposed to the p38 MAPK inhibitor SB203580 (10 μM) in the absence and presence of PKB inhibitor XI (1 μM). For comparison, the effects of 5-HD and SB203580 on IP-induced protection were determined for each study.

5. Study 7. To investigate whether PKB inhibition also protects the human myocardium (see Fig 14a1), myocardial slices from the right atrial appendage of patients undergoing elective cardiac surgery were exposed to the PKB inhibitor XI (1 μM) for 30 min or wortmannin (0.1 μM) for 20 min, both before the 90 min of ischaemia. For comparison, other myocardial slices were subjected to IP. In addition, to investigate whether PKB inhibition can reverse the unresponsiveness to protection of the diabetic myocardium (see Fig. 14a2), muscle slices from the right atrial appendage of patients with diabetes were also subjected to an identical protocol with the PKB inhibitor XI and wortmannin, with other myocardial slices being treated with IP or the mitoK_{ATP} channel opener diazoxide (100 μM) for 10 min before the 90 min of ischaemia.

6. Study 8. To determine the effect of PKB inhibition with the inhibitor XI on PKB Ser^{473} phosphorylation, rat ventricular slices (*n*= 4) were exposed to a 1 μM concentration of this agent for 20 min of aerobic incubation (e.g. no ischaemia). Other myocardial slices (*n*= 4/group) were exposed to wortmannin (0.1 μM) for 20 min or subjected to IP alone and in combination with the inhibitor XI and wortmannin. At the end of these periods, the slices were frozen in liquid nitrogen and kept at −80°C until analysis.

RESULTS

Study 1. Effect of PKB inhibitors on I/R-induced injury and IP in rat ventricular muscle

As shown in Figure 9 (panels B1 and 2), PKB inhibitors VIII and XI significantly reduces the ischaemia/ reoxygenation (I/R) myocardial injury (determined through CK release) and also the cardioprotection offered by them is comparable to that of IP. These inhibitors show similar reduction in necrosis and apoptosis (panels C1 and 2 & D1 and 2). It also demonstrates that the combination of PKB inhibition and IP did not result in additional benefit.

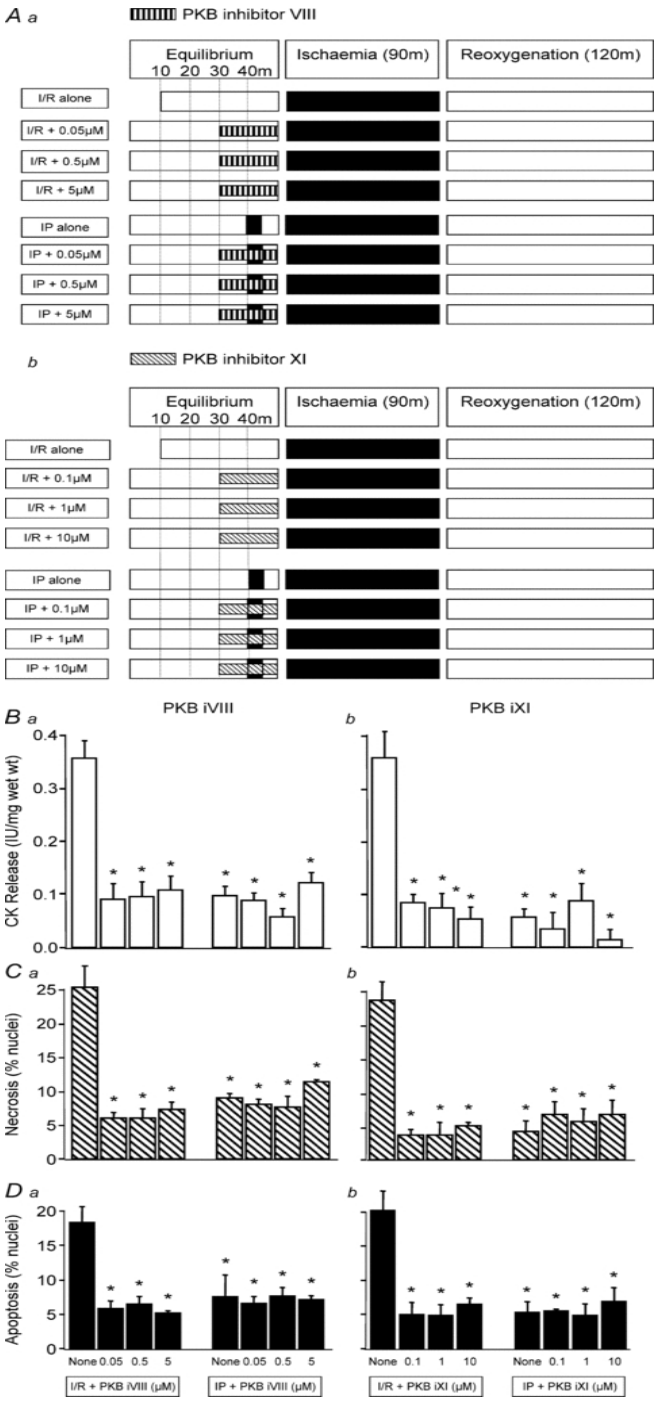

Figure 9. The effect of PKB inhibitors on I/R-induced injury and IP in rat ventricular myocardium.

Three different concentrations of PKB inhibitor VIII (0.05, 0.5, and 5μM) and PKB inhibitor XI (0.1, 1, and 10μM) were used to investigate their effect on ischaemia/reoxygenation (I/R)-induced injury and ischaemic preconditioning (IP) following the protocols shown in panels a1 and a2. In all cases tissue samples were analysed for CK release during the 120 minutes reoxygenation (panels b1 and b2), and for necrosis (panels c1 and c2) and apoptosis (panels d1 and d2) at the end of 120 minutes of reoxygenation. Data represent the mean ± SEM from 6 independent experiments. **P*< 0.05 vs I/R alone group.

Study 2. Effect of PI-3K inhibitors on I/R-induced injry and IP in rat ventricular muscle

After demonstrating cardioprotective properties of PKB inhibitors VIII and XI, we explored whether inhibiting PI-3K by LY294002 (10μM) and wortmannin (0.1 μM) would produce similar results. Figure 10 shows that the muscles subjected to I/R with these two inhibitors resulted in significant decrease in all the three parameters of myocardial injury (CK release, apoptosis and necrosis). No significant effect was demonstrated on IP.

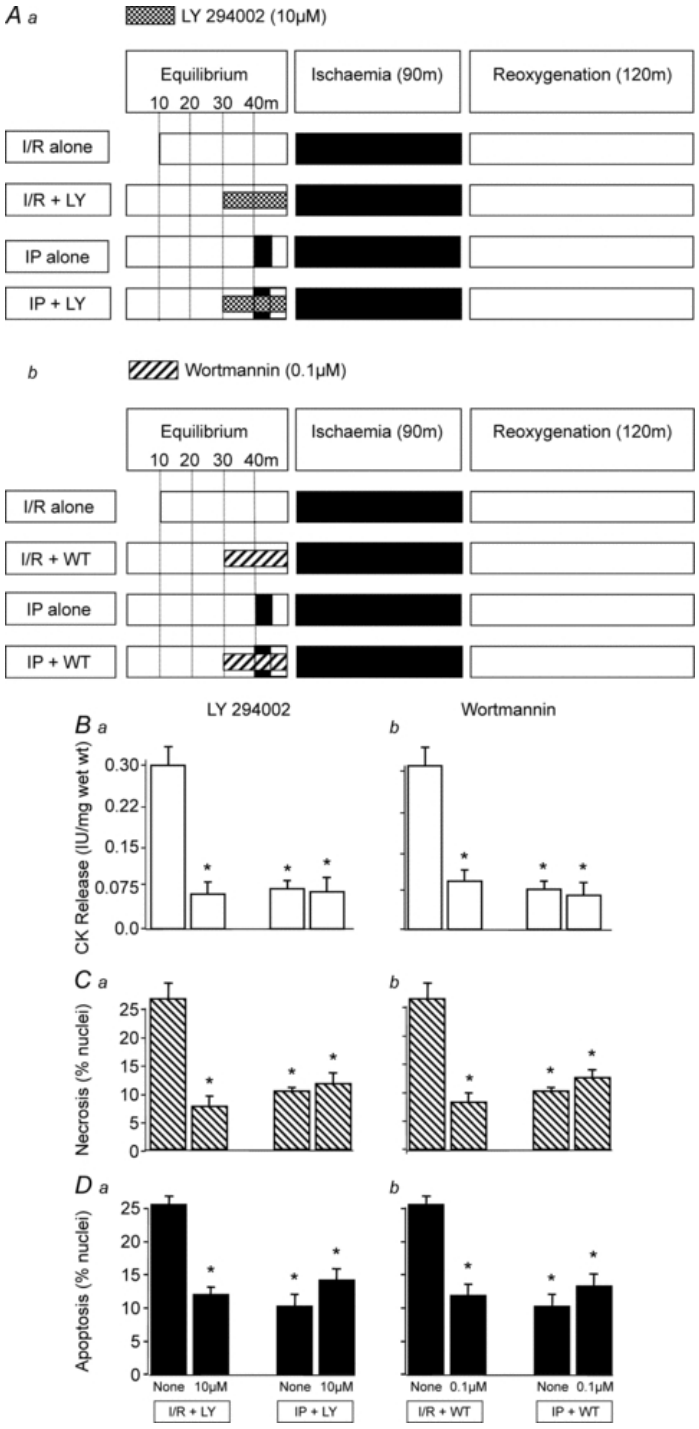

Figure 10. The effect of PI-3K inhibitors on I/R-induced injury and IP in rat ventricular myocardium.

LY 294002 (10μM) and wortmannin (0.1μM) were used to investigate the role of PI-3K in ischaemia/reoxygenation (I/R)-induced injury and ischaemic preconditioning (IP) following the protocols shown in panels a1 and a2. In all cases tissue samples were analysed for CK release during the 120 minutes reoxygenation (panels b1 and b2), and for necrosis (panels c1 and c2) and apoptosis (panels d1 and d2) at the end of 120 minutes of reoxygenation. Data represent the mean ± SEM from 6 independent experiments. *P< 0.05 vs I/R alone group.

Study 3 &4. Temporal effect of PKB and PI-3K inhibitor-induced cardioprotection

After demonstrating that PKB and PI-3K inhibitors are cardioprotective, we wanted to find out the optimal time of administrating these inhibitors.

Figure 11 shows that when PKB inhibitor XI was less effective during ischaemia or reoxygenation alone as compared to when it was given during both these phases.

Cardioprotection offered by inhibitor XI during ischaemia and reoxygenation is comparable to that when it was administered before ischaemia or when given throughout the entire experimental phases (before ischaemia, during ischaemia and during reoxygenation).

When PI3-K inhibitor wortmannin was used, similar results to those seen with inhibitor XI were seen (Figure 12).

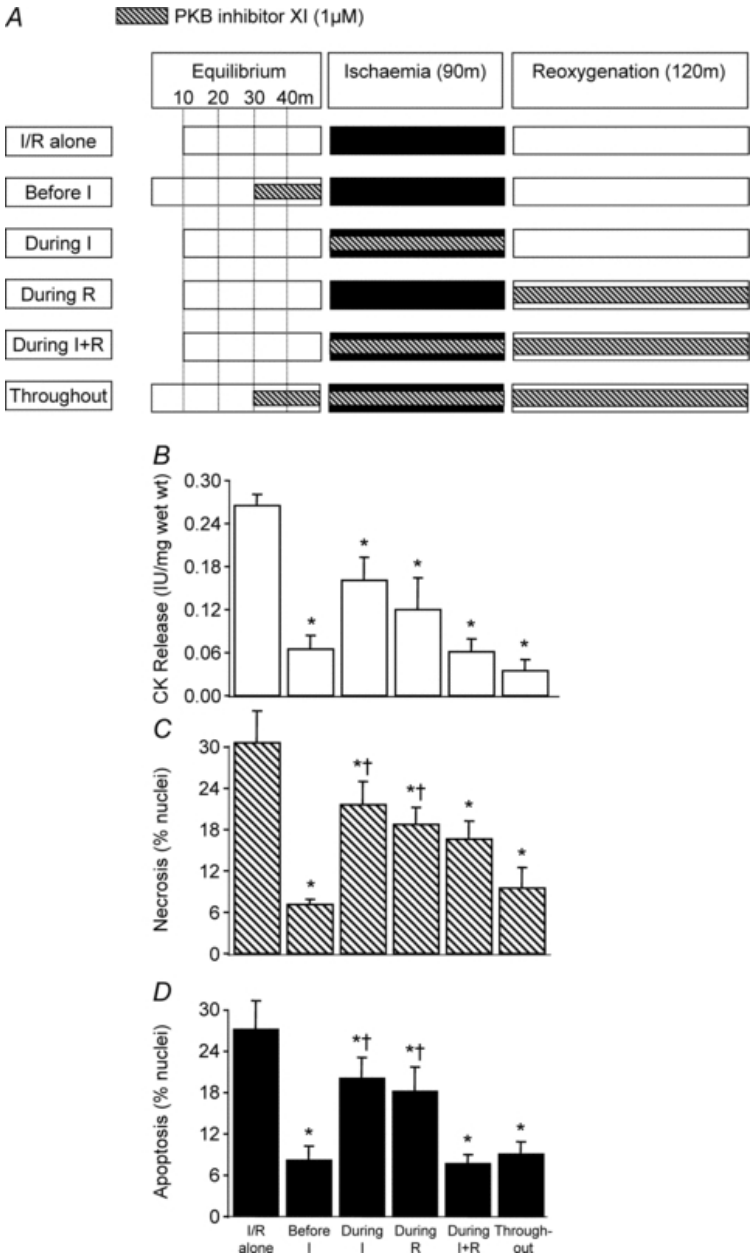

Figure 11. The temporal effects of PKB inhibitor XI administration during I/R.

As shown in panel a, PKB inhibitor XI (1μM) was administered at 5 different time points during the ischaemia/reoxygenation (I/R) protocol using rat left ventricular myocardial slices: 20 minutes before ischaemia; during ischaemia; during reoxygenation; during ischaemia and reoxygenation; and before ischaemia and throughout ischaemia and reoxygenation. In all cases tissue samples were analysed for CK release during the 120 minutes reoxygenation (panel b), and for necrosis (panel c) and (panel d) apoptosis at the end of 120 minutes reoxygenation. Data represent the mean ± SEM from 6 independent experiments. **P*< 0.05 vs I/R alone group; † *P*<0.05 vs before I and throughout groups.

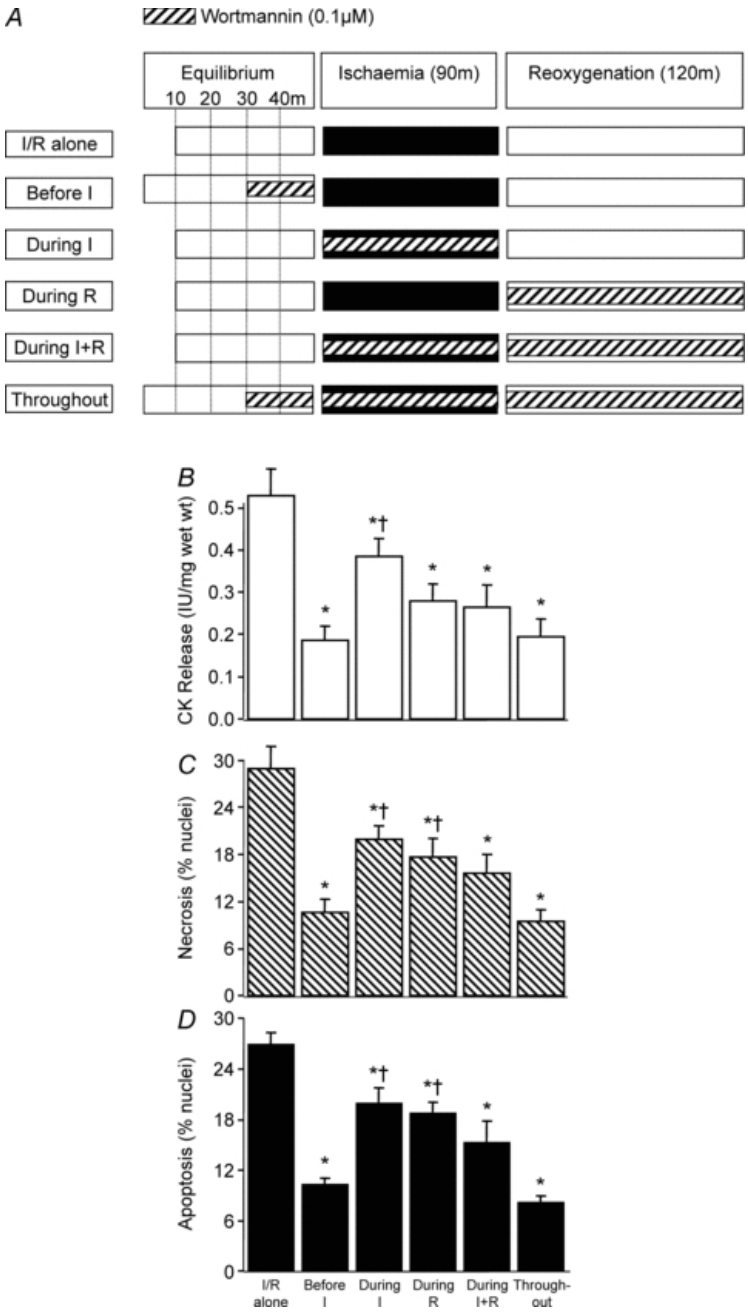

Figure 12. The temporal effects of wortmannin administration during I/R.

As shown in panel a, wortmannin (0.1μM) was administered at 5 different time points during the ischaemia/reoxygenation (I/R) protocol using rat left ventricular myocardial slices: 20 minutes before ischaemia; during ischaemia; during reoxygenation; during ischaemia and reoxygenation; and before ischaemia and throughout ischaemia and reoxygenation. In all cases tissue samples were analysed for CK release during the 120 minutes reoxygenation (panel b), and for necrosis (panel c) and (panel d) apoptosis at the end of 120 minutes reoxygenation. Data represent the mean ± SEM from 6 independent experiments. *P< 0.05 vs I/R alone group; † P<0.05 vs before I and throughout groups.

Study 5. Relationship between PKB inhibition and mitoK$_{ATP}$ channels

To determine the position of PKB in relation to the mitoK$_{ATP}$ channel, we used PKB inhibitor XI in the presence of mitoK$_{ATP}$ channel blocker 5-HD (10 μM). As shown in Figure 13, using inhibitor XI with 5-HD did not surpass the cardioprotective benefits induced by PKB inhibition alone. On the other hand, 5-HD abolished the IP induced cardioprotection. It can be therefore concluded that location of PKB is downstream to that to mitoK$_{ATP}$ channel because PKB inhibition induced cardioprotection is preserved in the present of mitoK$_{ATP}$ blocker 5-HD.

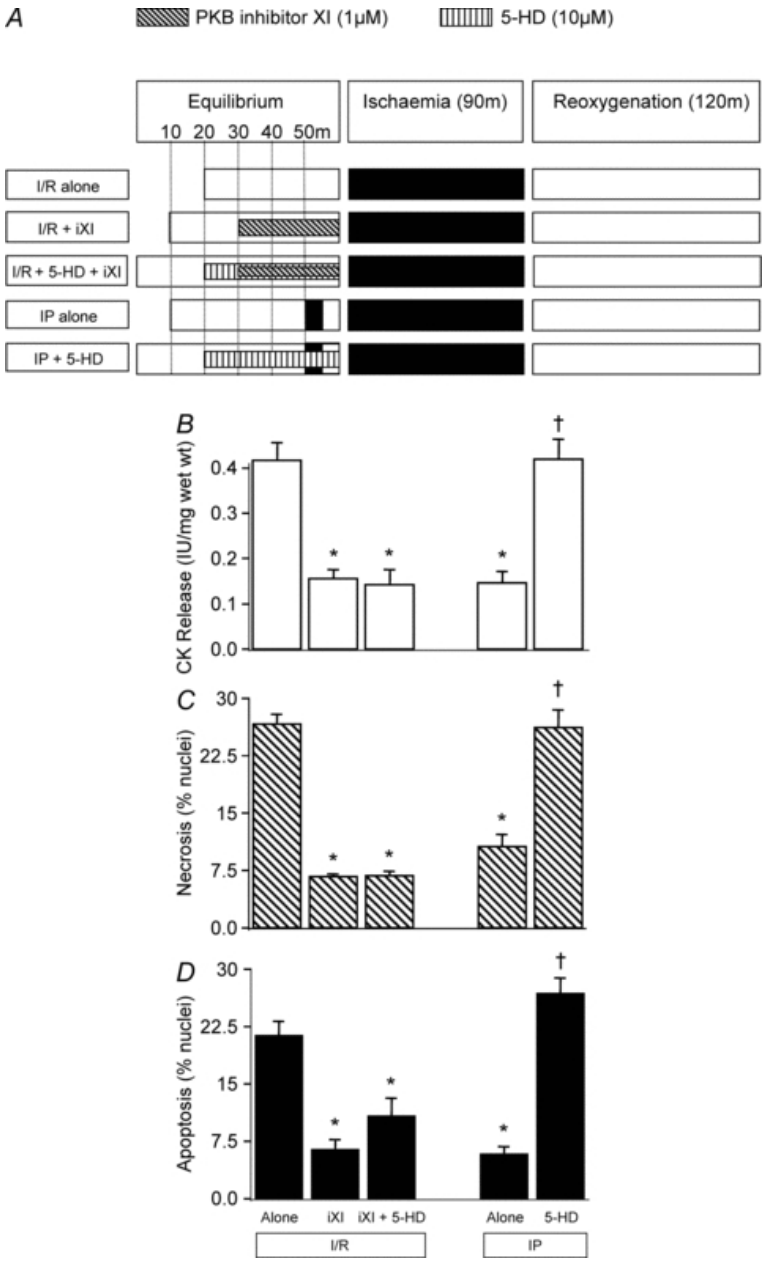

Figure 13. The relationship between PKB inhibition and the mitoK$_{ATP}$ channel.

The mitoK$_{ATP}$ channel inhibitor 5-HD (10μM) was used to determine if the mitoK$_{ATP}$ channel is upstream or downstream of PKB. For this, PKB inhibitor XI was encapsulated during 5-HD pre-treatment prior to ischaemia/reoxygenation (I/R) of rat left ventricular myocardial slices and compared to PKB inhibitor XI alone, as shown in panel a. For comparison, ischaemic preconditioning (IP) was also encapsulated during 5-HD administration prior to I/R. In all cases tissue samples were analysed for CK release during the 120 minutes reoxygenation (panel b), and for necrosis (panel c) and (panel d) apoptosis at the end of 120 minutes reoxygenation. Data represent the mean ± SEM from 6 independent experiments. *P< 0.05 vs I/R alone group; † P<0.05 vs IP group.

Study 6. Relationship between PKB inhibition and p38 MAPK

After determining the relative position of PKB and mitoK_{ATP}, we explored the relation of PKB with p38MAPK. Our laboratory has previously reported that they are downstream of the mitoK_{ATP} channel (72).

We used p38MAPK inhibitor SB203580 (10 μM) in various permutation and combination with PKB inhibitor XI on rat myocardial tissues. The effect of SB203580 on IP was taken as control with reference to previous studies where we have shown that cardioprotection can be reverse by inhibition of p38MAPK(72).

Figure 14 shows that inhibiting PKB along with p38MAPK was not cardioprotective. Also, p38MAPK inhibition alone had no effect on I/R induced myocardial injury but it blocked IP. These results imply that PKB is upstream of p38MAPK because cardioprotection attained by inhibiting PKB is abolished by p38MAPK inhibition.

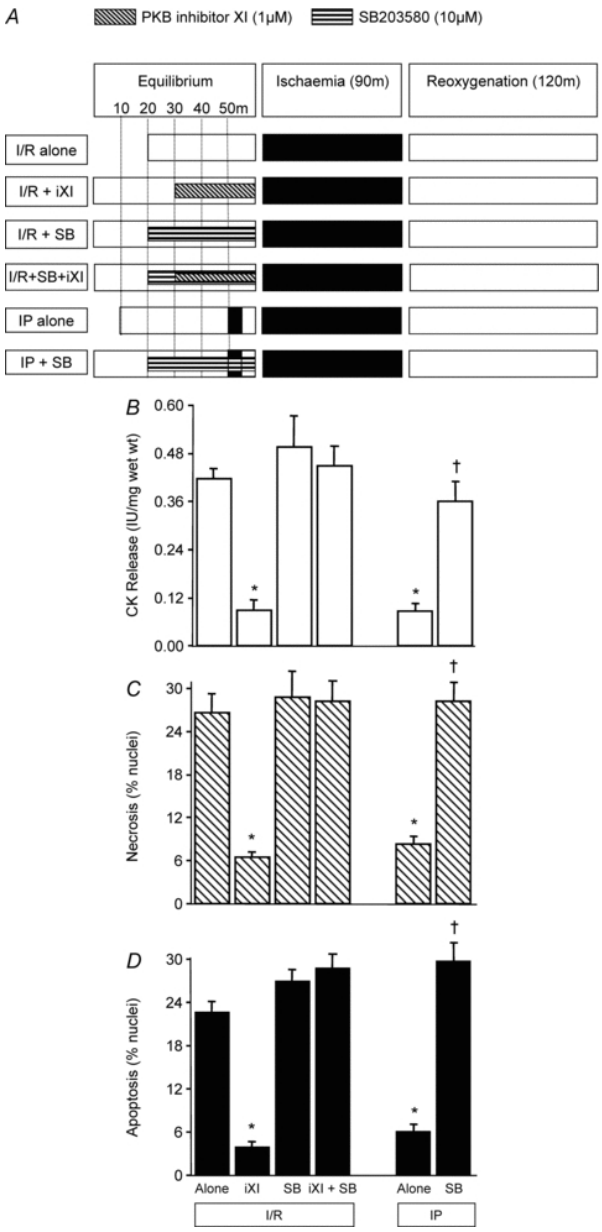

Figure 14. The relationship between PKB inhibition and p38 MAPK.

The p38 MAPK inhibitor SB 203580 (10μM) was used to determine if p38 MAPK is upstream or downstream of PKB. As shown in panel a, rat left ventricular myocardial slices were pre-treated with SB203580 or PKB inhibitor XI alone or encapsulated with SB203580 prior to ischaemia/reoxygenation (I/R). For comparison, ischaemic preconditioning (IP) was also encapsulated by SB203580 prior to I/R. In all cases tissue samples were analysed for CK release during the 120 minutes reoxygenation (panel b), and for necrosis (panel c) and (panel d) apoptosis at the end of 120 minutes reoxygenation. Data represent the mean ± SEM from 6 independent experiments. **P*< 0.05 vs I/R alone group; †*P*<0.05 vs IP group.

Study 7. Effect of PKB inhibition on I/R-induced injury in human myocardium

After demonstrating the cardioprotective effects of PKB inhibition on rat myocardium, we wanted to find out how human myocardium would respond to these inhibitors.

Right atrial muscles were obtained from non-diabetic and diabetic patients undergoing elective cardiac surgery and subjected to the same I/R protocol employed for rat myocardium.

As shown in Figure 15, PKB inhibition by inhibitor XI and wortmannin significantly decreased CK release, necrosis and apoptosis in non-diabetic human myocardium. All the three parameters of CK release, necrosis and apoptosis were decreased. Therefore it is safe to conclude that PKB and PI-3K inhibition offers cardioprotection in rat and human myocardium.

Moving on to next stage, we wanted to find out whether inhibiting PKB can offer cardioprotection in diabetic human myocardium. Our laboratory has demonstrated previously that diabetic human myocardium cannot be preconditioned (ischaemic and pharmacological) with mitoK_{ATP} channel opener diazoxide (150,151).

Figure 15 shows that PKB inhibition by inhibitor XI and wortmannin was able to offer cardioprotection in diabetic human myocardium. As expected, IP and diazoxide were not able to achieve it.

It shows that PKB inhibition cardioprotection is independent of functional mitoKATP channels

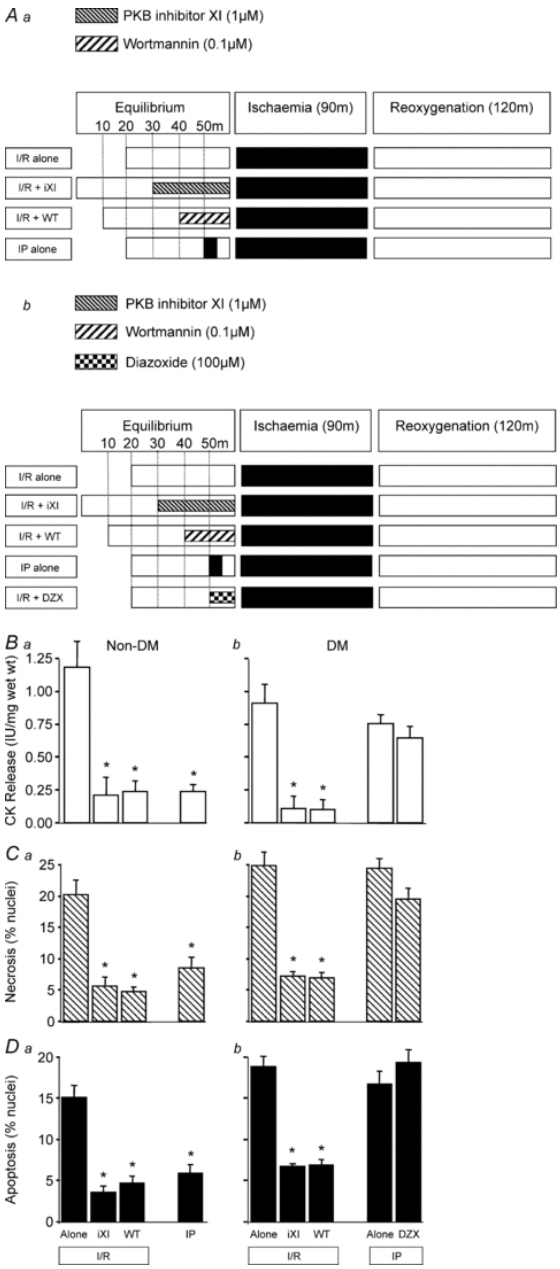

Figure 15. The effect of PKB and PI-3K inhibition on I/R-induced injury in non-diabetic and diabetic human myocardium.

Non-diabetic tissue (panel a1) was pre-treated with PKB inhibitor XI (1μM) or wortmannin (0.1μM) prior to ischaemia-re-oxygenation (I/R) and compared to ischaemic preconditioning (IP). A similar protocol (panel a2) was employed using diabetic human myocardium except with the inclusion of diazoxide (100μM; mitoK_{ATP} channel opener) to induce pharmacological preconditioning. In all cases tissue samples were analysed for CK release during the 120 minutes reoxygenation (panels b1 and b2), and for necrosis (panels c1 and c2) and (panels d1 and d2) apoptosis at the end of 120 minutes reoxygenation. Data represent the mean ± SEM from 6 independent experiments. *P< 0.05 vs I/R alone group.

Study 8. Effect of PKB inhibitor XI on PKB Ser^{473} phosphorylation

Western blot analysis of PKB Ser^{473} phosphorylation was performed using rat ventricular muscle in order to confirm PKB inhibition by PKB inhibitor XI. As shown in Fig. 16, PKB Ser^{473} phosphorylation was detectable in aerobic and ischaemic tissue. As expected, both PKB inhibitor XI and wortmannin reduced levels of PKB Ser^{473} phosphorylation in aerobic tissue following 20 min pre-treatment with the inhibitors. IP also induced a significant reduction in Ser^{473} phosphorylation. The reduction in PKB Ser^{473} phosphorylation following IP is in contrast to previous studies that have reported IP-induced increases in Ser^{473} phosphorylation (113,152). Treatment with inhibitor XI during the equilibrium period prior to IP (with inhibitor present) also reduced levels of PKB Ser^{473} phosphorylation. Overall these observations suggest that IP induces a marked reduction in Ser^{473} PKB phosphorylation, which is in line with the protection observed using the PKB inhibitors.

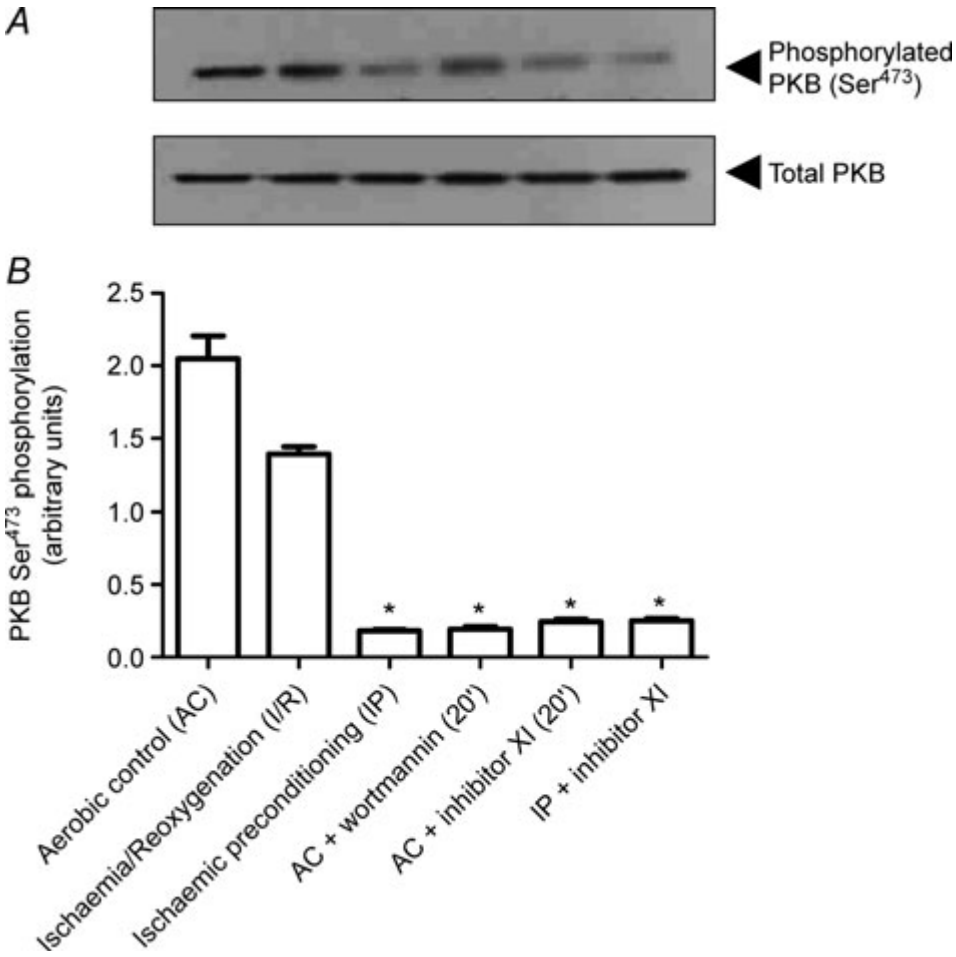

Figure 16. Western blot analysis of PKB Ser^{473} phosphorylation in rat ventricular myocardium.

Representative immunoblot (panel a) and quantitative analysis (panel b) of PKB Ser^{473} phosphorylation in samples obtained from the following protocols: aerobic control (AC); ischaemia/reoxygenation (I/R); ischaemic preconditioning (IP); 20 minutes pre-treatment with wortmannin (0.1μM) or PKB inhibitor XI (1μM) prior to AC and inhibitor XI treatment during the equilibration and ischaemic preconditioning (IP + inhibitor XI). Insulin-induced PKB Ser^{473} phosphorylation from DDT$_1$MF-2 cells was used as a positive control. Data represent the mean ± SEM from 4 independent experiments and are expressed as a percentage of the aerobic control values. *P< 0.05 vs aerobic control.

Discussion

Through these experiments we have shown the following:

1) PKB inhibition with specific inhibitors is a potent cardioprotector of rat and human myocardium
2) This cardioprotection can be duplicated by blocking PI-3K
3) PI-3K and PKB inhibition before ischaemia and during ischaemia & reoxygenation can result in maximal cardioprotection
4) The location of PKB is downstream of the mitoK_{ATP} channel and upstream to that of p38MAPK
5) PKB and PI3-K inhibition can still be cardioprotective in diabetic myocardium

These results are important in our understanding of cell signalling pathways of cardioprotection and therefore require detailed discussion.

Selectivity and action of PKB inhibitors

Recent bio molecular advances have made it possible to design specific PKB inhibitors (114-116) and have been experimented with in the field of oncology(149). Before these advancements, PI3-K inhibition through wortmannin and LY294002 were the principle compounds used for studying ischemic/reperfusion injuries in myocardium (152).

Our laboratory is one of first groups to use these novel inhibitors in studies pertaining to ischaemic injuries in myocardium. We selected PKB inhibitors VIII and XI for our experiments, which are cell permeable.

Inhibitor VIII is a selective inhibitor of PKB1/PKB2 activity and belongs to quinoxaline family of compounds. The IC_{50} for PKB1, PKB2 and PKB3 are 58nM, 210nM and 2.12 μM, respectively. The selectivity of this compound is due to its ability to interact with the PH domain of PKB (114). It does not interact, even at a higher concentration of 50 μM, with other non PKB PH domain such as PKA, PKC and GSK (114).

On the other hand, inhibitor XI interacts with the PH and kinase domains of PKB (IC_{50}=100nM) and is a copper based complex (115). It also reduces the PKB Ser473 phosphorylation in rat ventricular myocardium. On the basis of IC_{50}, we performed the dose response curves for each inhibitor. It was evident that PKB inhibition by inhibitors VIII and XI is cardioprotective in all the three measurable parameters of CK release, cell necrosis and apoptosis. This protection was comparable to that of IP. Furthermore, these inhibitors when used in addition to IP did not bring any added advantage. Therefore it may be deduce that these two mechanism i.e. PKB inhibition cardioprotection and IP use the same signalling pathway. Similar cardioprotection was obtained by blocking PI3-K by wortmannin and LY294002 in rat and human myocardium.

This is in apparent contradiction to the generally believed theory that PKB activation is cardioprotective (85,144,153). Tong et al. (152) reported that inhibition of PI3-K abolishes the protective effect of preconditioning whereas Ban et al. (154) demonstrated that genetic suppression of PI-3Kα isoform activity offers resistance against prolonged ischaemia. In recent studies, it has been shown that 3'-phosphoinositide-dependent kinase-1 (PDK1) knock out mice are prone to hypoxic injuries and die of heart failure at early stage of adult life

(155). Budas et al. have shown that IP induced cardioprotection is abolished by reduced expression of PDK1 in hypomorphic mutant mice (156).

Uchiyama et al. used PI-3K inhibitor LY 204002 at the concentration of 50 μM to demonstrate that PKB inhibition abolishes the cardioprotective effects of pharmacological preconditioning in rat myocytes. (148).

There are contradictory results in relation to mTOR also. mTOR is phosphorylated by PKB on Ser^{473}. Gross et al. reported that blocking mTOR with rapamycin when given before cancels the opioid mediated cardioprotection(157). Hausenloy et al. used rapamycin at the start of reperfusion to demonstrate similar results (158). On the contrary, Khan et al. have reported that rapamycin at the concentrations between 25-100nM using Langendorff model is cardioprotective in mice(159).

There is a wide variation in the material and methods used by various investigators to quantify myocardial injuries. It ranges from examining recovery of the left ventricular function (152-154) to measuring reduction in infarction size in isolated *in vitro* perfused hearts (156,158,159) to *in vivo* animals (157).

Mora et al. used intracellular Ca^{+2} overload and hyper contracture to benchmark myocardial injuries (155), whereas Uchiyama et al. used cell viability, lactate dehydrogenase and apoptosis as parameters to define myocardial injury (148). Our studies used CK release, apoptosis and necrosis in cardiac muscles to evaluate myocardial injury and protection. Therefore, contradictions in results by various investigators can be due to the variations in the experimental models and injury parameters.

It is important to emphasise that the novel inhibitors were not available previously to study the specific roles of PKB and therefore it can be deduced that PKB isoforms are major players in cell signalling cascade. This is an area of critical importance for therapeutic purposes and, certainly, a full elucidation would require further investigations.

'Cross talk' amongst various cell-signalling pathways has been postulated for sometime now. There is evidence that PKB phosphorylation inhibits Raf-1 leading to decrease in ERK1/2 signalling (102). In our studies, we have seen that blocking PKB and PI-3K results in cardioprotection. Applying the 'cross talk' theory in this instance could explain that inhibition of PKB leads to activation of ERK1/2 signalling cascade resulting in cardioprotection. PI-3K inhibitor LY294002 in isolate rat hearts enhances ERK1/2 activation but it did not enhance cardioprotection (158).

We also showed that blocking p38MAPK by SB203580 abolished the cardioprotection offered by PKB inhibition through inhibitor XI. Phosphorylation of PKB is known to activate ASK1, MLK3 and SEK1/MKKK4 that are involved in upstream activation of p38MAP/JNK (160). Thus, inhibiting PKB could also inhibit p38MAPK/JNK. Even then it does not explain our result of abolishing cardioprotection by blocking p38MAPK.

Clearly further detailed studies are required to explore the kinetics of PKB, ERK1/2, p38 MAPK and JNK activation during our experimental regime in the absence and presence of PKB inhibitors VIII and XI. The effect of PKB on GSK-3β activity, another important kinase in the signal transduction pathway of cardioprotection, is also unclear and needs to be elucidated, particularly in view of the reported conflicting results on the role of GSK-3β (161-164).

Importance of the time of administration

We are the first to demonstrate the importance of timing of PKB inhibition and its acute effect on myocardial protection. Maximal cardioprotection was obtained when inhibitor VIII inhibited PKB and XI before ischaemia or during entire period of ischaemia and reoxygenation. Also by blocking PI-3K by using wortmannin and LY294002 during these time points, the results were similar to that with PKB inhibition. This indicates that PKB status during ischaemia and reoxygenation plays a pivotal role in ascertaining the degree of protection to myocardial tissue against ischaemia.

Zhu et al. have shown that ischaemic post-conditioning involves activation of PKB (165). It further strengthens the argument that PKB activity is critical before, during and after ischaemia. Because of these controversies, it can be postulated that perhaps activation and inhibition of PKB can be either deteriorating or protective to myocardium depending upon the timing of such event.

Location of PKB in the cell signalling cascade for cardioprotection

Opening of mitoK_{ATP} channels enhances formation of ROS, which in turn activates kinases and eventually leads in preconditioning (166). This specific step has shown to be mediated by the inner mitochondrial membrane connexion-43(167).

Although not end effectors themselves, nevertheless they form an integral part of the cell-signalling network. There have been questions about the possible exploitation of mitoK_{ATP}

channels, using 5-HD as inhibitor and diazoxide as opener, as a selective tool to study signalling cascade in preconditioning of myocardium (168). There are conflicting reports on the position of mitoK$_{ATP}$ channels in relation to PKB. Garlid et al. proposed that PKB is located upstream of the mitoK$_{ATP}$ channel (169). However, through our experiments using mitoK$_{ATP}$ blocker 5-HD we have shown that the location of PKB is downstream to that of mitK$_{ATP}$ channels because 5-HD could not abolish the cardioprotection offered by inhibiting PKB by inhibitor XI. Other laboratories have shown that PKB phosphorylation can be induced by mitoK$_{ATP}$ channel openers BMS-191095 and diazoxide(170,171). Therefore, it can be inferred that the location of PKB is downstream of mitoK$_{ATP}$ channel. It is worth noting that the effect of diazoxide on PKB-phosphorylation reported by (170) was not blocked by 5-HD.

Our results also show that blocking p38MAPK by SB203580 can totally abolish the cardioprotection offered by PKB inhibition by inhibitor XI. This suggests that PKB is upstream to that of p38MAPK. We have earlier reported that p38MAPK is downstream of PKC and mitoKATP channels using human myocardial tissue in similar experimental techniques (72).

Also, there are contradictory reports on the role of p38MAPK in IP. It has been suggested that cardioprotection can be achieved by either activating or inhibiting p38MAPK(172,173).It has been shown that p38MAPKα isoform promotes cell death whereas cellular hypertrophy is mediated by the p38MAPKβ isoform (174). Shulz et al. have demonstrated that ischaemia results in increased activity of both α and β isoforms of MAPK but the activity level of β isoform returns towards baseline after prolonged ischaemia in non-preconditioned heart. In preconditioned heart it remains elevated (175).

To understand these discrepancies of p38MAPK physiology and its role in cardioprotection, it is important to identify that experimental protocols vary between laboratories. Also, *in vivo* and *in vitro* model observations are different. There are differences in the timing of administration of specific inhibitors/activators and of course their concentrations play a major role in pharmacological actions. Last but not least, 'cross-talk' amongst various cell signalling pathways takes place.

There are gaps in our understanding of the interactions amongst PKB, mitoK_{ATP} channels and p38MAPK. As discussed previously, reactive oxygen species production is stimulated through activation of mitoK_{ATP} (166). This in turn results in activation of PKCε (169). There is little evidence about the regulatory role of mitoK_{ATP} channels for PKB although there are reports that diazoxide activates PKB. However, it has been demonstrated that multiple mediators, depending on the stimuli, can interact with PKB (176).

The role of PKB is vital in cardioprotection and variation in experimental models and species can account for its differential effect caused(177). It is essential to carry out more research in the interactions between PKB, mitoK_{ATP} and p38MAPK to fill in the gaps of cell signalling mechanisms.

PKB inhibition overcomes the deficit of diabetes for cardioprotection

Previously we have demonstrated that mitochondrial dysfunction is the cause of the unresponsiveness of the diabetic myocardium to protection by IP (150)) and the present finding that PKB blockade overcomes this deficit confirms the location of PKB being

downstream of the mitoK_{ATP} channels. Similar results were obtained in our laboratory when activators of PKC and p38 MAPK were used (72), further supporting the location of these kinases beyond the mitochondria.

These results are important and can be exploited clinically. Pharmacological manipulation of protein kianses can decrease myocardial injuries in diabetic patients having ischaemic heart disease.

Study limitations

These experiments were based on *in vitro* model and its results therefore must be carefully interpreted for clinical conditions. Biological systems behave in a relatively complex manner as compared to in vitro studies where factors can be controlled tightly. The contradiction in the literature over the kinases can also be attributed to the inadvertent phosphorylation of survival kinases in the *in vitro* preparations (178). Also, alternative cell signalling pathways cannot be completely overruled inspite of using novel selective inhibitors of PKB.

To overcome these issues, a robust and multifaceted approach involving pharmacological and transgenic technology should be adopted.

Chapter 4

Role of MPTP in Cardioprotection

INTRODUCTION

In ischaemic reperfusion injury of myocardium, mitochondrion is a key player in apoptosis and necrosis. Mitochondria form a central platform around which the cardioprotective signalling pathways of ischaemic and pharmacologic preconditioning meet resulting in cell protection and survival.

The complex series of cytosolic signalling is initiated through G-protein coupled receptors (GPCR) or $Na^{+}K^{+}$ATPase that brings about cardioprotection. Garlid et al. demonstrated the transformation of signalling enzyme into signallosomes and their ultimate migration to the inner membrane of mitochondria. This event results in opening of mitochondrial ATP-sensitive K^{+}channel (mitoK_{ATP}) that produces ROS. ROS, in turn activates the mitochondrial protein kinase Cε (PKCε). This activation of PKCε is a direct inhibitor of mitochondrial permeability transition pore (MPTP) that decreases cell death. (169).

Mitochondrial Ca^{2+} overload and ROS formation are postulated mechanisms through which the mitoK_{ATP} are thought to be involved in cell survival (136,137).

Crompton et al. , Howarth and Hunter et al. (118,119) described the existence of a non-specific channel with a molecular cut off 1.5 kDa which was termed as mitochondrial permeability transition pore (MPTP). Amongst the major activators of MPTP are oxidative stress, phosphates and adenine nucleotide depletion, which are normally encountered during the ischemic/reperfusion phase of injury (120-122).

A lot of emphasis has been given on mitoK_{ATP} channel as a possible modulator of MPTP(179). MPTP has been shown to be sensitive to oxidative stress and mitochondrial Ca2+ levels (180). The opening of MPTP causes mitochondrial swelling and rupture releasing cytochrome *c* (181). Also, opening of MPTP causes uncoupling of oxidative phosphorylation resulting in ATP depletion and necrosis.

Crompton and Costi proposed the involvement of MPTP in Ca^{2+}overload and subsequent injury of myocardium (182). Furthermore, it was shown that MPTP inhibition during reperfusion could be converging point for cardioprotection through ischaemic and pharmacologic preconditioning and post conditioning(183-186). Subsequent pharmacological interventions targeted this event by inhibiting MPTP opening to for achieving cardioprotection(121). It was shown by Shanmuganatham et al. that suppression of MPTP opening at reperfusion stage is cardioprotective in human myocardium(187). Other laboratories showed that it is the transient opening of the MPTP that results in

cardioprotection(188,189). These data on the MPTP status indicate that the timing is crucial for the cell survival during ischemic/reperfusion injury.

The molecular structure of MPTP has been now thoroughly explored by various laboratories to understand the cell-signalling basis of cardioprotection. Earlier theories postulated voltage-dependent anion channel (VDAC) being an integral component of MPTP. Studies by Baines *et* al. demonstrated that mitochondrial phosphate and cyclophillin-D are the key players in the pore-forming unit of MPTP. Adenine nucleotide translocase (ANT) acts as a regulatory protein for this step(190).

Sanglifehrin A (SfA), cyclosporine A (CsA), N-methyl-4-isoleucine-CsA (NIM811) and D-3MeAla-4EtVal-CsA (Debio-25) were identified as direct inhibitors of MPTP (124-128). Furthermore, decreasing pH and decreasing oxidative stress were recognised as indirect inhibitors of MPTP.

Atractyloside has been used as pharmacological opener of MPTP to study the effects on myocardium. Interestingly, it also acts through ANT pathway (191).

Following were the objective of this study:

1. To investigate the effect of MPTP inhibition before ischaemia.
2. To investigate whether status of MPTP (opening and closing) during ischaemia and reoxygenation results in myocardial injury.
3. To investigate the effect of MPTP opening on myocardium.

Materials and Methods

Study Animals

This has been described in Chapter 2.

Experimental Preparation

This has been described in Chapter 2.

Solutions and Chemicals

This has been described in Chapter 2.

Assessment of Tissue Injury

This has been described in Chapter 2.

Assessment of Cell Death

This has been described in Chapter 2.

Assessment of Caspase 3 Activity

This has been described in Chapter 2.

Study Protocols

Myocardial slices (n = 6/group, unless otherwise indicated) were randomly allocated to different groups so that the tissues from each animal were not utilized more than once for the same group. Samples subjected to time-matched aerobic conditions only were used in each experiment and the rest of the myocardial muscles were subjected to 90 min ischemia/120 min reoxygenation.

The following studies were sequentially carried out:

(1) ***Study 1:*** To investigate the effect of MPTP inhibition before ischemia, rat ventricular muscles were exposed to CsA for 20 min prior to I/R and compared with IP (see Fig. 17A for protocol).

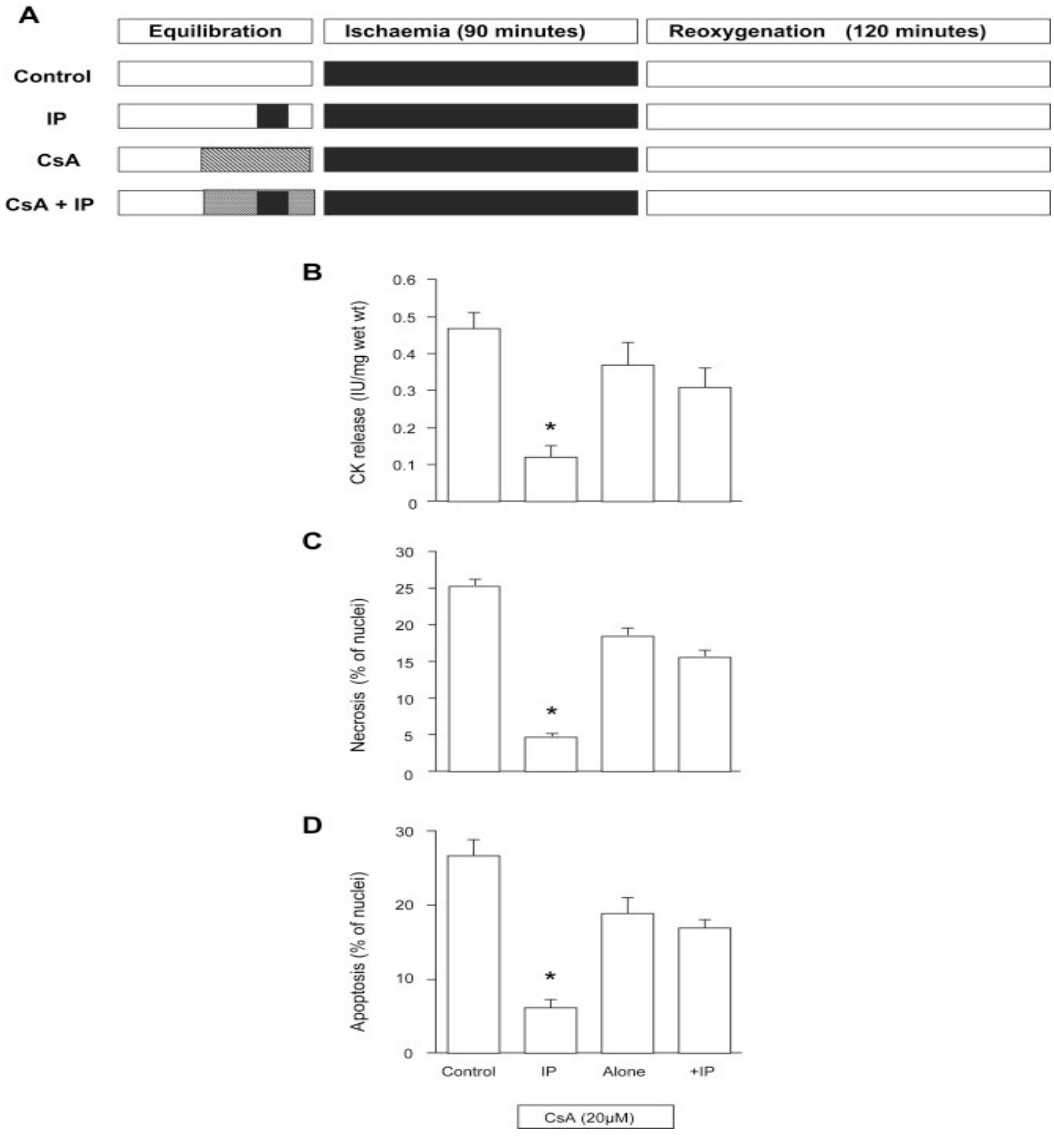

Figure 17. Protocol (A) and results on CK release (B), cell necrosis (C), and apoptosis (D) of rat left ventricular myocardium (n = 6/group) subjected to 90 min of ischemia followed by 120 min of reoxygenation and receiving several treatments before the induction of ischemia: ischemic preconditioning (IP), the MPTP inhibitor cyclosporine A (CsA; 20 μM), and IP + CsA (see text for details). *$P < 0.05$ *versus* control.

(2) ***Study 2:*** Having demonstrated that inhibition of MPTP by CsA prior to ischemia does not result in cardioprotection, this study was performed to elucidate whether the state of MPTP (opened/closed) during ischemia and the reoxygenation period results in myocardial injury or cardioprotection. For this, CsA was co-incubated with rat ventricular muscles for the entire ischemic period or for different time points during the reoxygenation period as shown in Figure 18. For comparison, some muscles were subjected to IP.

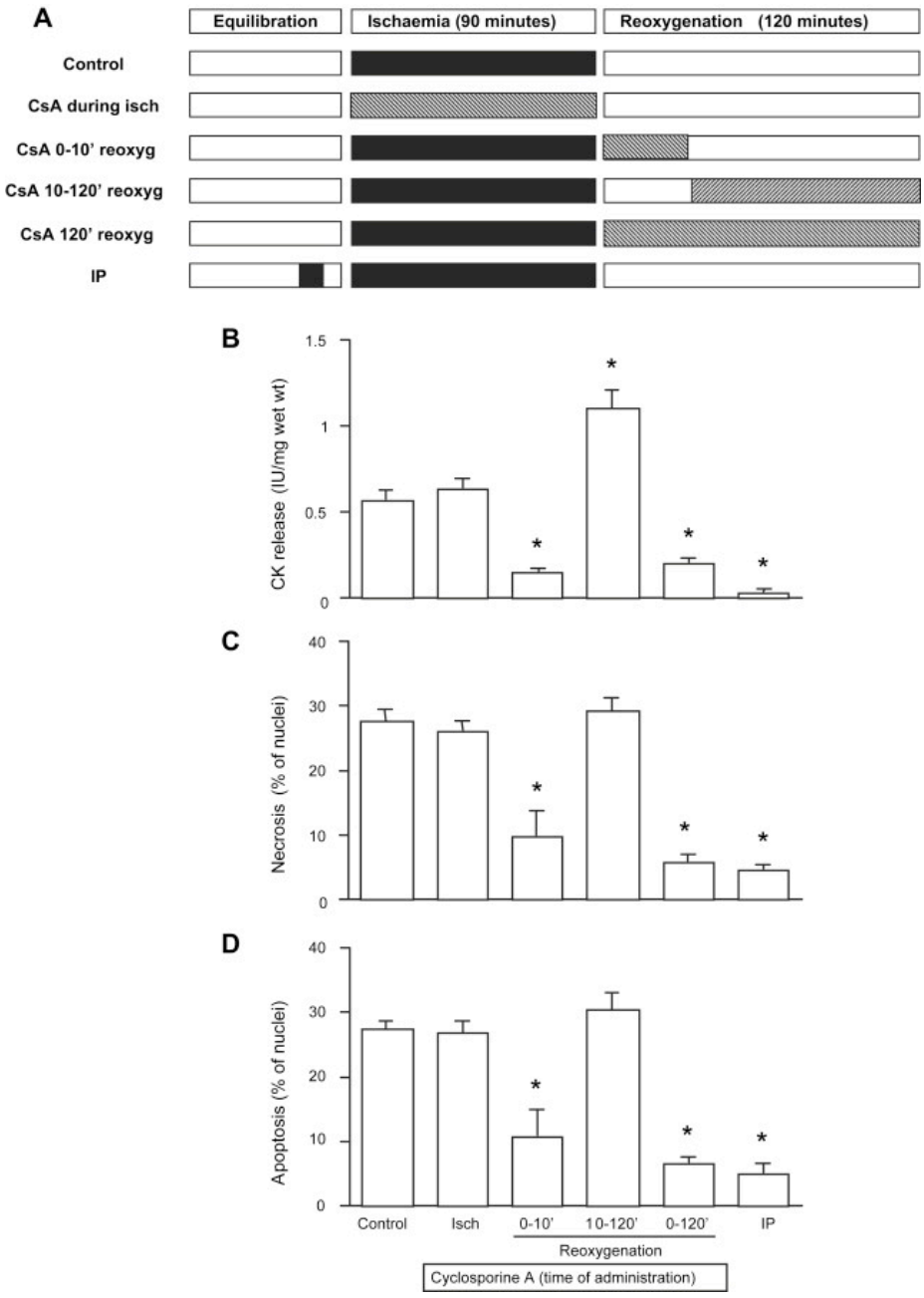

Figure 18. Protocol (A), and results on CK release (B), cell necrosis (C), and apoptosis (D) of rat left ventricular myocardium (n = 6/group) subjected to 90 min of ischemia followed by 120 min of reoxygenation and treated with the MPTP inhibitor cyclosporine A (CsA; 20 μM) at different time points during ischemia and reoxygenation (see text for details). Ischemic preconditioning (IP) was used for comparison. $*P < 0.05$ *versus* control.

(3) Study 3: The demonstration that blocking the MPTP with CsA during the early or the entire reperfusion period but not prior to or during ischemia was tested again in this study using an alternative MPTP inhibitor. For this we used BKA, which has a different mechanism of inhibition to that of CsA. In this study, BKA was co-incubated with the ventricular muscles prior to and during ischemia and also at various times during reoxygenation as described in Figure 19. Again, for comparison, some muscles were subjected to IP.

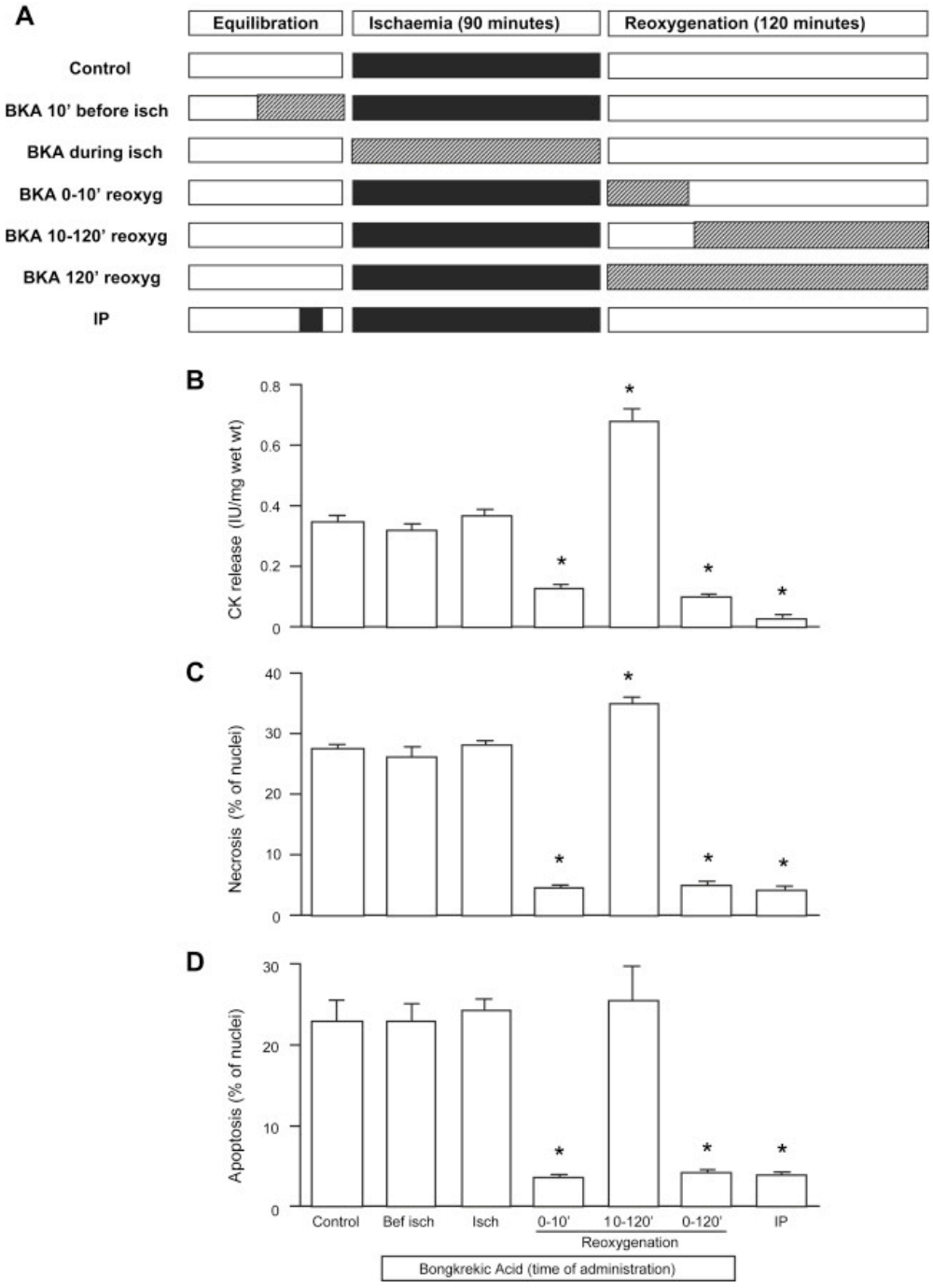

Fig. 19. Protocol (A), and results on CK release (B), cell necrosis (C), and apoptosis (D) of rat left ventricular myocardium (n = 6/group) subjected to 90 min of ischemia followed by 120 min of reoxygenation and treated with the MPTP inhibitor bongkrekic acid (BKA; 100 μM) at different time points before and during ischemia/and also during reoxygenation (see text for details). Ischemic preconditioning (IP) was used for comparison. $*P < 0.05$ *versus* control.

(4) Study 4: To investigate whether the effects of the opening MPTP are a mirror image of MPTP inhibition and whether they are determined by the time of administration (prior to or during ischemia, or during reoxygenation) the MPTP opener ATR was used, as described in Figure 20. As before, for comparison, some muscles were subjected to IP.

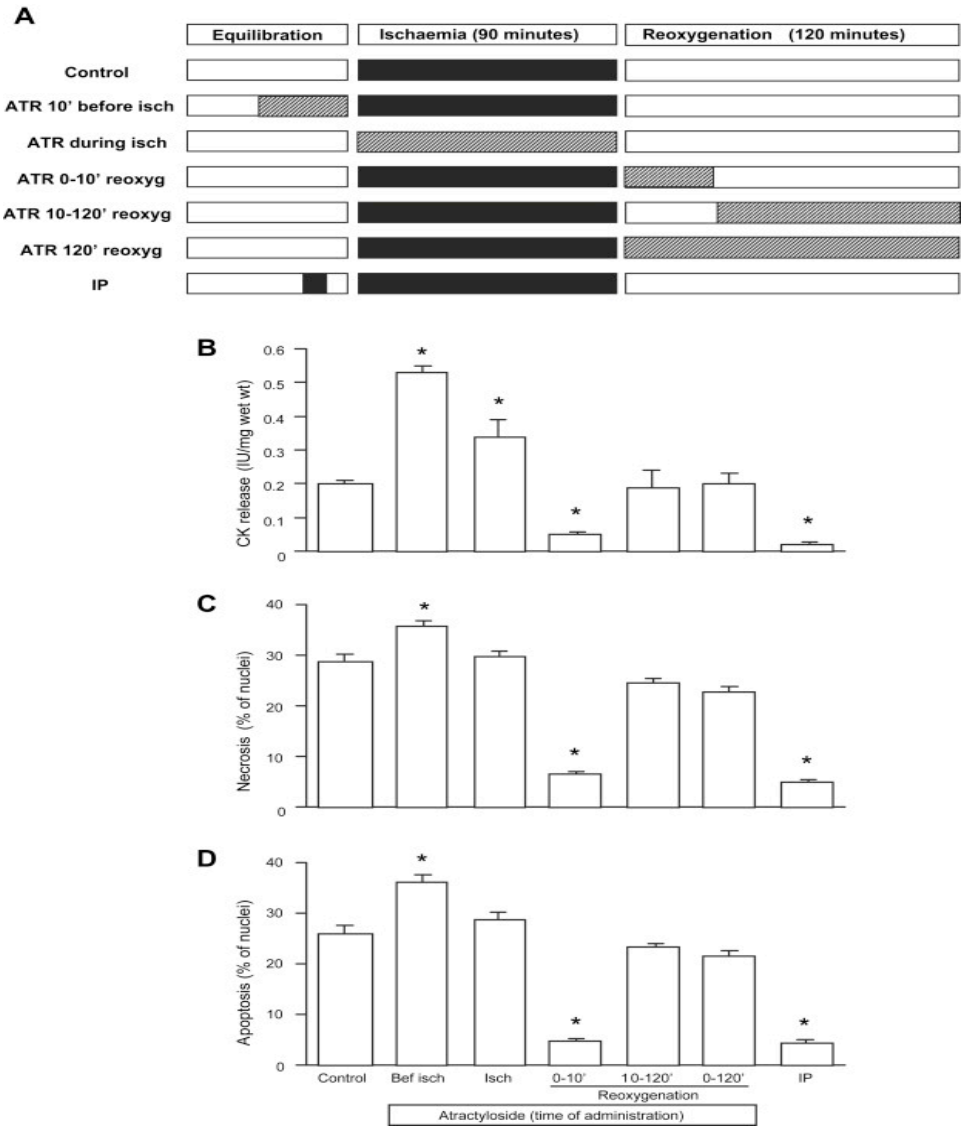

Fig. 20. Protocol (A), and results on CK release (B), cell necrosis (C), and apoptosis (D) of rat left ventricular myocardium (n = 6/group) subjected to 90 min of ischemia followed by 120 min of reoxygenation and treated with the MPTP opener atractyloside (ATR; 50 μM) at different time points before and during ischemia and also during reoxygenation (see text for details). Ischemic preconditioning (IP) was used for comparison. $*P < 0.05$ *versus* control.

(5) Study 5: Since inhibition and opening of the MPTP results in distinctive effects on myocardial ischemic injury and cardioprotection, the role of caspase 3 activity, a potential indicator of apoptosis was investigated. Rat ventricular muscles subjected to 90 min of ischemia followed by 120 min of reoxygenation were co-incubated for the first 10 min of reoxygenation with CsA or ATR. At the end of the 120 min reoxygenation period muscles were frozen and stored at −80°C until analysis. Muscles subjected to IP were also taken at the end of the preconditioning protocol.

Statistical Analysis

Data are expressed as mean ± SEM. Each reported value was obtained after subtracting the value from the corresponding time-matched aerobic sample. One-way ANOVA followed by Bonferroni's correction was used to compare the significance between groups. All the analyses were performed using the SPSS program (SPSS Inc., Chicago, IL) and differences were considered to be statistically significant at $P < 0.05$.

Results

Inhibition of MPTP with CsA Before Ischemia (Study 1)

Figure 17 B-D shows ischaemia/reperfusion (control) results in CK release, necrosis and apoptosis. Ischaemic preconditioning significantly offers protection in all three parameters of reperfusion injury. Interestingly, the inhibition of MPTP with CsA during equilibrium period (before ischaemia) did not alter the CK release, apoptosis and necrosis. CsA abolished the cardioprotection offered by IP alone. It shows that MPTP inhibition before ischaemia does not result in cardioprotection. Also, inhibiting MPTP before ischaemia abolishes the benefit of IP.

Inhibition of MPTP with CsA During Ischemia and During Reoxygenation (Study 2)

After demonstrating that MPTP inhibition does not result in cardioprotection before ischaemia, we wanted to explore the role of MPTP during ischemia and at various reoxygenation time points.

CsA when given during ischaemia did produce any notable effect (Figure 18B-D). Interestingly, when CsA was given during first 10 minutes of reoxygenation, it resulted in significant decrease in CK release and in cell necrosis and apoptosis. These results are comparable to when CsA was given for the entire 120 minutes of reoxygenation. There was significant increase in the CK release and in necrosis and apoptosis when CsA was

introduced after the first 10 minutes of reoxygenation. These result shows that the timing of MPTP inhibition plays critical role and that I/R injury is enhanced with MPTP inhibition. When given during ischaemia, CsA does not enhance the I/R injury. In summary, first 10 minutes of reoxygenation is vital in achieving cardioprotection and also blocks IP.

Inhibition of MPTP with Bongkrekic Acid (Study 3)

After using CsA to demonstrate the importance of first 10 minutes of reoxygenation, we wanted to find out whether these results could be duplicated by using another inhibitor of MPTP, Bongrekic acid (BKA). It binds to the m-conformation of ANT and inhibits MPTP. A dose response study was performed using 1,10 and 100 μM. 100 μM was shown to be optimum concentration for cardioprotection.

Figure 19 B-D shows that BKA was able to offer maximum cardioprotection when used during the first 10 minutes of reoxygenation. Furthermore, when given after 10 minutes of reoxygenation, it caused a significant rise in the CK release, necrosis and apoptosis. Like CsA, it was efficiently offering protection when given for the entire 120 minutes of reoxygenation and did not result in any effect when given during ischaemia.

Hence, BKA reproduced the results of CsA in similar experimental situations.

Opening of MPTP with Atractyloside (Study 4)

Having concluded that BKA and CsA are similar in their action, we wanted to explore the effect of MPTP opening at various time points of ischaemia and reoxygenation. Atractyloside

(ATR), a known MPTP opener, at the concentration of 50 μM was used in this experiment. This concentration has been shown to be effective in other studies (192).

ATR, when given before ischaemia, resulted in marked myocardial damage reflected through CK release, necrosis and apoptosis. When added during ischaemia, ATR remarkably increases CK release but does not affect necrosis and apoptosis of myocardial cells.

Strikingly, when ATR is given during first 10 minutes of reoxygenation, it offers cardioprotection similar to MPTP inhibition with CsA and BKA (Figure 20 B-D). Unlike MPTP inhibition, this cardioprotection is abolished when ATR is given throughout the reoxygenation period.

Also, it does not confer any protection when given after first 10 minutes of reoxygenation.

Effect of MPTP status on Caspase 3 Activity During the first 10 min of Reoxygenation (Study 5)

As shown in Figure 21, there is significant lack of activation of caspase-3 activity when MPTP is inhibited during the first 10 minutes of reoxygenation. Similar decrease in caspase-3 activity is shown in IP. The caspase-3 activity has been compared with the ischemia/reoxygenation control group. In contrast, when MPTP opener ATR is administered during the first 10 minutes, it causes a significant increase in caspase-3 activity.

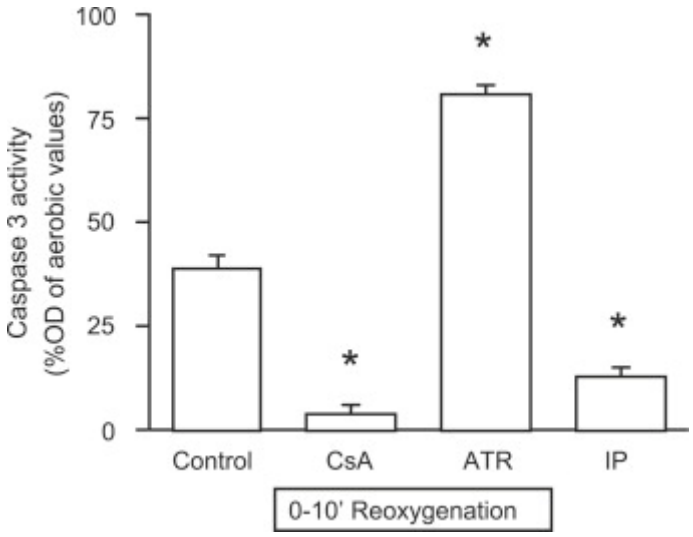

Figure: 21. Caspase 3 activity in rat left ventricular myocardium at the end of 120 min of reoxygenation after being subjected to 90 min of ischemia and treated with the MPTP inhibitor cyclosporine A (CsA; 20 μM) or the MPTP opener atractyloside (ATR; 50 μM) during the first 10 min of reoxygenation. Ischemic preconditioning (IP) was used for comparison. Data are expressed as percentage increase in optical density (OD) compared with aerobic controls. $*P < 0.05$ *versus* control.

Discussion

This study has explored the status of MPTP and its effect on ischaemia/reoxgenation induced injury in rat myocardium. Through our experiments, it is shown that both opening and closure of MPTP during first 10 minutes of reoxgenation can result in cardioprotection that is comparable to that of ischaemic preconditioning. These results are vital to understand the cellular mechanism of ischaemic/reoxygenation injury and cardioprotection. They open a wide arena of further explorations and can be manipulated to obtain clinical benefits.

MPTP During Reoxygenation

It is imperative to understand the molecular configuration of MPTP, which is permeable to molecules up to 1.5 kDa in size. When this pore opens, it results in swelling of the mitochondrial matrix and rupturing the outer membrane. This rupture causes release of cytochrome *c* turning on the apoptotic cascade of events (193-195). Therefore, opening of MPTP at the time of reoxygenation is an important factor in cellular apoptosis.

Similarly, if the mitochondrial membrane potential is disrupted through uncoupling of mitochondrial oxidative phosphorylation, necrosis sets in(192).

MPTP inhibition has been shown to be cardioprotective in various studies(121,184-186). Our laboratory is the first to report that both transient inhibition and opening during the early phase of reoxygenation are cardioprotective. This protection offered is comparable to that of

IP and has been measured on the measurable parameters of CK release, necrosis and apoptosis. To understand this contradiction, it can be imagined that there are two events taking place simultaneously. When the pore is inhibited, it stops the destruction of mitochondrial membrane and subsequent injurious cell signalling pathways. On the same note, downstream cardioprotective signals can be elicited by transient opening of the MPTP. Zorov et al. have demonstrated that transient opening of MPTP enhances ROS production and its release (196). This may result in the activation of parallel streams of signalling pathways such as PKB and ERK1/2(188).

Our results suggest that although MPTP inhibition and opening (by CsA and ATR respectively) during early reoxygenation results in cardioprotection, they do it through independent pathways and mechanism. The assessment of necrosis and apoptotic cell death was done through PI and TUNEL staining respectively.

Indeed, the finding that atractyloside administered during the first 10 min of reoxygenation promotes caspase-3 activation is in stark contrast to its effects on CK release and levels of necrotic and apoptotic cell death.

The cytochemical basis of TUNEL assay exploits DNA fragmentation of the cell and therefore shows positive staining for apoptosis (197). There is a considerable uptake of TUNEL assay by necrotic cells due to DNA laddering seen in these cells. Therefore to validate results, it is vital to use alternate methods of apoptotic detection such as caspase-3 activation.

Further simplifying, it is possible that MPTP opening through ATR can simultaneously decrease necrosis (measured by CK release and PI and TUNNEL assay) and increase apoptosis (measured by caspase-3 activity). Lamkanfi et al. suggested non-apoptotic roles of caspase activation (198) and it is widely accepted that caspases participate in cell proliferation, differentiation, migration and immune functions (199). Therefore, ATR activation of caspase-3 may not be linked to apoptotic cell death at all and instead it may be because of other described functions of it.

We showed that the MPTP inhibition for entire period of reoxygenation gives equal cardioprotection to that achieved by inhibiting it for initial 10 minutes of reoxygenation. However, blocking the pore formation after the first 10 minutes resulted in increased myocardial injury. It can be therefore, concluded that opening of MPTP in later phase of reoxygenation is beneficial in recovering for ischaemic assault. Similar results have been published from other laboratory using sanglifehrin A, MPTP inhibitor, administered 15 minutes after reperfusion did not decrease the size of infarction in isolated perfused rat hearts(200).

MPTP Before Ischemia

It has been shown previously by Hausenloy et al. that inhibiting MPTP formation by CsA and sanglifehrin A before ischaemia in an isolated perfused rat heart does not effect myocardial injuries(188). We have also shown that blocking the MPTP formation by CsA and BKA before ischaemia does not alter the expected myocardial damage. CsA has also been shown to cancel the beneficial cardioprotective effects provided by the release of reactive oxygen species such as H_2O_2 during IP using Langendorff-perfused rat hearts (189). It suggests the

involvement of transient opening of MPTP and notifying that opening of these pores are essential to obtain cardioprotection through IP. On the contrary, using the mitochondrial 2-deoxyglucose entrapment techniques have failed to detect any IP-induced increase in MPTP opening (201). Further exploration in these disputed territories is warranted.

MPTP Status During Ischemia

We showed that closure of MPTP during ischaemia had no influence on myocardial injury outcome. Bopassa et al. have demonstrated earlier that MPTP remains closed through the ischaemic period (82). Although there was a significant increase in CK release when MPTP was remained open during ischaemia, the necrosis and apoptosis markers were broadly unaffected. Therefore, it can be concluded that the MPTP status does not play significant role during ischaemia.

Cardioprotection and MPTP

Through our experiments with rat myocardium, we have shown that both transient opening and closing of MPTP during early reoxygenation results in comparable cardioprotection. These results challenge the widely accepted belief that MPTP is the end-effector of ischaemic preconditioning (202) and that PKA, PKB and PKC converge on GSK-3 β thereby inhibiting the formation of the MPTP(161,203).

The depolarization and Ca^{2+} efflux from the mitochondria is mediated through the transient opening of the MPTP resulting in the release of mitochondrial radical oxygen

species(188,196). In our experiments, blocking MPTP after 10 minutes of reoxygenation resulted in staggering myocardial injury whereas opening this pore during the same period does not have similar effect. This suggests that these pores participate in post-ischaemic recovery through mitochondrial Ca^{+2} efflux and containing mitochondrial Ca^{+2} overload.

In nutshell, these are important and relevant findings and further work is required to understand the physiology and signalling events of MPTP in relation to cardioprotection.

Study Limitations

The present studies have been performed in an *in vitro* model using myocardial slices from rat left ventricle. Whilst the use of such an *in vitro* model system has the distinct advantage of allowing a greater control and design of experimental manipulations, there are obvious disadvantages. For example, it would be useful to confirm in future work the observations reported in this study not only in *ex vivo* and *in vivo* animal models but crucially in human myocardium.

CHAPTER 5

Clinical Implications & Future Directions

In our laboratory, we have previously demonstrated that the diabetic myocardium does not respond to ischaemic preconditioning (150). Therefore, the results from this research work of inhibiting PKB overcome this barrier and protect diabetic myocardium, have opened immense area of novice clinical opportunity. It also shows that PKB is downstream of the mitoKATP channels and beyond the mitochondria. Manipulation of these protein kinases may reduce the ischaemic injury in subjects with diabetes and suffering an acute coronary syndrome or undergoing cardiac surgery.

This of course in my opinion is a major breakthrough in the available knowledge pool of diabetic myocardium. Further *in vivo* experiments with novel inhibitors of PKB are required to ascertain the clinical effects on human myocardium.

In recent times a newer version of myocardial protection called remote ischaemic peri conditioning has been in hot spot. It refers to the ability of conditioning of heart during ongoing ischaemia by brief period of ischemia and reperfusion. Botker et al. (204) has shown that remote periconditioning results in up to 33% increased myocardial salvage as compared to controls. More work need to be carried out to take this theory further on.

Similarly, transient opening of the MPTP has been implicated in ischemic preconditioning (188), we believe that this is the first report describing direct transient opening of the pore as a possible clinical target for cardioprotection. Present studies have shown that both formation and inhibition of the MPTP can be exploited for therapeutic purposes and that there is a defined therapeutic window, with the first few minutes of reoxygenation being a crucial period to achieve cardioprotection. To pursue this theory further, more studies, both *in vivo* and *in vitro*, need to be carried out with human myocardium.

Cyclosporine a has been studied in clinical trials and has been shown to decrease myocardial infarction when given prior to primary coronary intervention (PCI). Although these are encouraging findings, a larger multicentric clinical trial is needed to validate the results. Although MPTP inhibitors has been previously used as pharmacological tool for preconditioning of myocardium, the non desiring side effects have largely put them in back seat for further evaluation. New drugs need to be designed that could bring the beneficial effects of MPTP inhibition and opening.

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