The signaling pathways involved in the cardioprotection offered by insulin to the global low flow ischaemic/reperfused myocardium

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Declaration

I, the undersigned, hereby declare that the work in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:

Date: *07/02/2002*

Abstract

Introduction: It is well documented that insulin offers cardioprotection under ischaemic stress. In the past it was believed that the protective effects of insulin, such as the (a) recruitment of glucose transporters to enhance glucose entry into the cell, (b) stimulation of glycolysis, (c) enhancement of glycogen synthesis, (d) improved protein synthesis, and (e) positive inotropic and chronotropic properties, were metabolic of origin, but lately the emphasis has shifted towards the diverse signal transduction pathways elicited by insulin. Although these beneficial effects of insulin on ischaemia/reperfusion induced injury have been studied for many years, the exact protective mechanism is still not resolved. **Aim:** To investigate the influence of insulin on the signaling pathways as a possible protective mechanism against ischaemia/reperfusion and therefore to investigate the possible roles and cross signaling of cyclic adenosine monophosphate (cAMP), protein kinase B (PKB) and p38 mitogen activated protein kinase (p38 MAPK) in the cardioprotection offered by insulin to the reperfused, ischaemic myocardium. **Materials and methods:** Isolated rat hearts were perfused retrogradely in accordance with the Langendorff technique ($95\%O_2$, 5% CO_2). After 30 min of stabilization, hearts were subjected to 30 min global low flow ischaemia (0,2 ml/min), followed by 30 min of reperfusion. Hearts perfused with standard Krebs Henseleit solution containing 5 mM glucose were compared to hearts perfused with a perfusion solution containing 5 mM glucose and 0,3 µIU/ml insulin. Wortmannin was added during either ischaemia or reperfusion. Left ventricular developed pressure (LVDP), rate pressure product (RPP), tissue cAMP and PKB and p38 MAPK activation were measured. **Results:** Insulin treated hearts showed improved functional recovery ($P<0.05$) during reperfusion after ischaemia vs. non-

insulin treated hearts (85.5±4.6% vs. 44.8±4.9%). However, the addition of wortmannin (a PI3-kinase inhibitor) to the perfusion solution during either ischaemia or reperfusion abolished the improved recovery. At the end of ischaemia, cAMP levels of the insulin treated hearts were elevated significantly, while the cAMP content in the non-insulin treated hearts returned to control levels. Addition of wortmannin during ischaemia abolished this rise in cAMP. Wortmannin added during reperfusion only did not alter the levels of cAMP at the end of reperfusion. Activation of p38 MAPK was transient during ischaemia for both insulin and non-insulin treated hearts. Addition of wortmannin during ischaemia did not alter p38 MAPK levels at the end of ischaemia. P38 MAPK was activated significantly (P<0.001) in the non-insulin treated hearts vs. insulin treated hearts during reperfusion. Wortmannin, added at the onset of reperfusion, could partially abolish the effects of insulin to suppress p38 MAPK activation after 30 min of reperfusion. Activation of PKB in insulin treated hearts was significantly higher than non-insulin treated hearts during stabilization and early ischaemia. This activity was depressed by 30 min of ischaemia in both presence and absence of insulin. Wortmannin, when added before induction of ischaemia did not further lower this. The presence of insulin resulted in occurrence of strong PKB activation during reperfusion, peaking at 15 minutes and diminishing at 30 minutes. Wortmannin, added at the onset of reperfusion, abolished PKB activity measured at the end of reperfusion. **Conclusion:** Insulin exerted a positive inotropic effect and delayed the onset to ischaemic contracture. Inhibition of PI3-kinase by wortmannin abolished the protective effects of insulin, arguing for an insulin stimulated PKB involvement in cardiac protection. Insulin also increased cAMP production and attenuated activation of p38 MAPK, both associated with improved recovery. This evidence suggested possible cross signaling between different signaling pathways.

Uittreksel

Agtergrond: Insulin beskerm harte wat aan isgemiese stres blootgestel word. Alhoewel hierdie voordelige effekte van insulien reeds vir verskeie jare bestudeer is, is die presiese meganisme waarmee insulien die hart beskerm steeds nie duidelik nie. Navorsers het die beskermende effekte van insulien aan metaboliese gevolge soos: (a) verhoogde glukose transport d.m.v. inspanning van meer glukose transporters (b), stimulering van glikolise, (c) vebeterde glikogeensintese, (d) verhoogde proteiensintese, en (e) die positiewe inotropiese en chronotropiese eienskappe van insulien toegeskryf. Onlangs het die fokus verskuif na ander diverse seintransduksiepaaie. **Doel:** Die doel van hierdie studie was dus om die moontlike betrokkenheid van hierdie sientransduksiepaaie asook die interaksie tussen sikliese adenomonofosfaat (cAMP), proteïn kinase B (PKB) en p38 MAPK in die beskerming wat insulien aan die isgemiese, gereperfuseerde miokardium bied, te bestudeer. **Materiale en Metodes:** Geïsoleerde rotharte is geperfuseer in ooreenstemming met die Langendorff metode. Na 30 min van stabilisasie is harte blootgestel aan 30 min. globale lae vloei isgemie (0,2 ml/min), en daarna is harte vir 30 min. geherperfuseer. Harte wat geperfuseer is met 'n perfusaat wat 5mM glukose bevat is vergelyk met harte wat geperfuseer is met 'n perfusaat wat 5mM glukose en 0,3 µIU/ml insulien bevat. Sommige harte is geperfuseer met 'n perfusie oplossing waar wortmannin bygevoeg is tydens òf isgemie òf tydens herperfusie. Linker ventrikulêre ontwikkelde druk (LVDP), tempo-druk produk (RPP), weefsel cAMP-vlakke asook PKB en p38 MAPK aktiwiteit is gemeet. **Resultate:** Insulien-behandelde harte het funksioneel beduidend beter herstel tydens herperfusie na isgemie as harte wat nie met insulien behandel is nie ($85.5\pm4.6\%$ vs. $44.8\pm4.9\%$). Byvoeging van wortmannin by die

perfusie oplossing tydens òf isgemie òf reperfusie, het die toename in herstel wat gesien is in die insulien-behandelde harte, opgehef. Die cAMP vlakke in die insulien-behandelde harte het aan die einde van isgemie beduidend gestyg (P<0.001), terwyl vlakke in harte wat nie met insulien behandel is nie, na kontrole vlakke teruggekeer het. Die teenwoordigheid van wortmannin in die perfusie oplossing tydens isgemie, het die styging in cAMP voorkom , terwyl die byvoeging van wortmannin tydens herperfusie, nie die cAMP vlakke beïnvloed het nie. Die aktivering van p38 MAPK tydens isgemie was van verbygaande aard in beide die insulien-behandelde harte en harte wat nie met insulien behandel is nie. Die byvoeging van wortmannin tydens isgemie het nie die p38 MAPK aktivering beïnvloed nie. P38 MAPK is beduidend geaktiveer tydens herperfusie in harte wat nie met insulien behandel is nie vergeleke met die insulien-behandelde harte. Die byvoeging van wortmannin tydens reperfusie kon die effek van insulien om p38 MAPK aktivering te onderdruk, gedeeltelik ophef. PKB aktivering tydens die stabilisasie fase en vroeë isgemie was beduidend hoër in die insulien-behandelde harte vs. die harte wat nie met isulien behandel is nie. Die aktiwiteit is onderdruk deur 30 min isgemie ongeag die teenwoordigheid van insulien. Die byvoeging van wortmannin tydens isgemie het PKB aktivering nie verder verlaag nie. Die teenwoordigheid van insulien het 'n sterk aktivering van PKB tydens herperfusie veroorsaak met 'n piek na 15 min en 'n verlaging na 30 min. Wortmannin bygevoeg aan die begin van herperfusie, het PKB aktiwiteit opgehef aan die einde van reperfusie. **Opsomming:** Insulien het 'n positiewe inotropiese invloed gehad, en het die begin van isgemiese kontraksie vertraag. Die inhibisie van PI3-kinase deur wortmannin het die beskermende effekte van insulin opgehef, wat 'n insulin gestimuleerde PKB betrokkenheid aandui. Insulien het ook verhoogte cAMP produksie en verlaagde p38 MAPK aktivering tot gevolg gehad, en beide is geassosieer met

verbeterde herstel. Hierdie resultate dui dus op moontlike interaksie tussen die verskillende seintransduksiepaaie.

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Abbreviations

ADP	adenosine di-phosphate
adu	arbitrary densitometric units
AMI	acute myocardial infarction
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CF	coronary flow
CP	creatine phosphate
EGF	epidermal growth factor
ERK	extracellular signal-related kinases
FFA	free fatty acids
G3PDH	glyceraldehydes 3-phosphate dehydrogenase
G6P	glucose 6-phosphate
GIK	glucose-insulin-potassium
GPCR	G protein-coupled receptor
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
HR	heart rate
Hsp	heat shock protein
IRS-1	insulin receptor substrate 1
KH	Krebs Henseleit
K_i^+	intracellular potassium
K_o	extracellular potassium
LVDP	left ventricular developed pressure

MAPKAP-2	MAP kinase activated protein kinase-2
Na^+/Ca^{2+}	sodium-calcium exchanger
NGF	nerve growth factor
p38 MAPK	p38 mitogen activated protein kinase
PC	preconditioning
PDGF	platelet-derived growth factor
PDK	phosphoinositide dependent kinase
PFK	phosphofructokinase
PH	pleckstrin homology
PI3-K	phosphatidylinositol 3-kinase
PKB	protein kinase B
ROS	reactive oxygen species
RPP	rate pressure product
TOIC	time to onset of ischaemic contracture
wort	wortmannin

Chapter 1

INTRODUCTION

Insulin influences and improves all the aspects of metabolism of the heart, but the precise mechanism and signaling pathways by which it operates, is still not clear.

Insulin is a major regulatory hormone of carbohydrate metabolism in the heart (Taegtmeyer, 1994). Insulin promotes glucose entry into the cell and stimulates glycolysis, it enhances synthesis of glycogen, fatty acids and proteins, and inhibits glycogen and fat utilization (King & Opie, 1995). Insulin *per se* was shown to have cardioprotective properties. Van Rooyen et al. (1998) found that the administration of insulin improved the recovery of cardiac output during reperfusion through an increase in glucose uptake and stimulation of glycolysis during low flow ischaemia. The cardioprotective effect of insulin was also investigated by Jonassen et al. (1996) as part of the effect of a Glucose-Insulin-Potassium (Sodi-Pallares) cocktail. They found a reduction in infarct size when insulin was administered at the onset of reperfusion in both *in vivo* and isolated rat heart preparations. They subsequently focused their attention on insulin activated signal transduction pathways and the putative role of apoptosis in this cardioprotection exerted by insulin (Jonassen et al. 2000). In view of these interesting results, the aim of the present study was to further investigate the signal transduction pathways of insulin in the myocardium. However, our study differed from that of Jonassen and co-workers in that insulin was administered throughout the entire perfusion period, whereas they added insulin only during reperfusion. In addition, they used total global ischaemia compared to global low flow ischaemia in our study. We used recovery of mechanical function as endpoint compared to apoptosis and infarct size of the myocardium in their study.

It is known that insulin exerts some of its effects through the activation of the protein kinase B (PKB) signaling pathway, which in turn, mediates its metabolic effects. As this is a well-recognized survival mechanism (Downward, 1998) we investigated the involvement of insulin stimulated signaling pathways in cardioprotection of the rat heart during ischaemic stress.

During ischaemia and reperfusion, the metabolism of the myocardium changes, and it is characterized by an ATP shortage, the inhibition of glycolytic enzymes, altered membrane pump functioning, acidosis, and the accumulation of cyclic adenosine monophosphate (cAMP) (Opie, 1975; Wollenberger, 1969). However, the precise role of cAMP in the isolated perfused rat heart subjected to low-flow ischaemia, is not well documented. It is well established that cAMP production is directly related to myocardial contractility and accumulation thereof may even be harmful to the ischaemic heart. Furthermore, cAMP may lead to both PKB and p38 mitogen activated protein kinase (MAPK) activation. We therefore studied cAMP levels and possible links with other parameters and functional recovery in a model of low-flow ischaemia.

Several stress signal transduction pathways are activated by ischaemia and reperfusion. These pathways include the c-Jun NH_2-terminal kinase (JNKs), p38 MAPK and the extracellular signal-regulated kinases (ERKs) (Sudgen & Clerk, 1998). Whether these signaling pathways may play a protective or deleterious role during ischaemia and reperfusion is not well understood. We therefore investigated this controversial aspect of the stress signal transduction pathways, and a possible link with other cellular mechanisms and functional recovery of the hearts after low flow ischaemia.

The aim of this study was thus to investigate the possible roles for cAMP, PKB and p38 MAPK in the cardioprotection offered by insulin in the low flow ischaemic/reperfused myocardium.

Chapter 2

LITERATURE REVIEW

2.1 Ischaemia and reperfusion damage

Myocardial ischaemia occurs when the reduction of coronary flow is so severe that the supply of oxygen to the myocardium is inadequate for the demands of the tissue (Opie, 1998).

Myocardial ischaemia is a multifactoral process and is characterized by numerous changes in the tissue for example, loss of high energy phosphates (ATP + CP), accumulation of metabolites (eg. lactate), and acidosis (Opie, 1998). These changes are initially reversible but become irreversible when the period of ischaemia exceeds a certain limit. Should coronary flow be restored before the onset of irreversible injury, these ischaemic-induced changes can largely be reversed. Four main factors indicate irreversible ischaemic damage:

1) Critical ATP loss, 2) membrane damage, 3) mechanical effects such as cell swelling and membrane rupture as a result of elevated osmotic pressure, and finally 4) calcium overload (Opie, 1984; Opie, 1998).

Reperfusion improves the myocardial metabolism via increased washout of harmful metabolic products (Neely & Grotyohann, 1984), resumption of contractile activity, and by providing oxygen and nutrients to the ischaemic myocardium. While early reperfusion of the heart is essential in preventing further tissue damage due to ischaemia, reintroduction of blood flow can expedite the death of vulnerable, but still viable myocardial tissue, by initiating a series of events involving both intracellular and extracellular mechanisms, the so-called reperfusion injury (Park et al., 1999).

Reperfusion injury includes conditions such as postischaemic contractile dysfunction ("myocardial stunning"), reperfusion arrhythmias and lethal reperfusion injury (Piper et al., 1998).

It is suggested that reperfusion mediated injury is largely a consequence of injury during ischaemia, and can thus be effectively inhibited by the prevention of these ischaemic-specific events (Vanoverschelde et al., 1994).

The mechanism of reperfusion injury appears to be metabolic of origin, related to either free radical formation or excess accumulation of cytosolic Ca^{2+} (Opie, 1987), or a combination of these factors (Bolli, 1992). Reperfusion of postischaemic tissues is accompanied by generation of large amounts of oxygen radicals, formed by various mechanisms, which can overwhelm cellular defenses and induce cellular damage (Ambrosio et al., 1991; Zweier et al., 1987). According to Bolli (1990), compelling evidence indicates that the free radical damage occurs in the initial moments of reflow, so that myocardial stunning can be viewed as a sub-lethal form of oxyradical-mediated reperfusion injury.

The other crucial factor influencing reperfusion injury is the transient calcium overload during early reperfusion that contributes to postischaemic dysfunction ("stunning") (Tani & Neely, 1989; Bolli, 1990). Opie (1998) suggested that the internal calcium overload could damage the contractile apparatus to impair the normal physiological response to calcium, resulting in myocardial stunning. Application of calcium channel blockers or modulation of calcium flux via the sarcoplasmic reticulum during early reperfusion resulted in a decrease in the severity of stunning, and thus protection (Du Toit & Opie, 1992).

The calcium- and free radical-mediated components of the damage may be interactive. Free radicals may also inhibit the uptake of calcium by the sarcoplasmic reticulum,

inhibit the sodium pump, stimulate Na^+/Ca^+ exchange, and decrease the rate of inactivation of the calcium current (Opie, 1991).

The composition of the reperfusion solution may affect the incidence of reperfusion arrhythmias. During reperfusion, fatty acid oxidation quickly recovers and dominates as the source of energy. The main consequence of this is that glucose oxidation rates are markedly inhibited. As a result, high glycolytic rates, possibly due to increased glucose transport, and low glucose oxidation rates can result in a substantial uncoupling of glycolysis from glucose oxidation (Lopaschuk, 1997). This uncoupling of glycolysis from glucose oxidation contributes to the production of protons from glucose metabolism during reperfusion, which can contribute to reperfusion injury (Lopaschuk, 1994; Liu et al., 1996). Therefore it appears that the stimulation of glucose oxidation, either directly or by inhibiting fatty acid oxidation, results in a significant increase in cardiac function and efficiency (Lopaschuck, 1994). In this regard, the inhibitory effect on reperfusion ventricular arrhythmias of glucose oxidation versus fatty acid oxidation is well established (Manning & Hearse, 1984: Bernier & Hearse, 1988; Coleman et al., 1989).

2.2 Manipulation of the outcome of ischaemia-reperfusion

2.2.1 Glucose-Insulin-Potassium

2.2.1.1 Theory

Glucose-Insulin-Potassium (GIK) therapy was advocated for the treatment of acute myocardial infarction, initially as a polarizing agent to provide electrical stability and later as a metabolic support (Fath-Ordoubadi & Beatt, 1997).

2.2.1.2   The old rationale for GIK

In the early 1960's, Sodi-Pallares and his co-workers (1962) performed pioneering work on the effects of the administration of a GIK solution to the heart after infarction. They suspected that the cause of damage after myocardial infarction was a possible ionic disturbance, and thoroughly studied the effect of GIK on the electrocardiographic signs of myocardial insults.

They considered that the resting or diastolic polarization of the cardiac fibers was due to the ratio of the potassium inside the cell (K_i^+), to that on the outside (K_o^+) of the cell. Consequently, any disturbance of either the diastolic polarization or the K_i^+/K_o^+ ratio would influence one another.   Since potassium influences the excitability and contractility of cardiac muscle by means of the ionic interrelationship at the cellular level, it plays a major role in determining the state of polarization of the cell (Gautam, 1969), and any disturbance of the K_i^+/K_o^+ ratio would therefore result in disturbance of the resting membrane potential and generation of ventricular arrhythmias that could be fatal.

Sodi-Pallares and his co-workers thus hypothesized that forcing K^+ into the fibers should modify the trans-membrane potassium ratio, and normalize the ionic exchange. Addition of glucose and insulin to the so-called Sodi-Pallares solution was based on their known beneficial actions on K^+ movements and glucose uptake.   At that time Muntz (1959) concluded that insulin increased the intracellular accumulation of glucose and potassium, thereby enhancing the process of oxidative phosphorylation and the transfer of high-energy phosphate from phosphopyruvate to ADP and ATP.   During the sixties, the administration of this polarizing treatment was used successfully in at least two independent trials, showing its cardioprotective effect after a myocardial infarction (Mittra, 1965; Sodi-Pallares, 1962).

Subsequently, GIK was proven to be a safe and effective intervention, also for the treatment of patients in cardiogenic shock after hypothermic ischaemic arrest for coronary bypass surgery (Gradinak et al., 1989; Taegtmeyer & Villalobos, 1995). Clinical studies conducted by Diaz et al. (1998) furthermore showed a reduced mortality in patients with acute myocardial infarction (AMI) undergoing GIK reperfusion therapy.

Unfortunately, the notion of GIK treatment in acute myocardial infarction was received negatively by many scientists and clinicians because of subsequent studies that questioned the efficacy of this treatment. For example, a study by the Medical Research Council Working Party, found no significant difference in mortality between the GIK-treated and the untreated control groups (23,9% vs. 23,3%). This report played a significant role in discrediting GIK therapy (British Medical Research Council, 1968).

Fath-Ordoubadi and Beatt (1997) overviewed the randomized placebo-controlled GIK trials, and concluded that the results were inconclusive because of several factors, including a low number of recruits, poor design and methodological discrepancies between different clinical trials.

2.2.1.3 The new rationale for GIK

Despite the inconclusive and negative results described above, the interest in the concept of myocardial GIK-induced salvage in acute myocardial infarction did not disappear. The "old" and "new" rationales for the metabolic support of the ischaemic and postischaemic myocardium with GIK complement each other. Whereas earlier researchers focused more on the recovery of the disturbance of polarization of the

cells, later studies shifted their focus to prevent further tissue damage by improving the metabolic milieu of the heart by restoring energy flux (Taegtmeyer, 1995).

2.2.1.4 Mechanism of Action

During ischaemia, exogenous glucose has been shown to be a more efficient fuel than free fatty acids (FFA) or glycogen and is more likely to prevent ischaemic myocardial injury (Runnman & Weiss, 1988). In particular, glycolytic-derived ATP preferentially supports cell membrane function by protecting membrane ion transport and hence helps to preserve cell integrity (Lochner et al., 1996; Weiss & Lamp, 1987).

During myocardial ischaemia, high concentrations of FFA, provoked by high sympathetic activity (Gupta et al., 1969; Opie et al., 1977) have been shown to lead to increased myocardial oxygen requirements and depression of myocardial activity and contraction (Oliver & Opie, 1994; Mjøs, 1971). During ischaemia, β-oxidation of FFA is reduced, which in turn, results in intracellular accumulation of acylcarnitine and acyl coenzyme A. Acylcarnitine inhibits the sarcoplasmic reticulum Ca^{2+} pump, the sarcolemmal Na^+/Ca^{2+} exchanger, and the Na^+ pump. It can also activate Ca^{2+} channels and increase cyclic AMP levels (Apstein, 2000). Acyl coenzyme A might be involved in many of the deleterious effects of ischaemia, particularly arrhythmogenesis (Opie, 1975). Soboll et al. (1984) also found that long-chain acyl coenzyme A exerts an inhibitory effect on mitochondrial adenine nucleotide translocation (ATP-ADP translocase) in the cell.

These actions may cause the impairment of calcium homeostasis (Saman et al., 1988), and the production of free radicals, leading to instability and ventricular arrhythmias, and ultimately to cell membrane damage (Kurien & Oliver, 1970).

However, in the presence of a high glucose and insulin concentrations, the inhibition of glycolysis by FFA is minimal, while high circulating levels of glucose and insulin both depress plasma levels of FFA and decrease myocardial FFA uptake at any given plasma FFA level (Oliver & Opie, 1994; Opie, 1975).

GIK may therefore act beneficially via an antilipolytic mechanism, and by supporting membrane stability.

2.2.1.5 Reperfusion strategies

Studies conducted by Vanoverschelde and co-workers (1994) support the complementary roles of GIK and reperfusion therapy. They observed that through the reduction in the extent of myocardial ischaemic damage and suppression of FFA levels, GIK therapy helped to prevent reperfusion injury that may occur after successful revascularization.

GIK protects the cell membrane of ischaemic myocytes, as well as endothelial and vascular smooth muscle cells, and that may improve reflow after reperfusion and protect against no-reflow phenomenon by reducing cell swelling and microvascular compression (Eberli et al., 1991).

The sound theoretical rationale for the beneficial effect of GIK, its complementary role with reperfusion strategies, and very low incidence of serious side effects with proper monitoring provides a definite reason for further investigation and clinical application.

The mechanisms underlying GIK mediated cardioprotection are, however, still unclear. Recent data implicates insulin "alone" as the major protagonist of cardioprotection when administered at the time of reperfusion (Jonassen, 2000).

2.2.2 Glucose

2.2.2.1 Myocardial metabolism

During normoxia, the circulating free fatty acids (FFA) are the major fuel source for normal functioning of the heart (Bing, 1954; Brachfeld & Scheuer, 1967). During ischaemia, a shift occurs from aerobic to anaerobic metabolism (Oliver & Opie, 1994), since oxygen deficiency prevents the oxidation of non-glucose substrates such as fatty acids. The heart becomes totally reliant on anaerobic glycolysis during ischaemia. The rate of utilization of glucose or glycogen may determine the degree of survival of the ischaemic heart (Opie, 1998, Cascarano et al., 1968; Scheuer & Stezoski, 1969; Weissler et al., 1968). Therefore the provision of appropriate energy-giving substrates, such as glucose, may protect against ischaemic injury (Olivier & Opie, 1994).

The metabolism of the ischaemic myocardium is characterized by an ATP shortage, as well as inhibition of glycolytic enzymes by the accumulation of lactate and concomitant intracellular acidosis. Other detrimental changes include inadequate functioning of membrane pumps (Opie, 1990), potassium (K^+) loss in the ischaemic zone (Opie, 1970; Weiss and Lamp, 1987), sarcolemmal damage (Williamson et al., 1976), accumulation of cAMP (Wollenberger et al., 1969), and deficient Ca^{2+} homeostasis (Henry et al., 1977).

2.2.2.2 Glucose as energy bearing substrate

It is well established that increased provision of glucose is beneficial to the ischaemic myocardium (Opie, 1998). Indices of protection include a reduction of reperfusion-induced arrhythmias (Bernier & Hearse, 1988), the attenuation of stunning (Ferrari et

al., 1985), and a decrease in the occurrence of ischaemic contracture (Owen et al., 1990).

The mechanism underlying the protection conferred may be attributed to the increased production of glycolytic ATP (Owen et al., 1990), and the resultant improvement in cytosolic Ca^{2+} homeostasis (Opie, 1989). De Leiris et al. (1975) reported lower enzyme release in glucose perfused, ischaemic hearts, indicating a beneficial effect of glucose on sarcolemmal permeability. This was later confirmed by Lochner and co-workers (1996) who demonstrated the beneficial effects of glucose, using lanthanum to quantify sarcolemmal permeability.

To maximize glucose uptake into the myocytes, there must be adequate glucose delivery by the coronary blood flow and efficient transport of glucose from the blood to the interstitial space and finally the cytosol (King et al., 1995).

Anoxia, where the oxygen tension is reduced, promotes glucose transport into the cell (Morgan et al., 1961). Similarly, moderate ischaemia, during which oxygen deprivation is due to limited coronary flow, also enhances glucose uptake (Rovetto et al., 1973). However, during severe ischaemia, when the coronary flow is drastically reduced, glucose uptake is reduced since delivery to the myocardium is restricted (Achs & Garfinkel, 1977; Rovetto et al., 1973).

While anoxia accelerates glucose transport, the rate of anaerobic glycolysis is enhanced by reduced inhibition of glycolytic enzymes. Accumulation of free fatty acids and loss of high-energy phosphate compounds within the cell are responsible for this enzymatic inhibition during normoxia (Opie, 1975). In moderate ischaemia, the major portion of the glucose taken up enters the Krebs Cycle to produce energy via oxidative

metabolism. Severe ischaemia limits flux through the Krebs Cycle and lactic acid accumulates within the myocardium (Rovetto et al., 1973). Together with protons (generated by the breakdown of ATP), lactate inhibits glycolytic enzymes such as phosphofructokinase (PFK), hexokinase (HK), phosphorylase kinase C, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Neely et al., 1975).

Thus during ischaemia, glycolytic flux is regulated by both the rate of glucose delivery and the severity of enzymatic inhibition. As coronary flow is reduced, metabolic accumulation and the influence of thus metabolic inhibition become increasingly important, limiting glycolytic flux (King et al., 1995). This may explain why glycolytic flux is enhanced during mild ischaemia, and inhibited during severe ischaemia (Opie, 1990).

2.2.3 Insulin

Insulin is a major regulatory hormone of carbohydrate metabolism in the heart (Taegtmeyer, 1994). Insulin promotes glucose entry into the cell (see 1.2.3.2.2) and stimulates glycolysis (see 1.2.3.2.2.4), enhances synthesis of glycogen (see 1.2.3.2.2.3), fatty acids and proteins (see 1.2.3.2.2.5), and inhibits glycogen and fat utilization (King & Opie, 1995). Insulin was shown to have cardioprotective properties. Van Rooyen et al. (1998) found that the administration of insulin improved cardiac output recovery during reperfusion through an increase in glucose uptake and stimulation of glycolysis during low flow ischaemia. The cardioprotective effect of insulin as part of the effects of GIK (Sodi-Pallares) cocktail was further investigated by Jonassen et al. (1996). They found a reduction in infarct size when insulin was given at the onset of reperfusion in both the *in vivo* and the isolated rat heart preparation.

2.2.3.1 Insulin receptor, IRS-1, and Phosphatidylinositol 3-kinase (PI3-K)

Insulin receptor activation promotes an intrinsic tyrosine kinase activity, which leads to insulin receptor substrate 1 (IRS-1) phosphorylation. IRS-1 binds with Src homology 2 containing proteins (SH2-phosphotyrosine binding sites), including PI3-Kinase, Ras GTPase-activating protein, phospholipase C and others (Myers, 1994). PI3-kinase may eventually activate protein kinase B (PKB) and mediate translocation of the insulin sensitive glucose transporter, GLUT 4, to the sarcolemmal membrane (Myers, 1994; Sun et al., 1994), which in turn, leads to increased glucose uptake and glycogen synthesis.

2.2.3.2 Protein kinase B (PKB)

Protein kinase B/Akt was cloned by virtue of its homology to the A and C protein kinases, and is the cellular homologue of the product of the *v*-akt oncogene (Bellacosa et al., 1991; Coffer & Woodgett, 1991; Jones et al., 1991).

Protein kinase B is a member of the second messenger-dependent family of serine/threonine kinases that have been implicated in signaling pathways downstream of growth factor receptor tyrosine kinases (Meier et al., 1997).

2.2.3.2.1 Activation of PKB (Fig. 2.1)

PKB is activated in response to treatment of cells with a wide variety of growth stimuli, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, thrombin and nerve growth factor (NGF) (Downward, 1998). Several findings indicate that PI3-K is involved in the regulation of PKB, for example, growth factor as well as insulin-induced activation of PKB is inhibited by the PI3-K inhibitor wortmannin (Franke et al., 1995; Burgering & Coffer, 1995; Kohn et al., 1995). PKB is also inhibited by the

expression of a dominant negative form of PI3-K (Burgering & Coffer, 1995), and in the case of PDGF, it is inhibited by mutation of PDGF receptor tyrosines 740 and 751, which bind to the PI3-K regulatory subunit (Franke et al., 1995; Burgering and Coffer, 1995). PI3-K activity is thus required for the regulation of PKB by growth factors (Klippel et al., 1996; Marte et al., 1997; Franke et al., 1997; Didichenko et al., 1996). As PI3-K is stimulated by direct interaction with the p110 catalytic subunit (Rodrigues-Viciana et al., 1994; Rodrigues-Viciana, 1996) of the small GTP binding protein, Ras, PKB is also controlled by Ras (Klippel et al., 1996; Marte et al., 1997; Datta et al., 1996).

In addition to PI3-K-mediated regulation of PKB, another pathway for the regulation of PKB exists that is not sensitive to PI3-K inhibitors such as wortmannin (Downward, 1998). Cellular stresses such as heat shock and hyperosmolarity, or ischaemia/reperfusion, which also activate the p38 MAPK cascade, are able to stimulate the activity of PKB in a wortmannin insensitive manner (Konishi et al., 1997). Heat shock induces association of PKCδ with the pleckstrin homology (PH) domain of PKB (Konishi et al., 1994).

In addition, PKB can also be activated by increases in intracellular cAMP concentrations in a manner that is independent of PI3-Kinase. This activation does not appear to be due to a direct phosphorylation of PKB by PKA, but is ultimately related to the same mechanism as the one used by PI3-Kinase, that is phosphorylation of Thr308 and Ser473 of PKB (Sable et al., 1997).

PI3-K activation results in the production of PI(3,4)P_2 and PI(3,4,5)P_3. The binding of the pleckstrin homology (PH) domain of PKB to these phosphoinositides recruits PKB to the plasma membrane (Downward, 1998) where it undergoes a conformational

change (Hemmings, 1997) allowing the enzyme to become phosphorylated (activated) on Thr308 by PDK1 (Phosphoinositide dependent kinase)-1 and on Ser473 by PDK2. The activity, or possibly location, of PDK1, and perhaps PDK2, may also be regulated by PI3-Kinase products (Downward, 1998). Subsequently, PKB is released from the membrane to phosphorylate specific targets (Hemmings, 1997).

2.2.3.2.2 Downstream effects of PKB activation

2.2.3.2.2.1 Insulin and the metabolic effects of PKB

Activated PKB mediates a number of metabolic effects of insulin by phosphorylating its cellular substrates (Downward, 1998).

2.2.3.2.2.2 Glucose transport (GLUT 4 + GLUT 1)

One of the major metabolic responses invoked by insulin challenge is the stimulation of glucose transport in muscle and adipose tissue by recruiting glucose transporters (GLUT 1 and GLUT 4) to the cell surface from intracellular pools (Coffer et al., 1998). The insulin-sensitive glucose transporter, GLUT 4, is distributed between vesicles in the cytosolic pool and the plasma membrane (James et al., 1989).
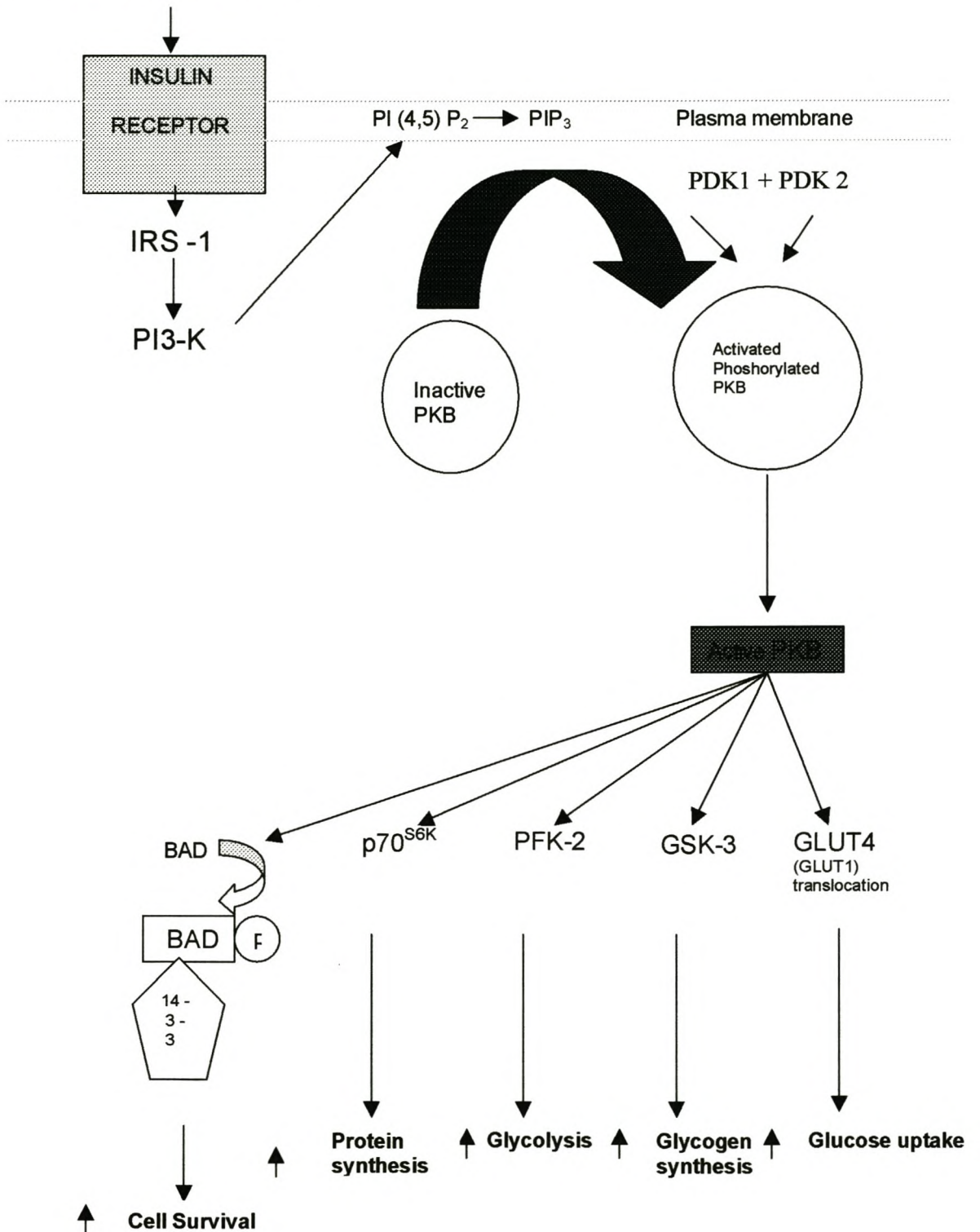
Insulin acutely stimulates glucose transport in muscle and fat by recruiting up to about 40% of cytosolic GLUT 4 to the plasma membrane, compared to a normal level of 1%. Glucose uptake can thus be increased 10-40 fold (King & Opie, 1998; Sun et al., 1994). GLUT 1 translocation may also be triggered by insulin (Elsas & Longo, 1992).

Figure 2.1   Protein kinase B pathway

While GLUT 1 has a lower affinity, but a higher capacity for glucose than GLUT 4, the latter is largely responsible for insulin-stimulated glucose uptake, and is far more efficient than GLUT 1 as a transporter of glucose into the cell (Nishimura et al., 1993).

Inhibitors of PI3-Kinase have been reported to block the ability of insulin to stimulate glucose uptake (Cheatham et al., 1994; Okada et al., 1994; Quon et al., 1995; Donthi et al., 2000). Overexpression of active forms of PI3-K at least partially mimic the effect of insulin (Katagiri et al., 1996; Martin et al., 1996; Tanti et al., 1996; Frevert et al., 1997), thus providing a link between activation of PKB and the stimulation of GLUT 4 translocation (Kohn et al., 1996; Tanti et al., 1997; Hajduch et al., 1998).

Furthermore, okadaic acid, a potent stimulator of GLUT 4 transport, induces PKB phosphorylation as efficiently as insulin without the activation of PI3-K. These observations demonstrate a parallel between the ability of agents to promote GLUT 4 translocation and activate PKB, suggesting an important function for PKB in this process (Coffer et al., 1998).

2.2.3.2.2.3 Glycogen synthesis

Glycogen synthesis is stimulated by insulin, as well as by increased intracellular glucose or glucose 6-phosphate (G6P) levels. Glycogen synthase is the most important enzyme in glycogen synthesis. Two forms of glycogen synthase, one acting on proglycogen and another on macromolecular glycogen, have been identified (Alonso et al., 1995), possibly accounting for different rates of synthesis of the different isoforms of glycogen.

Insulin stimulates glycogen synthesis by increasing glucose uptake and by stimulating glycogen synthase phosphorylation (Lawrence, 1992). Insulin also inhibits glycogen

breakdown by promoting dephosphorylation, and thus inactivation, of phosphorylase, the primary enzyme in glycogen breakdown (King & Opie, 1998).

Apart from regulation of glucose-transporter function, insulin stimulation also results in the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3) (Coffer et al., 1998). Stimulation of 293 cells (human embryonic kidney cells) with insulin or IGF-1 leads to PKB mediated phosphorylation of GSK3 at Ser9, resulting in its inactivation and consequent activation of glycogen synthesis (Shaw et al., 1997). GSK3 is involved in several intracellular signaling pathways, including the control of the transcription factors AP1 and CREB (the cyclic AMP response element binding protein), and the tumour supressor gene product APC (Welsh et al., 1996).

2.2.3.2.2.4 Glycolysis

Insulin treatment stimulates glycolysis by activating phosphofructose kinase-2 (PFK-2) via the PKB pathway in rat heart *in vivo* and in isolated rat cardiomyocytes. PFK-2 is responsible for generating fructose 2,6-bisphosphate, a key allosteric activator of 6-phosphofructo-1-kinase, the rate-limiting enzyme in mammalian glycolysis (Deprez et al., 1997).

2.2.3.2.2.5 Protein synthesis

Insulin contributes to protein synthesis via the PKB pathway. $p70^{S6k}$ plays a key role in cellular growth control mechanisms by coordinating protein biosynthesis via phosphorylation of the S6 subunit of 40 S ribosomes or via regulation of the activity of the eukaryotic initiation factor 4E binding protein, 4E-BP1 (Proud, 1996). Expression of

activated PKB can stimulate p70^{S6k} activity in T cells, indicating that PKB substrates are part of the p70^{S6k} activation pathways. Moreover, given the ability of PI3-Kinase signals to stimulate PKB and p70^{S6k}, it seems probable that PKB mediates the PI3-kinase activation of p70^{S6k} in T cells (Reif et al., 1997).

2.2.3.2.2.6 Anti-apoptotic signaling mediated by PKB

It is well known that PKB is involved in the regulation of cell survival (Downward, 1998). It has been demonstrated previously that inhibition of PI3-Kinase reduces the ability of survival factors to protect various cell types from programmed cell death or apoptosis (Yao and Cooper, 1995; Yao and Cooper, 1996). Jonassen et al. (2000) demonstrated that in rat neonatal cardiomyocytes, administration of insulin at the onset of reoxygenation after an ischaemic insult resulted in a significant reduction in total myocyte cell death and in the development of apoptosis. Their data suggest that a significant component of the insulin induced cell survival is based on the attenuation of apoptosis.

The mechanism of PKB protection against programmed cell death has been the subject of much investigation recently. PKB can phosphorylate the pro-apoptotic Bcl-2 family member BAD (Del Peso et al, 1997; Datta et al., 1997), both *in vitro* and in intact cells. The phosphorylation occurs at Ser136 of BAD (Datta et al., 1997), creating a binding site for 14-3-3 (a family of ubiquitous highly expressed adaptor proteins). When BAD binds to 14-3-3 it is unable to heterodimerise with and to inhibit the survival activity of the proteins Bcl-2 or Bcl-X$_L$ (Zha et al., 1996). The evidence suggests that PKB can act to reverse the death-inducing activity of BAD. However, it is far from clear whether this is the only, or even the primary, way in which the survival signal from PKB is mediated (Downward, 1998).

2.2.4 Stress kinases

2.2.4.1 Signaling pathways

Cells respond to extracellular signals by transmitting intracellular instructions to coordinate appropriate responses. Among the many pathways often used to transduce these signals are the highly conserved mitogen-activated protein kinase (MAPK) or extracellular signal regulated protein kinase (ERK) cascades (Robinson & Cobb, 1997). These cascades are present in all eukaryotic organisms and consist of a three-kinase module that includes a MAPK, which is activated by a MEK, which in turn is activated by a MEKK (Cobb & Goldsmith, 1995).

2.2.4.2 p38 MAPK activation by stress

A major advance in understanding signaling events in response to stress came from the discovery that stresses such as heat shock, protein synthesis inhibitors, UV, and DNA-damaging agents activate p38 MAPK (Robinson & Cobb, 1997). p38 MAPK may also be activated by reactive oxygen species (ROS) (Clerk et al. 1998; Aikawa et al. 1997), hypoxia/reoxygenation (Seko et al., 1997), hyperosmotic shock (Clerk et al., 1998), arsenite and proinflammatory cytokines (Wang et al., 1997). Cells respond to ischaemia/reperfusion by activating several members of the MAPK family. These kinases include c-Jun NH_2-terminal kinase (JNK), p38 MAPK and the extracellular signal-regulated kinases (ERK) (Sugden & Clerk, 1998). Studies by Bogoyevitch et al. (1996) showed that JNK is activated during reperfusion following ischaemia, whilst ERK is not activated during reperfusion following ischaemia. P38 MAPK and one of its downstream target substrates, MAPKAPK-2 (MAP kinase activated protein kinase-2),

are activated during ischaemia and this activation is sustained or increased during reperfusion (Bogoyevitch et al., 1996; Yin et al., 1995).

Ping et al. (1999) demonstrated that ischaemic preconditioning (PC) (short episodes of ischaemia) significantly increased the phosphorylation activity of the JNKs and ERKs via a PKC - dependent signaling pathway. This could be maintained in at least 6 cycles. They also indicated that the phosphorylation activity of the p38 MAPK cascade was increased by a brief ischaemic stimulus, but the activation was not sustained.

Release of endogenous catecholamines during ischaemia/reperfusion (Schömig et al., 1984) in the extracellular space of the ischaemic myocardium leads to activation of α_1- and β-adrenergic receptors which, in turn, may result in the stimulation of p38 MAPK, JNK and ERK. It is known that p38 MAPK is an important downstream target of the beta-adrenergic signaling pathway via the activation of cAMP and PKA (Cao, 2001).

2.2.4.3 Components of the p38 MAPK signaling pathway (Fig. 2.2)

Similar to stress, G protein-coupled receptor (GPCR) agonists may eventually lead to the activation of p38 MAPK's. Cellular stresses induce reorganization of the cytoskeleton and activation of the small GTP-binding proteins, Rac and Cdc-42, whereas GPCR agonists activate the small GTP-binding protein, Ras, through a PKC-dependent mechanism. Ras is associated with the activation of the ERK cascade and may either directly, or indirectly, activate the stress-responsive p38 MAPK. The upstream activators (MEKK's) of the stress-responsive MKK's have not been clearly defined but possibly include MEKK's (MAPK or ERK kinase kinases), mixed lineage kinases (MLK's), and/or p21-activated kinases (PAK's).

Figure 2.2   p38 MAPK pathway
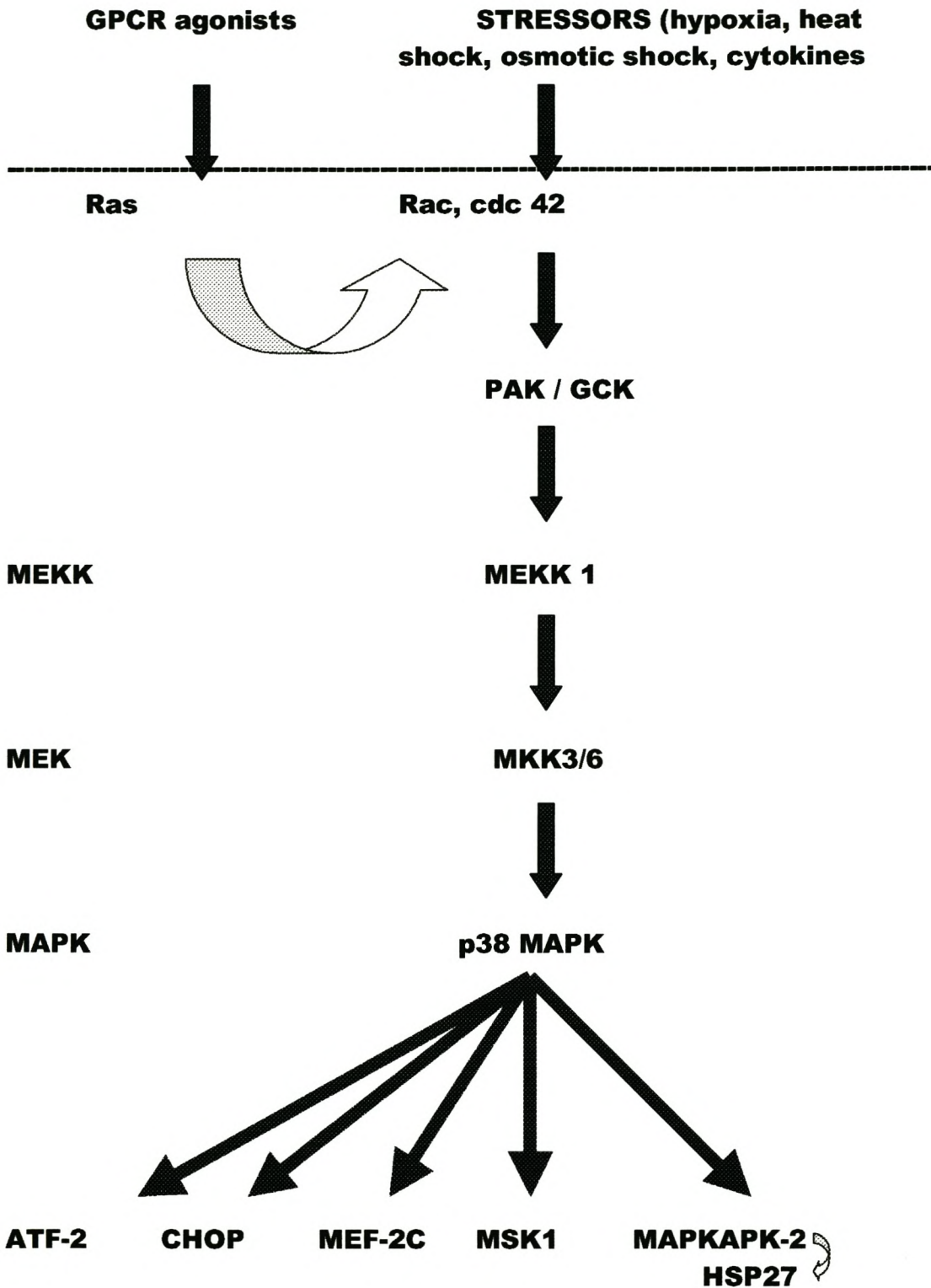
In the p38 pathway, the MEKKs activate MKK3 and MKK6 leading to p38 activation (Sugden & Clerk, 1998).

P38 MAPK has been reported to be located in both the cytosol and the nucleus, translocating from the cytosol to the nucleus in response to hyperosmotic stimulation (Raingeaud et al., 1996), or as a consequence of ischaemic stress adaptation (Maulik et al., 1998).

2.2.4.4   Downstream effects of p38 activation

2.2.4.4.1   Gene expression

The p38 pathway controls the activity of multiple transcription factors and the expression of many genes (Obata et al., 2000). The pathway leads to phosphorylation and enhancement of the activity of many transcriptional control factors, including:

- Activating transcription factor (ATF)-1/2 (Raingeaud et al., 1995; Raingeaud et al., 1996; Degols & Russell, 1997)

- CHOP/GADD153 (growth arrest and DNA damage) (Wang & Ron, 1996)

- MEF-2C (Myocyte enhancer-binding factor 2C) (Han et al., 1997)

- HSF (heat shock transcription factor)-1 (Kim et al., 1997)

- Mnk1/2, which may regulate the activity of the translational initiation factor eIF4E (Fukanaga & Hunter, 1997; Waskiewitz et al., 1997)

Some transcription factors are known to be direct substrates of p38 MAPK, while others are phosphorylated by downstream protein kinases that are themselves activated by p38 phosphorylation, such as MAPKAPK-2 (Obata et al., 2000, Bogoyevitch et al., 1996; Maulik et al., 1996; Zu et al., 1997)

2.2.4.4.2 Arachidonic acid signaling

The p38 MAPK pathway also influences the production of arachidonic acid metabolites (e.g., prostaglandins, leukotrienes, and other eicosanoids). The activation of Phospholipase A_2 (PLA_2), a primary regulator of arachidonic release from phospholipids, is enhanced via p38 MAPK-dependent phosphorylation upon TNF-α treatment of neutrophils (Waterman et al., 1996) or endothelin-1 treatment of smooth muscle cells (Husain & Abdel-Latif, 1999). After its release, arachidonate is metabolized via two main pathways, the cyclooxygenase (COX) and lipooxegenase pathways, that are largely constitutively active (Obata, 2000). The activity of the COX pathway is increased as a consequence of the gene expression of the first enzyme in the pathway, COX-2, a process that can be enhanced by p38 activity (Guan et al., 1998; Hwang et al., 1997; Pouliot et al., 1997). This should lead to increased production of prostaglandins at the expense of leukotriene generation, leading to selective enhancement or suppression of particular inflammatory responses (Obata et al., 2000).

2.2.4.4.3 Cytokine signaling

In addition to its recognized role in TNF-α and IL-1 production (Lee et al., 1994), p38 MAPK also plays a role in the production of IL-4 (Schafer et al., 1999), IL-6 (Beyaert et al., 1996), IL-8 (Marie et al., 1999), and IL-12 (Lu et al., 1999).

2.2.4.4.4 Nitric oxide signaling

The p38 MAPK pathway can enhance nitric oxide production by increasing the intracellular concentration of arginine, the substrate for nitric oxide synthetase via

stimulation of arginine transporter activity (Caivano, 1998). It is also required for increased gene expression of the inducible form of NO synthetase (iNOS, NOS II) (Guan et al., 1997). P38 may also be involved in the transduction of nitric oxide signaling. Activation of p38 MAPK in human neutrophils following LPS stimulation is attenuated by inhibitors of NO synthetase and by NO scavengers whereas treatment with NO releasing agents increased MKK3 and MKK6 activity and p38 phosphorylation (Browning et al., 1999). P38 is furthermore required for the NO-induced apoptosis in a neutrophil-like cell line (HL60) (Jun et al., 1999).

2.2.4.4.5 Cytoskeletal dynamics

Several cytoskeletal proteins are substrates of the p38 pathway, including the microtubule-associated protein tau (Goedert et al., 1997), the actin-associated protein, heat shock protein - 27 (hsp27), and the intermediate filament proteins h-caldesmon (Hedges et al., 1998), vimentin (Cheng and Lai, 1998) and keratin polypeptides 8,18 (Feng et al., 1999). The significance of most of these p38-catalyzed phosphorylations is unclear, but the effect of hsp27 phosphorylation has been extensively examined. After cells are exposed to hyperthermia, hsp27 levels increase and contribute to thermoresistance in a p38-dependent manner, likely through influencing actin reorganization (Landry & Huot, 1995; Lavoie et al., 1993). Under nonstress conditions, hsp27 forms large oligomers that, together with hsp70, act as molecular chaperones to stabilize and refold various proteins. Activation of p38 MAPK induces the phosphorylation of the small heat shock protein, hsp27 via MAPKAPK-2, causing a reduction in the size of hsp27 oligomers. These smaller oligomers are thought to be responsible for the stabilization of actin filaments (Lavoie et al., 1993).

2.3 Protective vs. deleterious effects of p38 MAPK stimulation

Controversy surrounds the ultimate role of p38 MAPK in myocardial ischaemia/reperfusion. Activated p38 MAPK may contribute to signal transduction pathways that culminate in cell death following stress, or they may act to prevent widespread cell death, limit damage or mediate adaptation (Bogoyevitch, 2000).

Studies conducted by Mackay and co-workers (Mackay & Mochly-Rosen, 1999), using chemical inhibitors, concluded that p38 MAPKs promote cardiac myocyte death during extended periods of ischaemia or exposure to hydrogen peroxide.

Marais et al. (2000) investigated the effect of preconditioning (PC) on the activation of p38 MAPK during 25 minutes sustained global ischaemia, and they noted that p38 MAPK was activated after 5 minutes of ischaemia in their perfusion protocol. Their findings indicated that the attenuation of p38 MAPK activity during sustained ischaemia and reperfusion was associated with improved functional recovery. They also showed that inhibition of p38 MAPK activation with SB 203580 could significantly improve both cell morphology and viability.

Saurin and his co-workers (2000) investigated the isoform selectivity of p38 MAPK by infecting myocytes with adenoviruses encoding wild-type p38α or p38β MAPK. They found that cells expressing a dominant negative p38α, which prevented ischaemic p38 MAPK activation, were resistant to lethal simulated ischaemia. Thus inhibition of p38α MAPK activation during ischaemia reduced injury.

Supporting these findings, Ma et al. (1999), concluded that p38 MAPK plays a pivotal role in promoting myocardial apoptosis in isolated perfused rat hearts. Similarly, cardiac injury in response to oxidative stress induced by hydrogen peroxide was

apparently mediated by a p38 MAPK-dependent production of TNF-α (Meldrum et al., 1999).

Cardioprotection may therefore be correlated with the ability of a compound or intervention to inhibit p38 MAPK activation (Bogoyevitch, 2000).

On the other hand Wang and co-workers (Wang et al., 1998) yielded more complex results by the overexpression of MAPK. Whilst p38 MAPK have been implicated in the survival response of cardiac myocytes, it may be the p38α MAPK isoform that is specifically involved in cell death. The results of overexpression of p38 MAPKs and constitutively - activated mutants of their specific activators, MKK3 and MKK6 indicate that this pathway plays a complex role in the cardiac myocyte. Whilst the overexpression of MKK3 or MKK6 increased surface area sarcomeric organisation and ANF expression, the co-expression of MKK3 with p38α MAPK prevented these changes and induced cell death. In contrast, p38β MAPK expression in the presence of MKK3 augmented hypertrophy and promoted cell survival (Wang et al., 1998). Thus p38α MAPK apparently promotes cell death whilst p38β MAPK promotes cell hypertrophy and survival. (Saurin et al., 2000)

The effects of the different p38 MAPK isoforms are therefore likely to be complex and dependent on stimulus and cellular context. The extent of activation of the different isoforms of p38 MAPK may dictate whether a cardiac myocyte is committed to death or survival (Nagarkatti & Sha'afi, 1998).

Further investigation to identify whether and how p38 MAPK might protect the myocardium (or whether it is good or bad) is important to resolve this controversy.

The focus of this study was therefore to examine the responses of the second messengers (PKB, p38 MAPK & cAMP) to insulin, and to correlate their respective activities with the functional parameters as a measure of cardiac performance.

Chapter 3

MATERIALS AND METHODS

3.1 Animals

Male Wistar rats weighing between 280g – 350g were used. The rats had free access
to food (standard lab chow) and water. They were maintained in animal quarters at a
constant temperature (22^0C) and humidity (40%) and were exposed to a twelve-hour
artificial light cycle. This investigation conforms to the "Guide for the care and use of
laboratory animals" published by the US National Institutes of Health (NIH publication no
85-23, revised 1985). The project was given ethical clearance by the Sub Committee C
of the University of Stellenbosch (animal ethics clearance number – 95\103).

3.2 Perfusion technique

The rats were anaesthetized by an intraperitoneal injection of Intramed Thiopentone
sodium (sodium pentobarbital) 0,1mg/g. The hearts were quickly excised and arrested
in ice cold Krebs – Henseleit solution containing 119 mM NaCl, 25 mM $NaHCO_3$, 4.7
mM KCl, 1.2 mM KH_2PO_4, 0.59 mM $MgSO_4.7H_2O$, 0.59 mM Na_2SO_4, 1.25 mM
$CaCl_2.2H_2O$ and 5mM glucose. The solution was aerated with 95% O_2; 5% CO_2 to
maintain the pH at 7.4. The isolated hearts were then mounted on a cannula via the
aorta and perfused with Krebs – Henseleit buffer (KH) (37^oC) within 60 seconds of
excision. The hearts were subsequently freed of excess non-cardiac tissue. All hearts
were perfused retrogradely in accordance with the Langendorff technique. A Sarns
Model 5500 pump kept the meniscus of the buffer at a constant level so as to maintain a
perfusion pressure of 100cm H_2O. The myocardial temperature of $37°$C was maintained

throughout the experiment by means of a Thermomix 1460 waterbath, which circulated warm water through the water-jacketed glass reservoirs. A thermistor probe was inserted into the right coronary sinus to monitor myocardial temperature during ischaemia.

The variation in systolic and diastolic pressure was monitored through the insertion of a compliant balloon into the left ventricle. The balloon was made of plasticised PVC and inflated with distilled water to establish a baseline pressure of 4-10mmHg. The balloon's volume and the baseline pressure, was maintained throughout the experiment. Any displacement of water, caused by the contraction of the heart against the balloon, was converted to a pressure reading by a pressure transducer and recorded on a Multitrace 2 chart recorder.

Global ischaemia was induced by reducing the coronary flow rate from 8-15 ml/min to 0,2 ml/min, by using a Gilson Minipuls 2 peristaltic pump.

3.3 Preliminary perfusion protocol (Figure 3.1)

In order to determine the optimum time of administion of insulin to the buffer to achieve its ultimate cardioprotective effect, insulin (0,3 μIU/ml) was added to the buffer in one group during reperfusion only (glu + ins$_{rep}$), and in the other group, insulin was added throughout the entire experiment (glu + ins).

Figure 3.1 Preliminary perfusion protocol

A) glucose (5mM) throughout experiment + insulin (0,3 µIU/ml) added only during reperfusion (glu + ins$_{rep}$).

Stabilization	Ischaemia	Reperfusion

0   15   30   45   60   65   75   90 min

B) glucose (5mM) + insulin (0,3 µIU/ml) throughout the experiment (glu + ins)

Stabilization	Ischaemia	Reperfusion

0   15   30   45   60   65   75   90min
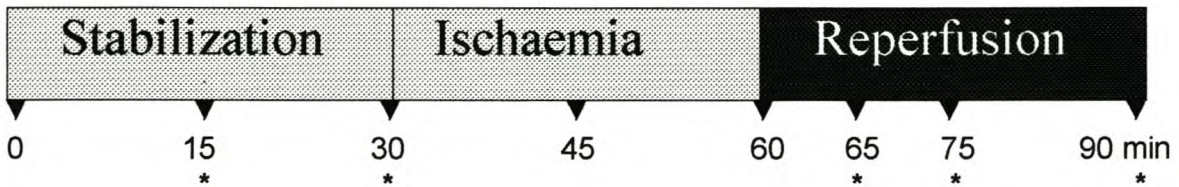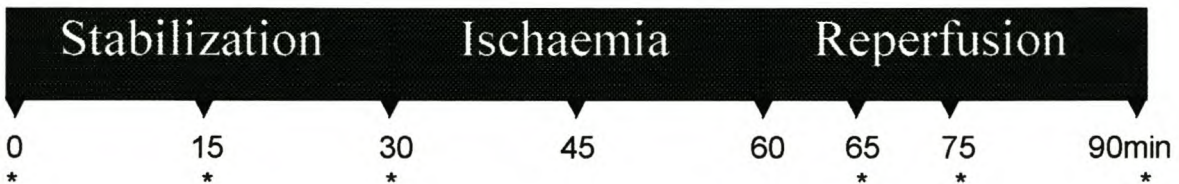
3.4 Perfusion protocol (Figure 3.2)

The protocol consisted of three sequential periods lasting 30 minutes each: (excluding control group: group 1)

1.) 0 – 30 min.: During the stabilization phase hearts were perfused under standard conditions at a pressure of 100 cm H_2O (coronary flow = 8-15 ml/min.).

2.) 30 – 60 min.: The hearts were subjected to global low flow ischaemia (0,2 ml/min). The myocardial temperature was kept between 36,8 °C and 37,3 °C.

3.) 60 – 90 min.: During the reperfusion phase, hearts were reperfused under standard conditions at a pressure of 100cm H_2O.

Five different groups were used:

(a) **Group 1:** 90 minutes of perfusion under standard control conditions at a pressure of 100 cm H_2O. **Group 1(a)** were perfused with a perfusion solution containing 5 mM glucose (n=4 hearts per group), while **Group 1(b)** were perfused with a perfusion solution containing 5 mM glucose and 0,3 μIU/ml insulin (n=4 hearts per group). For the purpose of biochemical analysis, hearts were freeze-clamped at t= 10, 30 and 90 minutes. (n=4 hearts per group)

(b) **Group 2:** Perfusion solution containing 5 mM glucose. Hearts were subjected to ischaemia and reperfusion. For biochemical analysis, hearts were freeze-clamped at t= 30, 45, 60, 65, 75, and 90 minutes. (n=6 hearts per group)

(c) **Group 3:** Perfusion solution contained 5 mM glucose + 0,3 μIU/ml insulin throughout the experiment. Hearts were subjected to ischaemia and reperfusion. Hearts were freeze-clamped at t= 30, 45, 60, 65, 75, and 90 minutes for the purpose of biochemical analysis. (n=6 hearts per group)

(d) **Group 4:** Perfusion solution containing 5mM glucose + 0,3 μIU/ml insulin throughout the experiment with wortmannin (100nM) (red block) present during ischaemia (30-60 min). Wortmannin is a phosphatidylinositol 3-kinase inhibitor, and consequently will inhibit PKB activation. Hearts were freeze-clamped at 60 min for biochemical analysis. (n=4 hearts per group)

(e) **Group 5:** Perfusion solution containing 5mM glucose and 0,3 μIU/ml insulin throughout the experiment, with wortmannin (red block) added during reperfusion (time 60 – 90 min). Hearts were freeze-clamped at 90 min (end of reperfusion). (n=4)

Figure 3.2 Perfusion protocol

* →	Mechanical function
□ →	Freeze clamping

A) Group 1(a), (b): glucose (5mM) ± insulin (0,3µIU/ml)

0	10	15	30	45	60	65	75	90min
	*		*			*	*	*
	□		□					□

B) Group 2: glucose (5 mM)

Stabilization   Ischaemia   Reperfusion

0	15	30	45	60	65	75	90min
	*	*			*	*	*
		□	□	□	□	□	□

C) Group 3: glucose (5mM) + insulin (0,3 µIU/ml)

Stabilization   Ischaemia   Reperfusion

0	15	30	45	60	65	75	90min
	*	*			*	*	*
		□	□	□	□	□	□

D) Group 4: glucose (5mM) + insulin (0,3 µIU/ml) + wortmannin (100nM), time 30-60 min

Stabilization   Ischaemia   Reperfusion

0	15	30	45	60	65	75	90min
	*	*			*	*	*
				□			

E) Group 5: glucose (5mM) + insulin (0,3 µIU/ml) + wortmannin (100nM), time 60-90 min

Stabilization   Ischaemia   Reperfusion

0	15	30	45	60	65	75	90min
	*	*			*	*	*
							□

3.5 Measured variables

In order to compare the functional recovery of the hearts in the different groups, the heart rate (beats/min), coronary flow (ml/min), left ventricular developed pressure (mmHg), and temperature ($^{\circ}$C) were monitored at t = 15, 30, 65, 75, and 90 min.

Coronary flow (ml/min) was measured by manually collecting the volume of the heart's effluent in one minute.

Functional recovery, as indicated by the left ventricular developed pressure (LVDP) at the end of the reperfusion period, was expressed as a percentage of the pre-ischaemic LVDP after 30 minutes of perfusion.

The rate pressure product (RPP) was calculated by multiplying the heart rate and the LVDP. The percentage rate pressure product recovery (%RPP) was reperfusion RPP expressed as a percentage of the pre-ischaemic RPP after 30 min of stabilization.

The time to onset of ischaemic contracture (TOIC) was determined by measuring the time from the beginning of ischaemia until a 4 mmHg rise in the left ventricular diastolic pressure was observed.

3.6 Exclusion criteria

Hearts with coronary flow rates outside the range of 8 – 15 ml/min, and heart rates outside of 200 – 340 beats/min during the stabilization phase, were excluded.

3.7 Tissue samples

For the purpose of biochemical analysis, hearts were freeze-clamped with Wollenberger metal thongs, which were pre-cooled in liquid nitrogen. The freeze-clamped ventricular tissue was then stored in liquid nitrogen (N_2) until analyzed.

3.8 Biochemical analysis

3.8.1 cAMP evaluation

0.1 gram of the ventricular tissue was fully pulverised in a pre-cooled metal homogenizer. The tissue was extracted with 1.2 ml 6% perchloric acid (PCA) in Greyward tubes on ice. All samples were kept on ice for 2 hours with occasional vortexing. After centrifugation for 20 minutes at 4000 rpm, 1 ml of the supernatant was transferred to an Eppendorff tube containing $5\mu l$ universal indicator. Each tube was neutralised with a KOH-Tris mixture (two parts 40% KOH-saturated KCL; three parts 0.2 M Tris-HCl, pH 7.5 [4°C]). The neutralisation volume was recorded and the samples left on ice to fully precipitate. Cyclic AMP levels were determined by Amersham's radioimmune assay (Amersham International plc, Amersham, UK). A standard curve ranging between 0.125 – 16 picomoles was included. Values obtained were presented as picomoles/g wet weight.

3.8.2 Western blotting for p38 mitogen activated protein kinases (MAPK) and Protein kinase B (PKB)

Cytosolic p38 MAPK was determined by homogenizing freeze-clamped hearts with a lysis buffer containing (in mM): Tris 20; p-nitrophenylphosphate 20; EGTA 1; NaF 50; sodium orthovanadate 0.1; phenylmethyl sulphonyl fluoride (PMSF) 1; dithiotreitol (DTT) 1; aprotinin 10 $\mu g/ml$; leupeptin 10 $\mu g/ml$, pH 7.4. For PKB, a lysis buffer containing (in mM): Tris-HCl (pH7.4) 20; NaCl 25; EGTA 1; NaF 10; sodium ortovanadate 1; phenylmethyl sulphonyl fluoride (PMSF) 0.1; Benzamidine 1; 1% Triton-X 100; aprotinin 10 $\mu g/ml$; leupeptin 10 $\mu g/ml$ was used.

The homogenates were spun down in a microfuge at 4°C for 15 minutes to remove particulate matter. The supernatant protein content was determined using the Bradford technique (Bradford, 1976). The supernatants were diluted in Laemmli sample buffer, boiled for 5 min. and 20 μg protein was separated by electrophoresis on a 12% polyacrylamide gel, using the standard Bio-RAD Mini-PROTEAN II system. The separated proteins were transferred to a PVDF (polivinylidene fluoride) membrane (Immobilon™-P, from Millipore). These membranes were stained with Ponceau S red (reversible stain) to visualize the proteins, and to assess the quality and quantity of the transfer. The membranes were laser-scanned and densitometrically analyzed (UN-SCAN-IT, Silkscience).

Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline, 0.1% Tween-20 (TBST). The activated p38 MAPK was visualized with an appropriate primary antibody (phospho-p38 MAPK [Thr 180/Tyr 182] Antibody, New England Biolabs) directed against the dual-phosphorylated, therefore activated enzyme. The activated PKB was visualized with phospho-Akt [Ser 473] antibody (New England Biolabs). Membranes were washed with large volumes of TBST (5 x 5 min.) and the immobilized primary antibody conjugated with a diluted horseradish peroxidase-labelled secondary antibody (purchased from Amersham LIFE SCIENCE). After thorough washing with TBST, membranes were covered with ECL™ detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through a non-radioactive method (ECL™ Western blotting from Amersham Pharmacia Biotech). Films were densitometrically analyzed, as described above.

3.8 Chemicals

All chemicals used for buffers were purchased from BDH or Merck, analytical grade, unless otherwise indicated.   The insulin (Humulin N) was obtained from Eli Lilly SA, (Fregersheim, France). The wortmannin was obtained from Sigma Chemicals Co.

3.9 Statistical analysis

Values are expressed as mean $\pm$ standard error of the mean (SEM).   Results were compared using a one-way ANOVA with Bonferroni Multiple Comparison as post test. P-values less than 0.05 ($P<0.05$) was considered as significant.

Chapter 4

RESULTS

4.1 Preliminary study

4.1.1 Left ventricular developed pressure (LVDP) glu+ins_rep vs. glu+ins (Figure 4.1)

Five minutes after reperfusion, the glu+ins group showed significant improvement compared to the glu+ins_rep group, with a LVDP of 83.9±4.6 mmHg vs. 47.4±9.1 mmHg (P<0.001). LVDP of the glu+ins group maintained reperfusion pressures from 5 min reperfusion to 30 min reperfusion, whilst the LVDP in the glu+ins_rep group improved from 47.4±7.5 mmHg to 65.1±5.6 mmHg.

Our results suggest that insulin administration throughout the protocol resulted in improved recovery.

4.1.1 Effect of insulin on control hearts (Figure 4.2)

Results presented in Figure 3.2 showed that insulin administration significantly improved LVDP in normoxic conditions, showing that insulin had a positive inotropic effect.

4.2 Functional data

4.2.1 Heart rate (Figure 4.3)

At the end of the stabilization period the HR for group 3 was 275.8±3.4 beats/min compared to 274.1±4.9 beats/min for group 2. During ischaemia heart rates were not measured. No statistical differences in reperfusion HR could be detected. There was therefore no significant difference in HR between the two groups.

4.2.2 Coronary flow (Figure 4.4)

Pre-ischaemic coronary flow (CF) was 11.3±0.3 ml/min for group 2 and 11.9±0.2 ml/min for group 3 at the end of the stabilization phase. During ischaemia, the CF was fixed at 0.2 ml/min. After 5 min and 15 min of reperfusion, there was a significant difference between the CF of the two groups (P<0.001). Group 3 returned to pre-ischaemic values (10.6±0.6 ml/min), while group 2 reached only 7.7±0.5 ml/min).

At the end of reperfusion, the CF of group 3 remained high at 9.5±0.7 ml/min compared to group 2 at 7.4±0.6 ml/min. There was no significant difference in CF between the two groups at this point.

4.2.3 Left ventricular developed pressure (LVDP) (Figure 4.5)

During the pre-ischaemic phase, LVDP was similar in both groups at 113.4±1.7 mmHg and 105.7±1.6 mmHg after 30 minutes of stabilization. After 5 minutes of reperfusion, group 3 showed significantly improved LVDP compared to group 2 (83.92±4.6 vs. 31.7±4.5 mmHg) (P<0.001). During the rest of the reperfusion phase, the developed pressure of group 3 remained significantly higher than that of group 2 at 84.4±5.2 mmHg and 46.2±7.4 mmHg respectively after 15 minutes and 86±3.9 mmHg and 44.7±7.04 mmHg at the end of reperfusion. (P<0.001)

4.2.4 Percentage recovery of left ventricular developed pressure (LVDP) (Figure 4.6)

The LVDP recovery of the hearts in group 3 was significantly higher than that of group 2 throughout the entire reperfusion period (P<0.001). After 5 minutes of reperfusion, group 3 recovered to 80.2±3.9% of pre-ischaemic values compared to 27.8±4.8% of those hearts in group 2. These values remained similar after 15

minutes of reperfusion (group 3 - 83.2±5.7 vs. group 2 - 37.3±7.0%), and at the end of reperfusion (group 3 85.5±4.6 compared to 44.8±4.9% of group 2).

4.2.5 Rate pressure product (RPP) (Figure 4.7)

The pre-ischaemic RPP for both groups were similar after 15 minutes of stabilization (group 3 - 30920±510.3 vs. group 2 - 28680±523.2), and remained constant throughout 30 minutes of stabilization (group 3 - 31340±522.1 vs. group 2 - 28590±578.2). The reperfusion RPP after 5 minutes for group 3 were significantly higher (20430±1448) than the values of group 2 (13010±2029), (P<0.001). The RPP of group 3 remained significantly higher (P<0.001) after 15 minutes of reperfusion (group 3 - 22820±1271 vs. group 2 - 9036±1628), and at the end of reperfusion with group 3 - 21450±1444 vs. group 2 - 8608±1828.

4.2.6 Percentage rate pressure product recovery (%RPP) (Figure 4.8)

Insulin treated hearts (group 3) showed reperfusion recoveries of 68±4.6% to the pre-ischaemic values compared to 44.6±5.4% of non-insulin treated hearts (group 2), (P< 0.001). In group 3, RPP values remained similar after 15 minutes of reperfusion (group 3 - 75.3±5.9%), while the RPP of group 2 increased only slightly to 37.8±6.5%. The significant difference between the insulin treated and non-insulin treated hearts persisted to the end of reperfusion (P<0.01).

4.2.7 Time to onset of ischaemic contracture (Figure 4.9)

Ischaemic contracture was significantly delayed in the presence of insulin (group 3 - 16.7±1.2 min) compared to 9.9±0.7 min (group 2) where insulin was not present, (P<0,0001).

4.2.8 Effect of wortmannin on LVDP (Figure 4.10 + Figure 4.11)

In the absense of insulin, administration of wortmannin had no effect on the reperfusion LVDP (Figure 4.10). The aim of the next experiment was to investigate the effect of wortmannin on the LVDP when added either before ischaemia or at the onset of reperfusion to a perfusion solution containing 5mM glucose and 0,3 μIU/ml insulin (Figure 4.11). After 5 minutes of reperfusion, the LVDP of group 3 was 83,9$\pm$4.6 mmHg with the LVDP of group 4 and group 5 significantly lower at 17$\pm$6.5 mmHg and 13$\pm$4.2 mmHg respectively (P<0.0001). After 15 minutes of reperfusion, the LVDP of group 4 and group 5 remained the same at 14$\pm$3.6 and 26.5$\pm$11.3; still significantly lower than that of group 3 (84.4$\pm$5.2). The conditions at the end of reperfusion remained similar with the LVDP of group 4 (33.5$\pm$9.6 mmHg) and group 5 (17.5$\pm$5.1 mmHg) significantly lower than the LVDP of group 3 at 86.0$\pm$3.9 (P<0.0001). This indicates that PI3-K may play a role in the protective effect of insulin.

4.3 Biochemical data

4.3.1 cAMP (Figure 4.12)

At the end of the stabilization phase, the myocardial cAMP levels of group 2 and group 3 were in the same range at 286.1$\pm$23.9 pmol\gwwt (picomole per gram wet weight) and 295.5$\pm$28.7 pmol/gwwt respectively. After 15 minutes of ischaemia, these values were elevated to 463.9$\pm$29.7 pmol/gwwt and 434.0$\pm$29.5 pmol/gwwt respectively. At the end of ischaemia, cAMP levels in group 2 were reduced to 326.6$\pm$35.8 pmol/gwwt, while that of group 3 were increased significantly to 639.6$\pm$27.3 pmol/gwwt (P<0,001). When wortmannin was added during ischaemia the rise in cAMP was abolished, and therefore group 4 presented similar values as group 2 (332.8$\pm$15 pmol/gwwt). After 5 minutes of reperfusion, cAMP levels in

group 3 decreased to the levels of group 2 (360.9±45.1 vs. 338.4±27.9 pmol\gwwt).

After 15 minutes of reperfusion, cAMP levels for group 2 and group 3 were similar

(273.2±32.5 and 266.4±14.5 pmol\gwwt respectively), but dropped even more after

30 min of reperfusion. Addition of Wortmannin during reperfusion only (group 5) did

not alter the levels of cAMP at the end of reperfusion.

### 4.3.2   p38 MAPK activation (Figure 4.13 & Figure 4.14)

After 30 minutes of perfusion, the amount of p38 MAPK activated in group 2 was

significantly higher than that of group 3 at 50±15 arbitrary densitometric units (adu)

vs. 30±2.6 adu (P<0.05). In the latter group, 15 minutes of ischaemia, elevated

activated p38 MAPK levels to 45±8.5 adu, while levels in group 2 remained constant

at 50±5 adu. This activation was transient because activated p38 MAPK levels

decreased in both groups at the end of 30 minutes ischaemia,. (25±2.7 and 15±3

adu, respectively). Addition of wortmannin before ischaemia did not alter p38

MAPK levels (30.3±5.5 adu) at the end of ischaemia (group 4). Five minutes of

reperfusion resulted in a significant activation of p38 MAPK in group 2 but not in

group 3 (55±5 adu vs. 30±5.3 adu),(P<0.05). However, after 15 min of reperfusion

the activation of p38 MAPK did not differ significantly between groups 2 and 3. At

the end of reperfusion, however, levels of activated p38 MAPK in group 3 dropped

to 15±3.8 adu, while it remained significantly higher in group 2 (55±3.0) (P<0.05).

Wortmannin added at the onset of reperfusion (group 5) could partially abolish the

effects of insulin to suppress p38 MAPK activation after 30 min of reperfusion.

Figure 4.14 is the representative Western blot of the activated p38 MAPK in insulin

vs. non-insulin treated hearts.

4.3.3 PKB activation (Figure 4.15 & Figure 4.16)

As insulin is known to activate PKB, the expected elevated levels of phosphorylated PKB were recorded in hearts from group 3. This was significantly higher than the group 2 levels at the end of the stabilization phase at 30 ± 9 vs. 5 ± 0.5 adu (P<0.0001). This remained similar during the first 15 minutes of ischaemia. This PKB activity was depressed by 30 min of ischaemia as group 2 had zero activated PKB at the end of the ischaemic period, while group 3 levels dropped to 5 ± 2.1 adu. The known inhibitor for PI3-kinase, wortmannin, when added before induction of ischaemia did not further lower this. The presence of insulin in group 3 resulted in occurrence of strong PKB activation during reperfusion, peaking at 15 minutes (50 ± 11.1 adu) and diminishing at 30 minutes (15 ± 3 adu). Reperfusion had no effect on PKB activation in group 2. Wortmannin added at the onset of reperfusion abolished PKB activity measured at 30 min reperfusion (group 5 vs. group 3). Figure 4.16 is the representative Western blot of activated PKB in insulin vs. non-insulin treated hearts.
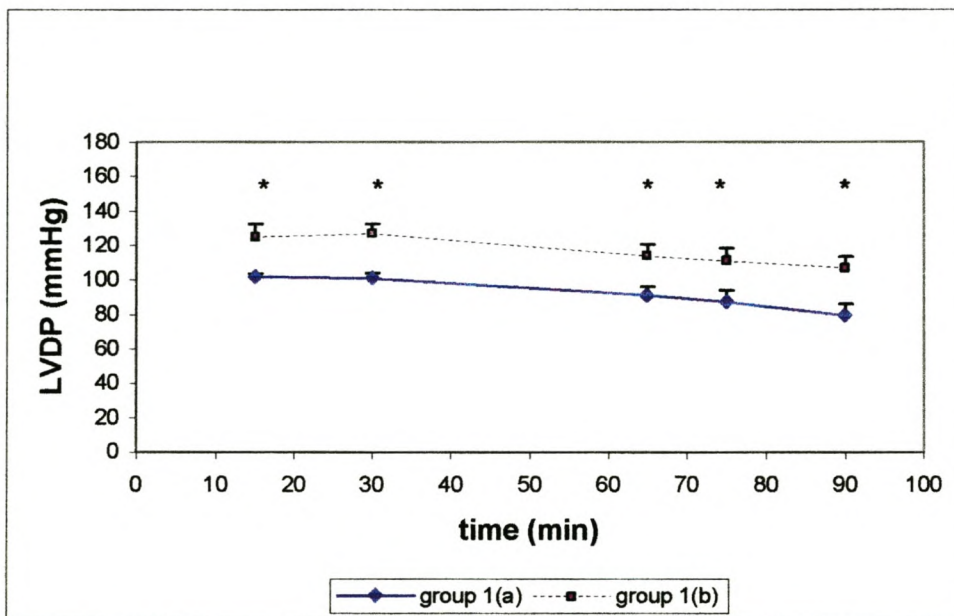
Chapter 4 – Addendum

Figure 4.1: Preliminary insulin trial
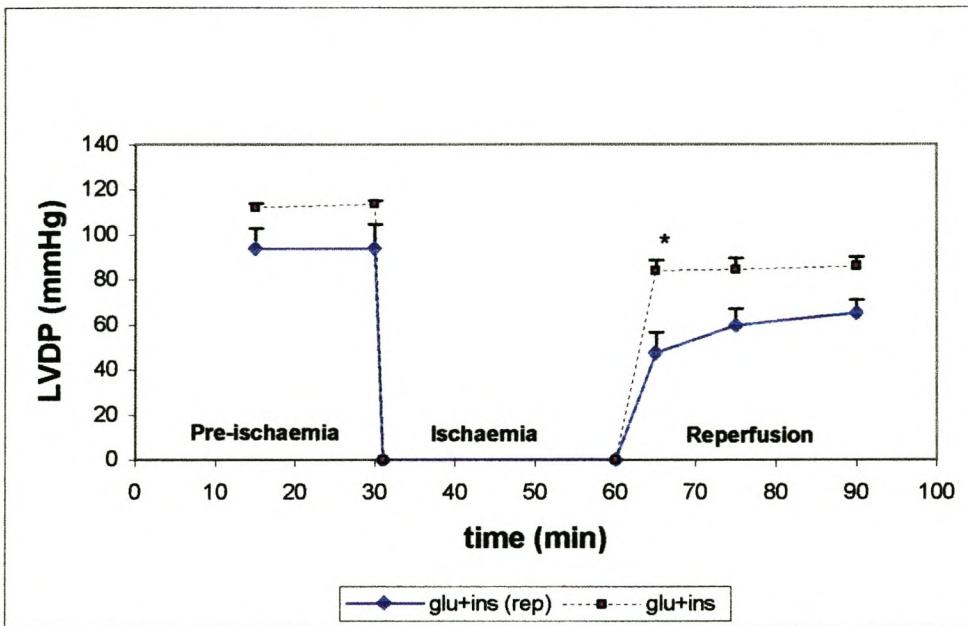
LVDP of hearts perfused with 5mM glucose and 0,3
μIU/ml insulin added either throughout the experiment
[glu+ins], or only during reperfusion [glu+ins(rep)].
n=4 hearts per group.
* P<0.001 for [glu+ins] vs. [glu+ins(rep)]

<u>Figure 4.2</u>: Effect of insulin on control hearts

LVDP of control hearts. Hearts from group 1 were divided into two groups: KH perfusion solution of group 1(a) contained 5mM glucose, while 5mM glucose and 0,3 µIU/ml insulin were added to the KH of group 1(b). Hearts were perfused under normal conditions – no ischaemia.
n=4 hearts per group
* P< 0.001 group 1(a) vs. group 1(b).

Figure 4.3: Heart rate
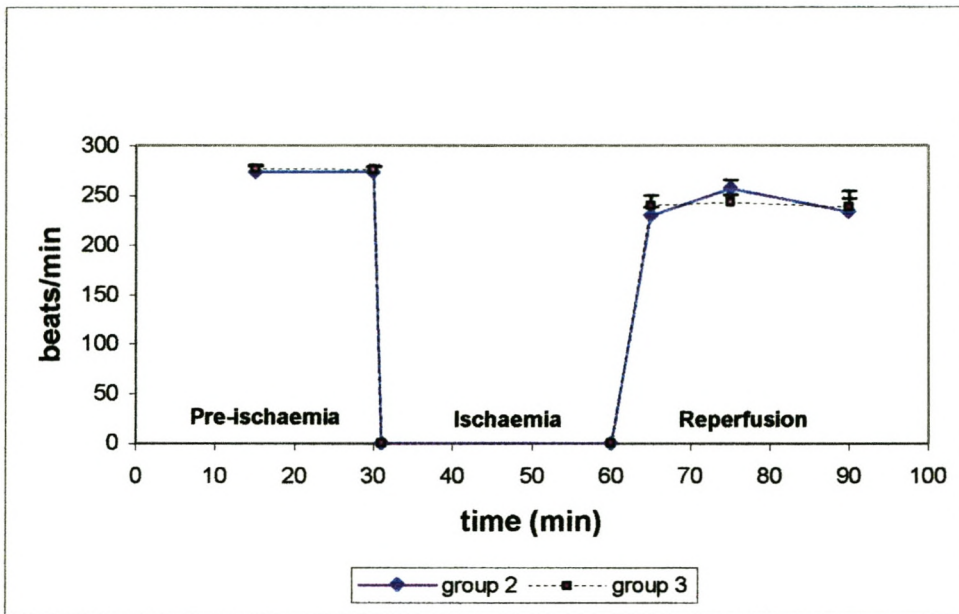
HR of hearts perfused with standard KH solution containing either 5mM glucose (group 2) or 5mM glucose and 0,3 μIU/ml insulin (group 3).
n=6 hearts per group.

Figure 4.4:Coronary flow
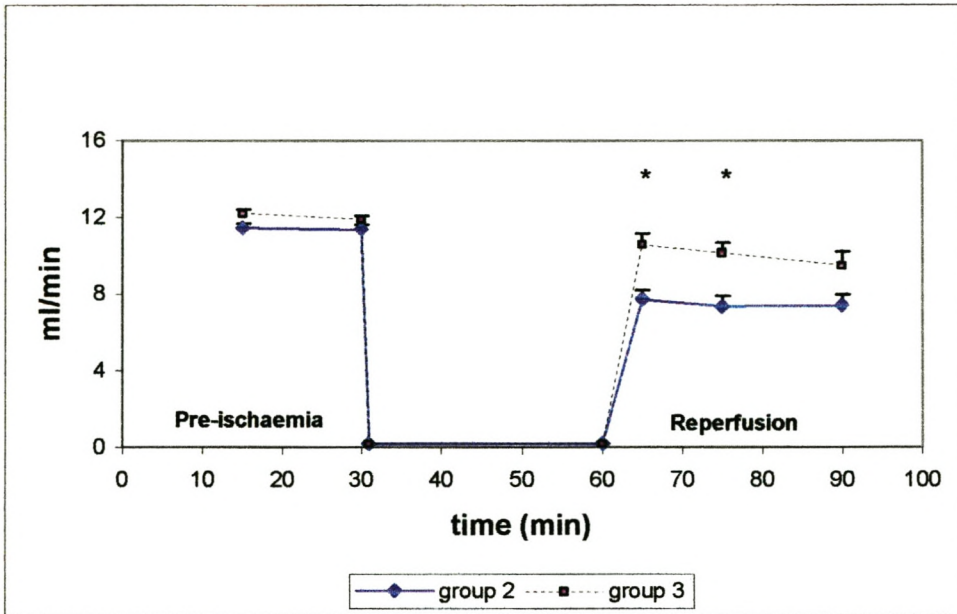
CF of hearts perfused with standard KH solution containing either 5mM glucose (group 2), or 5mM glucose and 0,3 μlU/ml insulin (group 3)n=6 hearts per group.
* P<0.001 vs. group 2.

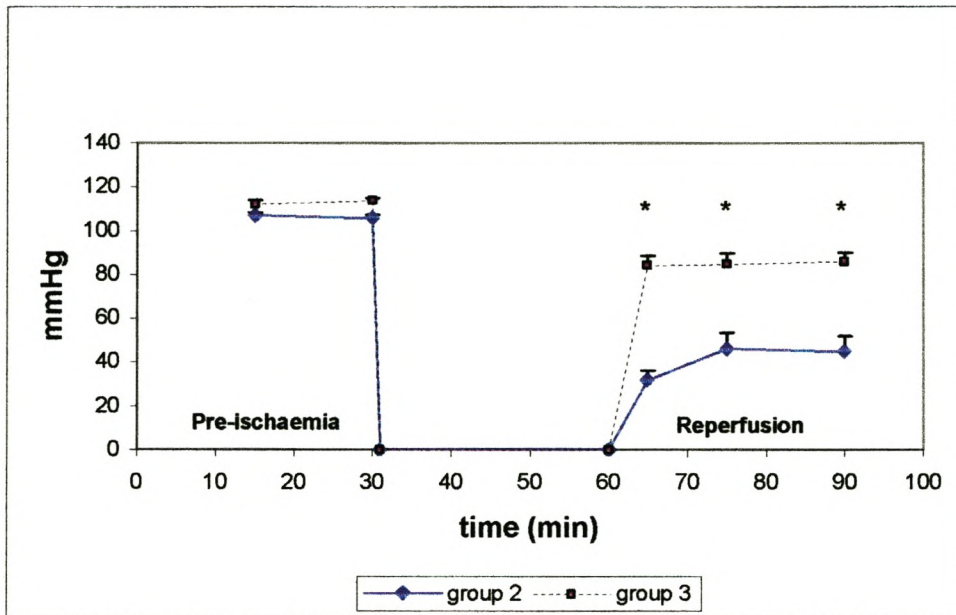**Figure 4.5: Left ventricular developed pressure**

LVDP of hearts perfused with standard KH solution containing either 5mM glucose (group 2) or 5mM glucose and 0,3 μIU/ml insulin (group 3). n=6 hearts per group
* P<0.001 vs. group 2

<u>**Figure 4.6**</u>: **% LVDP recovery**

% LVDP recovery of hearts perfused with standard KH
solution containing either 5mM glucose   (group 2) or 5mM
glucose and 0,3 μlU/ml insulin (group 3).
* n=6 hearts per group.*
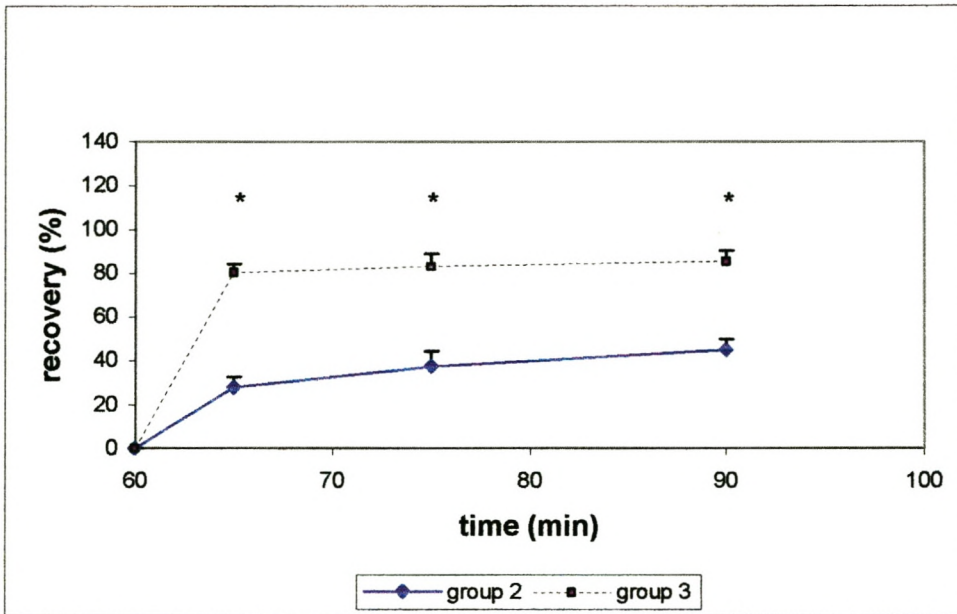* P<0.001 vs. group 2.*

<u>Figure 4.7</u>: **Rate pressure product**

RPP of hearts perfused with standard KH solution containing either 5mM glucose (group 2) or 5mM glucose and 0,3 µlU/ml insulin (group 3). The rate pressure product (RPP) was calculated by multiplying the heart rate and the LVDP.
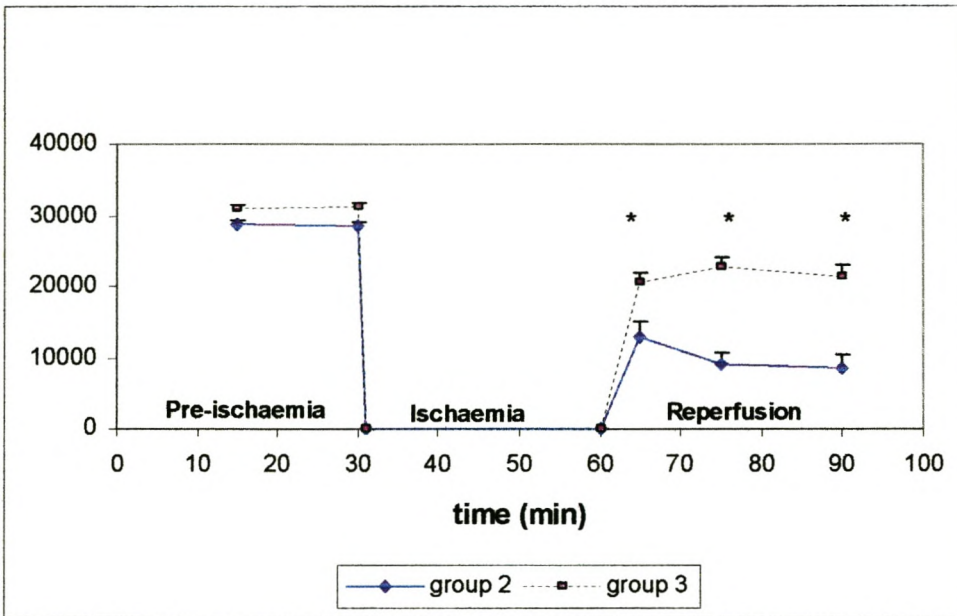*n=6 hearts per group.*
* P<0.001 vs. group 2.

Figure 4.8: **%RPP recovery**

%RPP of hearts perfused with standard KH solution containing either 5mM glucose (group 2) or 5mM glucose and 0,3 μlU/ml insulin (group 3). The percentage rate pressure product recovery (%RPP) was expressed as a percentage of the pre-ischaemic RPP after 30 min of stabilization.
n=6 hearts per group.
* P<0.001; ** P<0.01 vs. group 2.

Figure 4.9: Time to onset of ischaemic contracture

Time to onset of ischaemic contracture of hearts perfused with
standard KH solution containing either 5mM glucose (group 2) or
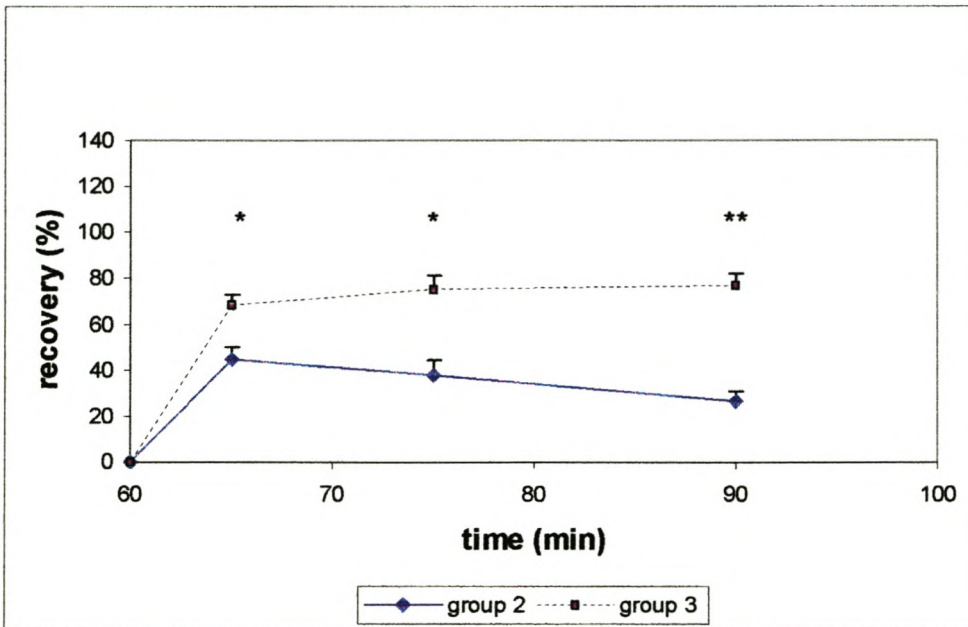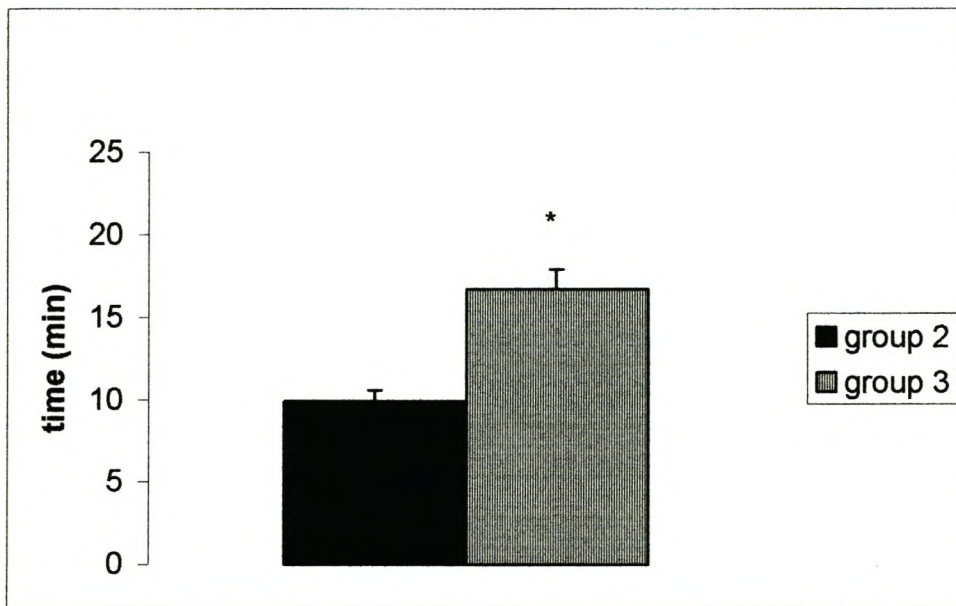*5mM glucose and 0,3 μIU/ml insulin (group 3).*
The time to onset of ischaemic contracture (TOIC) was
determined by measuring the time from the beginning of
ischaemia until a 4 mmHg rise in the left ventricular diastolic pressure was
observed.
n=6 hearts per group.
* P<0.001 vs. group 2.

Figure 4.10: Effect of wortmannin on LVDP
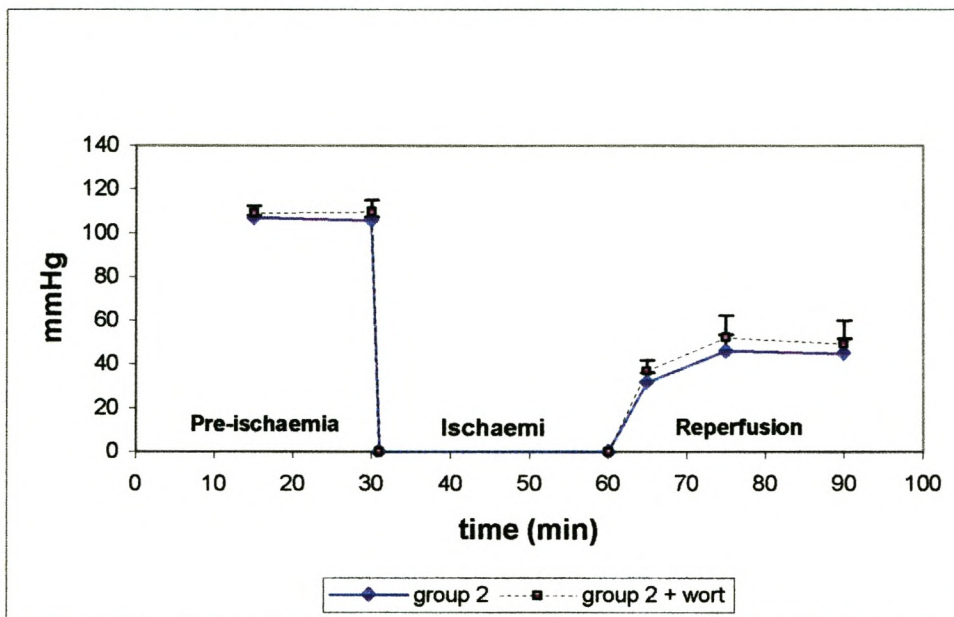
LVDP of hearts perfused with standard KH solution containing
either 5mM glucose (group 2) or 5mM glucose and 100nm
wortmannin (group 2 + wort).
n=6 hearts per group

Figure 4.11: Effect of wortmannin on LVDP
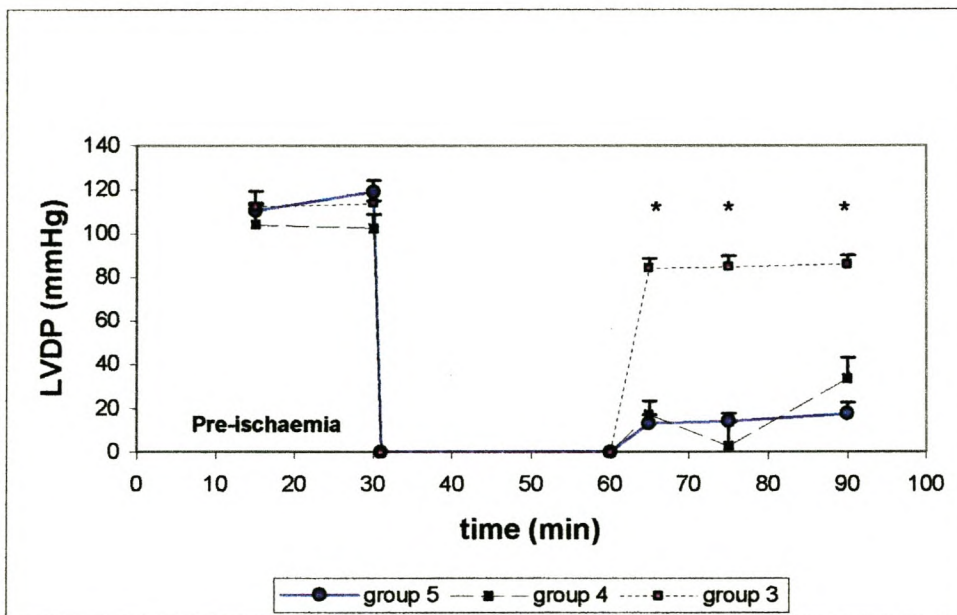
*LVDP of hearts perfused with standard KH solution
containing:
(a) 5mM glucose and 0,3 μIU/ml insulin (group 3),
(b) 5mM glucose, 0,3 μIU/ml insulin and 100 nm wortmannin
added during ischaemia (group 4)
(c) 5mM glucose, 0,3 μIU/ml insulin and 100 nm wortmannin
added during reperfusion (group 5) n= 6 hearts per group
* P< 0.001 between group 3 vs. group 4 and 5.*

Figure 4.12: cAMP
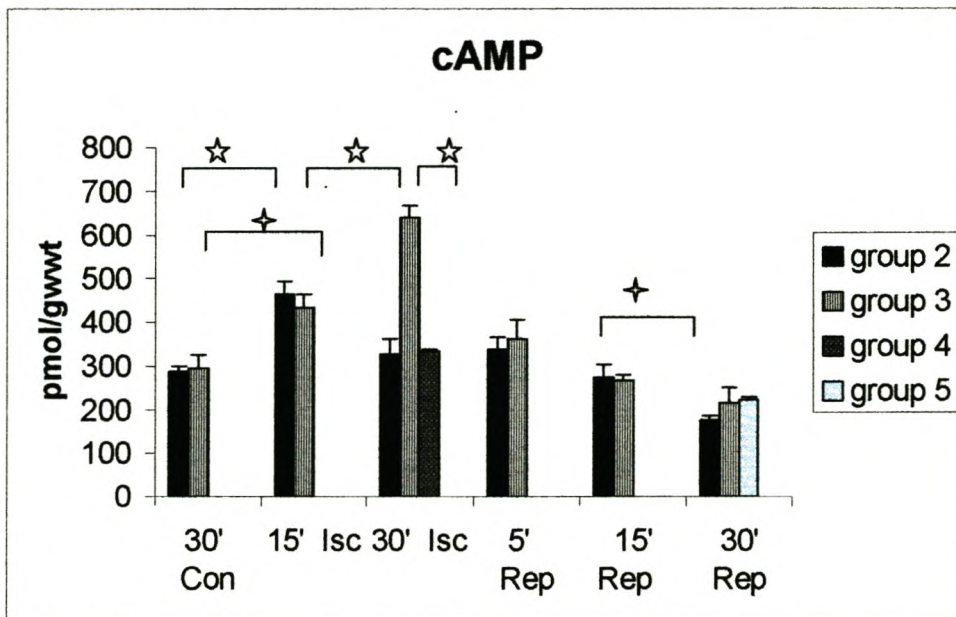
cAMP activation of hearts perfused with standard KH solution containing:

(a) 5mM glucose (group 2),

(b) 5mM glucose and 0,3 μIU/ml insulin (group 3),

(c) 5mM glucose, 0,3 μIU/ml insulin and 100 nm wortmannin added during ischaemia (group 4)

(d) 5mM glucose, 0,3 μIU/ml insulin and 100 nm wortmannin added during reperfusion (group 5)

cAMP activation was determined as described in Materials and Methods. n=6 hearts per group.

✛ $P<0.02$; ☆ $P<0.001$

Figure 4.13: p38 MAPK

Activated p38 MAPK of hearts perfused with standard KH solution
containing:
(a) 5mM glucose (group 2)
(b) 5mM glucose and 0,3 μIU/ml insulin (group 3),
(c) 5mM glucose, 0,3 μIU/ml insulin and 100 nm wortmannin added
 during ischaemia (group 4)
(d) 5mM glucose, 0,3 μIU/ml insulin and 100 nm wortmannin added
 during reperfusion (group 5)The activated p38 was visualized with
 an appropriate primary antibody (phospho-p38 MAPK
[Thr 180/Tyr 182] directed against the dual-phosphorylated,
activated enzyme.
n=6 hearts per group

✦ *P<0.02;* ☆ *P<0.001*

Figure 4.14: *Representative Western blot of p38 MAPK activation of hearts perfused with standard KH solution containing:*
(a) 5mM glucose (group 2)
(b) 5mM glucose and 0,3 μlU/ml insulin (group 3)

| 30' Con | 15' Isc | 30' Isc | 5' Rep | 15' Rep | 30' Rep |

Figure 4.15: Protein kinase B (PKB)

PKB activation of hearts perfused with standard KH solution
containing:
(a) 5mM glucose,
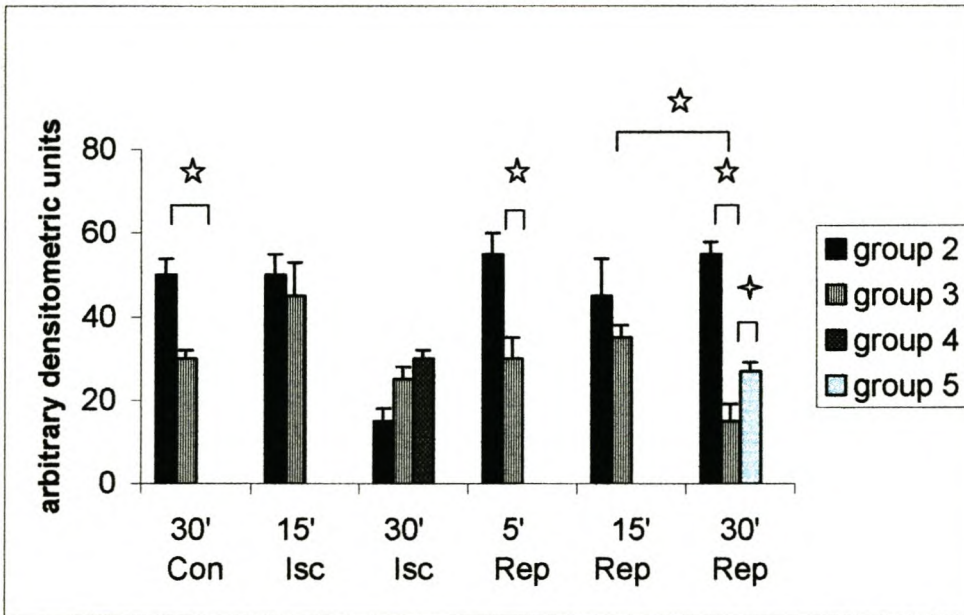*(b) 5mM glucose and 0,3 µIU/ml insulin (group 3),*
(c) 5mM glucose, 0,3 µIU/ml insulin and 100 nm wortmannin added
during ischaemia (group 4)
(d) 5mM glucose, 0,3 µIU/ml insulin and 100 nm wortmannin added
during reperfusion (group 5)
The activated PKB was visualized with an appropriate primary
phospho-Akt [Ser 473] antibody.
n=6 hearts per group.

 # P<0.02;   ☆ P<0.001

Figure 4.16: *Representative Western blot of PKB activation of hearts perfused with standard KH solution containing:*
(a) 5mM glucose (group 2)
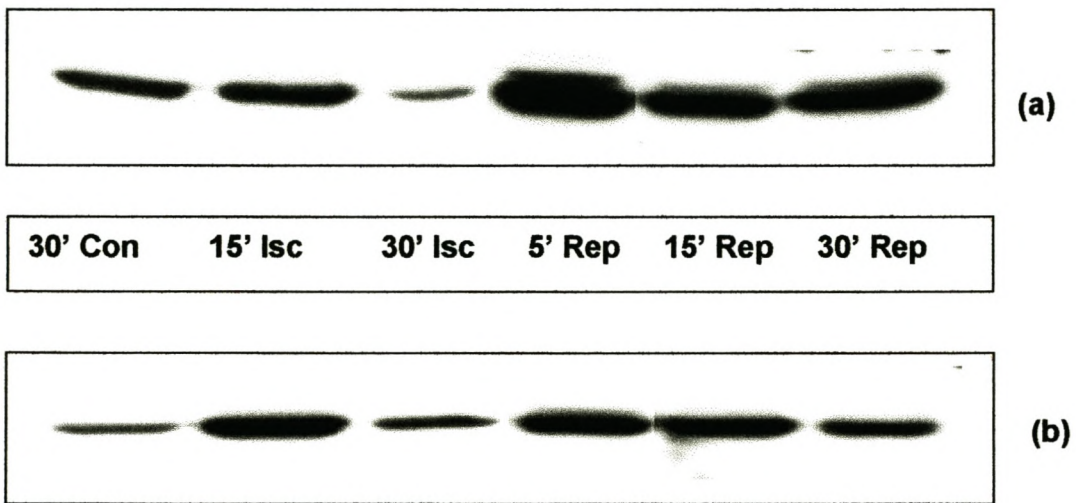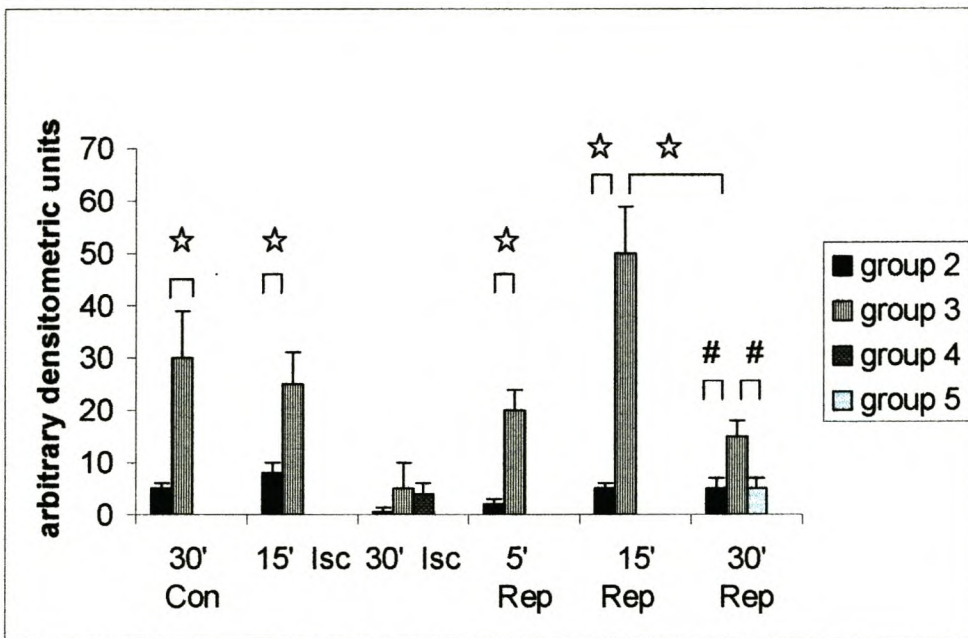*(b) 5mM glucose and 0,3 µIU/ml insulin (group 3).*

| 30' Con | 15' Isc | 30' Isc | 5' Rep | 15' Rep | 30' Rep |

(a)

(b)
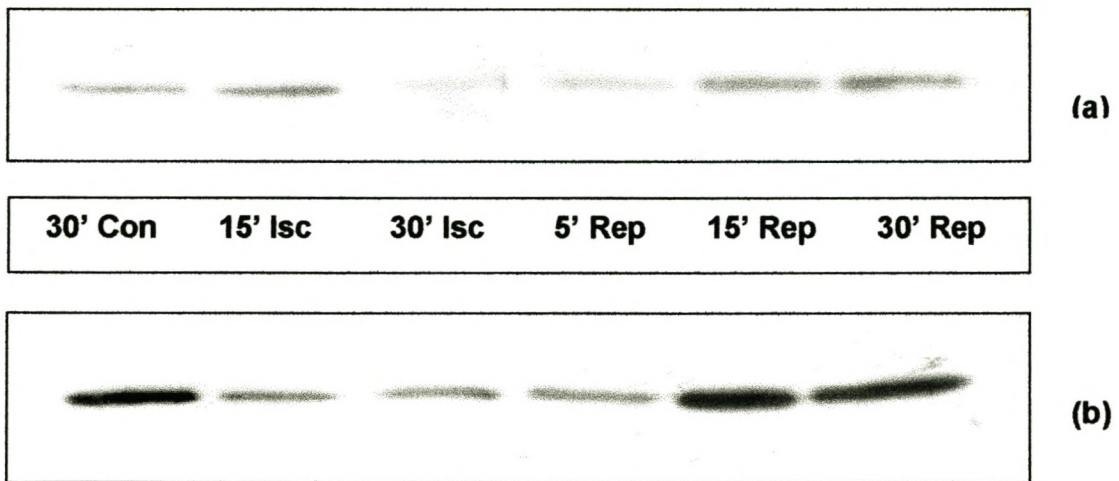
Chapter 5

DISCUSSION

It is well documented that insulin protects the heart against ischaemia. Pioneering work on the cardioprotective effect of insulin against ischaemia was performed by Sodi-Pallares in 1962, and sparked the interest in this field of study. The positive effect of insulin on the functional recovery and survival of the ischaemic heart has been the focus of several studies. Amongst the major findings are the improved cardiac output recovery during reperfusion (Van Rooyen et al., 1998), a reduction in infarct size with insulin given at the onset of reperfusion (Jonassen et al., 1996), and the attenuation of apoptosis in rat neonatal cardiomyocytes when insulin is administered during reoxygenation (Jonassen et al., 2000).

For long it was believed that the protective effects of insulin, such as the

(a) Recruitment of glucose transporters to enhance glucose entry (Coffer et al., 1998),

(b) Stimulation of glycolysis (Deprez et al., 1997),

(c) Enhancement of glycogen synthesis (Lawrence, 1992),

(d) Improved protein synthesis (Reif et al., 1997), and

(e) Positive inotropic and positive chronotropic properties (Kearney et al., 1998) were

metabolic of origin, but lately the emphasis shifted towards the diverse effects of signal transduction pathways elicited by insulin e.g. the different kinase cascades. Although these beneficial effects of insulin on ischaemia/reperfusion induced injury are well known, the precise mechanism by which insulin produces these effects are still not clear.

This study was therefore designed to investigate the possible roles for cAMP, PKB and p38 MAPK in the cardioprotection offered by insulin in the reperfused, ischaemic myocardium.

5.1 Preliminary study

5.1.1 Perfusion protocol

In order to resolve the protocol that best elicits the beneficial effects of insulin on the heart, we performed a preliminary study to determine the optimal time of administration to the perfusion solution. In a study conducted by Jonassen and co-workers, they concluded that glucose-insulin-potassium (GIK) administered at the onset of the postischemic reperfusion period was as cardioprotective as the administration of GIK during the entire ischemia/reperfusion period (Jonassen, 2000). We wanted to examine whether this was also true in our model using insulin in conjunction with low glucose (5mM), or if insulin was able to exert a protective effect even before stressful events, and thus 'prepare' the hearts to better withstand the consequences of ischaemia and reperfusion. We therefore, added insulin to the perfusion solution only during the reperfusion period in one group, and throughout the entire experiment in the other group. (Figure 4.1). On the basis of our results, insulin was added to the perfusion solution in all subsequent experiments in groups that were treated with insulin.

5.1.2 Effects of insulin on LVDP.

We observed that insulin increased the LVDP significantly under control conditions (Figure 4.2). This can be attributed to the positive inotropic ability of insulin that allows

improved contraction of the myocardium (Kearney et al., 1998), and thus a better developed pressure. Addition of insulin *in vitro* to bovine and rat hearts stimulates Na^+/Ca^+ exchange function (Kato & Kako, 1988). In isolated human atrial myocytes, the addition of insulin stimulates the Ca^{2+} current (Maier et al., 1999). Higher calcium stores and increased Na^+/H^+ antiport activity as a result of the addition of insulin enhance muscular performance and thus provide a crucial survival element (Aviv, 1994). These effects explain the positive inotropic effects of insulin.

5.2 The effect of ischaemia and reperfusion

5.2.1 Heart rate

Heart rates of both the insulin treated hearts (group 3) and non-insulin treated hearts (group 2) recovered to pre-ischaemic values during reperfusion. The improved recovery of the hearts in group 3, or the poorer recovery of the hearts in group 2 could thus not be attributed to an alteration of the heart rate (Figure 4.3).

5.2.2 Coronary flow

In non-insulin treated hearts (group 2) the reperfusion coronary flow was 25% below pre-ischaemic coronary flow. (Figure 4.4). Ischaemia is associated with a high sympathetic activity and a subsequent high concentration of FFA in the myocardium (Gupta et al., 1969). This has been shown to increase myocardial oxygen requirements and results in attenuation of myocardial activity and contraction (Oliver & Opie, 1994). The reduction of β-oxidation of the FFA during ischaemia, results in the intracellular accumulation of acylcarnitine and acyl coenzyme A (Apstein, 2000). This accumulation may cause impairment of the calcium homeostasis and the production of free radicals, which lead to

instability of the contractile function of the myocardium and subsequent decrease in coronary flow.

Addition of insulin (group 3) resulted in the recovery of the coronary flow to pre-ischaemic values. This can probably be accounted for by the ability of insulin in the perfusate to minimize the inhibition of glycolysis by FFA. As insulin may protect the cell membranes through a combined anti-lipolytic (Oliver & Opie, 1994) and a membrane-stabilization mechanism (Mittra, 1965), it is able to improve reflow after ischaemia and thus improve the ability to supply the nutrient and oxygen deficient myocardium with crucial elements to ensure better recovery and survival.

The recovery of the coronary flow of the insulin treated hearts (group 3) to pre-ischaemic volumes, may also be attributed to the ability of insulin to increase flow (Oltman, 2000).

5.2.3 LVDP and % LVDP recovery

The LVDP of the non-insulin treated hearts (group 2) showed a 30% recovery during reperfusion (Figure 4.5 and Figure 4.6). During ischaemia, oxygen deficiency prevents proper oxidation of non-glucose substrates, and the myocardium becomes totally reliant on anaerobic glycolysis and the rate of glucose utilization (Opie, 1998) for its energy requirements. As the coronary flow is impaired during ischaemia, the delivery of substrates to re-energize the cells is slowed down, and the functional recovery is impaired.

Reperfusion LVDP of the insulin treated hearts (in group 3) recovered to 80% of the pre-ischaemic values. This observation again confirms the well-established fact that the recovery of the myocardium is dependent on the hearts' ability to deliver glucose to the myocardial cells to promote survival. Insulin stimulated both glucose entry into the

myocardial cells and glycolysis (King & Opie, 1998). Combined with its ability to ensure an improved coronary reflow after ischaemia, this promoted the functional recovery.

Another factor that may influence glucose uptake is the stimulation of the $\alpha-$ and $\beta-$adrenergic receptors, which operate through a Ca^{2+} dependent mechanism (Clark & Pattern, 1984). Ischaemia results in a significant release of endogenous catecholamines, especially noradrenaline, which will stimulate both $\alpha-$ and $\beta-$adrenergic receptors (Mouton et al., 1992). Both $\alpha-$ and $\beta-$stimulation will enhance myocardial glucose uptake (Clark & Pattern, 1984). At the end of the ischaemic period, we also observed a significant rise in the cAMP content of the insulin treated hearts (group 3) (Figure 3.12, p.57). It is thus a possibility that this rise in the cAMP content triggered a mechanism in these hearts at the end of ischaemia that improved glucose uptake and inotropic ability, and thus assisted the hearts in a more successful recovery.

Another possible explanation for the higher LVDP in the insulin treated hearts (group 3), might be that the rise in cAMP leads to an increased intracellular Ca^{2+} release that would lead to stronger contractility (Aulbach, 1999) and a subsequent increase in LVDP. The addition of wortmannin to the perfusion solutions during both ischaemia (group 4) and reperfusion (group 5) resulted in a significant poorer LVDP recovery during reperfusion. Addition of wortmannin before the onset of ischaemia also abolished the rise in cAMP, confirming the suggestion that cAMP may be important in restoring contractile function. Wortmannin inhibits activation of PI3-Kinase, implicating this pathway in the improved LVDP as well as the elevated cAMP levels observed.

5.2.4 RPP and % RPP recovery

The RPP is HR multiplied by LVDP. The non-insulin treated hearts (group 2) had a 26% RPP recovery compared to a 68% recovery of the insulin treated hearts (group 3) (Figure 4.7and Figure 4.8).

Although the heart rates for both groups were similar, the LVDP of insulin treated hearts were significantly higher than that of the non-insulin treated hearts. That explains the higher RPP ($P < 0.05$) in those hearts treated with insulin.

5.2.5 Time to onset of ischaemic contracture (TOIC)

The TOIC has been used as a marker of myocardial ischaemic injury (Lasley & Mentzer, 1993). The TOIC was significantly delayed, and was sometimes absent in the insulin treated hearts (group 3) compared to the non-insulin treated hearts (group 2) (Figure 4.9). As the coronary flow in the hearts in group 2 was severely impaired at the onset of reperfusion, it may have contributed to the accumulation of ischaemia-induced metabolic products. The metabolism of the ischaemic myocardium is characterized by an ATP shortage, as well as inhibition of glycolytic enzymes by the accumulation of lactate and concomitant intracellular acidosis. Other detrimental changes include inadequate functioning of membrane pumps (Opie, 1990), potassium (K^+) loss in the ischaemic zone (Opie, 1970; Weiss and Lamp, 1987), sarcolemmal damage (Williamson et al., 1976), accumulation of cAMP (Wollenberger et al., 1969), and deficient Ca^{2+} homeostasis (Henry et al., 1977).

Several independent studies (Tani & Neely, 1989; Bolli, 1990; Opie, 1998) concluded that a crucial factor in reperfusion injuries following ischaemia is mitochondrial calcium accumulation, which may cause contractile dysfunction, like ischaemic contracture. As

insulin is a known vasodilator, it might allow for improved perfusion conditions, and thus less accumulation of deleterious substrates, even during limited ischaemic flow. Furthermore, because of insulin's ability to increase glucose uptake, it might deliver glucose better and quicker to the ischaemic myocardium, thus improving its metabolic milieu and possible survival. Improved glucose uptake *per se* has been shown to regulate TOIC (Owen, 1990).

5.2.6 Cyclic AMP (cAMP)

In the present study, elevated levels of cAMP were observed after 15 minutes of ischaemia in both insulin (group 2) and non-insulin treated (group 3) hearts (Figure 4.12). A possible explanation for this could be an ischaemia-induced activation of the beta-adrenergic signaling pathway, due to the release of endogenous catecholamines (Mouton et al., 1992), in conjunction with a decrease in phosphodiesterase activity (Lochner et al., 1998). The further increase in cAMP after 30 min of ischaemia in insulin treated hearts in comparison to non-insulin treated hearts was abolished by wortmannin. This indicates previously unreported cross signaling from the insulin stimulated signaling pathway via activation of PI3-Kinase to the generation of cAMP. Although evidence has previously been presented that cAMP can stimulate PKB activity in a wortmannin sensitive manner (Webster & Anwer, 1999), the opposite has not been reported previously. When wortmannin was added at the onset of reperfusion, cAMP levels during reperfusion, were unaffected.

An increase in cAMP content during ischaemia is usually associated with poorer functional recovery. As the second messenger of beta-adrenergic catecholamine stimulation, cAMP acts by increasing the cytosolic calcium content, which can potentially

have adverse electrophysiological effects (Lubbe et al., 1992). Evidence for an arrhythmogenic role of cAMP was proposed by Opie et al. (1979). They suggested that ischaemic ventricular fibrillation was associated with increased levels of tissue cAMP in the ischaemic zone. In contrast with their findings, Muller et al. (1986) showed that the prevention of ventricular fibrillation by beta-adrenergic blockade was not associated with decreased levels of cAMP, while Manning et al. (1985) found that markedly elevated tissue cAMP levels in the rat heart, obtained by stimulating adenylate cyclase with forskolin, did not promote ischaemic or reperfusion arrhythmias. Therefore, controversy still exists about the role of increased levels of cyclic AMP in the myocardium during ischaemia and reperfusion.

However, we observed significant increases in cAMP at the end of the ischaemic period in hearts treated with insulin compared to non-insulin treated hearts. These hearts showed improved mechanical reperfusion function despite high levels of cAMP at the end of ischaemia. Abolishing the elevated cAMP under these conditions with wortmannin was detrimental to recovery. These results therefore indicate that elevated cAMP during ischaemia in the presence of insulin may be beneficial. cAMP during reperfusion appears to be a less important parameter, as it may be washed out by reperfusion and never shows any significant changes.

5.2.7 p38 MAPK

Numerous biochemical changes occur within the heart during ischaemia and reperfusion, including increased oxidative stress and production of ROS (reactive oxygen species), changes in ion homeostasis and energy/fuel metabolism, decreases in intracellular concentrations of ATP and creatine phosphate, degradation of adenosine

nucleotides to adenosine and other nucleosides, and osmotic disturbances (Sudgen & Clerk, 1998). These biochemical changes are associated with the activation of the p38 MAPK pathway during ischaemia and reperfusion (Clerk et al., 1998; Seko et al., 1997; Bogoyevitch et al., 1996).

Perfusion of hearts with a perfusion solution containing 5mM glucose in the absence of insulin resulted in significantly higher levels of activated p38 MAPK, indicating that this perfusion condition stressed the heart (Figure 4.13). Increased p38 MAPK was abolished by the presence of insulin. p38 MAPK was transiently activated during ischaemia. During reperfusion, hearts perfused in the absence of insulin presented with high levels of activated p38 MAPK, while the presence of insulin significantly lowered this. Wortmannin added at the onset of reperfusion partly reversed the effect of insulin.

The functional recovery of insulin treated hearts was significantly better than that of non-insulin treated hearts, while addition of wortmannin reversed this. Functional recovery therefore correlated inversely with the levels of activated p38 MAPK measured during reperfusion. These results are in accordance with Bogoyevitch et al. (1996), Mackay & Mochly-Rosen (1999), Marais, (2000) and Saurin et al. (2000). Studies by Bogoyevitch et al. (1996) and Yin et al. (1995), showed that the activation of p38 MAPK during stress of ischaemia, may be sustained during reperfusion. In the present study we observed such a sustained activation of p38 MAPK during reperfusion in the non-insulin treated hearts, while activation was transient in the insulin treated hearts. As p38 MAPK is a stress-activated kinase, the lower activation found on reperfusion in insulin treated hearts, may be a reflection of protection and not the cause thereof. To prove that elevated p38 MAPK activation during reperfusion is detrimental, hearts would have to be pefused with a specific antagonist of p38 MAPK added at the onset of reperfusion. Addition of such an antagonist, SB203580, during ischaemia to cardiomyocytes

expressing wild-type p38α MAPK, resulted in protection (Martin et al., 2001). Conversely, cardiomyocytes expressing a SB203580 resistant mutant of p38α MAPK, were not protected against ischaemia, showing that activation of p38α MAPK during ischaemia is indeed detrimental to the myocardium.

5.2.8 PKB

The metabolic action of insulin affects every aspect of the cell, including the increase of glucose uptake from the blood, enhanced conversion to glycogen and triacylglycerol, the inhibition of glycogen and fat breakdown, and the promotion of protein synthesis (Moule & Denton, 1997). PKB is an important mediator of the physiological effects of insulin. Treatment of cells with insulin will lead to a 20- to 50-fold activation of kinase activity and phosphorylation of PKB (Hemmings, 1997). It is thus in accordance with these findings that treatment with insulin in the present study resulted in a significant increase in PKB content compared to non-insulin treated hearts (Figure 4.15).

Several studies indicate that PI3-K is involved in the regulation of PKB. These findings indicate that the PI3-K inhibitor, wortmannin, inhibits growth factor, as well as, insulin-induced activation of PKB (Franke et al., 1995; Burgering & Coffer, 1995; Kohn et al., 1995).

In agreement with the results that indicated an active role for PI3-K in the activation of PKB (Franke et al., 1995; Burgering & Coffer, 1995; Kohn et al., 1995), the current study showed that addition of wortmannin during reperfusion successfully inhibited the activation of PKB. This suggests that activation of PKB was thus mediated via a PI3-K pathway.

Konishi and colleagues (1997) investigated the effect of cellular stresses such as heat shock, hyperosmolarity and ischaemia/reperfusion on PKB activation. As activation of PKB by cellular stresses is insensitive to wortmannin, they concluded that it is possible that PKB is activated by the growth factors via a PI3-K dependent pathway, and by cellular stresses in a manner independent of PI3-K activation. As PKB was lower after 30 minutes than after 15 minutes, it is that stress-induced activity peaked at a shorter period of ischaemia.

When neonatal rat ventricular cardiomyocytes were subjected to simulated ischaemia and reperfusion, Punn and colleagues (2000) concluded that PKB was not activated after ischaemia alone, but only during reperfusion following ischaemia. They found a sustained phosphorylation of Ser^{473} of PKB that lasted for 2 hours. However, in the current study, no activation of PKB was found on reperfusion in the non-insulin treated hearts. A significant rise in the PKB activation of the insulin treated hearts after 5 and 15 min of reperfusion was documented (see Figure 4.15). This may be one way by which insulin could assert its protection when given only at the onset of reperfusion (Jonassen, 1996).

The functional recovery of the insulin treated hearts was significantly better than that of the non-insulin treated hearts, and this was accompanied by a higher PKB content. Furthermore, this improved functional recovery, similar to PKB activation could be abolished by the addition of wortmannin. This may be explained by the downstream effects of activation of PKB on glycolysis, glycogen synthesis, protein synthesis, glucose transporters, and its anti-apoptotic properties. The anti-apoptotic properties of PKB (which may be particularly important in the scenario of ischaemia-reperfusion) are mediated through the inhibition of at least four different pathways.

(i) PKB phosphorylates the pro-apoptotic protein BAD, and prevents it from forming a heterodimer with the anti-apoptotic proteins Bcl-2 or Bcl-X_L. As phosphorylated BAD is scavenged by the 14-3-3 scaffolding proteins, it prevents BAD from interfering with anti-apoptotic Bcl-2 or Bcl-X_L (Van Haesebroeck & Alessi, 2000).

(ii) PKB also phosphorylates and inhibits the actions of Human caspase-9, which is a crucial protease in the initiation of apoptosis (Alnemri, 1999). Indeed, evidence exists for the promotion of cell survival by PKB before activation of caspase 9. It intervenes in the apoptosis cascade, even before release of cytochrome c from the mitochondria and caspase-9 activation. This mechanism possibly operates by maintaining the integrity of the mitochondrial membrane (Kennedy et al., 1999).

(iii) PKB can interfere with cell-death by a transcription-based mechanism. It is believed that PKB can phosphorylate a large family of forkhead transcription factors (FKHR). FKHR, a human homologue of daf-16, contains three PKB phosphorylation sites and is expressed in the liver. Reporter gene studies in HepG2 hepatoma cells show that FKHR stimulates insulin-like growth factor-binding protein-1 promoter activity through an IRS (insulin response sequence), and introduction of IRSs confers this effect on a heterologous promoter. Insulin disrupts IRS-dependent transactivation by FKHR, and phosphorylation of Ser-256 by PKB is necessary and sufficient to mediate this effect. Antisense studies indicate that FKHR contributes to basal promoter function and is required to mediate effects of insulin and PKB on promoter activity via an IRS. FKHR stimulates promoter activity through an IRS and phosphorylation of FKHR by PKB mediates effects of insulin on gene

expression. Signaling to FKHR-related forkhead proteins via PKB may provide an evolutionarily conserved mechanism by which insulin and related factors regulate gene expression (Guo et al., 1999).

5.3 Possible cross-signaling

5.3.1 cAMP and p38 MAPK

Evidence suggests that there is a significant amount of cross talk between cAMP- and ERK-dependent signaling pathways in endothelial cell cytoskeletal organization and barrier regulation (Liu et al., 2001). The Raf kinases serve as central intermediates to relay signals from Ras to ERK. Cell-specific effects of these signals on growth, differentiation and survival can be observed due to the recruitment of different isoenzymes of the Raf family (Konig et al., 2001).

It has been proposed that the *in vitro* phosphorylation of a site unique to B-Raf (Ser429) is responsible for the negative regulation of the isoenzyme by PKB. Guan et al. (2000) showed that Ser429 is phosphorylated upon cAMP elevation in PC12 cells and proposed that PKA is a major kinase phosphorylating the B-Raf-specific site in vivo. Raf can thus be inhibited by both PKB and PKA. Similarly, Raf-1 is negatively regulated through phosphorylation by both PKB and PKA.

Raf-1 phoshorylates and activates MAPK kinase, which in turn leads to the activation of MAPK (Van Haesebroeck & Alessi, 2000). Sheth and colleagues (2001) investigated cross signaling between the p38 MAPK and ERK pathways in neutrophils. Their data show that p38 inhibition enhances ERK activity during endotoxemia. It is thus a possibility that cross signaling between these two pathways may result in the activation of the p38 MAPK pathway as a result of the inhibition of the ERK pathway via Raf by

cAMP. Our data does not support the above signaling pathway, as our results show a significant increase in cAMP in the insulin treated hearts at the end of the ischaemic period, but in the same hearts we recorded control values for p38 MAPK activation.

5.3.2 cAMP and PKB

PKB can be activated by increases in intracellular cAMP concentrations in a manner that is independent of PI3-Kinase (Sable et al., 1997). The phosphorylation of T308 and S473 appears to be responsible but direct phosphorylation of PKB by PKA (Sable et al., 1997) was not found. The present study noted that the significant rise in cAMP at the end of the ischaemic phase was accompanied by a transient activation of PKB at the beginning of the reperfusion phase. This activation peaked at 15 minutes of reperfusion, and attenuated towards the end of reperfusion.

The rise in the content of cAMP at the end of ischaemia could be fully abolished by wortmannin. This indicated cross signaling from the insulin stimulated signaling pathway to the cAMP pathway. Investigations should be performed to ascertain whether the activation of PKB on reperfusion occurring at a time when cAMP levels are low, is indeed a result of the PI3-K mediated rise in cAMP. As the levels of phosphorylated PKB were low at this time point, cross signaling must either occur via a different route, or a cascade of events must have been set in motion at an earlier stage.

4.3.3 p38 MAPK and PKB

Sweeney et al. (1999) investigated the mechanism underlying insulin-stimulated glucose transport in 3T3-L1 adipocytes and L6 myotubes. They found that in the presence of the p38 MAPK inhibitor, SB203580, insulin caused normal translocation and cell surface membrane insertion of glucose transporters without stimulating glucose transport. They

concluded that insulin stimulates two independent signaling pathways contributing to glucose transport: (i) The activation of PI3-K and PKB that leads to glucose transporter translocation, and (ii) a pathway that involves p38 MAPK that may lead to the activation of the recruited glucose transporter at the membrane. The inability of SB203580 to inhibit insulin-stimulated activation of all three isoforms of PKB suggest that p38 MAPK is not in the signaling pathway leading to the activation of PKB in response to insulin. It is thus a possibility that insulin exerts its cardioprotective effects by the parallel, complementary operation of both the PKB and p38 MAPK signaling pathways.

When wortmannin was added to the perfusion solution (group 5) during the reperfusion period, we observed a significant increase in the p38 MAPK activation, compared to insulin treated hearts (group 3). The functional performance of the hearts in group 3 recovered significantly better than the hearts in group 5. We can therefore argue that insulin might exert a cardioprotective effect by inhibiting the activation of p38 MAPK during reperfusion after an ischaemic insult. On the other hand, the lower p38 MAPK levels on reperfusion in the insulin treated hearts may only be an indication of lower levels of stress and not a direct consequence of insulin signaling. As stated previously, use of the specific p38 MAPK inhibitor SB203580 may clarify this matter.

Chapter 6

SUMMARY

In summary therefore this study set out to investigate the signaling pathways that insulin might use to protect the ischaemic/reperfused myocardium. We also investigated possible cross signaling between the different signaling pathways.

Our results demonstrated that:

(a)   Insulin exerted a positive inotropic effect in control perfused hearts.

(b)   Insulin, when added before, during and after low-flow ischaemia, results in significant cardioprotection, as reflected by improved functional performance during reperfusion.

(c)   The time to onset of ischaemic contracture was delayed or absent in the presence of insulin in the perfusate.

(d)   Wortmannin, and by implication inhibition of PI3-kinase, abolished the protection of insulin on LVDP recovery after ischaemia/reperfusion.

(e)   cAMP production, as seen at the end of ischaemia in hearts subjected to ischaemia in the presence of insulin, may play a role in the protective effects of insulin on LVDP as this was also abolished by wortmannin. To the best of my knowledge, this is the first demonstration of cross signaling from the insulin cascade to production of cAMP, a messenger normally associated with beta-adrenergic stimulation.

(f)   Improved recovery was associated with attenuated activation of p38 MAPK on reperfusion. This may be a reflection of the protected state of the heart and not the cause thereof.

(g)   On the other hand, improved recovery is associated with activation of PKB during early reperfusion, which might be linked to the attenuation of apoptosis (Jonassen, 2001).

Our data shows that insulin exerts a positive inotropic effect and delays the onset to ischaemic contracture. Inhibition of PI3-kinase by wortmannin abolishes the protective effects of insulin, arguing for an insulin stimulated PKB involvement in cardiac protection. Insulin also increase cAMP production and attenuate activation of p38 MAPK, both associated with improved recovery. Our findings thus suggest possible cross signaling between different signaling pathways.

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