University *of* Leicester

New Insight into The Signalling Pathways of Cardioprotection in The Non-Diabetic and Diabetic Human Myocardium

2001-2004

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Dedication:

This thesis is dedicated to my family who have been very supportive to me during hard times, who cheered me up when I was down, gave the strength when I was weak and without them this work would never have been done.

To my wife Mervat and to my children Mohamed and Merna

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- Does Alteration In Mitochondrial Membrane Potential And Superoxide Production Contribute To The Failure To Precondition The Diabetic Human Myocardium? A Hassouna, M Loubani, B.M. Matata, A Fowler, N Standen, M Galiñanes. Oral presentation, Midland Cardiothoracic Meeting, 27/02/04.

- Is the differential release of mitochondrial ROS a mechanism for Diazoxide mediated cardioprotection in human myocardium? ***A Hassouna***, B.M. Matata, M Galiñanes, accepted for the14th Annual Meeting of the Scandinavian Society for Research in Cardiothoracic Surgery, Geilo, Norway February 5th – 7th, 2004.

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- Failure to precondition the diabetic myocardium by diazoxide. ***A Hassouna***, B.M. Matata, A Fowler, N Standen, M Galiñanes. American society of cell biology in San Francisco, *USA*, Dec 2003.

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- Failure to precondition the diabetics' myocardium correlates with incomplete depolarization of mitochondrial membrane potential. ***A Hassouna***, B.M. Matata, A Fowler, N Standen, M Galiñanes. Presented at the Mediterranean Association of Cardiology and Cardiac surgery 15th annual meeting, *Lebanon* 10-13 September 2003.

- Failure to precondition the diabetic myocardium by diazoxide. ***A Hassouna***, B.M. Matata, A Fowler, N Standen, M Galiñanes. Presented at the Mediterranean Association of Cardiology and Cardiac surgery 15th annual meeting, *Lebanon* 10-13 September 2003.

- PKC activation is downstream and upstream of mitoK_{ATP} channel in ischaemic preconditioning of the human myocardium. ***A Hassouna***, B.M. Matata, M Galiñanes. Presented at The midland cardiothoracic surgical meeting, *UK*, September 2003.

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Mr. Ashraf Hassouna

Background: IPC is a well-recognised phenomenon in healthy hearts, however the occurrence of IPC in diabetics is controversial. In this thesis, I have investigated whether the diabetic myocardium can be preconditioned and the underlying mechanisms.

Methods and results: Right atrial sections from non-diabetic, IDDM, and NIDDM patients were randomised to one of the following protocols: aerobic perfusion, simulated ischaemia/ reoxygenation, IPC, and pharmacological preconditioning with phenylephrine, adenosine, diazoxide (mitoK_{ATP} channel opener), PMA (protein kinase C agonist) or anisomycin (p38 MAPK activator). CK leakage and MTT tissue viability endpoint measurements demonstrated that diabetic myocardium cannot be preconditioned by ischaemia or by diazoxide but that protection can be obtained by the activation of the pathway beyond mitoK_{ATP} channels (e.g. PKC and P38 MAPK). Using isolated mitochondria from the above groups of patients, MMP detection by JC-1 dye demonstrated that the partial depolarisation of MMP seen in non-diabetics after treatment with diazoxide was absent in the diabetic myocardium. The determination of ROS generation by the mitochondria of the non-diabetic and diabetic myocardium exposed to diazoxide showed an altered response in superoxide production in the diabetic myocardium. Finally, using specific PKC isoform inhibitors, I demonstrated that PKC α and PKC ε are involved in IPC of human myocardium with PKC ε being upstream and PKC α being downstream of mitoK_{ATP} channels.

Conclusions: The failure to precondition the diabetic myocardium is caused by mitochondrial dysfunction, possibly due to alterations in the mitoK_{ATP} channels as shown by the abnormal responses in MMP and superoxide generation following exposure of mitochondria to diazoxide. However, the diabetic myocardium can still be protected by activation of PKC and P 38 MAPK that are downstream of the mitoK_{ATP} channels. Since PKC α is the isoform beyond mitoK_{ATP} channels, this may represent potential clinical and therapeutic targets to protect the diabetic myocardium.

Abbreviations list:

A/C: aerobic control

CCCP: carbonylcyanide m-chlorophenylhydrazone

CK: creatinine kinase (CK-MB isoform)

CM-H_2DCFDA: Chloromethyl -dichlorodihydrofluorescein diacetate

DCFH: 2, 7-dichlorofluorescein diacetate

DXZ: diazoxide

HEPES: (N- [2-Hydroxymethyl] piperazine -N'- [2-ethanesulphonic acid])

IDDM: insulin dependant diabetes mellitus

IPC: ischaemic preconditioning

JC-1: 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazol carbocyanine iodide

Mito-K_{ATP} channel: ATP sensitive mitochondrial potassium channel

MMP: mitochondrial membrane potential

MnTBAP: Mn (III) tetrakis (4–Benzoic acid) porphyrin Chloride

MPG: mercaptopropionylglycine

MTT: 3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetiazolium bromide

NIDDM: non-insulin dependant diabetes mellitus

PKC: protein kinase C; PKCI: protein kinase C inhibitor

P38 MAPK: P38 MAP kinase

PMA: phorbyl myristate acetate

ROS: reactive oxygen species

SI/R: simulated ischaemia/ reoxygenation

SOD: superoxide dismutase

TMRE: tetramethyl-rhodamine ethyl ester

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Chapter 1
Introduction

1.1. Ischaemic preconditioning

In 1986 Murry and colleagues described the phenomenon of cardio protection by subjecting the dog heart to brief periods of ischaemia interspaced with perfusion prior to subsequent lethal ischaemic injury [1] . An early window of cardio- protection occurs within minutes and lasts for 1-3 hours and a further period of protection occurs 24 hours after the preconditioning stimulus and lasts up to 72 hours which is known as the second window of protection [2].

Cardioprotection by ischaemic preconditioning has been demonstrated in different experimental preparations including isolated myocytes [3], atrial muscle [4], [5], isolated heart perfusion models [6], [7] and *in vivo* animals [4] [1]. Ischaemic preconditioning is a powerful intervention that has been shown to reduce myocardial infarction [1], improve post-ischaemic functional recovery [8], attenuate myocardial cellular apoptosis [9] and reduce ventricular arrhythmias induced by ischaemia and reperfusion.

1.2. Evidence of preconditioning in human myocardium

The first evidence of preconditioning in humans arose from angioplasty studies where repeated balloon inflation and deflation lessened the severity of myocardial ischaemia [10].

Retrospective studies in humans have reported that a period of angina 24 to 48 hours before an acute myocardial infarction is cardioprotective, also lending support to the theory that preconditioning occurs in humans [11].

Ikonomidis et al were the first to provide *in vitro* direct evidence of ischaemic preconditioning in the human myocardium [3]. In their model of ventricular cardiomyocytes cell culture, protection was achieved using 20 minutes of simulated ischaemia followed by 20 minutes of reperfusion. Yellon et al [4] also demonstrated that preconditioning of the human heart during coronary artery bypass surgery reduces the depletion of ATP levels in preconditioned hearts as compared to non-preconditioned hearts. Later Walker et al demonstrated that preconditioning of the human atrial trabeculae had significant better post ischaemic recovery of contractile function than non preconditioned trabeculae [12].

1.3. Diabetes and cardiac disease

Diabetes increases the risk coronary artery disease by 2 to 4 fold [13]. Diabetes is also the cause of elevated morbidity and mortality in patients with coronary artery disease. Thus patients with diabetes but without previous MI carry the same level of risk for subsequent acute coronary events as non-diabetic patients with previous MI [14]. Diabetes also worsens early and late outcomes in acute coronary syndromes. In unstable angina pectoris compared with control, the presence of diabetes increases the risk of in-hospital MI, complications of MI, and mortality [15]. Regardless of the severity of clinical presentation, patients who have diabetes and coronary events experience increased rates of MI and death. Patients with diabetes also have an adverse long-term prognosis after MI, including increased rates of re - infarction, congestive heart failure, and death [16], so that the 5-year mortality rate following

MI may be as high as 50% for diabetic patients more than double that of non-diabetic patients [17].

Diabetic patients also experience higher risk of in-stent thrombosis than non-diabetic [18]. Several studies have demonstrated a greater long-term risk of restenosis after balloon angioplasty [19], [20]. Moreover, the severity of diabetes affects the outcome after stent implantation. In multivariate analysis, insulin requirement entailed a 2 fold increased risk of adverse cardiac events and target vessel revascularization at 1 year [21]. There is also significantly greater postoperative morbidity and mortality among diabetic patients undergoing CABG surgery when compared with non-diabetic patients [22]. The demonstration that cardiopulmonary bypass induces a greater oxidative stress in patient with diabetes than those without diabetes [23] may be a factor contributing to the greater rate of complications during cardiac surgery.

1.4. Preconditioning the human myocardium in diabetics

The literature on the ability to precondition the myocardium of diabetics is conflicting. Some studies have reported that the cardioprotection of ischaemic preconditioning is lost in the diabetics, whereas others have suggested that the diabetic heart can be protected against ischaemia to a greater degree than non-diabetic heart [24], [25], [26], [27]. The reported differences may be explained by the alteration in the function of cardiac subcellular organelles, including the sarcolemma, sarcoplasmic reticulum and mitochondria [28]. Diabetes is also associated [28] with several abnormalities in energy metabolism, depressed Na^+/Ca^{++} and Na^+/H^+ exchange activities, decreased Na^+/K^+ pump and sarcoplasmic reticulum Ca^{++} pump activities and elevated antioxidant defences. Diabetes also alters the function of vascular and myocardial ATP-dependent potassium channels (K_{ATP} channels) [29], [30], [31] and in addition, channel density appears to be diminished in diabetic hearts

[30], [32]. Since many authors have identified the K_{ATP} channels as major contributors to preconditioning protection against infarction and stunning [33], it is possible that the cardioprotective action afforded by this phenomenon is reduced in diabetic hearts. Glucose and insulin have been mentioned to have both deleterious and beneficial effects on cardiovascular function [34], [35], [36], [37] during ischaemia / reperfusion events and both hyperglycaemia and insulin have been described to have an effect on K_{ATP} channels [38], [39], [40].

The use of anti-diabetics agents may also effect preconditioning. It has been reported that human myocardium from patients without long-term exposure to oral sulfonylurea hypoglycaemic agents is functionally protected by preconditioning but that long-term oral intake of sulfonylureas blocks the protection by preconditioning [41]. These data suggest that ischaemic preconditioning in human myocardium relies on K_{ATP} channels, and long-term inhibition of K_{ATP} channels with oral hypoglycaemic agents has been suggested as an explanation for the excess cardiovascular mortality in this group of patients [41].

1.5. The signal transduction pathway of preconditioning

1.5.1. ***Introduction***

The mechanism of ischaemic preconditioning has been mainly investigated using pharmacological tools. In this way, it has been shown that adenosine A1 receptor stimulation [7], α1-adrenergic pathway [42], bradykinin release and nitric oxide production [43], ROS triggering process [44], glycogen depletion and lactate production [45], [46], protein kinase C (PKC) [47], [48] and tyrosine kinase [49] activation and ATP-sensitive potassium channel opening [50] are elements of the signal transduction pathway of preconditioning.

1.5.2. ***Potential mechanisms of IPC***

1.5.2.1. *Background*

The precise mechanism of IPC still remains unclear. One early theory explained that preconditioning was merely a manifestation of increased collateral blood flow. However, this cannot explain the preconditioning effect in isolated hearts preparation or myocardial tissue subjected to global ischaemic stimuli. This hypothesis was disproved by the demonstration that preconditioning was independent of collateral flow [1].

It was also suggested that sub-lethal preconditioning caused depressed contractility, that in turn would slow the metabolic demand, however this does not explain why a short period of ischaemia is cardioprotective whereas a longer ischaemic period is not. A second reason against the above hypothesis is that there is no correlation between the degree of the preconditioning stimuli and protection effect. In addition, reversal of the depressed myocardial contractility with dopamine does not prevent preconditioning [51].

A metabolism process might also be involved in IPC mechanism. During the ischaemic phase of IPC, glycogen stores are depleted, and lactate and protons accumulate. During the reperfusion phase of IPC, accumulated catabolites are rapidly washed out [52]. Since glycogen synthesis is slow and the glycogen-depleted myocardium is exposed to prolonged ischaemia, lactate accumulation is low. Complete catabolites washout by effective reperfusion seems to be an essential condition for IPC, and the restoration of glycogen after IPC coincides with the loss of anti-infarct effect [52]. Myocardial glycogen depletion appears to be necessary for the infarct-limiting effect of IPC. Furthermore, evidence suggests that lactate, which accumulates during IPC periods, can activate several triggers of

preconditioning. However, whether the transient exposure to external lactate improves contractile recovery as shown by IPC stimuli is controversial [53], [46]. It should be noted that the depletion of glycogen induced by IPC influences post-ischaemic functional and metabolic recovery, but are not the only determinant [54]. However, whether pre-ischaemic glycogen depletion and subsequent attenuation of ischaemic lactate accumulation play a major role in IPC protection against contractile dysfunction has been questioned [55].

1.5.2.2. *MitoK$_{ATP}$ as end effecter of IPC*

It is well known that brief episodes of myocardial ischaemia result in the production of substrates such as adenosine, bradykinins, opioids and endorphins. Adenosine is released from the heart as ATP is broken down to AMP and 5'nucleotidase releases free adenosine, which then occupies adenosine receptors on the surface of the heart. Bradykinin is also released and occupies bradykinin B2 receptors. Opioids and endorphins produced by the heart occupy the delta receptors. The activation of these receptors signals G- proteins that in turn activate the phospholipases C and D that eventually activate protein kinase C. In a positive feedback manner PKC increases 5'nucleotidase activity, which increases adenosine release and further activates the kinases [56]. Recently, there has been great interest in the mitochondrial K_{ATP} (mitoK_{ATP}) channels as the end-effectors of this protection [57], [49]. PKC translocate from the cytosol to the cell membrane after activation, mediating phosphorylation of target proteins, ion channels (including ATP-sensitive potassium channels) and myofilaments [58]. The opening of ATP-sensitive potassium channels leads to increased potassium conductance and shortening of action-potential duration, which might limit calcium entry and reduce energy metabolism [59].

1.5.2.3. *MitoK_{ATP} channels as a trigger*

It has been widely demonstrated that opening of mitoK_{ATP} channels with diazoxide is cardioprotective [60], [61], [62]. Opening of these channels causes the mitochondria to swell and to be slightly uncoupled. Neither effect should be protective. The observation that diazoxide protection is dependent on ROS production is significant in that both PKC as well as the p38 MAPK pathway are known to be activated by ROS. The above suggests that opening of the mitoK_{ATP} channels acts as a signal transduction step that triggers preconditioning protection rather than as the end-effectors. In this connection, Loubani and Galiñanes [63] have proposed that activation of the mitoK_{ATP} channels causes the mitochondria to produce free radicals that in turn trigger the kinases PKC and P 38 MAPK, which would then induce the phosphorylation of the as yet unknown end-effectors (Figure 1).

Figure 1: Schematic representation of the signal transduction of preconditioning

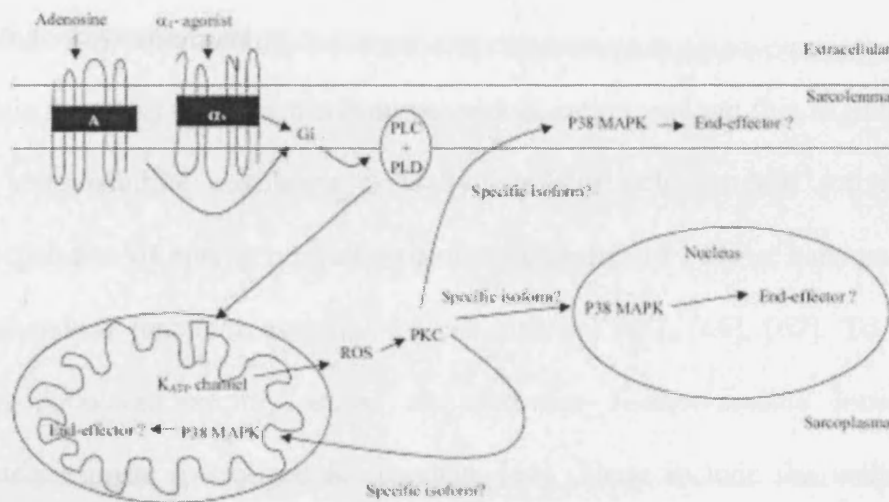


Figure 1: Proposed schematic representation of the signal transduction mechanism leading to cardioprotection of the human myocardium. Upon activation of sarcolemmal receptors mitoK_{ATP} channels are opened via G proteins. The opening of mitoK_{ATP} channels will activate PKC possibly via the production of radical oxygen species (ROS). PKC may then translocate to various cellular sites, where p38MAPK will be activated. In turn, p38MAPK may activate a single or multiple end-effectors directly or via MAPK intermediates [63].

1.6. Mitochondrial K_{ATP} channels and acute cardioprotection by preconditioning

*1.6.1. **K_{ATP} channels***

Potassium is the major cytoplasm and mitochondrial cation, and net flux of potassium across the inner mitochondrial membrane critically regulates mitochondrial activity [64]. This includes regulation of energy production and maintenance of cellular calcium homeostasis, two mitochondrial functions essential for cell survival [65], [66], [67]. To allow for bi-directional potassium cycling across an otherwise K-impermeable inner membrane, mitochondria express specialized K^+ conduits [64]. These include the well-characterized electro-neutral K^+/H^+ anti-porter responsible for K^+ efflux, along with less well known pathways for K^+ influx [68], [66]. A candidate mechanism for potassium entry is an ATP sensitive K (K_{ATP}) channel [66], [64]. This channel, known as the mitochondrial K_{ATP} channel, has been recently identified within the inner mitochondrial membrane [69], and the molecular identity of channel subunits has been partially characterized [64], [70].

*1.6.2. **The mechanism of cardioprotection induced by K_{ATP} channel opening***

In the past it was thought that opening the sarcK_{ATP} channel resulted in membrane hyperpolarization and shortening of phase 3 of the cardiac action potential, both effects causing a reduction in intracellular calcium levels which could produce a cardioprotective effect [71],[72]. However, it was demonstrated that enhanced shortening of the cardiac action potential was probably not the mechanism responsible for the cardioprotective effect of the K_{ATP} opener. It was shown that a low dose of K_{ATP} channel opening did not enhance action

potential shortening but still had a cardioprotective effect on the reduction of infarct size similar to higher doses which enhanced action potential shortening [73], [74]. In addition, it was shown that the cardioprotective effect of K_{ATP} channel opener in dogs was not blocked by an anti-arrhythmic agent that caused action potential shortening in treated hearts [75]. These studies suggested that the sarcK_{ATP} channel was most likely not the site of the cardioprotective effects of IPC and K_{ATP} openers and an intracellular site was proposed. Subsequently, a mitoK_{ATP} channel was identified by Inoue [69] in rat liver and Garlid's group [76] first isolated and characterized a mitochondrial K_{ATP} channel localized to the inner membrane in beef heart which had several properties similar to the sarcK_{ATP} channel. The same group later reported that the mitoK_{ATP} channel was possibly the site of action responsible for the cardioprotective effects of K_{ATP} openers [77].

1.6.3. Evidence of the role of mitoK_{ATP} channel in preconditioning

The evidence for mitoK_{ATP} channel involvement in cardioprotection has been obtained by using pharmacological methods showing that the mitoK_{ATP} of the channel opener diazoxide mimics IPC and the inhibitor 5-hydroxydecanoate (5-HD) blocks IPC. Using Patch clamp and flavoprotein fluorescence as an index of mitoK_{ATP} oxidation and membrane depolarization in rabbit myocytes it has been shown that diazoxide is a selective mitoK_{ATP} opener and that 5-HD is a selective antagonist [78], [79]. These studies also demonstrated that diazoxide was cardioprotective and reduced the number of myocytes killed in a cellular model of simulated ischaemia and that 5-HD abrogated protection. The role of mitoK_{ATP} in preconditioning gains additional support by the observation that HMR 1883, a sarcK_{ATP} channel antagonist, does not affect the increase in flavoprotein fluorescence produced by diazoxide and does not affect its cardioprotective effect in rabbit myocytes subjected to simulated ischaemia [80].

The mechanism by which mitoK_{ATP} opening might be protective remains uncertain; however it has been hypothesised that mitochondrial depolarization to limit Ca^{2+} accumulation, mitochondrial uncoupling, preservation of mitochondrial intermembrane architecture, and the release of reactive oxygen species (ROS) may play a role.

1.7. Mitochondrial membrane depolarization

The importance of mitochondria in the pathogenesis of cardiac diseases, particularly of ischaemia and reperfusion is well recognised (for review [81]). Besides the traditional role as "powerhouses" of the cell in generating ATP, mitochondria can play other physiological roles such as cell death and survival. The function and survival of cardiac cells, that have high-energy requirements, depend on the maintenance of the mitochondrial membrane potential (MMP). Measurement of MMP can therefore be used to investigate the molecular mechanisms controlling cardiomyocyte function [82].

The supply of energy by the mitochondria depends on the maintenance of the chemiosmotic gradient across its inner membrane [83]. This gradient, also known as the proton motive force, is generated by three respiratory enzyme complexes which use the free energy released during electron transport to translocate protons from the mitochondrial matrix into the intermembrane space. Proton motive force has two components: the MMP, which arises from the net movement of positive charge across the inner membrane and the pH gradient. Of these two components, MMP is the major contributor to the gradient, which typically is 150 mV. Hence, for practical purposes, MMP may be used as an indicator of the energization state of

mitochondria [82]. The effect of the opening of mitoK_{ATP} channels on MMP is however controversial.

1.8. ROS generation and cardioprotection

1.8.1. ***Introduction***

ROS are chemical species generated in small amounts during the normal metabolism of cells. ROS are transient, with 10^{-6} to 10^{-3} second half-lives and short radii of diffusion. The univalent pathway of oxygen reduction is the source of free radicals. ROS are highly reactive compounds which can oxidise many cellular components and cause post-ischaemic cardiac dysfunction [84] and tissue damage [85].

1.8.2. ***How, When And Why Are ROS Produced In Mitochondria?***

Electron transport chain (ETC) of mitochondria is a permanent source of reactive oxygen species (ROS) in cells. About 1 to 3 % of total mitochondrial oxygen consumed is incompletely reduced and leads to ROS production [86], [87]. By the transfer of one single free electron to molecular oxygen at the level of NADH CoQ reductase (Complex I) and CoQ cytochrome C reductase (Complex III), monoelectronic reduction of oxygen generates superoxide [88]. This superoxide anion is scavenged by intra mitochondrial Superoxide Dismutase (Mn SOD) leading to hydrogen peroxide and in the presence of reduced transition metals (such as iron) to the highly reactive hydroxyl radical.

It is generally accepted that following its generation in the inner mitochondrial membrane, at the level of the respiratory chain, superoxide ions are vectorially released into the

mitochondrial matrix and subsequently dismutated to hydrogen peroxide by the high content of the matrix enzyme Mn SOD. Conversely to the low diffusion capacity of the charged superoxide anion, hydrogen peroxide can easily cross the mitochondrial membrane towards cytoplasm [89], [90]. It should be added that a direct release of superoxide anion into the intermembrane space has been recently proposed [91]. Similarly to the mitochondrial superoxide ion pathway, in the cytosol the scavenging of superoxide to hydrogen peroxide occurs by an equivalent enzyme: CuZn SOD [92].

*1.8.3. **ROS and ischaemic preconditioning***

The generation of reactive oxygen species (ROS) has been shown to play an important role in the induction of IPC [93]. Reactive oxygen species at high doses have been reported to be toxic and damaging [94] but at low concentration it is protective [95], and this may be the reason why ROS are beneficial when produced during IPC and deleterious when generated during ischaemia / reoxygenation [44]. Another possible explanation is that the production of ROS during IPC is not accompanied by other aspects of the inflammatory reaction which could contribute to endothelial injury such as complement activation and expression of leukocyte adhesion molecules [96]. The relation between ROS and IPC was first suggested by Murry, in a study in which administration of oxygen radical scavengers during the first reperfusion period could block the beneficial effect of IPC on infarct size in the dog [97]. He suggested that the generation of low amounts of ROS during a short ischaemic episode is not sufficient to cause cell necrosis, but enough to modify cellular activity and induce preconditioning. Brief ischaemia may also increase the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase or glutathione peroxidase in isolated myocytes [44] and *in vivo* [98]. Oxidant stress can induce heat shock proteins in the heart [99] and lead to

delayed increases in the activity of SOD, catalase or glutathione peroxidase in endothelial cells [49]. It has been reported that, in the isolated rabbit heart, a low flux of ROS generated by purine/xanthine oxidase significantly improves post-ischaemic recovery of contractile function and reduces infarct size, an effect that was prevented by the scavenger SOD and the PKC inhibitor polymyxin B [100]. ROS produced during IPC also protect the coronary endothelium from reperfusion injury [96].

1.9. The role of Protein kinase C in ischaemic preconditioning

1.9.1. ***Introduction***

Protein kinase C (PKC) is a cyclic nucleotide-independent enzyme that phosphorylates serine and threonine residues in many target proteins. It was first identified in 1977 in bovine cerebellum by Nishizuka and co-workers [101] as a protein kinase that phosphorylates histone and protamine. Since then, its involvement in many biological processes has been demonstrated, including development, memory, differentiation, proliferation and carcinogenesis [102]. Once thought to be a single protein, PKC is now known to comprise a large family of enzymes that differ in structure, cofactor requirements and function.
At present, 10 isoforms have been identified (depending on the classification used), varying in tissue expression and cellular compartmentalization (allowing for specific interactions with substrates) [103].

The PKC family has been divided into three groups, differing in the enzymes' cofactor requirements [104]:

- Conventional (c) PKC isoforms (comprising α, β1, β2 and γ), that require calcium and diacylglycerol (DAG) for activation;
- Novel (n) PKC isoforms (comprising δ, ε, η, θ and μ) that require DAG;
- Atypical (a) PKC isoforms, namely λ, ζ, ι, that require neither calcium nor DAG.

All PKC possess a phospholipid-binding domain for membrane interaction. The general structure of a PKC molecule consists of a catalytic and a regulatory domain, composed of a number of conserved regions, interspersed with regions of lower homology, the variable domains [105].

1.9.2. ***PKC activation***

Under baseline conditions, PKC is rendered largely inactive by intramolecular interaction between 'pseudo-substrate' and catalytic sites [106]. PKC activation and subsequent translocation to membrane compartments is preceded by agonist-mediated stimulation of receptor-associated phospholipase C (PLC) [107]. PLC, upon stimulation, cleaves inositol-1, 4,5-trisphosphate and diacylglycerol from the membrane-bound phosphatydylinositol moiety. Diacylglycerol acts as a hydrophobic factor, and increases the affinity of PKC to the membrane-integrated structures within the subcellular compartments [108], [105]. In other words, diacylglycerol facilitates the translocation of PKC into these compartments. To activate PKC, PKC isoforms are phosphorylated at specific C-terminal serine/threonine residues by phosphatidylinositol-trisphosphate-dependent kinase (PDK1), with at least two additional phosphorylations and/or autophosphorylations of well-conserved sequences in each enzyme of the PKC family [109]. Each phosphorylation event induces conformational changes in the PKC molecule that site weakens conformational interactions between the

pseudosubstrate and the catalytic sites, and makes the pseudo substrate sequences less accessible to the PKC catalytic moiety [106] (Figure 2).

Until recently, it was generally accepted that PKC, upon its translocation, binds to lipid moieties within the membrane compartment. However, immunological and biochemical studies suggest that PKC may become attached to specific proteins within the membrane and nuclear subcellular fractions (RACK proteins; i.e. **r**eceptors for **a**ctivated **C**-**k**inase) [110], [111], [112].

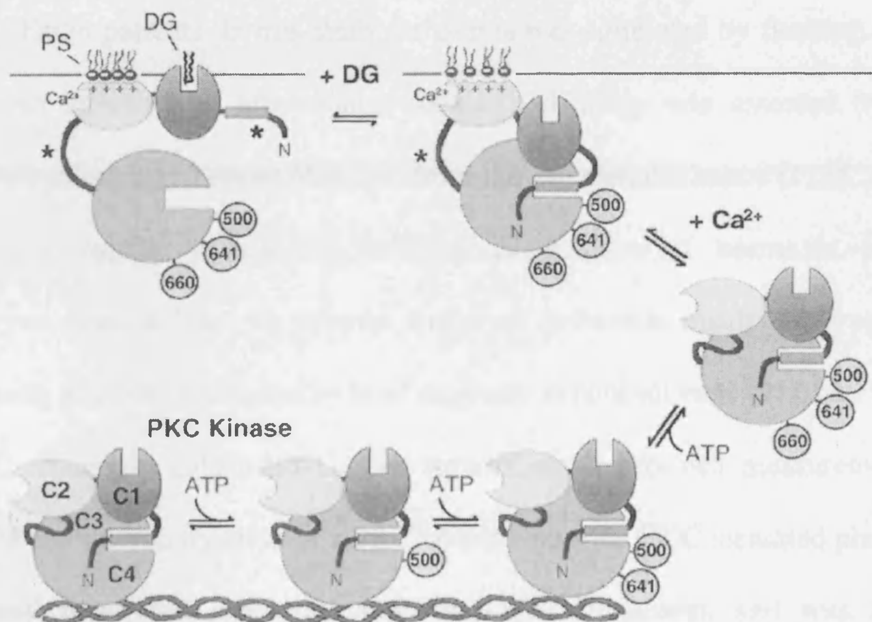


Figure 2: PKC is processed to the mature, cytosolic form by three functionally distinct phosphorylations: transphosphorylation at the activation loop to render the kinase catalytically competent (Thr-500); an autophosphorylation at the C terminus (Thr-641) that stabilizes the catalytically competent conformation; and a second autophosphorylation at the C terminus (Ser-660) that releases protein kinase C into the cytosol. PS=pseudosubstrate, DG= diacylglycerol

1.9.3. Evidence of the role of PKC in preconditioning

The first evidence for the role of PKC in preconditioning produced by Speechly-Dick and colleagues who subjected human right atrial trabeculae to a preconditioning stimulus consisting of 3 minutes of the simulated ischaemia followed by 7 minutes of reperfusion, and employed recovery of contractile function after 90 minutes of simulated ischaemia as their end point. Contractile recovery was significantly improved with both preconditioning, and exposure of the trabeculae to the PKC activator 1, 2-dioctanoyl glycerol, when compared with the non-preconditioned group [4]. The role of PKC in ischaemic preconditioning has also been assessed in cultures of paediatric cardiomyocytes obtained during surgery from Tetralogy of Fallot patients. In this study ischaemia was simulated by flushing cultures with 100% nitrogen followed by low-volume anoxia, and injury was assessed by monitoring cellular ability to extrude Trypan blue dye from the intracellular space [113]. A 20 minutes period of low volume anoxia followed by 20 minutes of normoxia protected the cardiomyocytes from a later 90 minutes sustained ischaemic insult. Low-volume anoxic preconditioning also was mimicked by brief exposure to phorbol ester [21], and was inhibited by the PKC antagonist calphostin C. The authors also performed measurements of PKC activity by in situ phosphorylation of a PKC-specific peptide. PKC mediated phosphorylation was increased both with preconditioning, and PMA treatment, and was abolished by calphostin C. By employing an isoform-nonspecific fluorescent antibody to PKC, the authors were further able to demonstrate an increase in fluorescence in the cell membranes and in the perinuclear area [113]. As the antibody recognizes sequences in the catalytic site of the PKC molecule, this method demonstrates that the catalytic PKC structure was indeed present, but does not provide information regarding the integrity of the entire PKC moiety or the activity status of the enzyme. Cleveland et al. reported that in the isolated human trabeculae the protective effect of ischaemic preconditioning could be abolished by α-adrenergic blockade

and/or PKC inhibition. PKC inhibition with chelerythrine also abolished the protection induced by stimulation of α-adrenoreceptors [48]. Since these protocols were performed *in vitro* settings and employed non-traditional preconditioning stimuli, their relevance to *in vivo* ischaemic preconditioning is not clear.

1.10. Hypothesis of the study

The loss of cardio protection in the diabetic heart is due to defective mitochondrial K_{ATP} channels or a defect involving mitochondrial metabolism.

1.11. Aims of the study

My aims in this thesis were: (i) to investigate the phenomenon of ischaemic preconditioning in the diabetic human myocardium and (ii) to dissect the signal transduction pathway of preconditioning in the non-diabetic and in the diabetic human myocardium. To achieve this I investigated the role of PKC isoforms in preconditioning, examined the role mitochondrial K_{ATP} channels and measured mitochondrial membrane potential and reactive oxygen species generated by the isolated mitochondria in response to activation of mitochondrial K_{ATP} channels by diazoxide.

Chapter 2

Methodology

2.1. Human atrial model

2.1.1. Introduction

In the clinical setting, studies on myocardial ischaemia are difficult to perform and are ethically unacceptable. Because of this the present studies were conducted using an established model for quantification of the effect of ischaemia and reperfusion on human cardiac muscle using thin slices of atrial appendages [114]. Atrial tissue was chosen because it is easily available as the atrial appendage is considered a waste during cardiac surgery. This model can avoid the problem of variable collateral flow and, more importantly, it can provide meaningful information on the human myocardium. The use of atrial tissue for studies of ischaemia may also offer advantages over isolated and cultured myocytes, because these are more difficult to obtain, they do not retain the normal cell-to-cell contact, ischaemia is more difficult to accomplish and they are viable only for short periods of time [115].

2.1.2. Methods

The study was conducted using an established model for quantification of the effect of ischaemia and reperfusion on human cardiac muscle using thin slices of atrial appendages. Sections circa 40mg wet weight and 300-400 μm thick were prepared from human right atrial appendages using a surgical skin-graft knife. These quiescent tissues maintain integrity, volume, ion gradients and ultra structure. Sections are immediately equilibrated in Krebs-

Henseleit Hepes buffer pH (7.4) by bubbling with 95% oxygen and 5% carbon dioxide in water bath maintained at 37°C throughout the experiments. Simulated ischaemia was induced by bubbling the medium with 95% N_2/5% CO_2 (pH 6.8–7.0) and by replacing D-glucose with 2-deoxy-D-glucose as described previously [114]. The ischaemic preconditioning protocol used consisted of 5 minutes ischaemia followed by 5 minutes reoxygenation. Reoxygenation is achieved by moving the tissue sections into 10 ml of oxygenated medium with added glucose.

All the muscles were equilibrated for a 30 minutes period before being randomly assigned to serve as time-matched aerobic controls or subjected to a 90 minutes period of simulated ischaemia (SI). The muscles were then reoxygenated (R) for another 120 minutes. At the end of the experimental protocols, samples from the incubation media used during the reoxygenation period were collected for the assessment of creative kinase (CK) leakage and the tissue was taken for the assessment of viability (reduction of 3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetiazolium bromide (MTT). All agents tested were added for 5, 10 or 20 minutes at the end of the equilibration period and before the induction of SI.

- *CK leakage*

Tissue injury was determined by measuring the leakage of CK into the incubation medium during the 120 minutes reoxygenation period. This was assayed by a kinetic ultraviolet method based on the reduction of NADP to NADPH and the increase in absorbance was measured at 340 nm [116]. The rate of change in absorbance is directly proportional to CK activity. The results are expressed as U/g wet wt.

- *Tissue viability*

The 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl-2*H*-tetrazolium bromide (MTT) assay was used to quantify tissue viability [117]. In this assay, the yellow MTT is reduced to a blue formazan product by the mitochondria of viable tissue. Briefly, at the end of each experiment, the slices were placed in 5 ml tubes, 2 ml of PBS (0.05 M) containing 3 mM MTT (final concentration) was added and the slices were incubated for 30 minutes at 37 °C. The slices were then homogenized (Ultra-Turrax T25, dispersing tool G8; IKA Laboratories, Staufen, Germany) in 2 ml of DMSO at 9500 rpm for 1 minute and then centrifuged at 1000 g for 10 minutes. Portions of the supernatant (200 μl) were dispensed into 98-well flat bottom microtitre plate (Nunc Brand Products, Denmark) and the absorbance of the blue formazan formed was measured on a plate reader (Benchmark, Bio-Rad Laboratories, California, USA) at 550 nm and the results expressed as mM/g wet wt.

2.1.3. ***Discussion***

There are differences between atrial and ventricular tissue, and results from one may not be applicable to the other, however data from Yellon's laboratory [12] and from our own group (unpublished data) has demonstrated that the response to ischaemia/ reoxygenation of ventricle and atrial tissue is similar. Adenosine receptors are present in both atrium and ventricle [118], as are K_{ATP} channels [119], although there may be differences in the density of these receptors. For example, Tung et al have shown in the guinea pig that the density of K_{ATP} channels is significantly lower in atrial than ventricular tissue [120]. In view of these similarities in both the response to experimental ischaemia and the presence of relevant channels and receptors, it seems probable that human atrial and ventricular tissue will respond in a similar way to ischaemic preconditioning. Rather than the "true" ischaemia used in the

classic preconditioning, the present study used simulated ischaemia. There is a great deal of experimental evidence that simulated ischaemia and hypoxia are as effective as ischaemia in inducing preconditioning [121], [122], [123], [124].

2.2. Mitochondrial isolation

*2.1.1. **Principle***

Mitochondria can be prepared easily from animal tissues by a simple method of homogenisation followed by low (600 x g) and high speed (11,000 x g) centrifugation [125]. The final pellet represents a mitochondrial fraction that may be used as the basis for further experiments.

*2.1.2. **Methods***

Myocardial sections were diced to small proportions and incubated in cold buffer (10 mM Hepes buffer ph 7.5 containing 200 mM mannitol and 70 mM sucrose and 1 mM EGTA, and 0.25 mg/ml trypsin) for 20 minutes. The sections were then homogenized using tissue glass homogeniser (Kontes Glass Company, New Jersey, USA) in cold buffer without trypsin. The samples were then centrifuged for 10 minutes at 600 g. The supernatant containing mitochondrial fraction was centrifuged for 15 minutes at 11,000 g. The pellet was then re-suspended in ice cold storage buffer (10 mM Hepes pH 7.5 containing 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4 and 1 mM DTT). The samples were assayed for protein concentration using Biorad protein assay kit based on the Bradford dye-binding procedure [126]. The isolated mitochondria were then immediately used for further analysis.

- *Electron microscopy*

The presence of the isolated mitochondria in the preparation was established using scanning electron microscopy. Mitochondria were fixed in 4% gluteraldehyde in phosphate buffered saline for 24 hours and post fixed in 1% osmium tetroxide for 1 hour. The sample was then dehydrated in graduated alcohols, cleared in acetone, impregnated with and embedded in epoxy resin (TAAB Laboratories.) Ultra thin 90nm sections were cut with a diamond knife on a Reichert-Jung Ultra cut E ultra- microtome and mounted onto a copper grid. The sections were stained with a saturated solution of uranyl acetate in 40% alcohol for 10 minutes and Reynold's lead citrate for 5 minutes. The sections were examined on a Jeol 100CX electron microscope fitted with an AMT digital camera system (Figure 3).

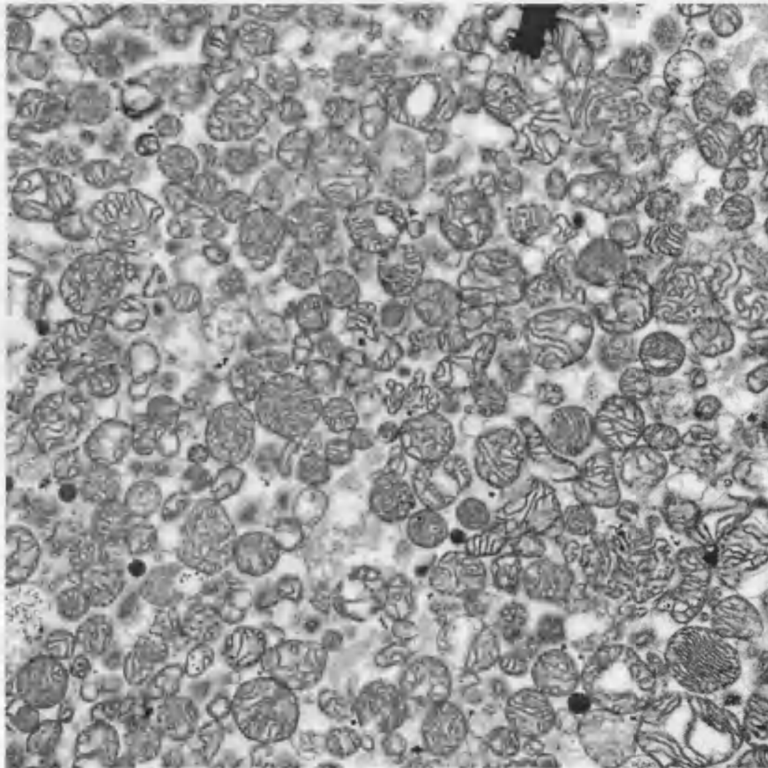


Figure 3: A scanning electron microscope image of the isolated mitochondria

- *Bio-Rad's protein assay*

Bio-Rad's protein assay, based on the Bradford dye-binding procedure [126] is a colorimetric assay for measuring total protein concentration.. Bio-Rad's protein assay is based on the colour change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. The dye binds to primarily basic (especially arginine) and aromatic amino acid residues. The assay is useful for measuring proteins and polypeptides with M.W greater than 3,000–5,000, depending on the charged groups.

Dilutions of a protein standard containing from 0.2 mg/ml to about 1.5 mg/ml protein of bovine human albumin was prepared. A standard curve was prepared each time the assay was performed. 5 μl of standards and samples were pipette into a clean, dry microtitre plate. 25 μl of reagent A and 200 μl reagent B were added into each well.

After 15 minutes, absorbance can be read at 750 nm.

- *Cytochrome c measurements*

Cytochrome c measurement was used to assess the quality of the isolated mitochondria. Cytochrome c oxidase is located on the inner mitochondrial membrane and it has been used as a marker for integrity of the outer membrane of the mitochondria [127], [125], [128], [129].

Two parallel aliquots of the mitochondrial suspension were diluted to 0.1 - 0.2 mg protein/ml with either 5 mM Tris-HCl buffer (cytochrome c oxidase activity in intact mitochondria) or

with the buffer containing the detergent 1 mM n-Dodecyl ß-Maltoside to lyse the mitochondria to release the total pool of cytochrome c oxidase (the total cytochrome c oxidase activity). Reduced ferrocytochrome c (0.22 mM) is then added to both samples. The decrease in absorbance of ferrocytochrome c measured at 550 nm caused by its oxidation to ferricytochrome c by cytochrome c oxidase is measured using spectrophotometry. The results, expressed as a percentage of (total cytochrome c activity - cytochrome c oxidase activity in intact mitochondria) to the total cytochrome c activity, show that more than 80% of the mitochondria were intact (Figure 4).

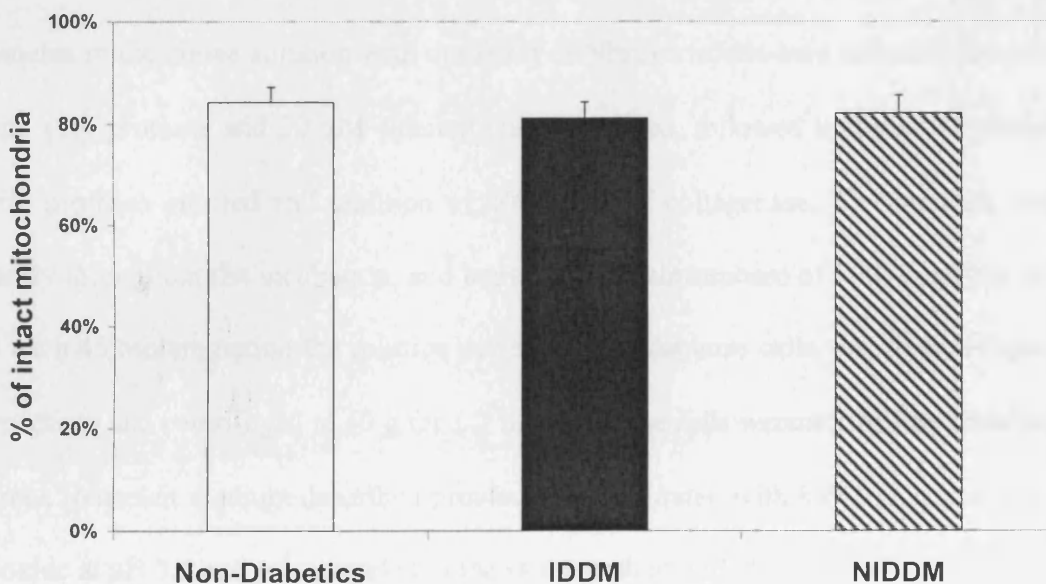


Figure (4): The percentage intact mitochondria obtained following mitochondria isolation. IDDM: insulin dependant diabetes mellitus, NIDDM: non-insulin dependant diabetes mellitus

2.3. Human myocytes isolation

*2.3.1. **Methods***

Human tissue was obtained from right atrial appendage removed during routine cardiac surgery and transported to the laboratory in 2-5 minutes in cold K-H buffer.

The atrial tissues 400-500 mg were chopped quickly into chunks of approximately 1 mm^3 using an array of razor blades. The chunks were incubated for a total of 12 minutes at 35 ^0C in 40 ml of a low calcium medium of the following composition (in mM): NaCl 120, KCl 5.4, $MgSo_4$, Pyruvate 5, Taurine 20, Glucose 20, Hepes 10, Nitrilotriacetic acid 5, pH 6.96, and containing 1-2 μM calcium chloride. The medium was changed 3 times during this period. The chunks were stirred by bubbling with 100% oxygen. The low calcium medium was removed by straining with 300 μm gauze. The chunks were then incubated at 35 ^0C for 45 minutes in the above solution with omission of Nitrilotriacetic acid and addition of 4 u/ml of type xxiv protease and 30 μM calcium chloride added, followed by two 45 minutes periods with protease omitted and addition of 400 iu/ml of collagenase. The medium was shaken gently throughout the incubation, and kept under an atmosphere of 100% oxygen. At the end of each 45 minute period the solution containing the disperse cells was filtered through a 300 μm gauze and centrifuged at 40 g for 1-2 minutes. The cells were washed by centrifugation in Krebs Henseleit medium described previously equilibrated with 95% oxygen and 5% carbon dioxide at pH 7.4 and re suspended in the same medium [130].

- *Myocytes viability*

Myocytes were placed in a perspex superfusion chamber mounted on the stage of a Nikon Diaphot inverted microscope and continuously superfused with Tyrode at a rate of 5 ml/minutes. Cells were viewed with the aid of a CCTV camera and continuously stimulated at 1 Hz by electrical field stimulation. Changes in cell morphology were assessed from video images and cells were classified as either contractile or non-contractile in response to stimulation (Figure 5).

Figure (5): High-resolution picture of an isolated myocytes from right human atrium.

2.4. Mitochondria membrane potential measurements

2.4.1. The use of JC-1 stain in isolated mitochondria

- **Principle**

Major advances in this field came with the development of fluorescent indicators, which could be localized into the mitochondrial compartment. One of most commonly used fluorescent indicators for measuring MMP either in single cells or in isolated mitochondria is carbocyanine JC-1. This compound accumulates in the mitochondrial matrix because of their charge and solubility in both the inner mitochondrial membrane and matrix space. In many cases, the distribution of the free dye across the inner membrane has been shown to follow the Nernst equation [131].

JC-1 emits light at red and green wavelengths according to its concentration: at high concentrations, J-aggregates form and emit red light, whereas at low concentrations the monomer form emits green light [132].

- **Methods**

The mitochondria suspensions were diluted to 1 mg protein per ml in 20 mM MOPS buffer pH 7.5 containing KCl (110 mM), ATP (10 mM), $MgCl_2$ (10 mM), sodium succinate (10 mM) and EGTA (1 mM). Changes in the mitochondrial membrane potential were then measured using the dye JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazol carbocyanine iodide) [133]. This fluorescent cationic molecule redistributes between the inside and outside the mitochondrial matrix according to the membrane potential across the separating membranes. When excited at 490 nm, the fluorophore JC-1 has the property that the emission spectrum shift will be dependent on the concentration of the molecule. JC-1

stain (final concentration 0.3 μM) then added to 2 ml of diluted mitochondria suspension (approx 10- 50 μg) in 20 mM MOPS buffer pH 7.5. The fluorescence of the sample was then read at excitation wavelength of 490 nm and emission wavelength of 590 nm using spectrofluorimeter (Fluostar, BMG Labtech, Offenburg, Germany).

2.4.2. The use of TMRE staining in isolated myocytes

- **Principle**

Mitochondrial membrane potential was measured using the mitochondrial membrane potential-sensitive dye tetramethyl-rhodamine ethyl ester (TMRE). TMRE fluorescence was excited at 475 nm and images collected at >510 nm. TMRE is positively charged and partitions between mitochondria and cytoplasm. Under the loading conditions used in my studies, TMRE accumulates in mitochondria because of the negative potential of the matrix relative to the cytoplasm, where it causes auto quenching of fluorescence.25, 26 Mitochondrial depolarisation leads to redistribution of TMRE from mitochondria to cytoplasm, resulting in a decrease in quenching and an increase in the whole-cell fluorescence signal. The potential across the plasma membrane prevents significant loss of TMRE from the cell.

- **Methods**

Cells were loaded at room temperature (20°C) with the TMRE (5 μm) for 10 minutes. To limit the photo damage that can be caused when TMRE is used, the light exposure was minimised by reducing light intensity as far as possible with an iris and limiting the exposure time to 33 ms at 0.2 Hz. Experiments were carried out in a light-proof chamber that resulted in very low background fluorescence. The imaging system enabled the fluorescence signal

from several cells in the field of view (usually 3–5 myocytes) to be measured independently and simultaneously and also allowed regions of interest to be set which included the whole of each cell throughout the experiment [134].

- **Discussion**

Several criteria must be met for these probes to be useful in the estimation of MMP: they should not cause loss of mitochondrial function and/or depletion of MMP and the compound must be easily detected to estimate the distribution across the inner membrane. Mathur et al. have compared and re-evaluated the use of the dyes in neonatal cardiomyocytes using confocal microscopy of individual cells, and, for the first time, flow cytometry of cell cultures [82]. The study of MMP by flow cytometry has several advantages over other techniques. In particular, it allows the analysis of heterogeneous cell populations and is amenable to multiparametric measurements. Mathur *et al.* concluded that JC-1 is the best dye for detecting c hanges in MMP; however, they questioned the use of JC-1 as a ratiometric indicator, and found that much information could be gained by studying changes in the fluorescence of the individual wavelengths.

2.5. ROS Measurements

2.5.1. Hydrogen peroxide/hydroxyl radicals measurements

- **Principle**

Hydrogen peroxide and hydroxyl radicals produced by mitochondria causes oxidation of 2, 7-dichlorofluorescein (DCFH) diacetate, yielding the fluorescent product dichlorofluorescein (DCF) (λ ex 500nm– λ em 520nm). The dichlorofluorescein (DCFH) diacetate (i.e. the acetyled reduced form) derivative is permeable and diffuses into mitochondria, but once internalised, it is cleaved by intracellular esterases [135]. Oxidation of the reduced dyes can then occur in the presence of hydrogen peroxide and hydroxyl radicals, causing the dyes to fluoresce, however the oxidation of DCFH may not easily discriminate between the various reactive oxygen species. Chloromethyl -dichlorodihydrofluorescein diacetate, (CM-H_2DCFDA) is another derivative of dichlorofluorescein but it is better retained within the cell or mitochondria because its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols [136].

- **Methods**

Mitochondria were isolated from non-diabetics, IDDM and NIDDM according to the method described above.

- Using hydrogen peroxide (500 μM) as a positive control, mitochondrial aliquots (0.1-0.2 mg protein /ml) were loaded with 1 μM of DCFH (Sigma; St. Louis, MO). DCF fluorescence was detected in a 10 mM Hepes buffer pH 7.5 containing the mitochondrial suspension. The fluorescence was measured using a spectrofluorimeter (Fluostar, BMG Labtech, Offenburg, Germany) at an excitation and emission wavelengths of 500 and

530 nm, respectively. DCF fluorescence was normalized to maximal values obtained by exposing mitochondrial suspension to 500 μM hydrogen peroxide.

- Using hydroxyl radicals generating system consisting of xanthine/ xanthine oxidase (25μm/0.01 U per ml) and ferrous ammonium sulphate (100 μM) as a positive control, mitochondria aliquots isolated from diabetics and non-diabetic's atrial tissue were loaded with CM-H2DCFDA to final concentration of 1 μM. Mercaptopropionylglycine (MPG) (2.5 mM) a specific hydroxyl scavenger was used as negative control. The fluorescence was read at excitation of 504 and emission 530 nm. The results were normalized to that of positive control.

*2.5.2. **Superoxide production measurements***

- **Principle**

The measurement of superoxide can be achieved with the use of chemiluminescence resulting from energy transfer between molecules during redox reactions. Lucigenin have been used extensively as chemiluminescent substrates in various cellular systems. After reduction of these compounds, a reaction with superoxide leads to an unstable intermediate which emits light during the spontaneous decomposition to its ground-state electronic configuration [137]. Relatively high concentrations of lucigenin (~100 μM) are required to obtain fluorescent staining; however, low concentrations reportedly yield a chemiluminescence response to stimulated superoxide generation within the mitochondria [138]. Such a method is extremely sensitive and has been used to measure superoxide in intact cells, isolated mitochondria and submitochondrial particles [138], [139], [140], [141].

- **Methods**

Superoxide generation in isolated mitochondria was detected using lucigenin chemiluminescence. Mitochondria were isolated from diabetics and non-diabetic myocardium. Mitochondrial samples (1-2 mg/ ml) were then loaded with chemiluminescence lucigenin (15 μM). Production of superoxide was measured using luminometry (Microlumat Plus LB 96 V, Berthold technologies, Bad wildbad, Germany). The peak relative luminescence per second (RLU/s) was used as the index of the superoxide production measurements. The results were normalized to that of positive control.

2.6. Western Blotting

- **Principle**

PKC immunoblotting represents a valuable alternative to biochemical assessments. This technique utilizes electrical current to isolate proteins present in processed tissue samples and/or sub cellular fractions, and to transfer them (i.e. to blot) to nitrocellulose filter, or nylon membrane. Non-specific sites of blotted proteins are blocked with milk proteins, and primary antibodies against individual PKC isoforms are then added. After incubation and washing, all proteins blotted and tagged with primary antibody are incubated with secondary horseradish peroxidase tagged antibody, allowing the investigator to perform qualitative and/or semi quantitative analysis of the immunological reaction between individual PKC isoforms and the primary antibody. The bound antibody can be detected by reading the luminescence produced after the oxidation of luminol by horseradish peroxidase (ECL Immunoassay Signal Reagent, Amersham, Arlington Heights, IL).

- **Methods**

At the end of each protocol, the tissue sections were quickly frozen in liquid nitrogen and stored at -80°C until analysis. Samples were homogenized in RIPA buffer containing protease inhibitor, PMSF (1 mM), DDT (0.5 mM), Glycerophosphate (25μM), sodium orthovanadate (1 mM). The homogenate was then centrifuged at 10000 g for 30 minutes. The supernatant obtained was analysed for protein concentration using the Bio-Rad protein assay kit.

Then 20 μg of the tissue supernatant were elecrophoresed on 10% SDS/ PAGE and blotted onto nitrocellulose membrane. Membranes were blocked in TBS buffer containing 5% fat free dry milk, followed by incubation with the appropriate primary antibodies at 1 in 1000 dilution overnight at 4°C. Membranes were then detected by the appropriate HRP-conjugated secondary antibody (anti rabbit goat antibody and anti-mouse goat antibody at 1 in 5000 dilution). The signals were then developed using an ECL western blotting detection kit (Amersham; Buckinghamshire, UK). Densitometry analysis of bands was performed by Scion image ™ (Scion, Frederick, MD, USA).

Chapter 3

Preconditioning Of The Diabetic Myocardium

3.1. Introduction

To date the phenomenon of ischaemic preconditioning has been extensively studied in healthy hearts, however the occurrence of IPC in diseased hearts such as diabetics is less known. Conflicting evidence on preconditioning in diabetics has been reported in the literature with some studies demonstrating failure of preconditioning in diabetics [5], [142], [25]; whilst others have reported that diabetic myocardium may benefit more from ischaemic preconditioning than normal myocardium [27].

Several reasons have been suggested to explain the failure to precondition the diabetic myocardium including the effect of anti-diabetic medication [41], altered mitoK_{ATP} channels function [143] and sarcolemmal K_{ATP} channel dysfunction [144], [145]. Potassium sensitive ATP channel function, an integral part of ischaemic preconditioning, has been reported to be altered in diabetic rat ventricular myocytes [29].

Aims: In these studies, my aims were to investigate the phenomenon of ischaemic preconditioning in diabetics and dissect the signal transduction pathway of preconditioning in non-diabetics and in diabetic human myocardium.

3.2. Materials And Methods

3.2.1. ***Study subjects***

The study was conducted with patient consent and approved by the local ethics committee in accordance with the Helsinki declaration. Atrial biopsies were collected from patients undergoing elective coronary bypass surgery and/or aortic valve surgery prior to the initiation of cardiopulmonary bypass. Three groups of patients were included in the study, non-diabetics, IDDM, and NIDDM. Patients with atrial fibrillation, poor left ventricular function (EF<30%) and right ventricular failure were excluded from the study. Patients on mitoK_{ATP} channels openers, oral opioid or catecholemines were also excluded.

3.2.2. ***Processing of samples and experimental preparation***

The study was conducted using an established model described previously (section 2.1.2)

3.2.3. ***Measurement of tissue injury and viability***

End point measurements: CK leakage and MTT reduction were measured to assess tissue injury and viability, respectively, as described in section 2.1.2.

3.2.4. ***Study protocols***

3.2.4.1. *To elucidate whether the intensity of the IPC stimulus influences cardioprotection in the diabetic myocardium.*

Myocardial sections obtained from diabetic patients were randomized to one of the following protocols (n=6 per group): (i) aerobic control, (ii) simulated ischaemia for 90 minutes and then reoxygenation for another 120 minutes, (iii) one or three cycles of IPC (5 minutes ischaemia followed by 5 minutes reoxygenation) followed by a period of 90 minutes

ischaemia and 120 minutes of reoxygenation. Tissue viability and damage were assessed by CK leakage and reduction of MTT at the end of the experimental protocol.

3.2.4.2. *To investigate whether alterations in the signal transduction pathways of preconditioning are responsible for the failure to protect the diabetic myocardium.*

Myocardial tissue was obtained from patients without diabetes, with IDDM, and with NIDDM on glibenclamide or on metformin. Right atrial appendages from these groups were randomly assigned (using the uniform discrete distribution, MiniTab Corp.) to one of the following protocols (n=8 each group): (i) aerobic perfusion, (ii) simulated ischaemia for 90 minutes then reoxygenation for another 120 minutes and (iii) IPC or pharmacological preconditioning. Pharmacological preconditioning was induced by phenylephrine (0.1μM), adenosine (100μM), diazoxide (mitoK_{ATP} channel opener at 100μM), PMA (protein kinase C agonist at 1μm) or anisomycin (p38 MAP kinase activator at 1nM) each administered for 10 minutes as shown in the protocols below (Figure 6). The concentrations used were found to optimally protective in our laboratory using an identical experimental model.

30 min	10 min	90 min	120 min
Aerobic perfusion			
Equilibration		Simulated ischaemia	Reoxygenation
Equilibration	IPC (5 min SI/5 min R)	Simulated ischaemia	Reoxygenation
Equilibration	Adenosine (100 μM)	Simulated ischaemia	Reoxygenation
Equilibration	Phenylephrine (0.1 μM)	Simulated ischaemia	Reoxygenation
Equilibration	Diazoxide (100 μM)	Simulated ischaemia	Reoxygenation
Equilibration	PMA (1 μM)	Simulated ischaemia	Reoxygenation
Equilibration	Anisomycin (1 n M)	Simulated ischaemia	Reoxygenation

Figure (6): Protocol to investigate the signal transduction pathway of preconditioning in the non-diabetics and diabetic myocardium

3.2.4.3. *Studies to investigate the effect of metformin on tissue injury and viability of human myocardium*

Right atrial appendages from non-diabetic patients (n=6) were randomised to receive one of the following protocols: aerobic perfusion, simulated ischaemia-reoxygenation and ischaemic preconditioning, metformin for 10 minutes followed by SI/R. Doses selected were based on the blood concentration of therapeutic doses of metformin used to treat NIDDM. At the end of each protocol CK leakage and MTT reduction was measured.

3.2.5. Materials

The incubation medium was prepared daily with de-ionised distilled water and contained (in mM): NaCl (118), KCl (4.8), $NaHCO_3$ (27.2), $MgCl_2$ (1.2), KH_2PO_4 (1.0) $CaCl_2$ (1.25), HEPES (20) and D-glucose (5) or 2-deoxy-D-glucose (5). The aerobic medium (pH 7.4) was bubbled with 95% oxygen and 5% carbon dioxide. Simulated ischaemia was induced by bubbling the medium with 95% N_2/5% CO_2 (pH 6.8–7.0). All the chemicals including diazoxide, adenosine, phenylephrine, PMA and anisomycin were purchased from Sigma Chemicals.

3.2.6. Statistical analysis

All data are presented as mean ± SEM. Mean values were analysed by ANOVA and paired Student's *t*-test where appropriate. Statistical significance was taken at $P < 0.05$.

3.3. Results

3.3.1. The effect of increasing the intensity of the IPC stimulus in the diabetic myocardium:

As shown in Figure 7, IPC of diabetic myocardium with one cycle did not result in the reduction in CK leakage or in increase of MTT values when compared to those seen with SI/R alone. Increasing the intensity of the IPC stimulus to 3 cycles did not significantly alter the CK and MTT mean values obtained with SI/R alone. These results suggest that the failure to precondition the diabetic myocardium does not depend on the intensity of the IPC stimulus.

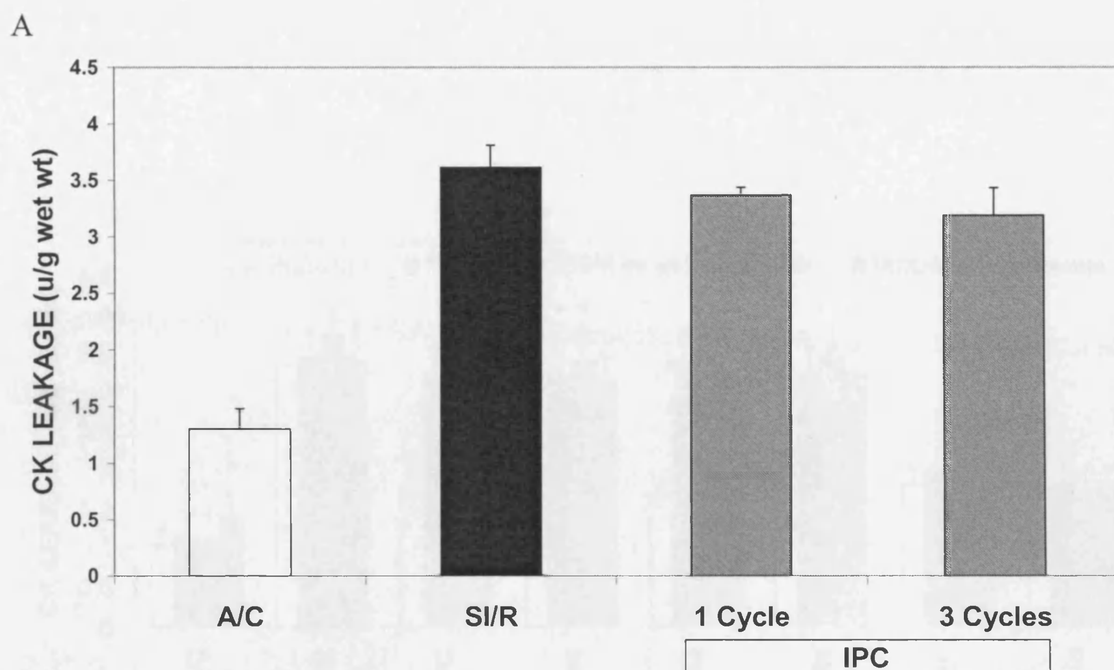
3.3.2. *Identification of the alteration in the signal transduction pathway of preconditioning responsible for the failure to protect diabetic myocardium:*

Figure 8A and 8B show that SI/R induces similar degrees of injury, as assessed by the CK leakage and MTT reduction, in the non-diabetic and diabetics groups, and that whereas IPC protected the non-diabetic group it was ineffective in all forms of diabetes. Similarly preconditioning with phenylephrine and adenosine resulted in equivalent protection to that of IPC in the groups without diabetes but failed to protect the diabetic groups. Importantly, the mitoK_{ATP} channel opener diazoxide induced protection in non-diabetics and did not protect IDDM and NIDDM cardiac tissue, however, the PKC activator PMA and the P38 MAP kinase activator anisomycin were equally protective in the diabetic and non-diabetic groups, a benefit that was similar to that obtained with IPC. The results in the IDDM groups were identical to those in the NIDDM groups independently of whether they were treated with glibenclamide or metformin. These results suggest that a dysfunction in the mitoK_{ATP} channels may be responsible for the failure to precondition the diabetic myocardium; however cardioprotection can still be obtained by the activation of PKC and P38 MAPK. The results also suggest that PKC and P38 MAPK are downstream of mitoK_{ATP} channels in the signal transduction pathway of preconditioning, thus confirming previous results obtained in this laboratory in non-diabetic myocardium [63].

3.3.3. *Effect of metformin on ischaemic preconditioning*

Figure 9 shows that SI/R resulted in significant injury in all tissue slices. It also shows that IPC alone was cardioprotective and that metformin at any of the concentrations used during IPC did not block cardioprotection induced by IPC and did not cause tissue damage as compared to SI/R

A



B

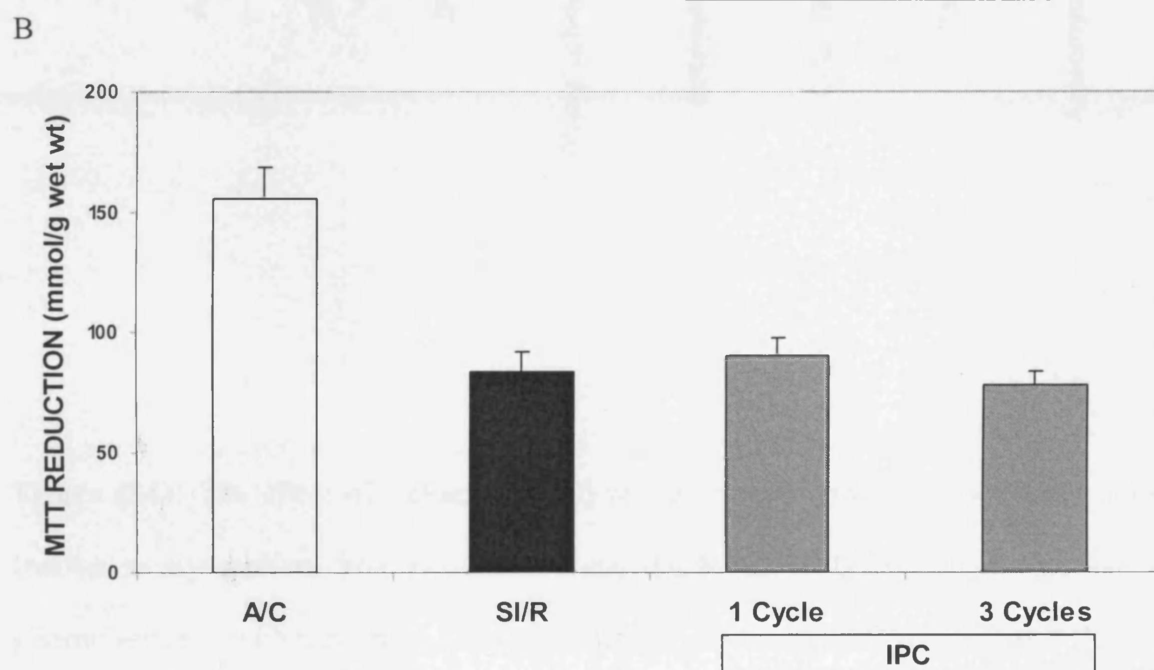


Figure (7): The effect of one and 3 cycles of ischaemic preconditioning (IPC) on CK leakage (A) and MTT reduction (B) in the diabetic myocardium. Data are expressed as mean ± SEM of n=6/group. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

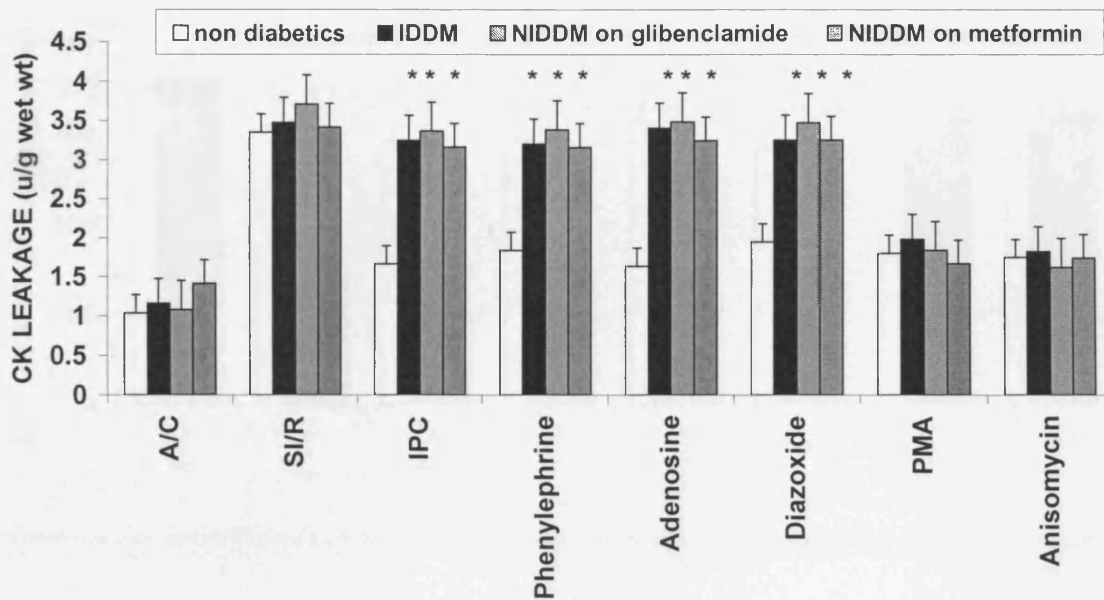


Figure (8A): The effect of ischaemic (IPC) and pharmacological preconditioning on CK leakage in myocardium from non-diabetic and IDDM and NIDDM subjects treated with glibenclamide or with metformin. Data are expressed as mean ± SEM of n=6/group. *P< 0.05 vs. IPC in non-diabetics.

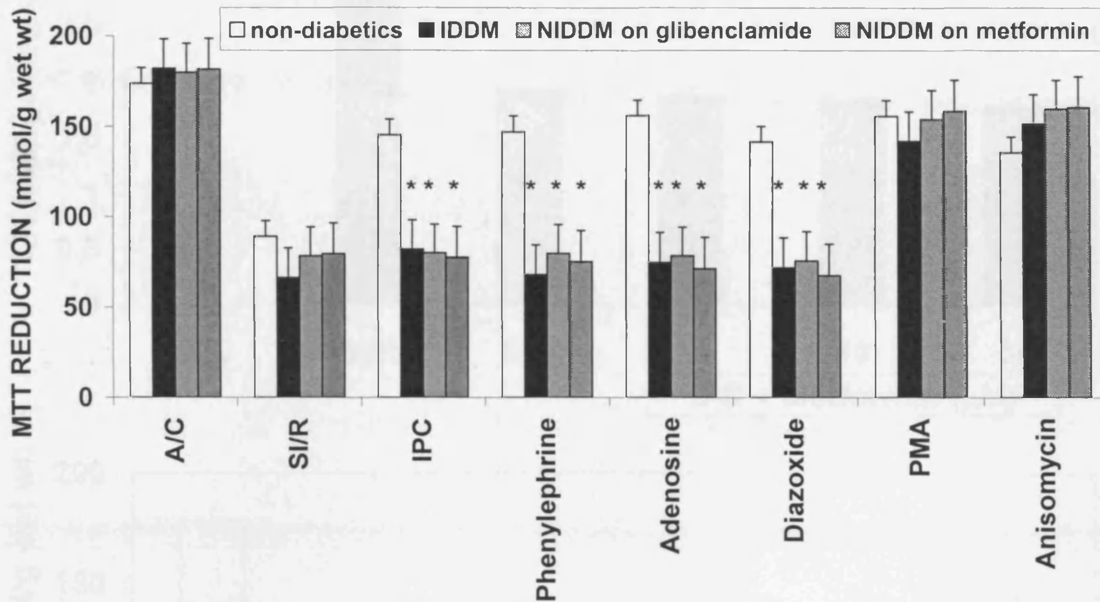


Figure (8B): The effect of ischaemic (IPC) and pharmacological preconditioning on the MTT reduction in myocardium from non-diabetic and IDDM and NIDDM subjects treated with glibenclamide or with metformin. Data are expressed as mean ± SEM of n=6/group. *P< 0.05 vs. IPC in non-diabetics.

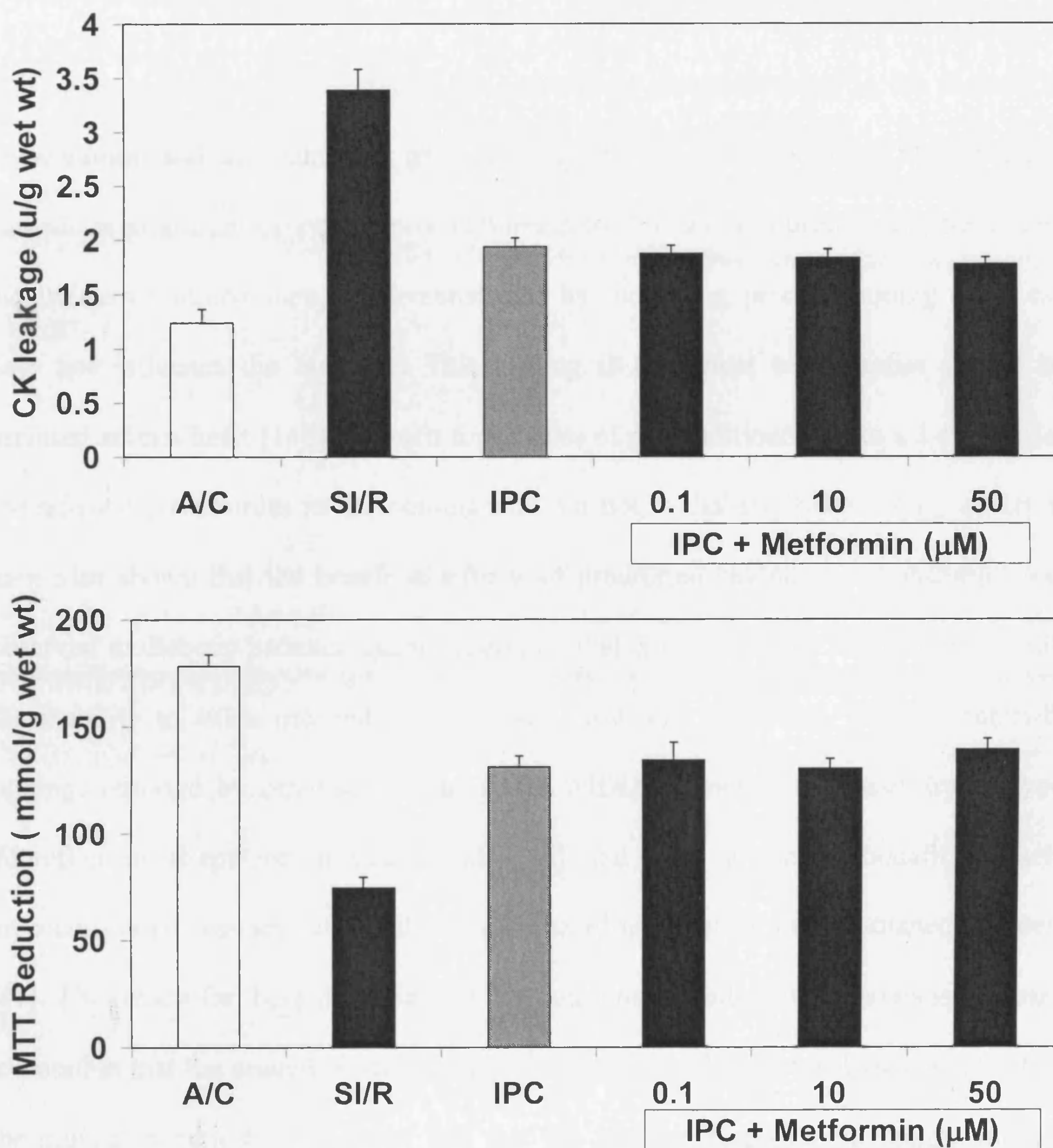


Figure 9: The effect of metformin added 10 minutes during IPC on CK leakage (A) and tissue viability MTT (B) in non-diabetic myocardium. Data are expressed as mean ± SEM of n=6/group. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

3.4. Discussion

This study is the first to investigate the pathway of preconditioning in the diabetic human myocardium, and also confirms an earlier report from this laboratory[5] that the human diabetic myocardium cannot be preconditioned by IPC alone. Furthermore, the intensity of the ischaemic intervention, as demonstrated by increasing preconditioning cycle number, does not influence the outcome. This finding is consistent with studies in the isolated perfused animal heart [146] in which four cycles of preconditioning reduced the incidence of re-perfusion arrhythmias in the non-diabetic but not in diabetic hearts. Angioplasty studies have also shown that the beneficial effects of prodromal angina in non-diabetics were not observed in diabetic patients, again suggesting that diabetes per se might be responsible for the inability to attain preconditioning [142]. However, the above findings contradict the findings reported by other studies in which NIDDM protects the heart from myocardial infarction in streptozotocin-treated rats [26] and even a greater benefit of ischaemic preconditioning was seen in the diabetic myocardium than in the non-diabetic myocardium [41]. The reason for these discrepancies between human and animal studies is unclear, but it is possible that the animal model of streptozocin- induced diabetes does not reflect entirely the clinical condition of diabetes and that the variable responses in animal models may depend on the duration of the pharmacologically-induced diabetes [147].

It is presently unclear whether the failure to precondition the diabetic hearts arises as a result of anti diabetic treatment or due to diabetes as a disease. A recent study has shown that human myocardium from patients with diabetes on long-term oral hypoglycaemic drugs intake blocks the protection conferred by preconditioning [41]. In addition glibenclamide administered before or immediately after preconditioning completely abolished the protective effects in barbital-anaesthetized open-chest dogs [148]. Therefore anti-diabetic medication

itself can affect the protection against ischaemic injury and this may be difficult to separate from the effect of diabetes. In the present studies, the myocardium from diabetics on insulin, on glibenclamide and on metformin, a drug that is not known to affect mitoK_{ATP} channels were investigated. Metformin on its on own in non-diabetics did not affect ischaemic/ reperfusion injury and did not abolish the protection of ischaemic preconditioning (Figure 9). These results are in contrast with other studies which have reported that in fact metformin can be cardioprotective by improving myocardial energy metabolism during myocardial ischaemia in the isolated rabbit heart [149].

I have shown in this study the inability of the diabetic myocardium to be protected by ischaemic preconditioning in patients receiving insulin, glibenclamide or metformin suggesting that diabetes per se is the cause of the failure to cardio-protect the myocardium. This hypothesis is supported by the finding that in patients undergoing coronary angioplasty, glimepiride-treated diabetics could not be cardio-protected by ischaemic preconditioning whereas glimepiride did not block cardioprotection in non-diabetics [150]. Other studies have reported that insulin is cardioprotective [151] and that insulin restores cardioprotection that was blocked by the induction of diabetes in the rabbit heart *in vivo* [152]. This suggests that the reason for the inability to cardio-protect the diabetic heart is not due to insulin and should be attributed to alteration of other endogenous factors. The inability to precondition the diabetic myocardium with diazoxide has lead me to therefore propose that the failure to precondition diabetic tissue may be due to a dysfunction in the mitochondrial K_{ATP} channels. This interpretation is in agreement with the studies on infarct size conducted on diabetic dogs [143] in which diazoxide failed to protect the diabetic myocardium, an observation that was attributed to hyperglycaemia. In the model used in my studies, the tissue from diabetics and non-diabetics was perfused with identical glucose concentration so the inability to precondition the diabetics was not due to hyperglycaemia. It is of interest to note that Del

Valle et al [145] have suggested that sarco-K_{ATP} channel dysfunction in diabetic hearts is the cause of failure to precondition. However the involvement of mitoK_{ATP} and sarcoK_{ATP} channels in ischaemic preconditioning is the subject of controversy.

An important finding of my studies with clinical implications is that although diabetic myocardium cannot be preconditioned by activation of mitoK_{ATP} channels, it can be preconditioned by the activation of PKC or P38 MAPK, however, since both PKC and P38 MAPK have several isoforms and are involved in various biological processes, it would not be possible to use non-specific activators, similar to those utilised in the present studies, for clinical purposes. Because of this it will be important to identify the specific PKC and P38 MAPK isoforms involved in cardioprotection and to develops specific agents that would activate the relevant isoforms in order to elicit cardioprotection alone and spare other unwanted biological effects.

Having demonstrated that the cause of the failure to precondition the diabetic myocardium is a dysfunction of the mitochondria, my next aim was to investigate whether the reason of this is an alteration in the response of the mitochondrial membrane potential.

Chapter 4

Mitochondrial Membrane Potential And Cardioprotection

4.1. Introduction

The cardioprotective effect of diazoxide is well documented in various animal models of ischaemia-reperfusion situations [77], [153], [154]. The mechanism and extent by which mitochondrial K_{ATP} channels could contribute to cardiac protection against ischaemia is not clear. Work with isolated cardiac mitochondria has shown that putative mitochondrial K_{ATP} channel openers depolarise the inner membrane, which could stimulate respiration and promote Ca^{2+} efflux from the matrix [155]. It has been postulated that depolarization of the inner mitochondrial membrane by activation of K_{ATP} channels, or other means, may protect these organelles from the deleterious effects of Ca^{2+} overload, which include the formation of the mitochondrial permeability transition pore[155]. The results from the previous chapter suggest that a dysfunction of the mitochondria is the cause of the failure to precondition the diabetic myocardium. Therefore, in this chapter my aim was to investigate whether alteration in mitochondria membrane potential are the reason for the altered response in diabetics. To achieve this, the specific mitochondrial K_{ATP} channel opener diazoxide was used in isolated mitochondria and isolated myocytes from human diabetic and non-diabetic myocardium.

4.2. Materials And Methods

4.2.1. ***Study subjects***

The study was conducted with patient consent and approved by the local ethics committee in accordance with the Helsinki declaration. Atrial biopsies were collected from patients undergoing elective coronary bypass surgery and/or aortic valve surgery prior to the initiation of cardiopulmonary bypass. Three groups of patients were included in the study, non-diabetics, IDDM, and NIDDM. Patients with atrial fibrillation, poor left ventricular function (EF<30%) and right ventricular failure were excluded from the study. Patients on mitoK_{ATP} channels openers, oral opioid or catecholemines were also excluded.

4.2.2. ***Processing of samples and experimental preparation***

Isolation of the mitochondria and myocytes was described previously (Section 2.2.2 and section 2.3.1).

4.2.3. ***End point measurements***

At the end of the experimental protocols, mitochondrial membrane potential was measured as previously described (section 2.4.1).

*4.2.4. **Study protocols***

4.2.4.1. *To investigate the effect of mitoK_{ATP} channel opening on the mitochondrial membrane potential of isolated myocytes from **non-diabetic tissues***

- Isolated myocytes were randomized to receive either 100 μM of diazoxide treatment or carbonyl cyanide m-chlorophenylhydrazone (CCCP, 1 μM). Cell were loaded with 5 μM of TMRE as described previously (section 2.4.2) and the fluorescence signal from several cells in the field of view was recorded. TMRE fluorescence were normalised to the initial fluorescence intensity recorded in normal Tyrode and are expressed as the relative fluorescence.

4.2.4.2. *To investigate the effect of mitoK_{ATP} channel opening on the mitochondrial membrane potential of isolated mitochondria from **diabetic and non-diabetic tissues***

Isolated mitochondria were diluted to 1 mg protein per ml in 20 mM MOPS buffer pH 7.5 as described previously. Aliquots of mitochondria isolated from non-diabetics and diabetics, both insulin dependent and NIDDM, were randomized to receive one of the following protocols for 10 minutes at 30 ^{0}C: (i) diazoxide (100 μM in DMSO); (ii) carrier alone (DMSO x 1%), (iii) or CCCP (1 μM), an uncoupler of the mitochondrial electron transport chain, as positive control. Mitochondria were loaded with JC-1 stain as described previously. The fluorescence of each sample was then read at excitation wavelength of 490 nm and

emission wavelength of 590 nm using spectrofluorimeter. The MMP mean values were expressed as a percentage of the control (sample alone without diazoxide treatment).

4.2.5. ***Materials***

Tyrode solution for myocytes preparations and K-H solution for isolated mitochondria were prepared daily. The chemicals diazoxide at 100 μM, CCCP at 1 μM (carbonylcyanide m-chlorophenylhydrazone), JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazol carbocyanine iodide) at 0.3 μM and TMRE stain at 5μM were used.

4.2.6. ***Statistical analysis***

All data are presented as mean ± SEM. Mean values were analysed by ANOVA and paired student's *t*-test where appropriate. Statistical significance was taken at $P < 0.05$.

4.3. Results

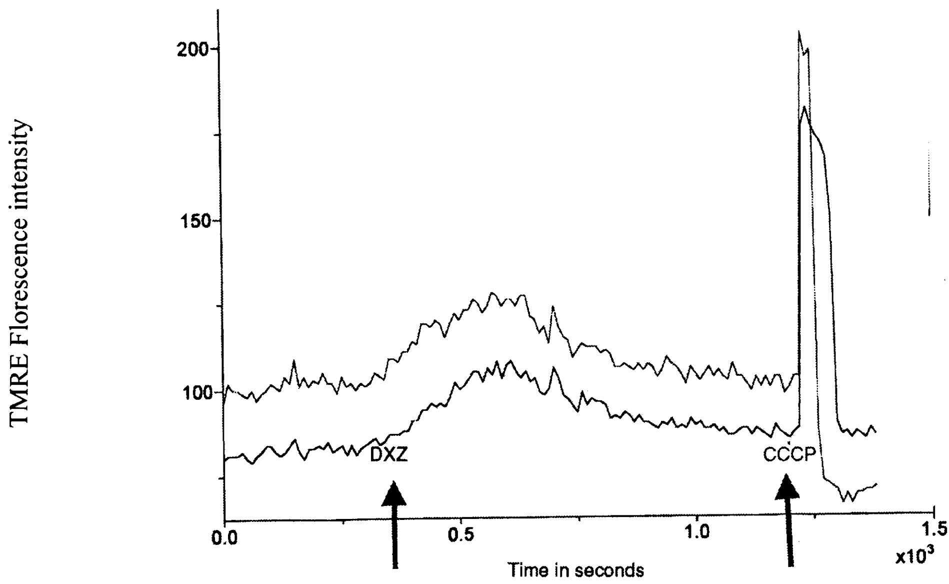
4.3.1. ***Detection of mitochondrial membrane depolarization in isolated myocytes***

In isolated myocytes from non-diabetic myocardium, 3 myocytes showed evidence of membrane depolarization and this response was reversed when myocytes were washed with substrate free Tyrode while 3 other myocytes did not show any response to diazoxide. All myocytes showed evidence of irreversible depolarization in response to CCCP (Figure 10). Because of the inconsistency obtained with the myocytes preparation, this preparation was abandoned; instead I used the isolated mitochondria preparation to measure MMP.

4.3.2. ***The effect of diabetes on mitochondrial membrane depolarization by diazoxide:***

The mitochondria membrane potential as detected by the fluorescence of JC-1 dye was decreased significantly by diazoxide in the non-diabetics but not in the IDDM and NIDDM groups, and as expected the mitochondrial electron chain uncoupler CCCP fully depolarised the mitochondria in all 3 groups (Figure 11).

A:



B:

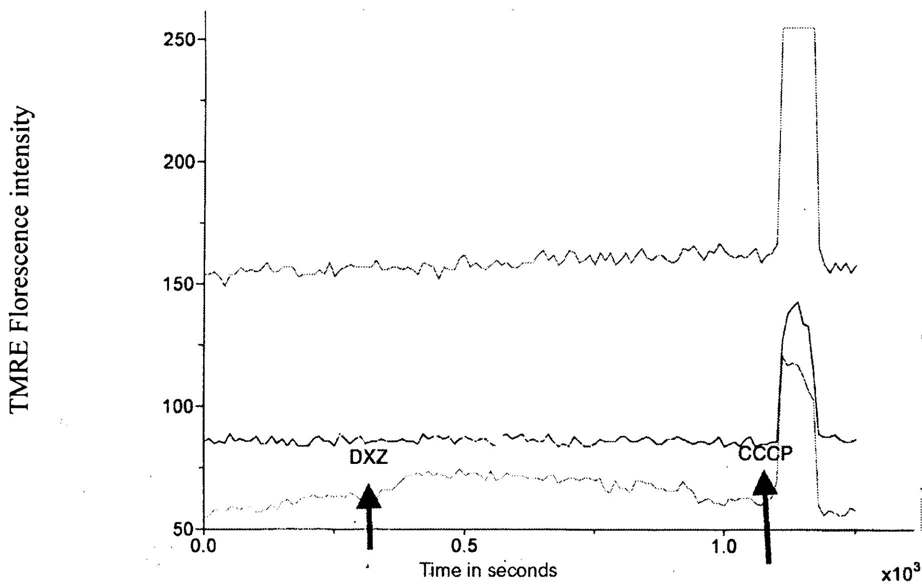


Figure (10): Diazoxide (100 μM) caused partial depolarisation of MMP in (A), whereas in (B) diazoxide had no effect. CCCP (1 μM) was used as a positive control and resulted in an increase in TMRE fluorescence. This inconsistency in the results leads us to abandon measurement of MMP in isolated myocytes

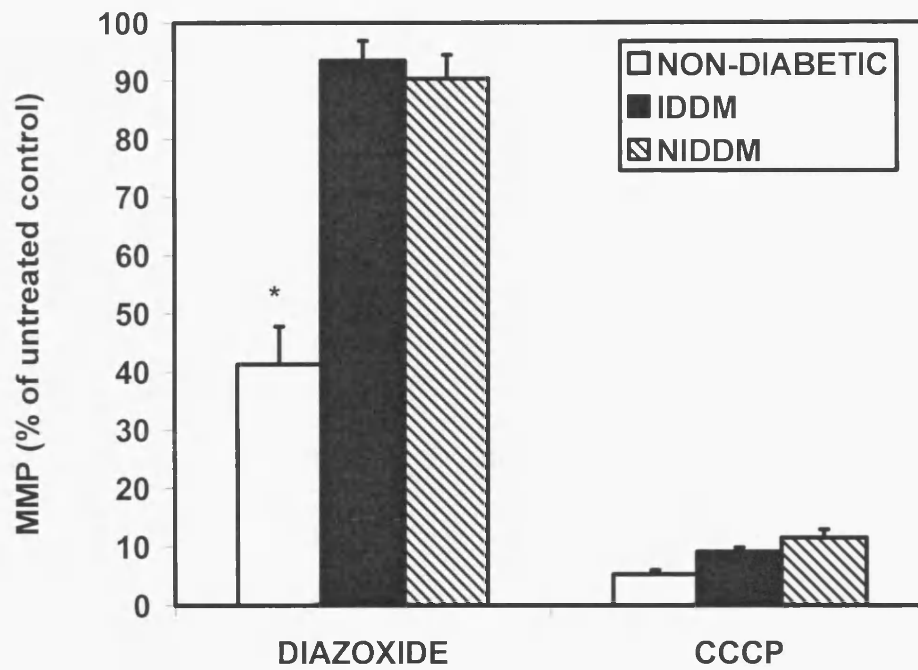


Figure 11: The effect of diazoxide on mitochondria membrane depolarization in non-diabetics, IDDM, NIDDM. Data expressed as a percentage of control (untreated groups) ± SEM of n=8 per group. *P< 0.05 vs. control. CCCP is known mitochondria uncoupler, used as a positive control.

4.4. Discussion

In order to investigate how the opening of mitoK_{ATP} channels is cardioprotective in the non-diabetics myocardium but fails to protect the myocardium in the diabetics, I have examined in this chapter the effect of diazoxide on mitochondria membrane potential in both diabetic and non-diabetic tissue. It is well known that the maintenance of MMP is essential for ATP synthesis in normal cell function. In this thesis and other investigators before me have demonstrated that diazoxide caused preconditioning in non-diabetic myocardium. In the isolated myocytes preparation, I observed depolarization of some myocytes whilst others were not responsive. Previously it was reported that in isolated rat ventricular myocytes, diazoxide at concentrations that are highly cardioprotective (100, 200 μM), causes no detectable increase in TMRE fluorescence [156]. The inconsistency in my results obtained with isolated myocytes could be related to the quality of the isolation and also to the metabolic state of these isolated cells. However, using isolated mitochondria preparation, I have clearly demonstrated that diazoxide causes partial depolarization of the mitochondria membrane potential in the non-diabetics. Therefore one could hypothesize that modest mitochondrial uncoupling may be a critical cellular event in orchestrating preconditioning. Indeed it has been shown that short-term administration of 2, 4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation and cyclosporin A, an inhibitor of mitochondrial respiration, trigger preconditioning-like cardioprotection in the isolated rat heart [157]. By contrast, trimetazidine, a known mitochondrial 'protector', attenuated both drug-induced and ischaemic preconditioning [157]. These data support the hypothesis that modulation of mitochondrial homeostasis may be a common downstream cellular event linking different triggers of preconditioning.

My results are in agreement to those reported in the literature where it has been shown in rat liver mitochondria that the potassium channel opener RP 66471 induces depolarization of the

mitochondrial membrane [158]. Furthermore, Terzic's group has shown in isolated cardiac mitochondria, that the K_{ATP} channel openers cromakalim and pinacidil, depolarise the mitochondrial membrane, accelerate respiration, slow ATP production, release accumulated Ca2+, produce swelling, and stimulated efflux of intermembrane proteins [159], [160]. Other workers have suggested that the primary effect of K+ flux through mitoK_{ATP} channels is to regulate mitochondrial volume and that reported changes in membrane depolarisation and Ca2+ uptake are in fact due to very high drug doses [161]. The hypothesis that mitochondria membrane potential partial depolarization is needed for preconditioning is however contradicted by observation that diazoxide attenuates the loss of MMP seen during ischaemia/reperfusion, an action that was accompanied by remarkable recovery of ATP and absence of calcium accumulation in mitochondria [162] .

I have demonstrated in the previous chapter that diazoxide failed to precondition the diabetic myocardium. In the present study I have also shown that diazoxide failed to partially depolarise the mitochondria in the diabetic myocardium which may result in impaired ATP synthesis. Taken together, it can be concluded that the unresponsiveness of the mitoK_{ATP} channel to partial depolarisation upon activation may be the cause of the failure to precondition the diabetic myocardium. The effect of diazoxide on the mitochondrial membrane potential in diabetic myocardium is not previously reported and the reason that diazoxide depolarises MMP in non-diabetics but not in the diabetic is unknown and will require further investigation. It should be noted that other studies have shown that the MMP of diabetic myocytes was slightly but significantly decreased from non-diabetic controls [163] and also that the MMP in liver mitochondria from chronic diabetic rats is lower than in normal mitochondria [164]. This may suggest that the MMP is already depolarised in diabetics and that therefore diazoxide cannot act as a trigger for preconditioning. Of interest

is that CCCP, which is known mitochondria uncoupler, has a similar effect in diabetics and non-diabetics.

The concept of the existence of mitoK_{ATP} remains controversial with some authors suggesting that the sulfonylurea-inhibitable mitoK_{ATP} channel may not exist [165], whereas others have reported the existence of native cardiac mitoK_{ATP} channels based on pharmacological and histochemical studies [166], [167]. The specificity of diazoxide has also been questioned with diazoxide being inferred to activate the sarcolemmal, not the mitochondrial K_{ATP} in mice and providing cardioprotection in this way [168], however others have shown that diazoxide selectively opens and 5-HD selectively blocks mitoK_{ATP} channels in isolated mitochondrial preparations [169] and intact cardiac myocytes. It has also been suggested that diazoxide induces protection of the mouse heart and in the guinea pig heart by the opening of the sarcolemmal K_{ATP} channel and decreasing succinate oxidation in a dose-dependent manner without affecting NADH oxidation and that even at a concentration of 100μm diazoxide does not decrease mitochondrial membrane potential as assessed by tetramethylrhodamine ethylester [170]. By contrast, our laboratory has demonstrated that mitochondrial and not sarcolemmal K_{ATP} channels are responsible for the cardioprotection of preconditioning in the human myocardium [171]. It has been argued that diazoxide possesses non-specific metabolic effects on the mitochondria, in particular inhibition of succinate dehydrogenase, which could account for its cardioprotective effects [170, 172, 173]. Further, the K_{ATP} blocker 5-hydroxydecanoate (5-HD) can also have metabolic effects by being activated to a CoA derivative and serving as a substrate in the β-oxidation pathway for fatty acids or, alternatively, depending on the isomer, inhibit this pathway [173, 174]. Therefore, it has been argued that the effects of diazoxide and 5-HD in inducing and blocking IPC, respectively may reflect actions on mitochondrial metabolism that do not necessarily involve mitoK_{ATP}

channels. My studies showing that diazoxide causes partial depolarization of the MMP, as assessed by JC-1 stain, would support the argument that diazoxide induces preconditioning through its action on mitoK_{ATP} channels but a metabolic effect cannot be ruled out. It is possible that species differences and variations in experimental conditions may account for the differing results, however, it is clear that further investigation is required to fully elucidate the role played by K_{ATP} channels.

Having investigated the effect of diazoxide on the MMP of the diabetic and non-diabetic myocardium, I embarked in the next chapter in the study of the effect of the MMP depolarisation in terms of ROS generation, which may be the link between mitochondria and downstream pathway of preconditioning.

Chapter 5

The Effect Of MitoK_{ATP} Channel Opening On ROS Generation

5.1. Introduction

The effect of MMP depolarisation remains controversial. On the one hand, it has been reported that activation of the mitoK_{ATP} channel elicits an increase in ROS generation that is required for preconditioning protection [93] [175] [176]. Moreover, ROS generation is abolished when the K_{ATP} channel is blocked, further suggesting that ROS are generated as a consequence of channel activation [177]. On the other hand, other studies have shown that K_{ATP} channel blockers abolish cardioprotection without attenuating the generation of ROS [178]. Moreover mitochondrial depolarization have no effect or reduce mitochondrial ROS generation in intact cells [86], [179].

It is generally accepted that ROS play a major role in IPC. Thus, hearts exposed to superoxide or hydrogen peroxide causes preconditioning [176], [100] and administration of antioxidants abolish preconditioning [180], [181]. Because of the potential relationship between MMP and ROS and the differences in response to MMP in the in diabetics and non-diabetics seen in the previous chapter, my aim in this chapter was to investigate the effect of MMP depolarisation by the mitoK_{ATP} channel opener diazoxide on the generation of the ROS in the mitochondria isolated from the myocardium of diabetics and non-diabetics.

5.2. Methods And Materials

5.2.1. ***Study subjects***

The study was conducted with patient consent and approved by the local ethics committee in accordance with the Helsinki declaration. Atrial biopsies were collected from patients undergoing elective coronary bypass surgery and/or aortic valve surgery prior to the initiation of cardiopulmonary bypass. Three groups of patients were included in the study, non-diabetics, IDDM, and NIDDM. Patients with atrial fibrillation, poor left ventricular function (EF<30%) and right ventricular failure were excluded from the study. Patients on mitoK_{ATP} channels openers, oral opioid or catecholemines were also excluded.

5.2.2. ***Processing of samples and experimental preparation***

The isolation of the mitochondria was described previously in section 2.2.2.

5.2.3. ***End point measurements***

At the end of the experimental protocols, mitochondrial generations of hydrogen peroxide/hydroxyl radicals and superoxide radicals were performed as described previously (section 2.5).

5.2.4. ***Study protocols***

5.2.4.1. *To investigate the effect of diazoxide on ROS generation*

Using specific ROS generators as a positive control we investigated the effect of mitoK_{ATP} channel opening by diazoxide on ROS generation in isolated mitochondrial from the myocardium of diabetics and non-diabetics as follows:

- ***Determination of hydrogen peroxide/hydroxyl radicals generation:***

Mitochondria were isolated from non-diabetics, IDDM and NIDDM according to the method described previously.

A. Using DCFH as a detector of hydrogen peroxide/hydroxyl radicals generation, mitochondrial aliquots (0.1-0.2 mg protein /ml) were randomised to receive one of the following protocols at 30 ^{0}C: hydrogen peroxide (0.5 mM) as a positive control, diazoxide (100 μM in DMSO), carrier only (DMSO x 1%) or Catalase (200 U/ml) +MnTBAP (2.4 μM) as negative control. Mitochondria were then loaded with 1 μM of DCFH (Sigma; St. Louis, MO). DCF fluorescence as a measure of hydrogen peroxide/hydroxyl radicals generation was detected in a 10 mM Hepes buffer pH 7.5 containing the mitochondrial suspension. The fluorescence was measured using a spectrofluorimeter (Fluostar, BMG Labtech, Offenburg, Germany) at an excitation and emission wavelengths of 500 and 530 nm, respectively. DCF fluorescence yield was expressed as a percent of the positive control (maximal values obtained by exposing mitochondrial suspension to 500 μM hydrogen peroxide).

B. Using CM-H2DCFDA as a second detector of hydrogen peroxide/hydroxyl radicals generation, mitochondria isolated from non-diabetic and diabetic atrial tissue were subjected to the following treatments; 30^{0}C: diazoxide (100 μm in DMSO), carrier only (DMSO x 1%), a hydroxyl radical generating compounds composed of xanthine (25μm) / xanthine oxidase (0.01 U per ml) and ferrous ammonium sulphate (100 μM) that was used as a positive control, and mercaptopropionylglycine (MPG) (2.5 mM) a specific hydroxyl scavenger that was used as negative control. The mitochondria suspension was loaded

with 2', 7'-dichlorodihydrofluorescein acetate (CM-H2DCFDA) to a final concentration of 1 μM. The fluorescence was read at excitation of 504 and emission 530 nm. Results were expressed as a percent of the positive control (CM-H2DCFDA fluorescence values obtained by exposing mitochondrial suspension to hydroxyl radical generating compounds).

- ***Determination of superoxide production*:**

Superoxide generation in isolated mitochondria was detected using the chemiluminescence of the probe lucigenin. Diluted mitochondria suspension isolated from diabetics and non-diabetics myocardium were subjected to one of the following treatments at 30 ^{0}C: xanthine/ xanthine oxidase (25 μM/ 0.01 U per ml) as a positive control, diazoxide (100 μM), carrier only (DMSO x 1%) or MnTBAP (2.4 μM) as a negative control. Cu, Zn SOD was not used as a negative control as it does not influence chemiluminescence of the probe lucigenin because of mitochondrial membrane impermeability to Cu, Zn SOD (34). Mitochondrial samples were then loaded with chemiluminescence lucigenin (15 μM). Production of superoxide was measured using luminometry (Microlumat Plus LB 96 V, Berthold technologies, Bad wildbad, Germany). The peak relative luminescence per second (RLU/s) was used as the index of the superoxide production measurements. The results were expressed as a percentage of the positive control.

5.2.4.2. *To investigate the role of superoxide radicals scavenging on mitochondrial membrane depolarization by diazoxide*

Aliquots of isolated mitochondria at equal protein concentration were obtained from non-diabetic myocardium and randomised to one of the following protocols for 10 minutes at 30^0C: diazoxide (100 μM in DMSO), carrier alone (DMSO x 1%), and diazoxide (100 μM) in combination with superoxide dismutase at various concentrations (200, 400 and 600 Units/ml) to evaluate the role of superoxide scavenging on the MMP. The mitochondrial electron transport chain uncoupler CCCP (1 μM) was used as positive control and measurement of the MMP was made as described above.

5.3. Results

5.3.1. ***The effect of diazoxide on hydrogen peroxide/hydroxyl radicals generation by diabetic and non-diabetic mitochondria***

Using DCFH as a detector and hydrogen peroxide as a positive control, exposure of isolated mitochondria to 500μM hydrogen peroxide resulted in similar maximal fluorescence yield in both non-diabetic and diabetic groups. Mitochondria isolated from diabetic and non-diabetic myocardium exhibited no significant difference among the measured values (in arbitrary fluorescence units) for the positive control groups. However, the mean values in the untreated groups (carrier only) expressed as a percentage of the positive control were significantly higher in diabetics than in non-diabetics (Figure 12). Additionally, as shown in the Figure 13, diazoxide treatment significantly reduced hydrogen peroxide/hydroxyl radicals generation in both the non-diabetic and diabetic groups ($p<0.05$ versus positive controls).

However using CM-H2DCFDA as a detector and hydroxyl radicals generating compounds as a positive control, as shown in Figure 14, diazoxide caused a significant increased in

hydrogen peroxide/hydroxyl radicals generation when compared to the untreated control (carrier only). Interestingly, the carrier alone (DMSO) produced a similar response to that of MPG indicating that DMSO may be a hydroxyl radical scavenger (Figure 14).

These inconclusive and surprisingly opposite effects of diazoxide on hydrogen peroxide/hydroxyl radicals generation could be due to the differences in experimental protocols.

*5.3.2. **The effect of diazoxide on superoxide generation by diabetic and non-diabetic mitochondria***

The xanthine/xanthine oxidase generating system employed produced the optimal rate of chemiluminescence and therefore was used as a positive control. There was no significant difference among the measured values (relative luminescence unit per second) for the positive control groups in the diabetic and non-diabetic isolated mitochondria. However, mean values of superoxide generation in the untreated groups (carrier only), expressed as a percentage of the positive control, were again significantly higher levels of superoxide generation in the diabetic than in non-diabetic groups (Figure 15). As shown in Figure 16, the addition of diazoxide (100 μM) significantly increased superoxide generation in the non-diabetic but not in the diabetic groups.

5.3.3. The influence of superoxide scavenging on the effect of diazoxide on mitochondria membrane potential depolarization

As shown in Figure 17, diazoxide-induced depolarization of mitochondrial membrane and the presence or absence of superoxide dismutase in diazoxide-treated preparation had no effect on mitochondrial membrane potential. This observation may suggest that superoxide generation occurs downstream of mitochondrial membrane depolarization.

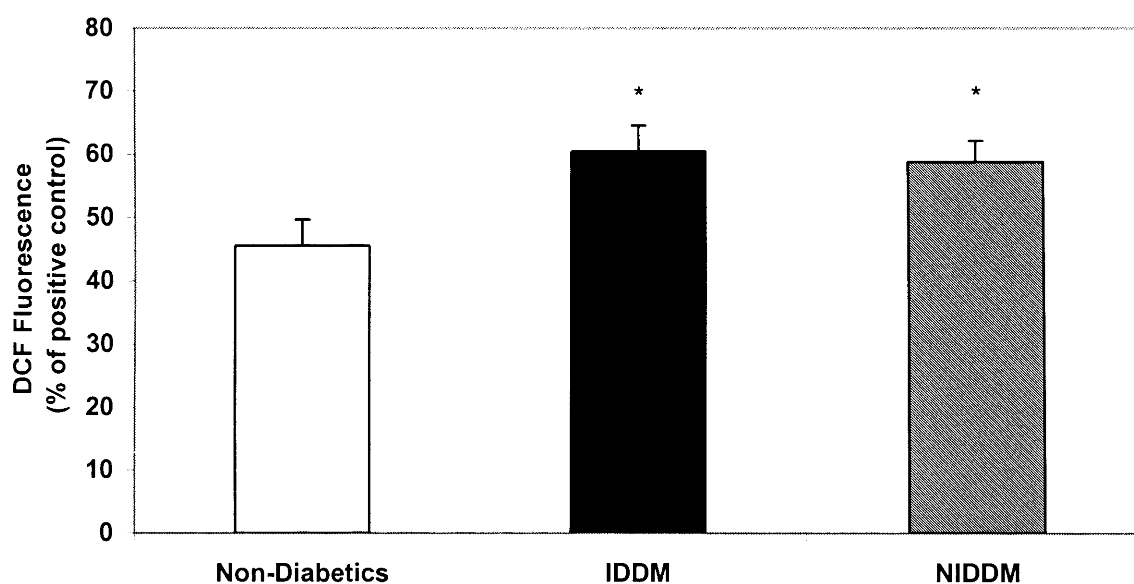


Figure 1 2: Hydrogen peroxide/hydroxyl radicals generation expressed as a percentage of positive control ± SEM. of n=8 per group. *P< 0.05 vs. non-diabetics. DCFH compound was used a detector of hydrogen peroxide/hydroxyl radicals generation. Data are expressed as a percentage of positive control ± SEM of n=8 per group. *P< 0.05 vs. non-diabetics.

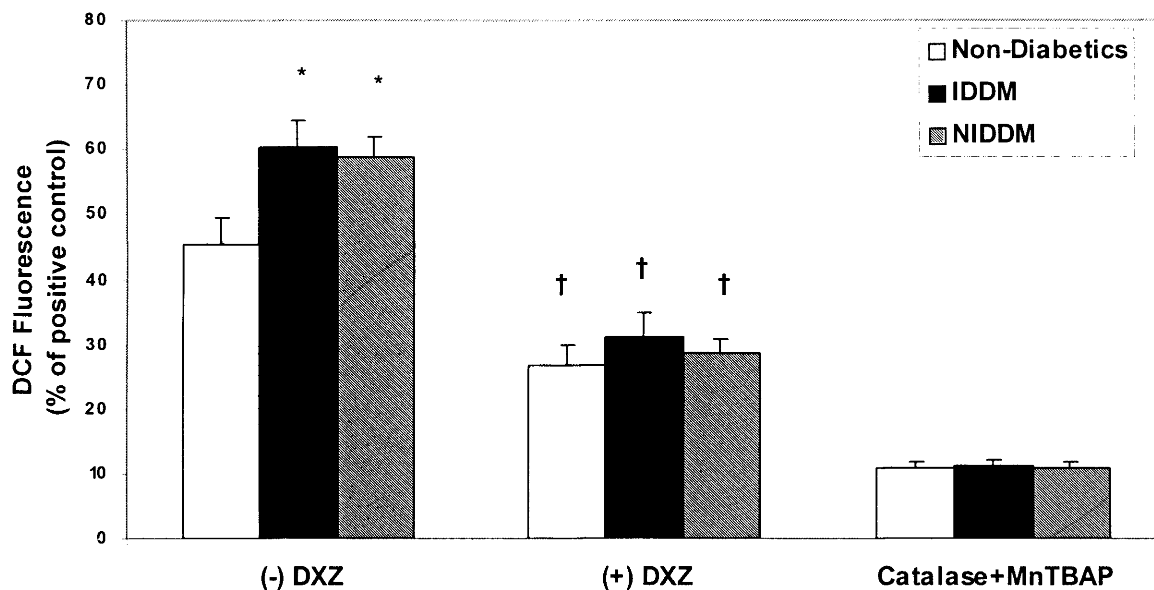


Figure 13: The effect of diazoxide (DXZ) on hydrogen peroxide/hydroxyl radicals generation by isolated mitochondria from the diabetic and non-diabetic myocardium. MnTBAP and catalase in combination were used as negative control. DCFH compound was used a detector of hydrogen peroxide/hydroxyl radicals generation. Data are expressed as a percentage of positive control ± SEM of n=8 per group. *P< 0.05 vs. non-diabetic corresponding group; †P<0.05 vs. the corresponding groups without diazoxide.

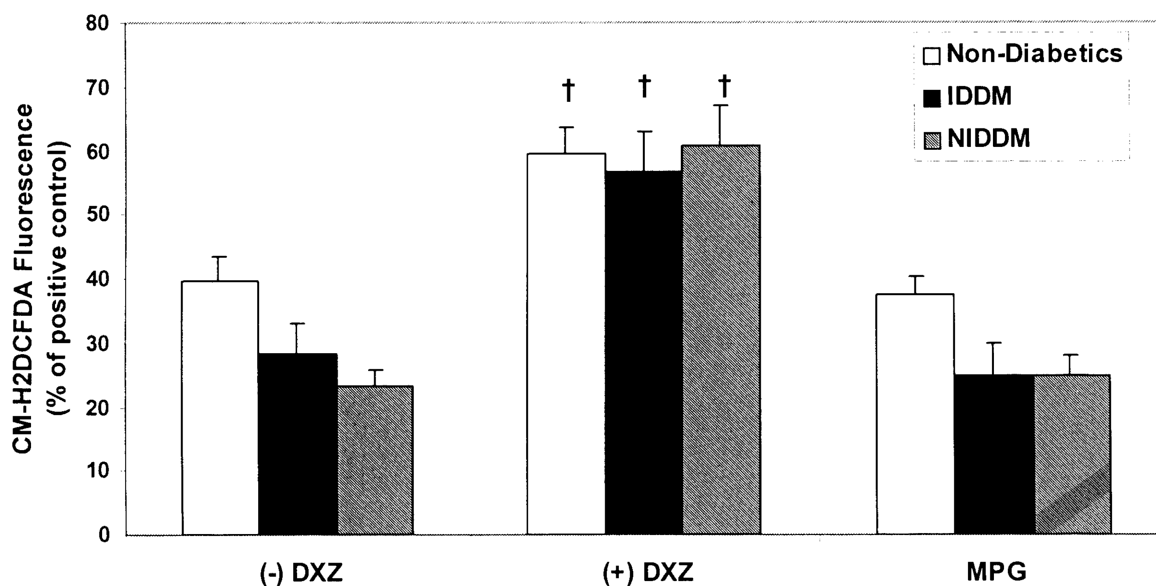


Figure 14: The effect of diazoxide (DXZ) on hydrogen peroxide/hydroxyl radicals generation by isolated mitochondria from the myocardium of non-diabetics, IDDM, NIDDM. MPG was used as negative control. CM-H2DCFDA was used as a detector of hydrogen peroxide/hydroxyl radicals generation. Data are expressed as a percentage of positive control ± SEM of n=8 per group. †$P<0.05$ vs. the corresponding groups without diazoxide.

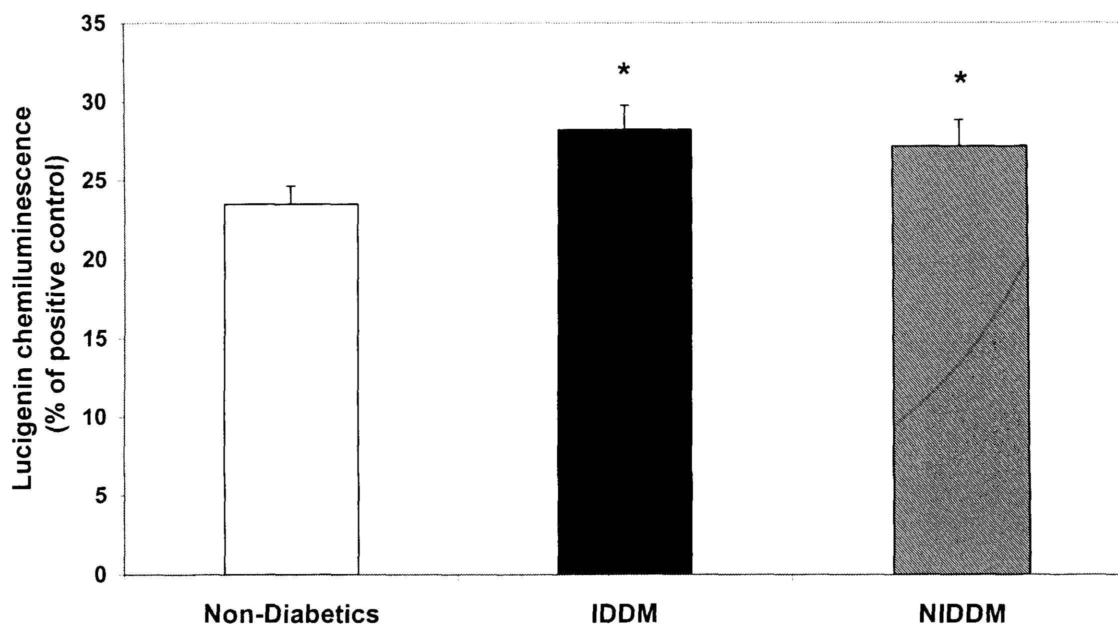


Figure 15: Superoxide generation by the mitochondria from diabetics and non-diabetics. Data are expressed as a percentage of positive control ± SEM of n=8 per group. *P< 0.05 vs. non-diabetics.

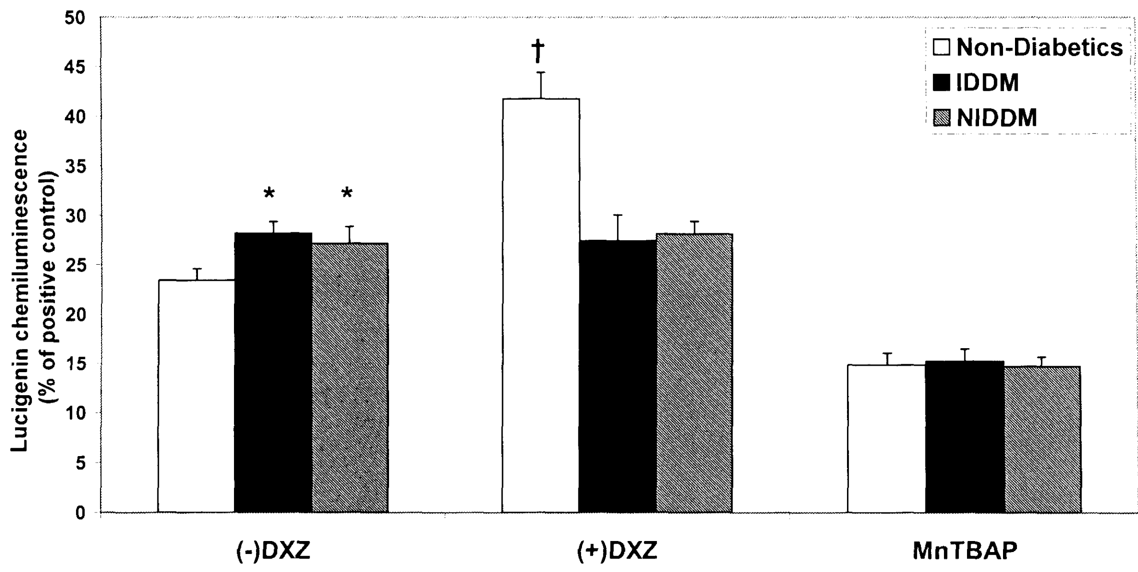


Figure 16: The effect of diazoxide (DXZ) on superoxide generation by isolated mitochondria from the myocardium of diabetics (IDDM, NIDDM) and non-diabetics. MnTBAP and catalase were used as negative control. Data are expressed as a percentage of positive control ± SEM of n=8 per group. *$P < 0.05$ vs. non-diabetic corresponding group; †$P<0.05$ vs. the corresponding groups without diazoxide.

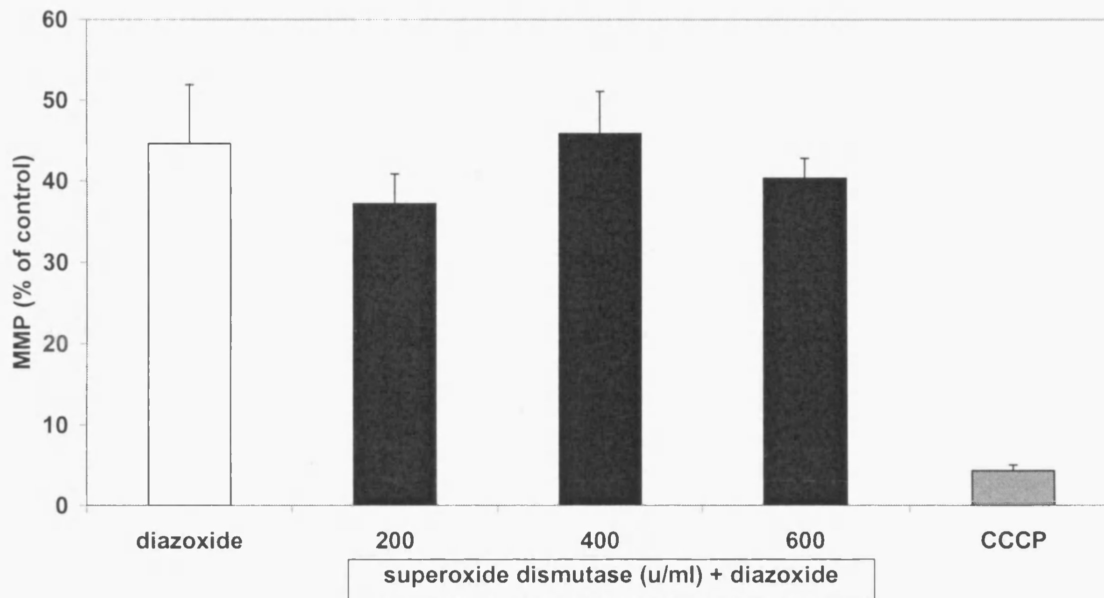


Figure 17: The effect of superoxide scavenging on mitochondria membrane potential depolarization in mitochondria from the myocardium of non-diabetics. Data expressed as a percentage of control (untreated groups) ± SEM of n=8 per group. CCCP a known mitochondrial uncoupler was used as a positive control.

5.4. Discussion

Forbes et al [175] have provided the first direct evidence that opening of mitoK_{ATP} increases ROS production in isolated rat ventricular myocytes. Using a ROS sensitive fluorescent probe DCF, they showed that diazoxide as well as pinacidil (a non-selective K_{ATP} opener) increased DCF fluorescence, implying an elevated ROS production. The use of a selective mitoK_{ATP} blocker 5-hydroxydecanoate (5-HD) abolished the increase and the antioxidant 2-mercaptopropionylglycine (MPG) also blocked the fluorescence increase. Here I have provided a novel insight on the effect of mitoK_{ATP} channels opening on the generation of the ROS. I have shown that diazoxide increases the generation of superoxide seen in non-diabetics but not in diabetics. This might imply that superoxide generation is a key step in the signalling downstream of the mitochondria.

The role of superoxide generation in preconditioning has been demonstrated in animal studies. Thus, there is evidence that infusion of hypoxanthine or xanthine oxidase separately in lieu of preconditioning had no effect on infarct size, but induced protection when combined [176]. Similarly, it has been reported [182] that in isolated heart the superoxide scavengers MnTBAP and superoxide dismutase abrogate the effects of ischaemic preconditioning. It is of interest that in these experiments brief preconditioning pulses resulted in increased superoxide generation during the ischaemic but not during the reperfusion phase. In isolated cardiomyocytes, Vanden Hoek et al found that hypoxia increased mitochondrial superoxide generation which initiated preconditioning [183], which is in agreement with my finding in the non-diabetic human myocardium mitochondria. My

studies also demonstrated that superoxide scavenging did not affect MMP implying that MMP depolarisation is upstream of superoxide production.

The response to mitoK$_{ATP}$ activation in terms of hydrogen peroxide/hydroxyl radicals generation was inconclusive. In one hand using DCFH as a detector and hydrogen peroxide as positive control, diazoxide reduced the generation of hydrogen peroxide/hydroxyl radicals. On the other hand using CM-H2DCFDA as a detector and hydroxyl radicals generating compounds as a positive control, diazoxide increased the generation of hydrogen peroxide/hydroxyl radicals. These opposite effects of diazoxide could be due to the different experimental protocols used and may indicate the lack of specificity of DCF compounds. CM-H_2DCFDA and DCFH are both derivatives of dichlorofluorescein but CM-H_2DCFDA is better retained within the cell or mitochondria. The generation of hydrogen peroxide/hydroxyl radicals has been controversial. Ozcan et al [184] have shown a decrease in DCFH fluorescence in response to diazoxide, others have reported a small burst of oxidant stress during preconditioning phase in a model of embryonic ventricular cultured myocytes [178]. The reason for this discrepancy is unclear but it is possible that experimental differences (e.g. mitochondria versus myocytes) may be responsible.

In summary, we have demonstrated the diazoxide caused partial depolarisation of the MMP in the non-diabetics. We have also demonstrated that diazoxide can increase superoxide production in non-diabetics. In the diabetic myocardium, diazoxide fail to depolarise the mitochondria and also failed to increase superoxide production. However it has been also reported that mild uncoupling may lead to decrease in superoxide radicals production [185]. Therefore diazoxide effect on superoxide production could also result from respiratory chain inhibition by diazoxide. In either case, the mitochondrial target of diazoxide is dysfunctional

in the diabetic myocardium, so that superoxide production is compromised and cardioprotection cannot be elicited.

Having described the effect of diazoxide on MMP in terms of ROS production and identified the lack of MMP depolarisation and the production of superoxide in the diabetic mitochondria, I focused my attention on the PKC isoforms that may be involved in the signalling transduction pathway of IPC and their relationship with the mitoK_{ATP} channels.

Chapter 6

PKC Signalling In Ischaemic Preconditioning

6.1. Introduction

Protein Kinase C plays an important role in the signalling pathway of ischaemic preconditioning (IPC) in human [171], [63], [4] and animal studies [186], [187], [188], [189], although this concept has been disputed by some investigators [190], [191]. The reason for these discrepancies may be related at least in part to the complexity of PKC biology and pharmacology [105], [101]. Different PKC isoforms are known to be important in IPC; however the precise role of specific isoforms appears to vary between animal species. For example PKC ε has been shown to be important component of IPC in rabbits [192], whereas in rats both PKC ε [193] and PKC δ [189] have been reported to be essential for cardioprotection by IPC. In contrast, in the canine heart model it has been shown that PKC α is important to provoke IPC [194]. However with the exception of the study by Julier et al [195] there is little information in the literature regarding studies on human hearts.

The putative mitoK_{ATP} channels also play a key role in the signal transduction mechanism of IPC [148], [4], [93], [79], [77]. It has been reported that opening of the mitoK_{ATP} channels can activate PKC by generation of radical oxygen species [93]; although it has also been

suggested that opening of these channels can be potentiated by PKC [79]. This may suggest that PKC isoforms may exist upstream and downstream of mitoK_{ATP} channels but direct evidence of this does not exist.

The present study was designed to investigate the role of different PKC isoforms in IPC of the human myocardium and to determine how they are related to the mitoK_{ATP} channels.

6.2. Materials And Method

6.2.1. *Study subjects*

The study was approved by the local ethical committee and was conducted with the consent of patients in accordance with the Helsinki Declaration. Atrial biopsies were collected from patients undergoing elective coronary artery bypass surgery and/ or aortic valve surgery during cannulation of the right atrium prior to initiation of cardiopulmonary bypass. Patients with enlarged atriums, atrial arrhythmias, poor left ventricular function (ejection fraction <30%) and right ventricular failure were excluded. Other criteria for exclusion were diabetes mellitus and the treatment with oral opioid analgesia, K_{ATP} channel openers or catecholemines.

6.2.2. *Processing of samples and experimental preparation*

The study was conducted using the tissue perfusion model described in section 2.1.2.

6.2.3. *Measurement of tissue injury and viability*

Tissue injury was determined by measuring the leakage of CK into the incubation medium during the reoxygenation period and tissue viability was assessed by the reduction of MTT as described in section 2.1.2.

6.2.4. *Study protocols*

6.2.4.1. *Identification of PKC isoforms involved in ischaemic preconditioning*

To identify the PKC isoforms that are involved in IPC, dose response studies were performed using specific PKC inhibitors. Following equilibration at 37°C in aerobic medium for 30 minutes, myocardial sections (n=6 per group) were randomised to receive one of the protocols summarized in Figure 18A. To serve as controls, some of the myocardial sections were subjected to the highest dose of the various PKC inhibitors used for 10 minutes followed by the induction of 90 minutes of simulated ischaemia/ 120 minutes of reoxygenation (SI/R). The rest of sections were treated with the various PKC inhibitors added 10 minutes prior to and 10 minutes during IPC followed by SI/R at various concentrations as follows: myristoylated V1-2 peptide (0.1, 1, 10 μM) a PKC ε inhibitor; rottlerin (1, 10, 100 μM) a PKC δ inhibitor; LY333531 (1, 10, 100 nM) a PKC (β1 + β2) inhibitor; and GO6976 (1, 10, 100 nM) and Ro 32-0432 (0.1, 1, 10μM) both of which have been identified as PKC (α + β) inhibitors. In the view of the on-going controversy on the use of rottlerin as a specific PKC δ inhibitor, the selective PKC δ activator bistratene A (1, 10, 100 nM) was administered for 10 minutes prior to simulated ischaemia/ reoxygenation.

6.2.4.2. *The sequence of involvement of PKC isoforms in the mechanism of preconditioning and their relation to the activation of mitoK$_{ATP}$ channels*

To determine the sequence of events leading to the induction of IPC via activation of PKC isoforms, myocardial sections (n=6 per group) were equilibrated for 30 minutes before they were randomised into any of the groups of the protocol summarized in Figure 18B. The mitoK$_{ATP}$ channel opener diazoxide was used alone or in combination with specific PKC isoform inhibitors. The concentration of 100 μM of diazoxide has been used as it has been shown in our laboratory to be optimally protective [171].

6.2.4.3. *Phosphorylation of PKC isoforms*

The phosphorylation of the PKC isoforms found to participate in IPC in the above studies was measured in myocardial sections (n=4 per group) that were randomised after 30 minutes of equilibration to receive any one of the following protocols: time matched aerobic control for additional 10 minutes, IPC alone using 5 minutes of simulated ischaemia followed by 5 minutes reoxygenation and 10 minutes preconditioning with diazoxide (100μM). At the end of each protocol myocardial sections were quickly frozen and then phosphorylation of PKC ε and PKC α was measured using western blotting described above in section 2.6.

A:

30 minutes	10 minutes	10 minutes	90 minutes	120 minutes
Aerobic control				
equilibration			Simulated ischaemia	reoxygenation
equilibration		PKC Inhibitors	Simulated ischaemia	reoxygenation
equilibration		IPC	Simulated ischaemia	reoxygenation
equilibration	Go6976	IPC+ GO6976	Simulated ischaemia	reoxygenation
equilibration	V1-2 peptide	IPC+ V1-2 peptide	Simulated ischaemia	reoxygenation
equilibration	LY333531	IPC+ LY333531	Simulated ischaemia	reoxygenation
equilibration	Rottlerin	IPC+ Rottlerin	Simulated ischaemia	reoxygenation

B:

30 minutes	10 minutes	10 minutes	90 minutes	120 minutes
Aerobic control				
equilibration			Simulated ischaemia	reoxygenation
equilibration		IPC	Simulated ischaemia	reoxygenation
equilibration		Diazoxide (DXZ)	Simulated ischaemia	reoxygenation
equilibration	V1-2 peptide	DXZ+V1-2 peptide	Simulated ischaemia	reoxygenation
equilibration	GO6976	DXZ+ GO6976	Simulated ischaemia	reoxygenation

Figure 18: Protocol to identify PKC isoforms involved in ischaemic preconditioning of the human myocardium.

6.2.5. *Solutions and chemicals*

Samples were incubated in Krebs medium composed of (in mM): NaCl (118), KCl (4.8), $NaHCO_3$ (27.2), $MgCl_2$ (1.2), KH_2PO_4 (1.0) $CaCl_2$ (1.25), HEPES (20) and D-glucose (10) or 2-deoxy-D-glucose (10) to induce simulated ischaemia, and prepared in deionised distilled water. All these chemicals, creatine kinase assay kit, MTT, diazoxide and bistratene A were purchased from Sigma Chemicals (Dorset, UK). The specific PKC isoforms inhibitors Go6976, V1-2 peptide, Rottlerin were obtained from Biomol Research Laboratories (Pennsylvania, USA) and LY333531 was supplied by AG Scientific Inc. (San Diego, USA). The antibodies for PKC α and PKC ε, phospho PKC α (serine 657) and phospho PKC ε (serine729) were obtained from Upstate Cell Signalling Solution (Virginia, USA).

6.2.6. *Statistical analysis*

All data are presented as mean ± SEM. Mean values were analysed by ANOVA with a post hoc Tukey's test. Statistical significance was taken at $P < 0.05$.

6.3. Results

6.3.1 *Identity of PKC isoforms involved in ischaemic preconditioning*

The results shown in Figures 19 to 22 indicate that IPC significantly reduced CK leakage and increased the MTT reduction of muscles subjected to ischaemia/ reoxygenation. None of the PKC inhibitors (at the highest doses used) in the absence of IPC had any significant effect on tissue damage or tissue viability. They also show that the PKC ($\alpha + \beta$) inhibitor GO6976 at 100 nM blocked preconditioning whereas PKC β inhibitor LY333531 failed to block preconditioning. The effect of PKC ($\alpha + \beta$) inhibition was also confirmed with the use of Ro 32-0432 (data not shown). This would suggest that PKC α but not PKC β is involved in signalling pathway of IPC. Furthermore, the PKC ε inhibitor V1-2 peptide completely blocked protection by IPC at a concentration of 10 μM, while the PKC δ inhibitor rottlerin had no effect at any of the study concentrations. Activation PKC δ isoforms with bistratene A failed to induce protection in our model (Figure 23). These results indicate that PKC ε participate in the protection by IPC of the human myocardium with no involvement of PKC δ.

6.3.2. *Sequence of involvement of PKC α and PKC ε in relation to the mitoK_{ATP} channel*

Figures 24A and 24B demonstrate that, as expected, IPC and diazoxide conferred similar degree of cardioprotection as shown by the reduction of CK leakage and increased MTT viability. As seen previously, PKC ε inhibitor V1-2 peptide (10 μM) blocked protection by IPC but, interestingly, it did not affect the protection induced by diazoxide. This would suggest that PKC ε is upstream of mitoK_{ATP} channels. Figures 25A and 25B demonstrate that the PKC (α + β) GO6976 (100 nM) blocked the protection seen with both IPC and diazoxide, which indicates that PKC α is downstream of mitoK_{ATP} channels (note that the studies presented above suggested the absence of a role for the PKC β isoforms in IPC).

6.3.3. *Phosphorylation of PKC isoforms*

To demonstrate the phosphorylation of PKC ε and α in relation to the opening of mitoK_{ATP} channels, tissue extract were detected by western immunoblotting. As shown in Figure 26, basal level of 30-35% phosphorylation of PKC ε and PKC α were observed in aerobic controls. IPC increased the phosphorylation of both PKC α and PKC ε; however, diazoxide caused significant increase in PKC α phosphorylation but failed to induce significant increase in the phosphorylation of PKC ε which confirms that PKC ε lies upstream and PKC α lies downstream of mitoK_{ATP} channels.

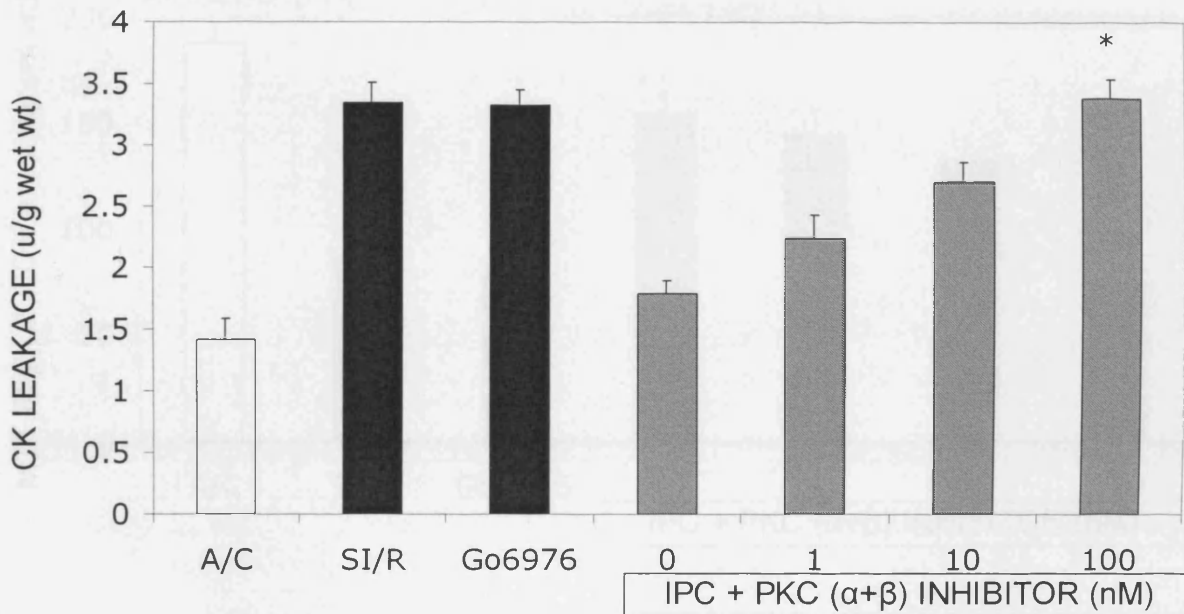


Figure (19A): Dose-response to PKC (α+ β) inhibition of ischaemic preconditioning (IPC) using GO6976 on CK leakage. Data are expressed as mean ± SEM of n=6/group. *P< 0.05 vs. IPC alone. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

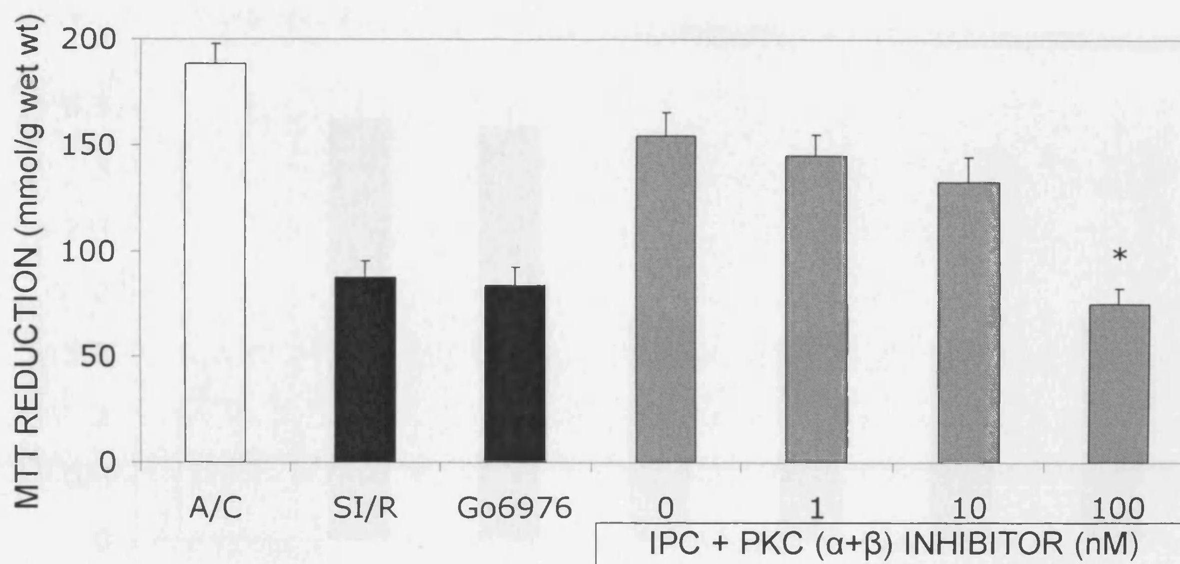


Figure (19B): Dose-response to PKC (α+ β) inhibition of ischaemic preconditioning (IPC) using GO6976 on MTT reduction. Data are expressed as mean ± SEM of n=6/group. *P< 0.05 vs. IPC alone. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

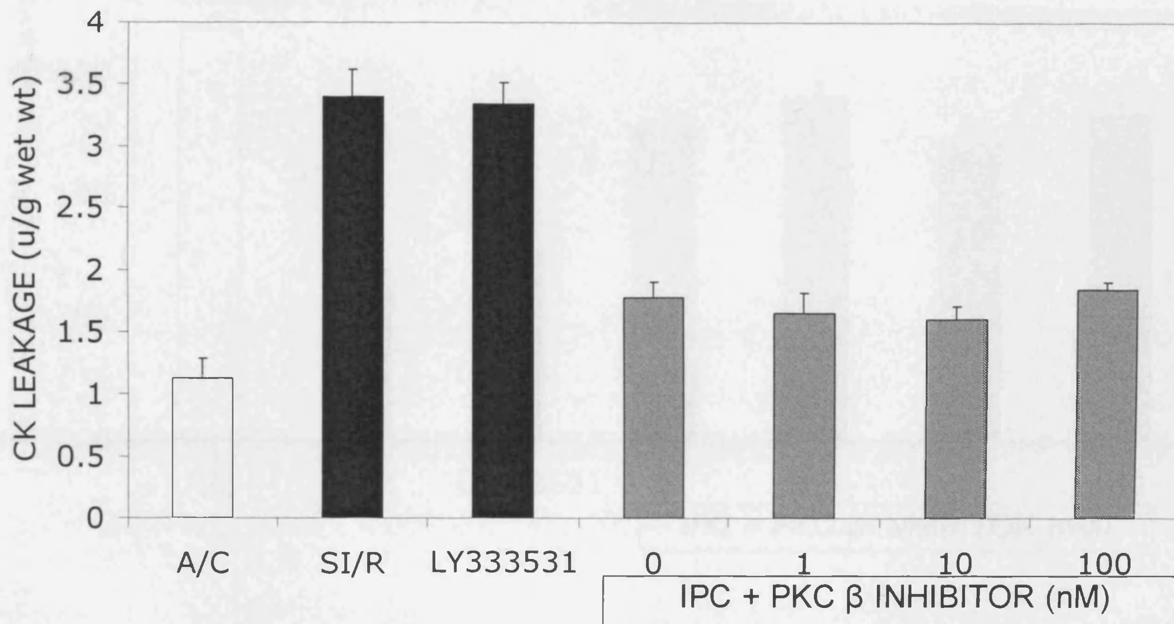


Figure (20A): Dose response to PKC β inhibition of ischaemic preconditioning (IPC) with LY333531 on CK leakage. Data are expressed as mean ± SEM of n=6/ group. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

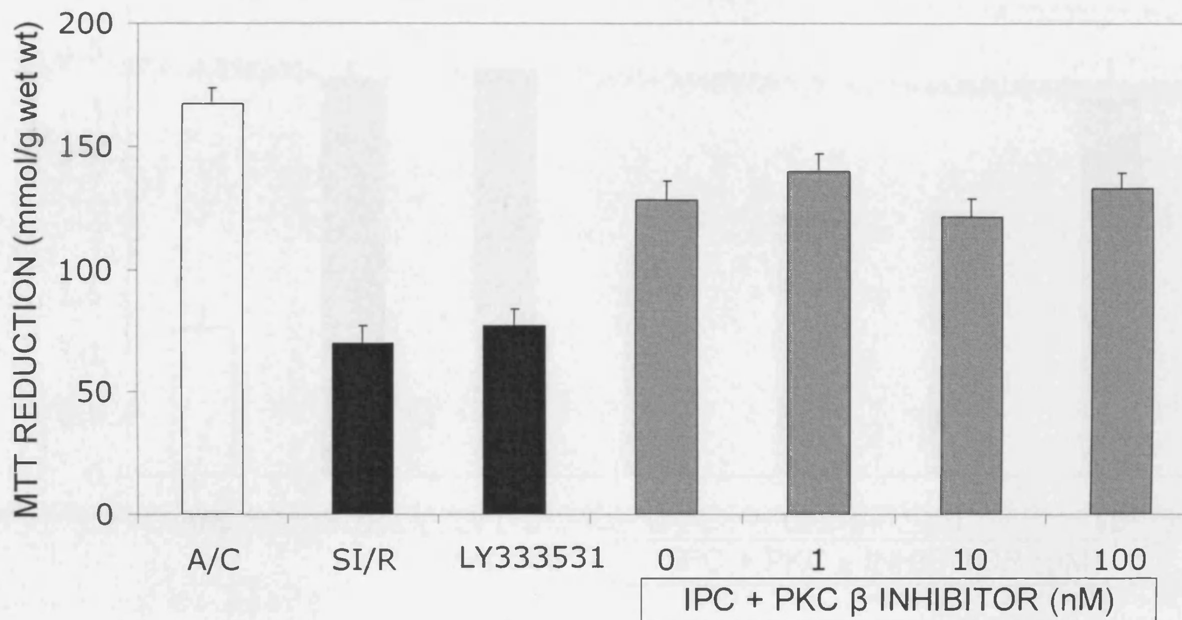


Figure (20B): Dose response to PKC β inhibition of ischaemic preconditioning (IPC) with LY333531 on MTT reduction. Data are expressed as mean ± SEM of n=6/ group. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

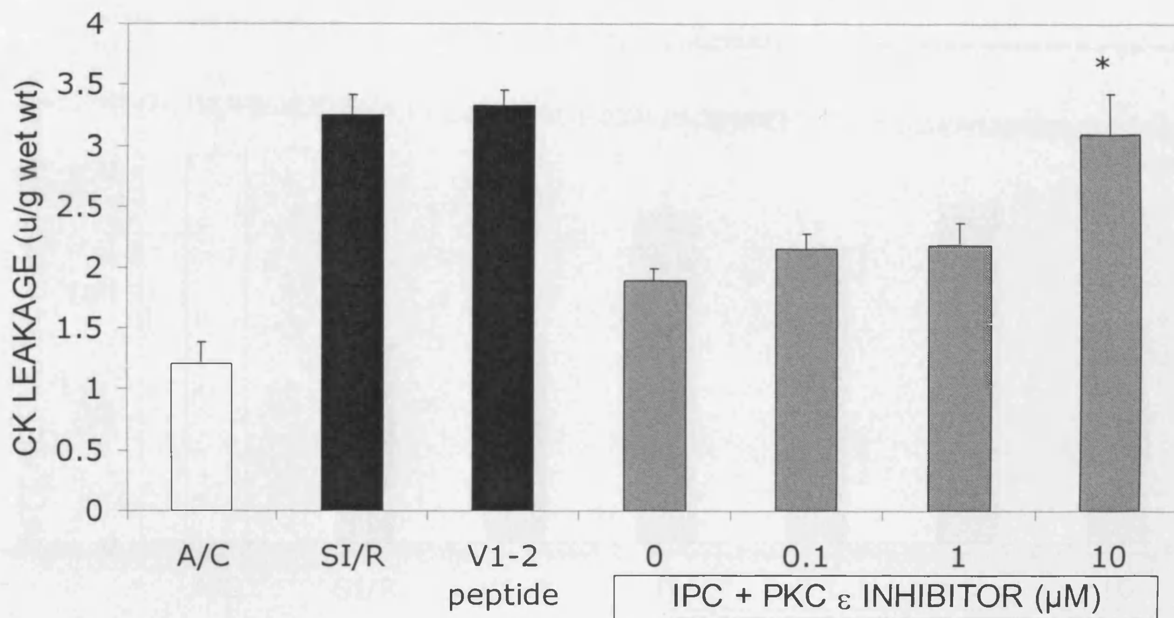


Figure (21A): Dose response to PKC ε inhibition of ischaemic preconditioning (IPC) with V1-2 peptide on CK leakage. Data are expressed as mean ± SEM of n=6/group. *P<0.05 vs. IPC alone. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

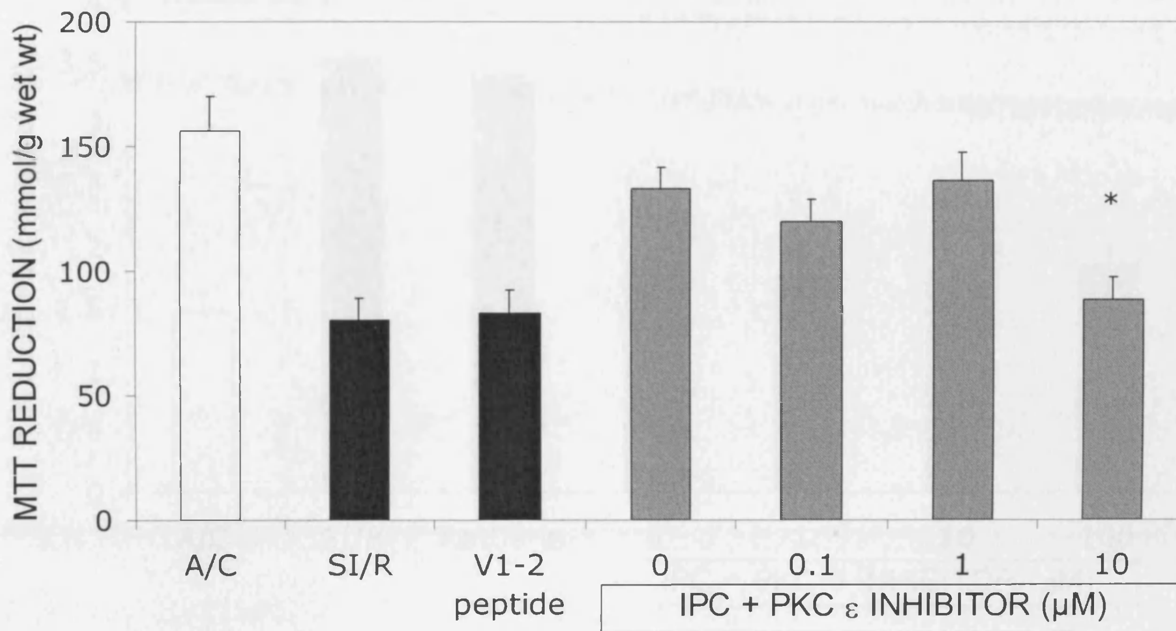


Figure (21B): Dose response to PKC ε inhibition of ischaemic preconditioning (IPC) with V1-2 peptide on MTT reduction. Data are expressed as mean ± SEM of n=6/group. *P<0.05 vs. IPC alone. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

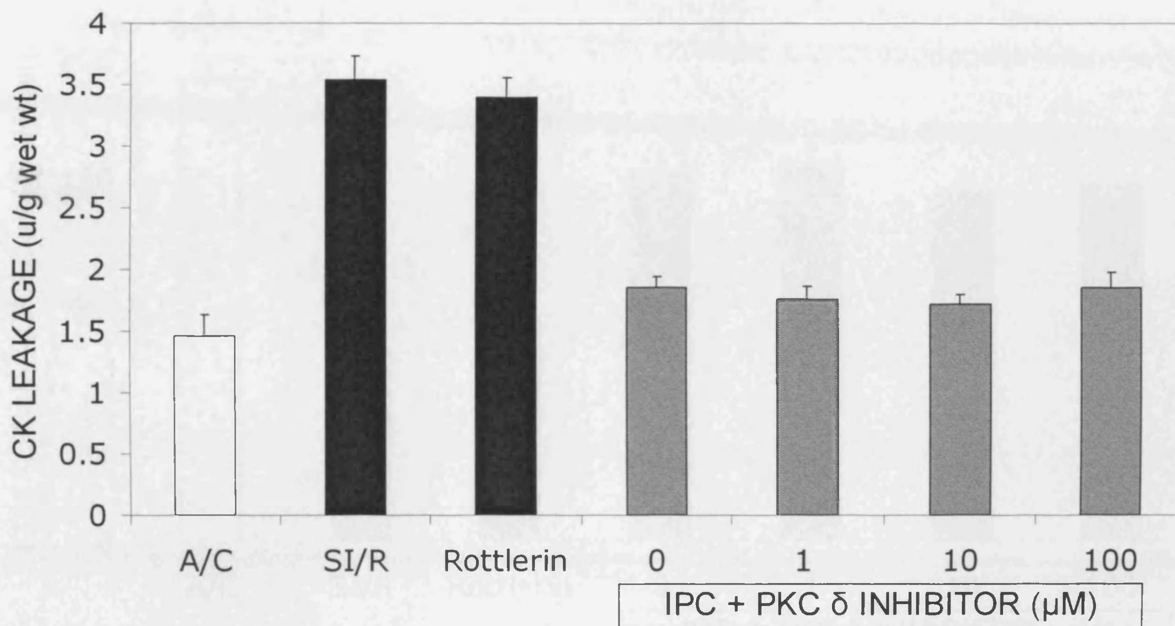


Figure (22A): Dose response to PKC δ inhibition of ischaemic preconditioning (IPC) with rottlerin on CK leakage. Data are expressed as mean ± SEM of n=6/group. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

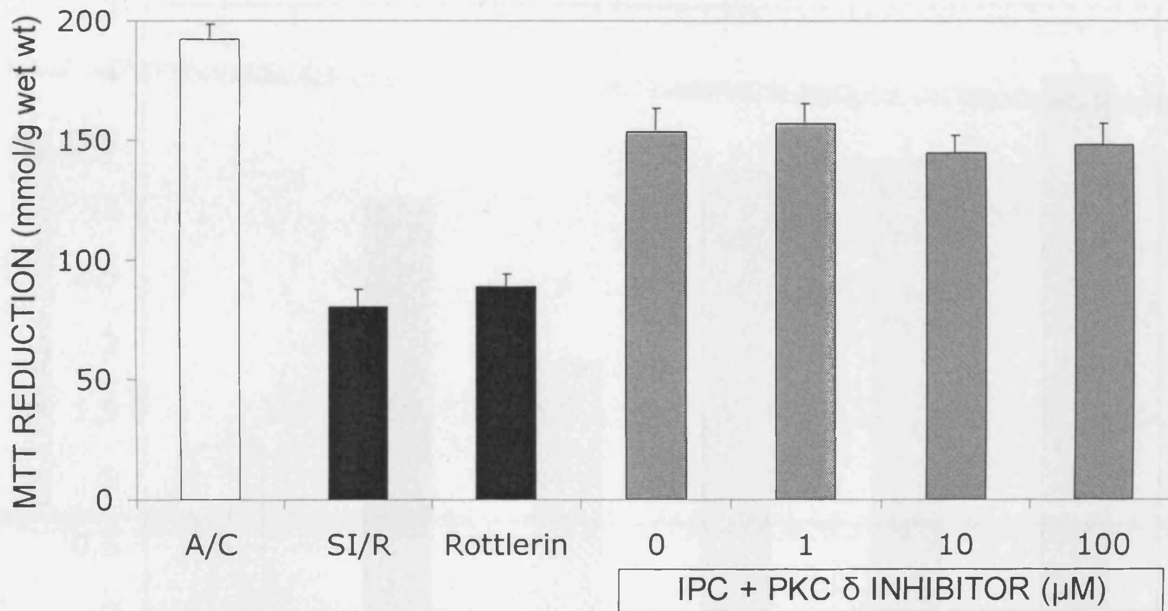


Figure (22B): Dose response to PKC δ inhibition of ischaemic preconditioning (IPC) with rottlerin on MTT reduction. Data are expressed as mean ± SEM of n=6/group. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

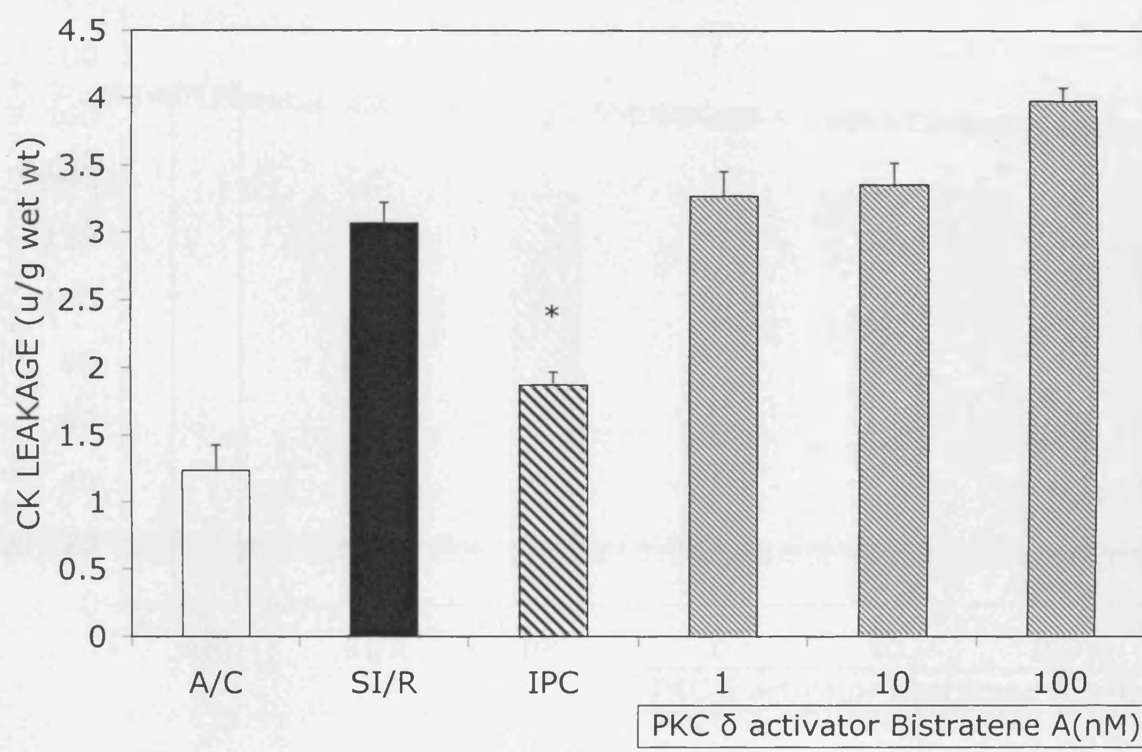


Figure (23A): Dose response to PKC δ activation with bistratene A on CK leakage. Data are expressed as mean ± SEM of n=6/group. *P<0.05 vs. SI/R. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

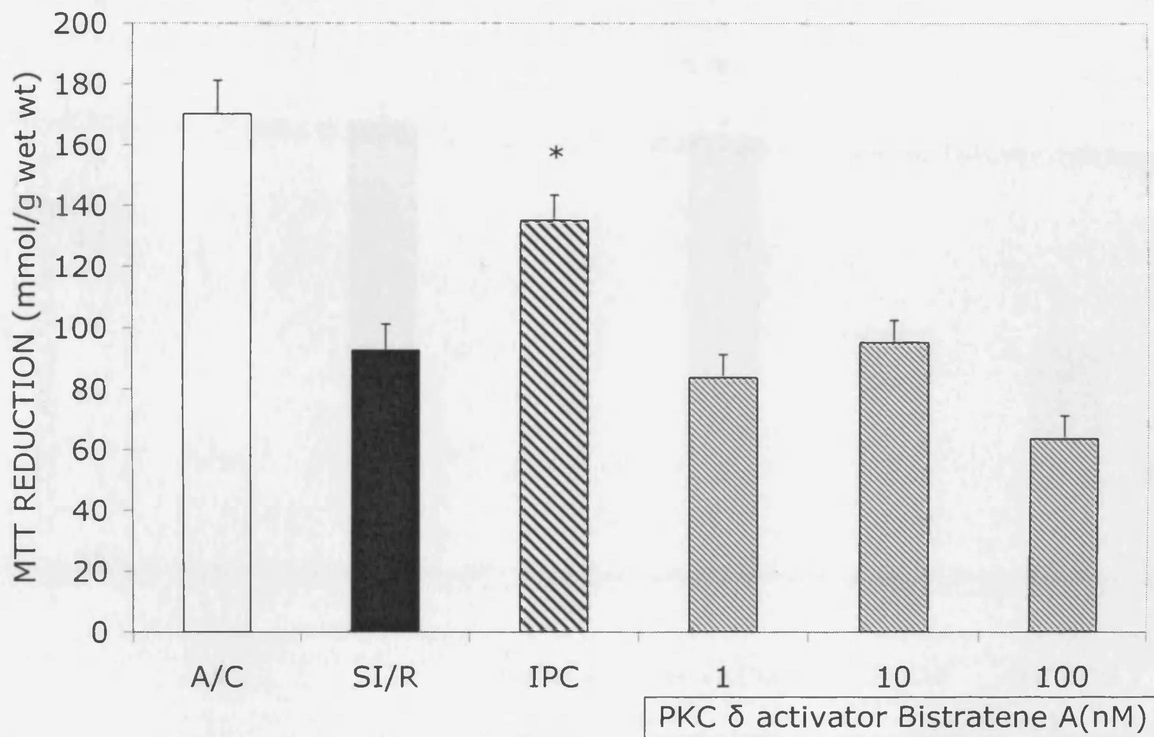


Figure (23B): Dose response to PKC δ activation with bistratene A on MTT reduction. Data are expressed as mean ± SEM of n=6/group. *P<0.05 vs. SI/R. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

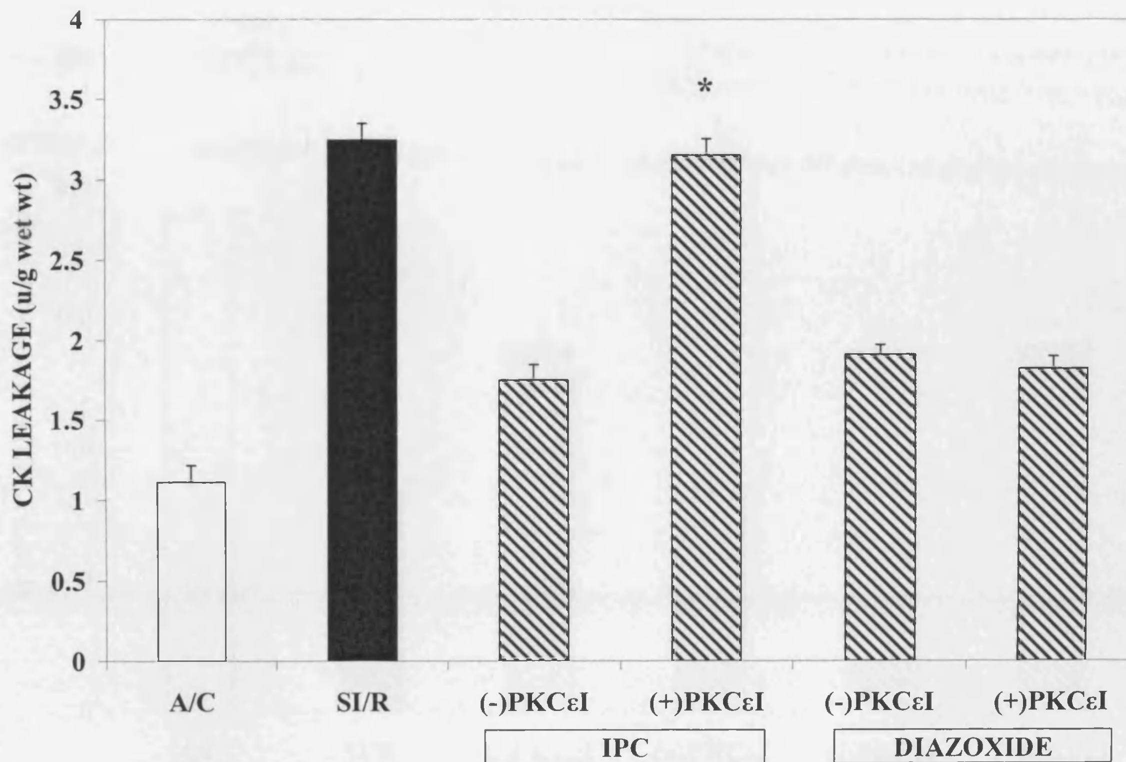


Figure (24A); Effect of the PKC ε inhibitor V1-2 peptide (10μM) on the protection of ischaemic preconditioning (IPC) and diazoxide on CK leakage. Data are expressed as mean ± SEM of n=6/group. *P<0.05 vs. IPC without PKC ε inhibition (PKCεI). A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

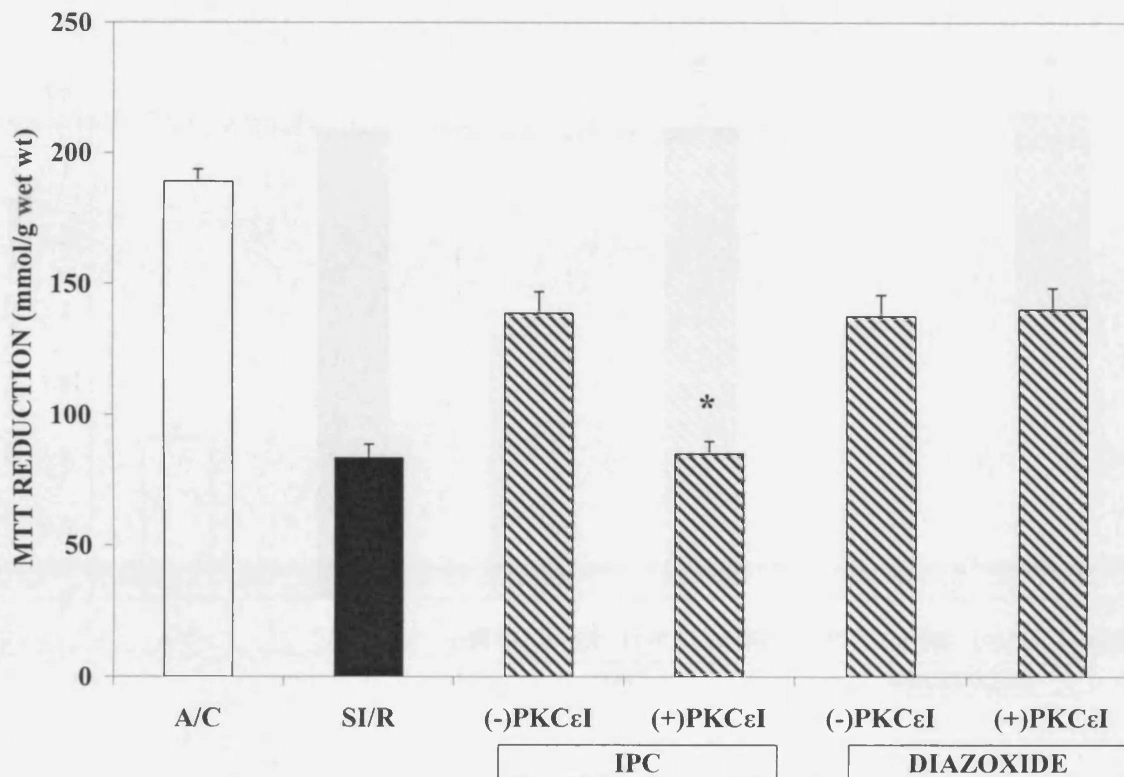


Figure (24B): Effect of the PKC ε inhibitor V1-2 peptide (10μM) on the protection of ischaemic preconditioning (IPC) and diazoxide on MTT reduction (B). Data are expressed as mean ± SEM of n=6/group. *P<0.05 vs. IPC without PKC ε inhibition (PKCεI). A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

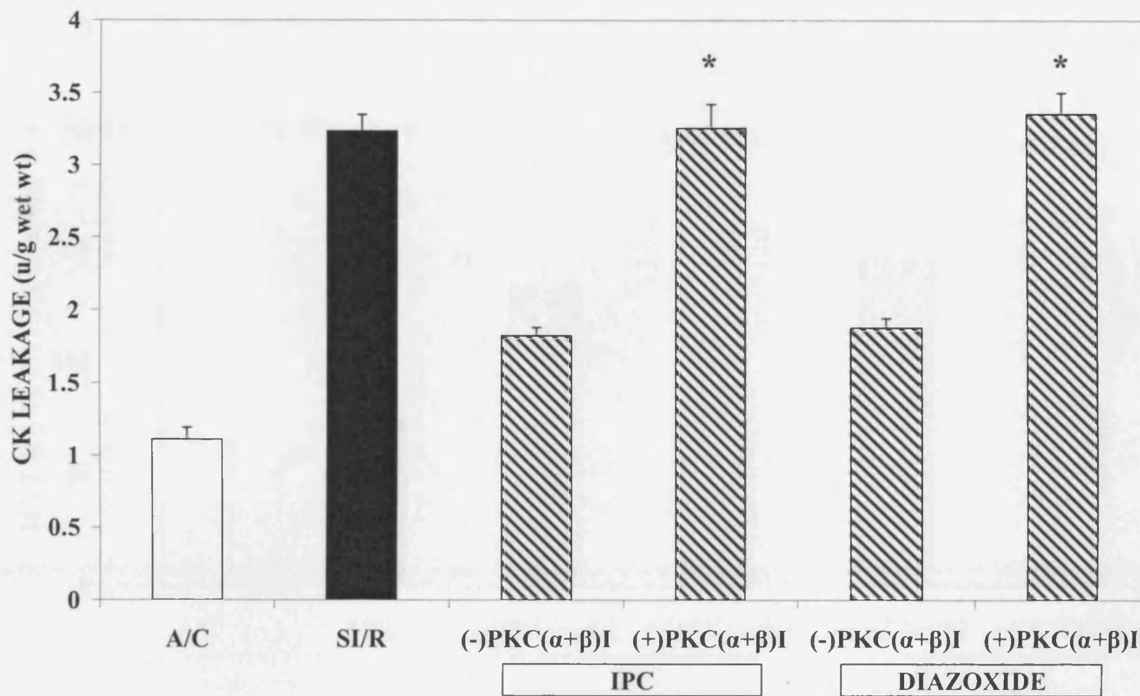


Figure (25A): Effect of the PKC (α +β) inhibitor GO6976 (100 nM) on the protection of ischaemic preconditioning (IPC) and diazoxide on CK leakage. Data are expressed as mean ± SEM of n=6/group. *P<0.05 vs. IPC without PKC (α + β) inhibition [PKC (α + β) I]. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).

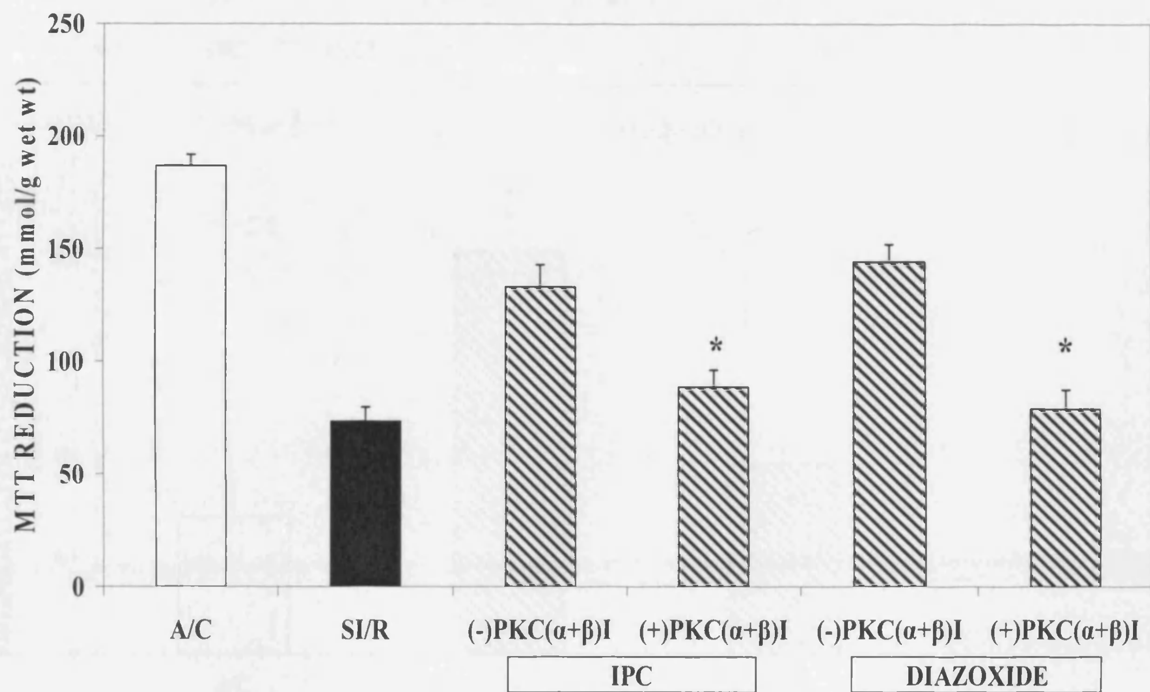
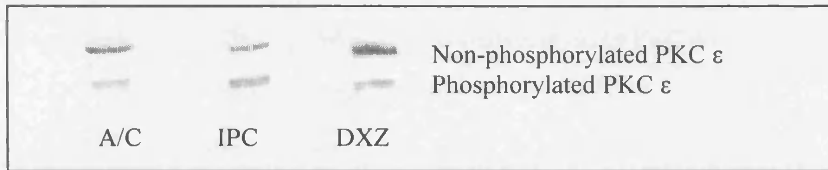


Figure (25B): Effect of the PKC (α + β) inhibitor GO6976 (100 nM) on the protection of ischaemic preconditioning (IPC) and diazoxide on MTT reduction (B). Data are expressed as mean ± SEM of n=6/group. *P<0.05 vs. IPC without PKC (α + β) inhibition [PKC (α + β) I]. A/C (aerobic/ control), SI/R (simulated ischaemia/ reoxygenation).



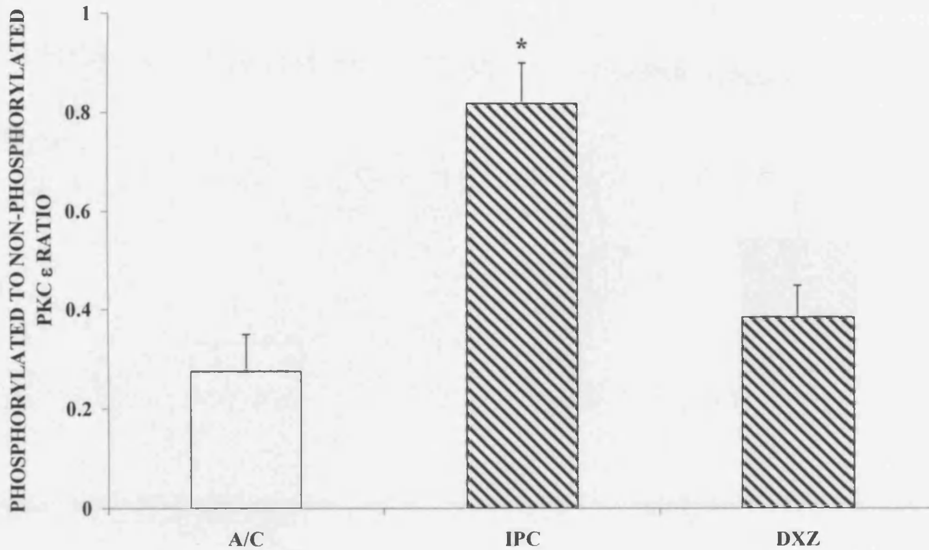
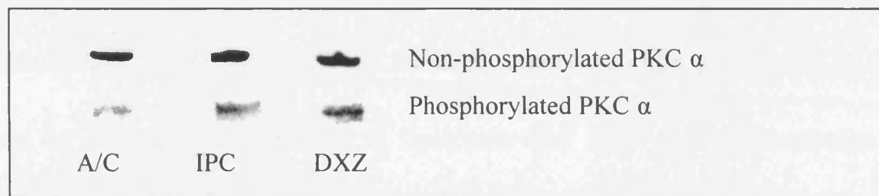


Figure (26A): The ratio of phosphorylated to non-phosphorylated PKC ε in response to ischaemic preconditioning (IPC) and to diazoxide by western blotting and compared to aerobic control (A/C). Data are expressed as mean ratio ± SEM of n=4/group. *$P<0.05$ vs. A/C. The top panels are representative blots.



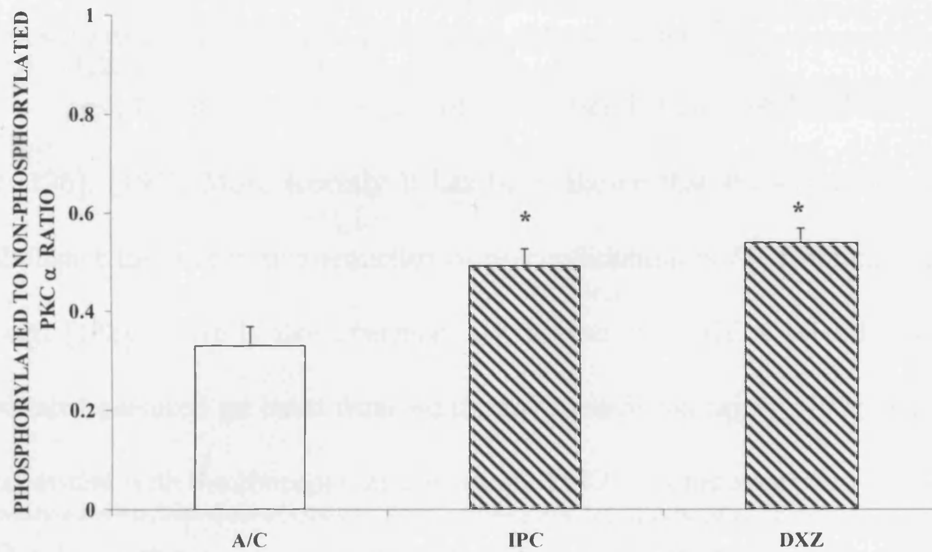


Figure (26B): The ratio of phosphorylated to non-phosphorylated PKC α (B) in response to ischaemic preconditioning (IPC) and to diazoxide by western blotting and compared to aerobic control (A/C). Data are expressed as mean ratio ± SEM of n=4/group. *P<0.05 vs. A/C. The top panels are representative blots.

6.4. Discussion

The present studies provide novel information on the signal transduction pathway of IPC by demonstrating the involvement of PKC ε and α in the protection of the human myocardium and by showing that PKC ε is upstream and PKC α is downstream of mitoK_{ATP} channels. These results in the human myocardium may find some support in reported animal studies. Thus for example, Gray et al using a cell culture model of hypoxic preconditioning found that V1-2 peptide not only abolished cardioprotection but also inhibited the translocation of PKC ε [196], [197]. More recently it has been shown that the disruption in the PKC ε gene abolishes the infarct size reduction of preconditioning in the isolated buffer-perfused mouse heart [198]. There is also evidence that the use of a PKC ε selective activator protects the isolated perfused rat heart from ischaemia/ reperfusion injury [193]. The above findings are consistent with the concept that activation of PKC ε is necessary for cardioprotection by IPC. The demonstration in our studies that PKC α also participates in the cardioprotection by IPC in the human myocardium is in agreement with the demonstration that GO6976, a potent inhibitor of PKC α, significantly attenuates the protection of hypoxic preconditioning on cell death [178] and blocks the reduction of necrosis and apoptosis afforded by three 1 minute cycles of simulated ischaemia [199] in embryonic chick cardiomyocytes. Since GO6976 is a potent inhibitor for α and to a lesser extent of the β isoforms, it may be postulated that the results of the above studies may be due, at least in part, to PKC β inhibition [200]. However in our studies we have demonstrated that in the human myocardium PKC β inhibition by LY333531 had no effect on preconditioning, thus suggesting that the effect of GO6976 is largely attributed to the inhibition of PKC α rather than the PKC β isoforms.

The lack of a role for the PKC β and PKC δ isoforms in IPC of the human myocardium is also supported by similar findings in adult rabbit cardiomyocytes [201] although conflicting

results have been published regarding the role of PKC δ. In one study, the PKC δ inhibitor rottlerin was found to block the cardioprotection induced by the activation of opioid receptors in the *in vivo* rat heart model [202]. Another study by the same investigators reported that rottlerin did not abolish the reduction in infarct size induced by IPC in the same species and experimental model [203]. The most plausible explanation for the different results is that the protection elicited by activation of opioid receptors may use different PKC isoforms than the ones used in IPC. Certainly the elucidation of this issue would require further investigations.

The timing of the administration of the inhibitors is controversial. We have administered the inhibitors 10 minutes prior and 10 minutes during IPC without washing with the aim of interrupting the preconditioning protocols. It has been reported that staurosporine blocked the protective effect of ischaemic preconditioning only when it was present in the perfusion medium following the episode of preconditioning ischaemia (i.e. during the intervening reperfusion) and during the early phase of sustained occlusion [204]. In other studies the protective effect of IPC was lost even when staurosporine was given prior to the preconditioning ischaemia and washed out before the sustained ischaemic insult in the rat model [189]. The fact that our model involved different species and different perfusion protocols may be an explanation for these discrepancies.

The possibility that the degree of stimulation from our simulated ischaemia is lower than that for diazoxide such that diazoxide can overcome the PKC ε block while IPC cannot does not explain the inability of diazoxide to overcome the inhibitory effect of PKC α blocker. The identification of the sequence of the involvement of the two PKC isoforms with PKC ε being upstream and PKC α being downstream of mitoK_{ATP} channels, demonstrated by pharmacological manipulation and by biochemical means, is of biological relevance and may

have important clinical implications. In experimental models the use of non-selective PKC inhibitors such as chelerythrine, staurosporine and calphostin C are insufficient to dissect this relationship and any conclusion on the location of PKC isoforms in relation to mitoK_{ATP} channels using these tools should be interpreted with caution [205], [63], [186]. My laboratory has previously demonstrated that protection of the human myocardium can be elicited by the PKC activator PMA in the presence of the mitoK_{ATP} closer 5-hydroxydecanoate suggesting the PKC is downstream of mitoK_{ATP} but the PKC responsible isoforms and whether other isoforms were involved upstream of mitoK_{ATP} channels was not investigated in those studies [63]. In the literature there is also evidence that mitoK_{ATP} channels are modulated by PKC [93].

To clarify the relation between mitoK_{ATP} channel activation and PKC ε and PKC α, the phosphorylations of PKC isoforms is used as an assay of its involvement as phosphorylations is an essential step towards the activation of PKC [109], [206]. In our studies we measured the phosphorylation of Protein Kinase C α on Serine 657 and PKC ε serine 729 which play a role in the control of active enzyme [207], [208]. The above sites are thought to be autophosphorylated sites and this may explain our finding of 30-35% autophosphorylation in the aerobic control but it does not exclude the possibility of another kinase/mediators involved in the reaction. PDK1 has been demonstrated to phosphorylate Ser-473 which is an autophosphorylated site of PKB [209]. PDK1 was also shown to play a role in the regulation of PKC α [210] and that over expression of wild type PDK1 resulted in increase in Ser-729 phosphorylation of wild type PKC ε but not in Ser-729 of a kinase-negative mutant PKC ε [211]. The mechanisms by which mitoK_{ATP} channels opening affect PKC α autophosphorylation is unknown. Opening of mitoK_{ATP} may lead to the generation of ROS, which in turn may affect autophosphorylation of PKC α. There is evidence that SOD blocked

the autophosphorylation of PKC [211]. It has also been shown in isolated perfused rat hearts that diazoxide causes PKC α translocation [212] but fails to induce translocation of PKC ε [192].

The above findings support the view that PKC α and PKC ε isoforms act independently. As shown in the diagram depicted in the Figure 27, the activation of sarcolemmal receptors triggers a G-protein mediated signalling that via the activation of PKC ε leads to the opening of mitoK_{ATP} channels and that in turn and possibly through the release of reactive oxygen species (ROS) would activate PKC α, this leading to the phosphorylation of p38 MAPK which then effect a single or multiple end-effectors of ischaemic preconditioning. The proposal by Garlid et al [213] that PKC may be proximal and distal to the mitoK_{ATP} channels and the demonstration that PKC ε interact with and inhibit the permeability transition pore in the cardiac mitochondria and contributes to PKC ε induced cardioprotection [214] would support the present findings. However, the exact place and action of these PKC isoforms would require further investigations.

Potential limitations:

The selectivity of the some of the agents used to block the PKC isoforms may be questioned. V1-2 peptide is known to be a PKC ε specific inhibitor [215], however the selectivity of rottlerin to block PKC δ has been controversial and whereas some investigators have reported selective PKC δ inhibition [216] others have not [217]. To overcome this problem we used the selective PKC δ activator bistratene A [218], [219], which failed to precondition the tissue and thus confirming the lack of a role of PKC δ in preconditioning. Although GO6976 [200] and Ro 32-0432 [220] are selective inhibitors of both PKC α and PKC β the separation

of the action of these two isoforms was carried out with the use of PKC (β_1 + β_2) specific inhibitor LY333531 [221].

Evidence in the literature has shown that the activation of PKC leads to translocation of different isoforms into various subcellular sites but has not shown that this is an important mechanism of cardioprotection by ischaemic preconditioning. In this study, I did not investigate the subcellular translocation of phosphorylated PKC isoforms because the heart tissue model used in this study was not appropriate for such assays and this is a potential pitfall.

The present findings may have important clinical implications since it may be possible to target specific PKC isoforms to induce cardioprotection without activating other isoforms that may be involved in other cellular processes. In particular it may be useful for eliciting cardioprotection in the diabetic heart that can not be preconditioned with ischaemia [5] and this could result in a reduction of the cardiac complication associated with ischaemic injury in diabetics.

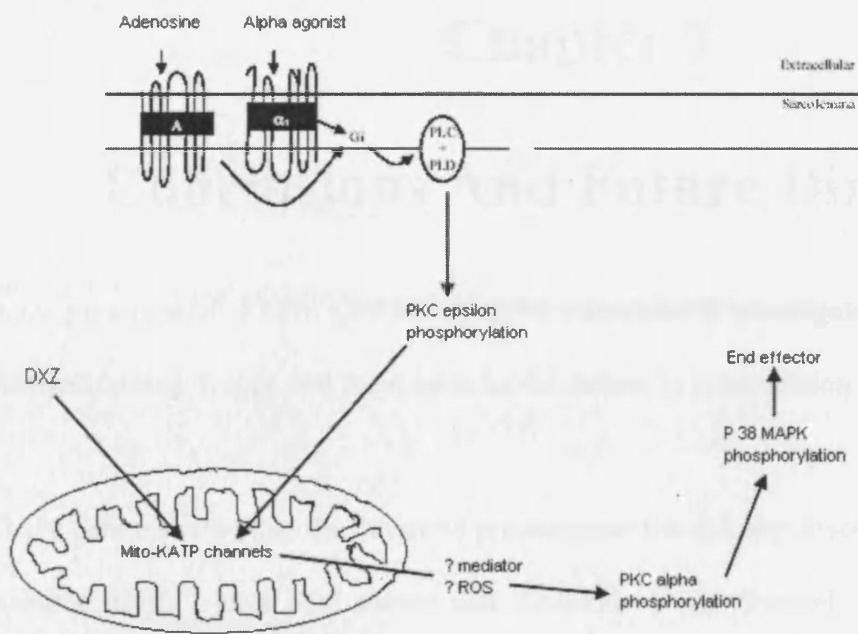


Figure 27: Proposed schematic representation of the signal transduction mechanism leading to cardioprotection by diazoxide and ischaemic preconditioning of the human myocardium. Upon activation of sarcolemmal receptors mitoK_{ATP} channels are activated via PKC ε. The opening of mitoK_{ATP} channels will activate PKC α possibly via the production of radical oxygen species (ROS). PKC α may then translocate to various cellular sites and activate p38MAPK. In turn, p38MAPK may activate a single or multiple end-effectors directly or via MAPK intermediates. PLC: phospholipase C, PLD: phospholipase D.

Chapter 7

Conclusions And Future Directions

In the present study I have used human atrial trabeculae to investigate the mechanism of preconditioning in man and the reason for the failure to precondition the diabetic heart.

I have demonstrated that the failure to precondition the diabetic myocardium by IPC is due to diabetes itself. I have also shown that diazoxide (K_{ATP} channel opener) failed to induce cardioprotection in the diabetic myocardium, however cardioprotection can still be induced by activation of the downstream elements in the signal transduction pathway of preconditioning PKC and P38 MAPK. The inability of diazoxide to precondition the diabetic myocardium has lead me to conclude that a dysfunction of the mitochondria, probably at the level of mitoK_{ATP} channels, is the failure to precondition the diabetic myocardium. Further studies carried out on isolated mitochondria have shown that the diazoxide caused MMP partial depolarisation in non-diabetic but not in the diabetic myocardium. In addition diazoxide failed to increase superoxide generation in the diabetic myocardium but it may have affected other ROS. Our hypothesis that diazoxide failure to depolarise the MMP may lead to failure to increase in superoxide production in the diabetic myocardium is supported by evidence that generation of superoxide radicals trigger preconditioning [176]. However this hypothesis is contradicted by reports suggesting that mild uncoupling of the MMP lead to reduction in superoxide generations [185]. Therefore diazoxide effect on superoxide production in non-diabetic may be independent of its action on the MMP. Diazoxide effect on other ROS in the diabetic myocardium may be differential however these radicals are very rapidly inter-converted by mitochondria and to discriminate between them is difficult. Further

investigations with the use of more specific ROS detectors and/or specific ROS scavengers could you useful to define the relationship between diazoxide, MMP and ROS. More experiments are needed to explain the lack of mitochondrial depolarization in the diabetics and to demonstrate the direct relation of mitochondrial uncoupling to the different ROS generation and whether the activation of partial uncoupling of the mitochondria in diabetics can induce preconditioning and improve the outcome in the diabetics.

I also have demonstrated for the first time that the specific PKC isoform PKC α and ε are involved in ischaemic preconditioning with PKC ε being upstream and PKC α being downstream of mitoK_{ATP} channels. This relation was confirmed using specific PKC ε and α inhibitors given during preconditioning with diazoxide. This hypothesis was reinforced by the finding of increased PKC α phosphorylation but not PKC ε in response to treatment with diazoxide.

More studies are needed to investigate the subcellular localisation of the PKC isoforms involved and the mechanism by which PKC ε may affect mitoK_{ATP} channels. For these studies we suggest that the use of specific activator of PKC ε (when available) on the activity of mitoK_{ATP} channels may provide more information on the mechanism of preconditioning. The mechanism by which mitochondria K_{ATP} channels may phosphorylate PKC α phosphorylation should also be investigated and this may involve ROS species such as superoxide, as suggested by my studies. Certainly the elucidations of these mechanisms would require further investigations.

Clinical implications:

The present studies may have important clinical implications since they suggest that it is possible to target specific PKC isoforms to induce cardioprotection without activating other isoforms that may be involved in other cellular processes, thus avoiding unwanted side effects. In particular it may be useful for eliciting cardioprotection in the diabetic heart, which cannot be preconditioned by IPC or by diazoxide, by the specific activation of PKC α, which is downstream of the defective mitochondria. A better cardioprotection of the diabetic heart may result in reduction of the greater morbidity and mortality observed in diabetic patients with ischaemic cardiomyopathy and in saving of health resources.

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