UNIVERSITY OF LONDON THESIS

Degree MD Year 2006 Name of Author BOSE, Amal Krishna

Glucagon Like Peptide –1 (GLP-1) in Myocardial Ischaemia-Reperfusion Injury

Thesis submitted by

Amal Krishna Bose BSc (Hons), MB.,BS MRCS

For the degree of

Doctor of Medicine

University of London

The Hatter Institute and Centre for Cardiology
University College London Hospitals and Medical School
Grafton Way
London WC1E 6DB

2006

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Acknowledgments

I wish to express my thanks and gratitude to those at the Hatter Institute, in particular my special thanks for all their hard work, guidance and support to Dr Mihaela Mocanu and Professor Derek Yellon.

I would also like to acknowledge Dr Richard Carr for his support, encouragement and financial support and also the Wellington Hospital.

I thank my family for their undoubted support and especially Tanya who has been a ray of sunshine throughout my time at the Hatter. This work is dedicated to my beautiful daughter Mia.

Declaration

I confirm that the work presented in this thesis is my own.

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Abstract

Glucagon-Like Peptide–1 (GLP-1) is an incretin hormone released by enteroendocrine cells lining the intestine in response to the presence of nutrients. GLP-1 is known to cause increased secretion of insulin from the pancreas and has been identified as one of the crucial components of insulin and in turn glucose homeostasis. GLP-1 has a very short half life of 1-2 minutes, being rapidly degraded by a ubiquitous enzyme called dipeptidyl dipeptidase IV and also undergoing renal excretion. Interestingly GLP-1 mRNA transcripts have been identified in several organs outside of the expected enteropancreatic axis including the heart. Insulin has been shown to reduce cell death in the ischemic-reperfused rat myocardium and in isolated rat myocytes via its ability to activate prosurvival kinase signalling pathways. We propose that GLP-1 could protect the myocardium against ischaemia-reperfusion injury by activating similar prosurvival signalling pathways.

Both in-vivo (open chest) and in-vitro (Langendorff perfused) rat heart models of regional ischaemia and reperfusion were used. In-vivo treatment with GLP-1 produced a significant reduction in infarction (% infarct/risk zone) compared to valine pyrrolidide (VP), (an inhibitor of the enzyme dipeptidyl peptidase), and control groups (20.0 ± 2.8, vs. 47.3 ± 4.3, and 44.3 ± 2.4, respectively $P<0.001$).

In isolated perfused hearts (where there is no circulating insulin) GLP-1 significantly reduced infarct size compared to VP and control (26.7 ± 2.7 vs. 52.6 ± 4.7 and 58.7 ± 4.1, $P<0.001$) groups respectively. Protection was abolished in the presence of the PI3kinase inhibitor, LY294002 (58.6 ± 4.1), the ERK 1/2 MAPK inhibitor, U0126 (48.3 ± 8.6), the p70s6K inhibitor, Rapamycin (57.1 ± 4.9%) and by the GLP-1 receptor antagonist exendin-9-39 (57.3 ± 3.8). GLP-1 protects the myocardium against ischaemic - reperfusion injury when given throughout ischaemia - reperfusion or when given just five minutes prior to the onset of reperfusion or as a preconditioning mimetic.

To further elucidate the mechanism of GLP-1 mediated myocardial preservation we carried out Western blot studies examining the phosphorylation of components of the RISK pathway which showed an increase in the phosphorylation of BAD. The

increased phosphorylation of the pro-death peptide BAD, confirmed the potential anti-apoptotic effect of GLP-1.

In conclusion we have demonstrated for the first time that GLP-1 protects the rat myocardium against ischaemia-reperfusion injury, both in vivo and in vitro. GLP-1 appears to protect via the up regulation of specific prosurvival kinase pathways. This may represent a new therapeutic potential for this class of drugs currently undergoing trials in the treatment of non-insulin dependent diabetes.

Publications arising from this thesis

Abstracts

- Glucagon-Like Peptide-1 mediated cardiac protection against ischaemia/reperfusion injury involves multiple pro survival pathways. **Journal of Molecular and Cellular Cardiology** 2004. 37 (1) 288-289

- Glucagon-Like Peptide-1 (GLP-1), A Gut Incretin, Protects the Myocardium Against Ischaemic/Reperfusion Injury through the Myocardial GLP-1 Receptor and Activation of Prosurvival Pathways. **Circulation.** 2004(110) 17 SIII-68.

- Glucagon-Like Peptide-1 (GLP-1) Provides Protection Against Cardiac Ischaemia/Reperfusion Injury In Both in vivo and in vitro Experimental Models in the Rat. **Heart.** 2004 (90) SII; A23

Presentations

- Glucagon Like Peptide-1 (GLP-1) Provides Protection Against Cardiac Ischaemia / Reperfusion Injury in Both In Vivo and In Vitro Experimental Model in the Rat **British Cardiac Society Annual Scientific Meeting May 2004**

- GLP-1 Protects Ischemic and Reperfused Myocardium via PI3Kinase and ERK 1/2 MAPK Signalling Pathways. **The American Diabetic Association Annual Scientific Meeting Orlando June 2004**

- Glucagon Like Peptide-1 (GLP-1) Mediated Cardiac Protection Against Ischemic/Reperfusion Injury Involves Three Individual Pro Survival Pathways **ISHR Global Meeting Brisbane 2004**

- Myocardial infarct size attenuation by glucagon like peptide-1 (GLP-1) in both in vivo and in vitro rat heart. **European Association for Study of Diabetes Annual Meeting, Munich Germany September 2004**

- Glucagon-Like Peptide-1 (GLP-1), A Gut Incretin, Protects the Myocardium Against Ischaemic/Reperfusion Injury through the Myocardial GLP-1 Receptor and Activation of Prosurvival Pathways. **American Heart Association, New Orleans October 2004**

- Glucagon Like Peptide-1 (GLP-1) mediated cardiac protection against ischemic / reperfusion injury involves multiple pro-survival pathways. **World Congress of the World Society of Cardiothoracic Surgeons. Ottawa Canada August 2006**

Full papers

- Bose AK, Mocanu M, Brand C, Carr RD, Yellon DM. Glucagon like peptide-1 (GLP-1) can directly protect the heart against ischaemia/reperfusion injury. **Diabetes 54 (1): 146-51; 2005**

- Bose AK, Mocanu M, Carr RD, Yellon DM. Glucagon-Like Peptide-1 is protective against myocardial ischaemia/reperfusion injury when given either as a preconditioning mimetic or at reperfusion in an isolated rat heart model. **Cardiovascular Drugs and Therapy. 19 (1):9-11; 2005**

- Myocardial Ischaemia-Reperfusion Injury is Attenuated by Glucagon Like Peptide –1 (GLP-1) through a p70s6K Dependent Pathway_Bose AK, Mocanu M, Carr RD, Yellon DM. **In preparation**

List of abbreviations

ACE-I	Angiotensin-converting enzyme inhibitor
ADP	Adenosine diphosphate
cAkt	Cellular Akt (also known as protein kinase B)
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocase
APD	Action potential duration
ATP	Adenosine triphosphate
ATPase	ATP synthase
B2	Bradykinin B2 receptor
Bad	Bcl-X_L /Bcl-2-associated death promoter
Bax/BAX	Bcl-associated X protein
CABG	Coronary Artery Bypass Grafts
CAD	Coronary Artery Disease
Caspase	Cystein specific aspartate directed protease
CFR	Coronary flow rate
cGMP	Cyclic guanine-5-monophosphate
CK	Creatine kinase
Da	Dalton

DPP-IV	Dipeptidyl peptidase 4
DMSO	Dimethyl sulphoxide
ECLA	Estudios Cardiologicos Latinoamerica
EDTA	Ethylene diamine tetracetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-tetra acetate
eNOS	Endothelial nitric oxide synthase
Erk	Extracellular signal-regulated kinase
FFA	Free Fatty acids
GLP-1	Glucagon-Like Peptide-1
GLP-1 R	Glucagon-Like Peptide-1 Receptor
GPCR	G-Protein Coupled Receptor
HSP	Heat shock protein
gp130	Glycoprotein 130
IC_{50}	50% inhibitory concentration
IDDM	Insulin Dependent Diabetes Mellitus
IGF-1	Insulin-like growth factor 1
iNOS	Inducible nitric oxide synthase
IPC	Ischaemic preconditioning
I/R%	Infarct-risk volume ratio

ISIS-IV	The fourth International Study of Infarct Survival
K_{ATP}	ATP-sensitive potassium channel
kDa	Kilodalton
K_i	Dissociation constant for inhibitor binding
LDH	Lactate dehydrogenase
LAD	Left anterior descending
MAPK	Mitogen activated protein kinase
MAPKAPK	MAPK- activated protein kinase
MAPKK/MKK	MAPK- kinase
MI	Myocardial Infarction
mPTP	Mitochondrial permeability transition pore
NADH	Nicotinamide adenine dinucleotide
nm	Nanometres
NF-κB	Nuclear factor kappa B
NHE	Na^+-H^+ exchanger
NIDDM	Non Insulin dependent Diabetes Mellitus
NO	Nitric oxide
PBS	Phosphate buffered saline
PG	Prostaglandin
PI3K	Phosphatidyl inositol 3-OH kinase

PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLSD	Protected least significance difference
p70s6K	70-kDA ribosomal protein S6 kinase
RISK	Reperfusion injury salvage kinase
ROS	Reactive oxygen species
RPP	Rate pressure product
RTK	Receptor tyrosine kinase
SDS	Sodium dodecylsuphate
STEMI	ST elevation myocardial infarction
VP	Valine pyrrolidide

List of figures and tables

Chapter 1 Introduction

1.1 Coronary Artery Disease

Coronary Artery Disease (CAD) is the largest killer in the western world and will, in the future, become the leading cause of mortality worldwide. CAD is caused by the build up of atheromatous plaques in the lining of the coronary arteries, known as atherosclerosis, causing limitation of coronary blood flow to the myocardium and hence tissue oxygen deprivation or ischaemia. Myocardial ischaemia can clinically present, acutely as a Myocardial Infarction (MI) or with a spectrum of disease ranging from chronic stable angina to heart failure. The disease may be silent or may lead to symptoms such as angina. In the UK, data from the British Heart Foundation (BHF) indicate that CAD is the most common cause of mortality. It accounts for more than 125 000 deaths per year. Mortality rates vary by gender and account for one in four deaths in men and one in six in women. Morbidity is also considerable. Statistics indicate that approximately 1.5 million people suffer from angina and 246 000 patients present every year with an acute myocardial infarction in the UK. Thankfully the majority of these are non fatal at presentation. A large percentage of patients require revascularisation whether performed percutaneously, surgically or with thrombolysis to improve their prognostic outcome. Revascularisation implies the restoration of coronary artery blood flow by reperfusion and hence tissue oxygenation. This can be achieved by the use of thrombolytic agents such as Streptokinase, percutaneously through procedures such as angioplasty and intra-coronary stent placement or by surgical means by construction of Coronary Artery Bypass Grafts (CABG).

Myocardial ischaemia can lead to myocardial infarction and cell death with resultant morbidity and mortality. To salvage the myocardium from acute ischaemia, reperfusion is mandatory. Reperfusion brings with it life giving tissue oxygenation but also harbours, like a Trojan horse, deleterious consequences which have been collectively termed as ischaemic-reperfusion injury.

Therefore, in order to realise an improvement in the mortality and morbidity of this disease, it is necessary to discover novel treatment strategies which provide protection to the myocardium from ischaemia-reperfusion injury.

1.2 Coronary Artery Disease and Diabetes Mellitus

Diabetes Mellitus, whether insulin dependent (IDDM or type 1) or non insulin dependent (NIDDM or type 2) is, recognised as, a significant risk factor for the development of CAD (Kannel et al. 1979) A large proportion of diabetic patients suffer from myocardial ischaemia and its consequences. Diabetics have more severe, more aggressive, more complex and more diffuse coronary artery disease than do age matched controls. In general, coronary disease develops at a younger age than in the non diabetic patient. In insulin dependent diabetes, premature coronary artery disease is detectable in population studies from the fourth decade and by age of 55 years up to one third of patients have died from the complications of CAD (Fuller et al. 1983). The relative risk for fatal coronary heart disease associated with diabetes is 50% higher in women than in men (Huxley et al. 2006).

The risk of developing CAD in the patient with non insulin dependent diabetes is two to four times higher than the general population and does not appear to relate to either the severity or the duration of the diabetes, possibly because the presence of insulin resistance may predate the onset of clinical symptoms by 15-25 years.

Diabetes although an independent risk factor for CAD, is also associated with the presence of abnormalities of lipid metabolism, obesity, systemic hypertension and an increase in thrombogenesis (increased platelet adhesiveness and elevated levels of fibrinogen). Late results of coronary artery bypass grafting are less favourable in diabetics (Cho et al. 2006), and diabetics have both an increased early mortality and higher risk of re-stenosis following angioplasty.

1.3 Preconditioning

One of the most powerful mechanisms for protecting the myocardium before the acute coronary artery occlusion occurs, is to ischaemically precondition the myocardium. Ischaemic Preconditioning (IPC) is a phenomenon which was first described in the seminal study by Murry and colleagues in 1986. IPC is protection against lethal prolonged ischaemia afforded by repeated short bursts of sub-lethal ischaemia with intervening reperfusion. This manoeuvre renders the myocardium more resistant to the ensuing lethal ischaemic period, by inducing an innate cellular adaptation response to stress. This, approach, however depends crucially on intervening *before* the ischaemic event, which is difficult, given the unpredictable timing of an acute coronary artery occlusion.

Several years after the description of acute cardiac protection by IPC, a second window or delayed cardioprotective effect was described (Marber et al. 1993). The first window of cardioprotection develops within minutes of IPC and lasts 1-3 hours, whereas the second window opens 12- 24 hours after the first phase has subsided and lasts for 3-4 days (Bolli. 2000).

IPC has shown to protect every animal species to date, including rat, rabbit, dog, pig and more importantly in human myocardium during CABG (Jenkins et al. 1997, Yellon and Downey 2003).

Since IPC unambiguously limits infarct size in animal models a huge amount of research has been directed into understanding the mechanisms involved. The goal of this research has been not only to contribute to the understanding of the IPC mechanisms, but also ultimately to investigate a pharmacological agent which can be clinically useful in mimicking IPC and producing a protected state as well as having a hypoglycaemic effect.

Preconditioning Signalling

IPC has been shown to be receptor mediated (Liu et al. 1991). These receptors are trans-membrane G-protein coupled receptors (GPCR). A huge number of surface receptors capable of inducing IPC, or contributing have been confirmed. The multitude of GPCR are linked to a smaller number of secondary messengers such as

Protein Kinase C (PKC) and end effectors such as the mitochondrial K-ATP channel, whilst other signal cascades implicated in IPC such as the Mitogen Activated Protein kinases (MAPKs) may lead to the modification of gene expression and be more important for delayed cardioprotection.

The PI3K-Akt Kinase Cascade

Recent studies have implicated signalling through the pro-survival, phosphatidyl inositol 3-OH kinase (PI3K)-Akt cascade, during the preconditioning phase before the index ischaemic episode, in IPC-induced protection. Tong and colleagues (Tong et al 2000) were the first to demonstrate that IPC activates the PI3K-Akt kinase cascades prior to the index ischaemic episode, and they showed that inhibiting Akt activity, using the PI3K inhibitor, wortmannin, abolished IPC-induced protection, using the recovery of function as the end-point. Yellon's group confirmed these findings in the isolated perfused rat heart infarct model. Interestingly, an earlier study (Baines et al.1999) had demonstrated that insulin-induced preconditioning also required the activation of the PI3K-Akt kinase cascade prior to the index ischaemic episode.

The PI3K-Akt signalling cascade has been implicated as a trigger of preconditioning (Krieg et al. 2003), such that it relays the preconditioning signal from the GPCR at the cell membrane to the mitochondrial K -ATP channel, where the opening of the latter mediates the mitochondrial release of Reactive Oxygen Species (ROS). The intracellular signalling pathway through which the activation of the PI3K-Akt kinase cascade results in the opening of the mitochondrial K-ATP channel is not clear although nitric oxide has been implicated. (Oldenburg et al. 2004).

Mitogen-Activated Protein Kinases (MAPKs)

The MAPKs comprise 4 major kinase cascades: the p38-MAPKs, the c-Jun N-terminal kinases (JNKs), the 42 and 44-kDa extracellular signal-regulated kinases 1/2 (Erk1/2) MAPK, and big MAPK kinase 1 (BMK1 also known as Erk5). These 4 major kinase pathways exhibit the same conserved three-tier module in which a series of three protein kinases phosphorylate and activate one another (Johnson et al. 2002).

These kinase cascades are activated in response to stress such as ischaemia-reperfusion and receptor protein tyrosine kinases, GPCR's and PKC.

Erk1/2 MAP Kinase

Whether the Erk1/2 MAPKs contribute to cardio-protection associated with classical preconditioning is unclear, with studies demonstrating that they are activated prior to the index ischaemic period (Mocanu et al. 2002) but not all of them show these MAPK's contributing to IPC-induced protection (Strohm et al. 2000) .A recent study suggests that diazoxide-induced mitochondrial ROS release may activate Erk1/2 (Samavati et al. 2002). Erk1/2 can mediate cellular protection by phosphorylating recruiting several anti-apoptotic mechanisms. Erk1/2 can phosphorylate and activate the ribosomal kinase p90RSKs which in turn can phosphorylate and inactivate the pro-apoptotic factor, BAD. (Harada et al. 2001)

The Reperfusion Injury Salvage Kinase Pathway: The RISK-Pathway

Yellon's laboratory (Yellon and Baxter 1999) formulated and tested the hypothesis that the pharmacological activation of the signalling kinase pathways, phosphatidylinositol-3-OH kinase (PI3K)-Akt, and extra-cellular signal-regulated kinases (Erk 1/2), at the time of reperfusion protects the myocardium against lethal reperfusion injury. Activation of these kinase cascades (which have been termed the Reperfusion Injury Salvage Kinase Pathway [The RISK-Pathway] (Hausenloy et al .2004) protects the heart by recruiting several anti-apoptotic pathways of cellular survival (Figure 1.1).

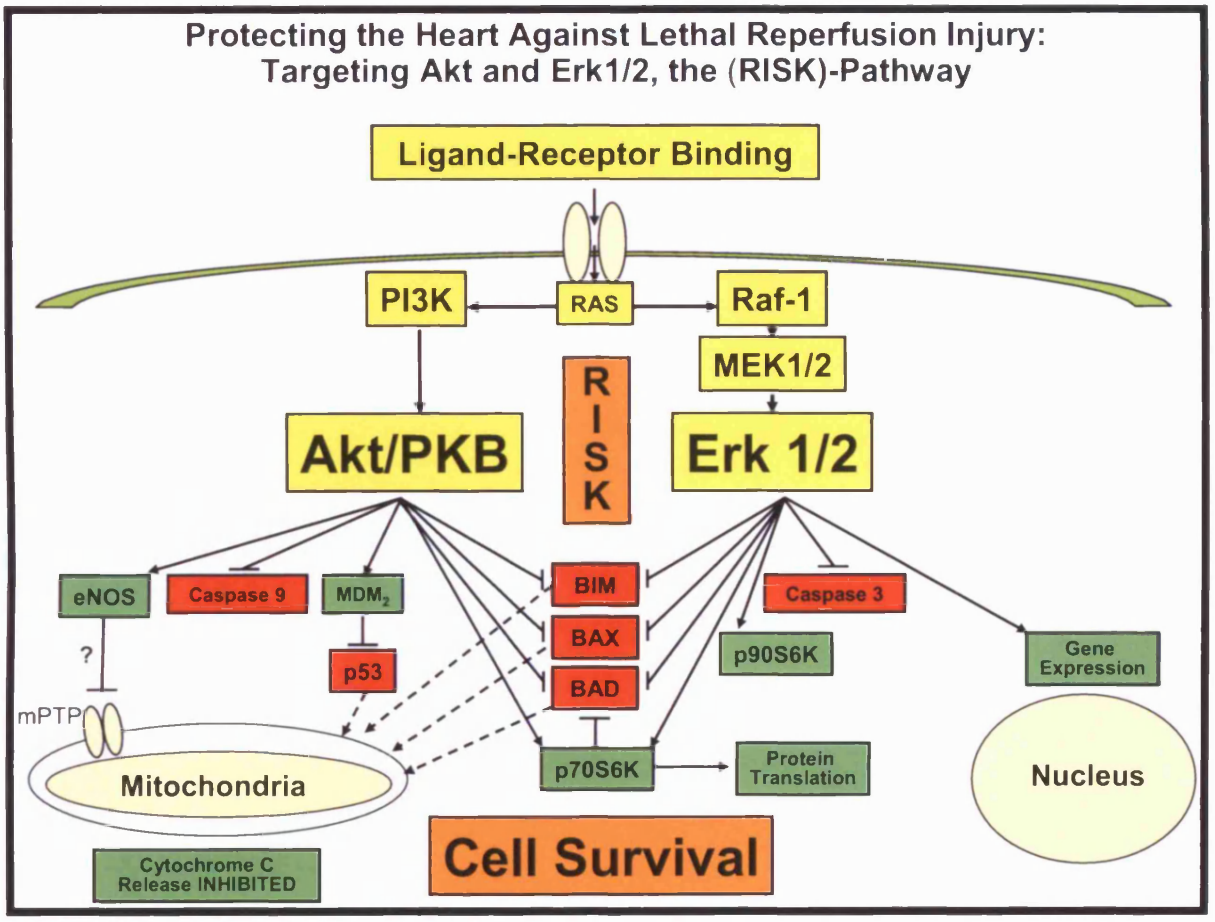
Jonassen and colleagues (Jonassen et al. 2000a) demonstrated using an in vivo rat heart model of ischaemia-reperfusion injury, a reduction in infarct size associated with the administration of glucose-insulin-potassium (GIK) at the time of reperfusion. In studies using rat cardiomyocytes subjected to hypoxia, Yellon's laboratory (Jonassen et al. 2000b) demonstrated that insulin given, at the time of reoxygenation, attenuated the apoptotic and necrotic components of cell death. In order to determine the mechanism of insulin-induced protection at the time of reperfusion, Yellon's

group (Jonassen et al. 2001) demonstrated using the isolated perfused rat heart, that administering insulin at the time of reperfusion activated the PI3K-Akt-BAD pathway, and that inhibitors of this pathway such as wortmannin (the PI3K inhibitor), and rapamycin (the mTOR inhibitor), abrogated insulin-induced protection. Administering insulin at the time of reperfusion activated this kinase cascade. The early perfusion of insulin was essential for protection, as delaying its administration to 15 minutes after the onset of reperfusion, was not associated with protection, suggesting that the protection via the PI3-Akt kinase cascade was mediated in the first few minutes of reperfusion. (Jonassen et al. 2001) In an in vivo rat heart model of ischaemia-reperfusion injury, eNOS, another downstream target of Akt phosphorylation, has been implicated (Gao et al. 2002), in insulin-mediated cardio-protection at reperfusion.

The activation of the pro-survival PI3K-Akt and MEK 1/2-Erk 1/2 cascades at the time of reperfusion, by ligands to growth factor or G-protein coupled receptors, protects the heart against lethal reperfusion injury. The protective mechanisms induced by these kinase cascades are mediated by the diverse array of substrates which these kinases phosphorylate.

The PI3K-Akt kinase signalling cascade is activated in response to the activation of a wide range of receptors, including those for growth factors and G-protein coupled receptors (Cross et al. 2000). In response to ligand-receptor binding, the p85 regulatory subunit of PI3K is phosphorylated, which results in the activation of the p110 catalytic subunit of PI3K, which in turn phosphorylates the membrane lipid, phosphatidylinositol 4,5 biphosphate (Franke et al. 1997) to phosphatidylinositol 3,4,5-triphosphate, which then recruits Akt (also known as PKB) to the membrane. Upstream kinases such as 3-phosphoinositide-dependent protein kinases 1 and 2 (PDK1 and 2) then phosphorylate and activate Akt at Thr-308 and Ser-473. (Datta et al. 1996).

Figure 1.1 : Scheme Outlining Proposed Pathways leading to cell Survival .



Legend: Agents which protect bind to receptors, which via the small G-protein, RAS, activate the PI3K-Akt and Raf1-MEK1/2-Erk1/2 kinase cascades, which together comprise the Reperfusion Injury Salvage Kinase (RISK)-pathway. Protection against lethal reperfusion injury is then mediated by the: (1) phosphorylation and inactivation of pro-apoptotic factors, caspases 3 and 9, BIM, BAX, BAD and p53, one consequence of which is to prevent the release of mitochondrial cytochrome C in response to an apoptotic stimulus (shown by dashed arrows); (2) phosphorylation and activation of eNOS (endothelial nitric oxide synthase), producing nitric oxide which may protect by inhibiting opening of the mitochondrial permeability transition pore (mPTP); (3) phosphorylation and activation of p70s6K and p90S6K which can protect by inactivating BAD or regulating protein transcription/translation; and (4) regulating the expression of genes concerned with cellular survival.

The activation of Akt phosphorylates a diverse array of substrates, influencing numerous cellular processes, many of which are anti-apoptotic in action. Downstream targets of PI3K-Akt are shown in Figure 1.1 which mediate anti-apoptotic effects.

The evidence lends itself to the suggestion that the pro-survival kinase cascades may therefore constitute a common pathway, mediating the cardio-protection induced by IPC on the one hand, as well as protecting the myocardium through their recruitment at the time of reperfusion, on the other hand. However, the only direct evidence for this rests with insulin-induced cardio-protection, and therefore more research is needed to elucidate whether the pro-survival kinase cascades actually constitute the common pathway for cardio-protection in these two settings.

The significant action of insulin in cardioprotection led us to take an interest in insulinotropic agents such as GLP-1.

1.4. Glucagon-like peptide-1

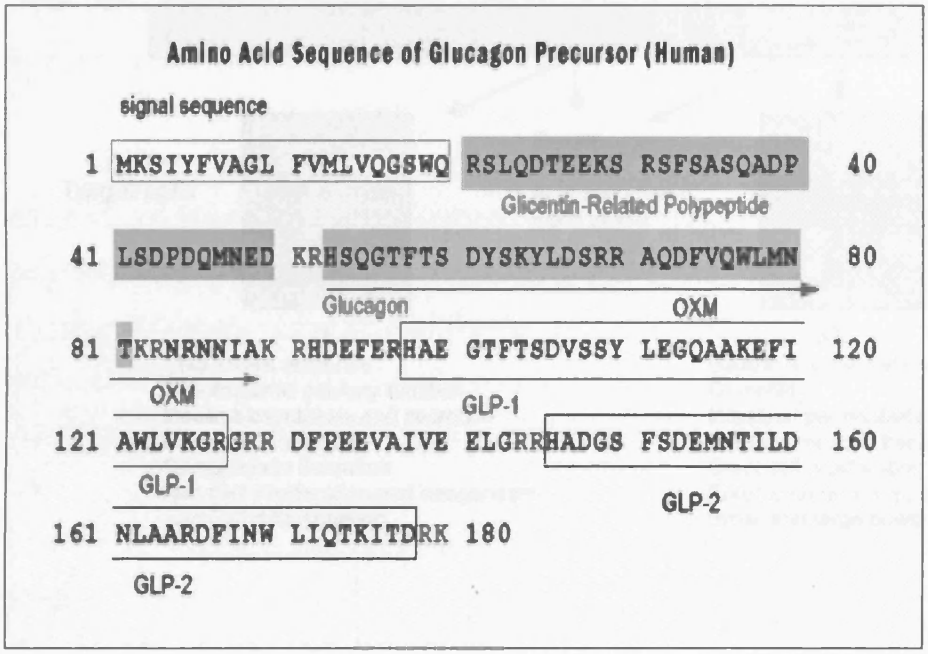
1.4.1 Introduction

In the early 1980s, the structure of mammalian pre-proglucagon was deduced from the cloned DNA, and found to contain, in addition to glucagon itself, the sequences of two related peptides, which were termed glucagon-like peptide (GLP) -1 and -2 because of their considerable sequence homology to glucagon (Bell et al. 1983). Fig. 1.2 A single glucagon gene encodes a larger biosynthetic precursor, proglucagon, in mammals. Tissue-specific processing of proglucagon gives rise to glucagon in the brain, and glicentin, oxyntomodulin, GLP-1, and GLP-2 in the intestine, with an intermediate profile of peptides liberated in the central nervous system. GLP-1 and GLP-2 are produced in the same gut endocrine cells and liberated, following posttranslational processing of a single proglucagon precursor. The available evidence suggests that only one proglucagon gene is expressed in mammals that gives rise to an identical mRNA in brain, pancreas, and intestine (Mojov et al. 1986). The tissue-specific liberation of proglucagon is controlled by cell-specific expression of prohormone convertase (PC) enzymes. An essential role for PC2 in the processing of islet proglucagon is revealed by studies of the PC2 knockout mouse. This mouse has mild hypoglycemia, elevated proinsulin, and exhibits a major defect in the processing of proglucagon to mature pancreatic glucagon, and the murine islet α cells secrete proglucagon from atypical secretory granules. Fig.1.3

Thereafter a search for the biological function of these additional peptides began, but initial progress was slow. Based upon the presence of pairs of basic amino acids, typically sites at which prohormone processing occurs, a 37 amino acid peptide, GLP-1 (1-37) was predicted to be secreted, but this peptide turned out not to affect glucose or insulin concentrations significantly, even when administered in high doses (Ghiglione et al. 1984). It was for a further several years before two naturally occurring truncated forms, GLP-1 (7-36) amide (Ørskov et al. 1986) and GLP-1 (7-37) (Mojsov et al. 1987) were identified. With the demonstration that these truncated

forms were probably the most potent insulinotropic agents hitherto known (Holst et al. 1987; Ørskov et al. 1988, Mojsov et al. 1987), interest in GLP-1 research grew explosively.

Figure 1.2 Amino Acid sequence of human glucagon precursor

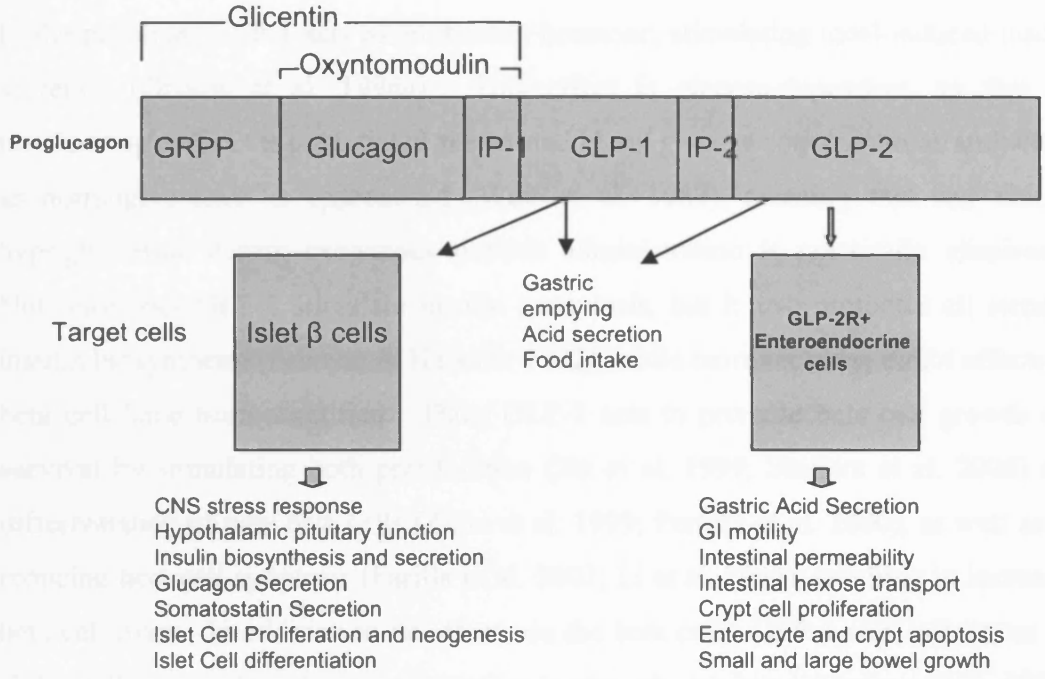


Legend

Primary accession number P01275

Structure of the human glucagon gene. White J.W., Saunders G.F.; Nucleic Acids Res. 14:4719-4730(1986).

Figure 1.3 Structure of proglucagon and biological actions of GLP-1 and GLP-2.



The principal target cell types for GLP-1 (islet ß cells) and GLP-2 (intestinal endocrine cells) are shown below the peptide sequences. GRPP, Glicentin-related pancreatic polypeptide; IP, intervening peptide; GLP-2R, glucagon-like peptide-2 receptor. (Drucker et al. 2003)

1.4.2 Pancreatic Actions

GLP-1 possesses a number of properties which make it a potentially ideal anti-diabetic agent. It is released from the intestinal L-cell in response to orally ingested nutrients (Elliott et al. 1993; Herrmann et al. 1995) and has potent effects upon the endocrine pancreas, the gastrointestinal tract and in the brain.

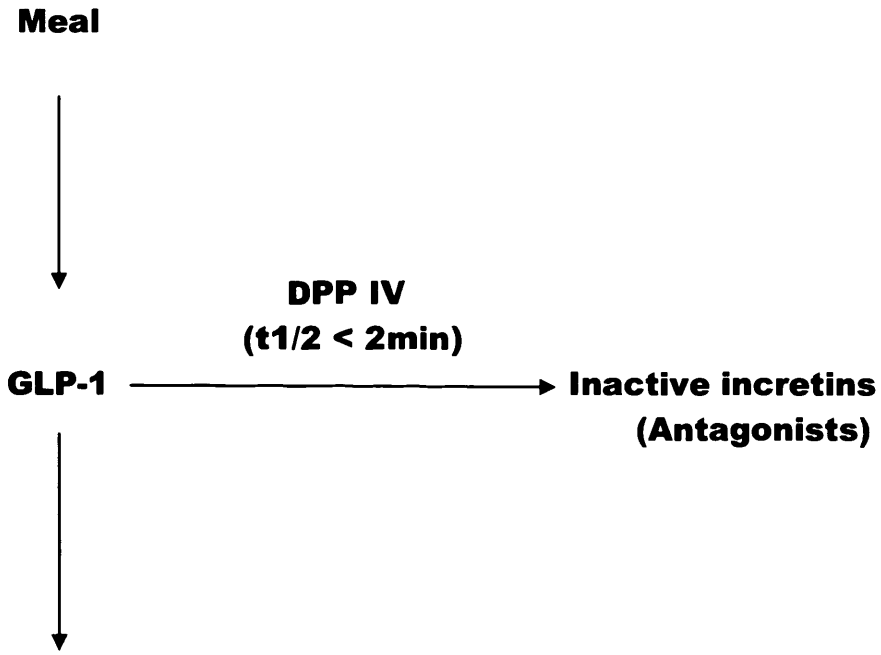
In the pancreas, GLP-1 acts as an incretin hormone, stimulating meal-induced insulin secretion (Ørskov et al. 1996a). This effect is glucose-dependent, so that the insulinotropic effect is potentiated at elevated blood glucose concentrations and wanes as normoglycaemia is approached (Weir et al. 1989), meaning that any risk of hypoglycaemia during exogenous peptide administration is practically eliminated. Not only does GLP-1 stimulate insulin exocytosis, but it also promotes all steps in insulin biosynthesis (Fehman & Habener 1992), while more recently, direct effects on beta cell have been identified. Thus, GLP-1 acts to promote beta cell growth and survival by stimulating both proliferation (Xu et al. 1999; Stoffers et al. 2000) and differentiation of new beta cells (Zhou et al. 1999; Perfetti et al. 2000), as well as by reducing beta cell apoptosis (Farilla et al. 2002; Li et al. 2003), resulting in increased beta cell mass. In addition to its effects on the beta cells, GLP-1 also influences the alpha cells, such that glucagon secretion is strongly inhibited (Ørskov et al. 1988). Notably, this glucagonostatic effect also is glucose-dependent (Nauck et al. 2002), meaning that GLP-1 administration is unlikely to impair the glucagon counter-regulatory response to hypoglycaemia. In the gastrointestinal tract, GLP-1 inhibits motility and secretion (Wettergren et al. 1993), thereby contributing to reduce the glucose excursion by delaying the passage of nutrients to the small intestine. Indeed, under physiological circumstances in healthy subjects, delayed gastric emptying appears to outweigh the insulinotropic effect of GLP-1 (Nauck et al. 1997). In humans, peripherally administered GLP-1 has a satiating effect, not only in healthy normal weight subjects (Flint et al. 1998), but also in obese subjects (Naslund et al. 1999) and in patients with type 2 diabetes (Gutzwiller et al. 1999a). Thus, when given over a prolonged period (6 weeks) by continuous subcutaneous infusion, patients with type 2 diabetes reported a reduction in appetite, which led to a significant reduction in body weight by the end of the study (Zander et al. 2002). Part of the mechanism appears to be related to a reduced gastric emptying rate (Verdich et

al. 2001), but a reduced sensation of appetite during GLP-1 in the fasting state, before meal ingestion (Gutzwiller et al. 1999b), suggests other mechanisms may also contribute. Central administration of GLP-1 inhibits food intake in rodents (Tang-Christensen et al. 1996; Turton et al. 1996), raising the possibility that peripherally released GLP-1 may have a direct effect on the brain. This is because circulating GLP-1 can access GLP-1 receptors in areas of the brain (subfornical organ, area postrema) which participate in the regulation of appetite and energy homeostasis (Ørskov et al. 1996b). However, it is also relevant that gastric distension activates GLP-1-containing neurons in the caudal nucleus of the solitary tract, suggesting a role for centrally expressed GLP-1 as an inhibitor of food intake (Vrang et al. 2003). Interestingly, central administration of the GLP-1 receptor antagonist, exendin (9-39), increases food intake (Turton et al. 1996; Meeran et al. 1999), suggesting that GLP-1 produced locally within the brain may exert a tonic satiating effect.

In 1992, it was demonstrated that GLP-1 was also effective in patients with type 2 diabetes (Gutniak et al. 1992; Nathan et al. 1992), and could normalise blood glucose in both the fasting and the fed state in these subjects when given as a continuous intravenous infusion (Nauck et al. 1993; Rachman et al. 1996; Larsen et al. 2001), even in those patients with advanced type 2 diabetes long after sulphonylurea secondary failure (Nauck et al. 1998).

However, unexpectedly, the effects of a single subcutaneous injection of GLP-1 were disappointing. Although high plasma levels of immunoreactive GLP-1 were achieved (Nauck et al. 1996), insulin secretion rapidly returned to pre-treatment values and blood glucose concentrations were not normalised (Nauck et al. 1996; Juntti-Berggren et al. 1996; Todd et al. 1997). However, the effect of repeated subcutaneous administration on fasting blood glucose is as good as that of intravenous administration (Nauck et al. 1996), while continuous subcutaneous administration for 6 weeks reduces fasting and post-prandial glucose concentrations and lowers HbA1c concentrations (Zander et al. 2002).Fig 1.3 and 1.5.

Figure 1.4 Scheme illustrating the known physiological actions of GLP-1



- **Improves Insulin synthesis and secretion**
- **Stimulates β cell growth**
- **Promotes satiety**
- **Delays gastric motility and secretion**
- **Increased glucose disposal in fat, muscle and liver**
- **Improves insulin receptor binding**
- **Decreases glucagon and gluconeogenesis**

1.4.3 The GLP-1 receptor

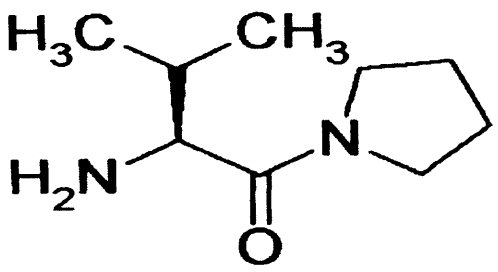
The GLP-1 receptor (GLP-1 R) is widely expressed in pancreatic islet β-cells, kidney, lung, the brain particularly in the hypothalamic paraventricular and arcuate nucleus, the gastrointestinal tract, and interestingly, also in the heart (Wei et al. 1995). The presence of the GLP-1 receptor in various organs suggests the possibility of an extended physiological role for GLP-1 outside of its primary incretin action. The receptor for GLP-1 is coupled to heterotrimeric $G\alpha_s$ proteins. Thus the GLP-1 R is a GPCR, and is a distinct member of the glucagon-secretin receptor super family that has been shown to function by causing intracellular calcium influx in addition to up-regulating cyclic-adenosine monophosphate(c-AMP) (Holz et al. 1995). Interestingly, c-AMP has been demonstrated, in several cell types other than myocardial, to protect against apoptosis (Kwon et al. 2003 and Hui et al. 2003). Although considerable effort has been expended in searching for a second GLP-1 receptor, only a single receptor has been isolated to date.

Specific agonist and antagonists of the GLP-1 R have now been isolated as discussed later in section 5.1.2

1.4.4 GLP-1 metabolism

Native GLP-1 has a short half life of minutes, being rapidly degraded by dipeptidyl peptidase-IV (DPP-IV), to generate an N-terminally truncated metabolite. In addition, GLP-1 undergoes renal excretion from the circulation. Dipeptidyl peptidase-IV, (EC 3.4.14.5), also known as the T-cell antigen CD26, is a serine exopeptidase. DPP-IV rapidly ($T_{1/2}$<2 min) cleaves off a dipeptide at the penultimate alanine near the amino terminus of GLP-1 (7–36) amide and generates the inactive or antagonistic GLP-1 (9–36) amide (Pauly et al. 1996). Therefore, in order to assess the roles of intact GLP-1, it is necessary to use a DPP-IV inhibitor, namely valine pyrrolidide (VP), as a means of preventing its degradation (Deacon et al. 1998).

Figure 1.5 Chemical Structure of VP



$C_9 H_{18} N_2 O$
(+)-(S)-2-Amino-3-methyl-1-(pyrrolidin-1-yl)butan-1-one

1.4.5 Cardiac Effects of GLP-1

A number of different groups have looked at differing aspects of GLP-1 in the myocardium and cardiovascular physiology. However the role of GLP-1 in ischaemic-reperfusion injury has not previously been explored. Cellular studies in isolated cardiac myocytes have demonstrated elevated c-AMP levels as a result of exposure to GLP-1 as well as demonstrating chronotropic effects (Vila Petroff et al. 2001). In vivo studies have been performed examining the effect of GLP-1 on blood pressure and heart rate (Barragan et al. 1994 and Yamamoto et al. 2002). Fascinatingly, a centrally mediated component to the effect of GLP-1 on haemodynamic parameters has been clearly identified (Barragan et al. 1999 and Isbil-Buyukcoskon et al. 2004). The majority of the literature demonstrates a role for GLP-1 in cardiovascular physiology with most in vivo experiments showing elevated blood pressure and heart rate as a result of GLP-1 infusion (Yamamoto et al. 2002). However some studies have failed

to demonstrate any haemodynamic effects with GLP-1 (Deacon et al. 1996). This may be due to species differences, the dose of GLP-1 used in the studies, and the use of inhibitors of the enzymatic degradation of GLP-1 such as DPPIV. Studies using knock-out mice have also suggested a role for the GLP-1 R in the control of cardiac structure and function (Gros et al. 2003). GLP-1R knock out mice were found to have reduced body weight and heart weight with thicker than expected ventricular walls. There were no overt cardiac histological differences between the wild type mice and knockouts.

More importantly, a recent clinical study demonstrated that GLP-1 improved left ventricular function in patients with acute myocardial infarction and left ventricular dysfunction (Nikolaidis et al. 2004). The same group have now gone on to demonstrate improved recovery of left ventricular function in a large animal model of ischaemia-reperfusion induced stunning (Nikolaidis et al. 2005).

The metabolic effects of GLP-1 in the myocardium are also of great interest as the insulin / glucose axis in cardioprotection has been the cornerstone of many academic departments. In particular, the balance between free fatty acid (FFA) oxidation and glucose oxidation remains crucial. Although free fatty acids are preferred as metabolic substrate for the heart under normal conditions, glucose becomes the preferred substrate during ischaemia. Initially it was thought that the provision of glucose benefits the myocardium by increasing glycolytic ATP and by decreasing free fatty acids usage. This results in a more efficient use of oxygen for the production of ATP, thus easing the heart's workload. Thus, developed, the hypothesis that clinical infusions of glucose-insulin-potassium (GIK) would be of benefit in myocardial infarction or induced ischaemia as occurs during cardiopulmonary bypass procedures. Indeed GIK treatments have had some success in the reduction of mortality and morbidity (Opie 1999, Sack and Yellon 2003), albeit at the cost of hypoglycaemia, volume overload or hypokalaemia.

The actions of GLP-1 as an insulinotropic agent and any effects on glucose homeostasis could directly influence this axis.

Another potential cardiovascular action for GLP-1 has been as a vascular endothelial relaxant. A randomised cross over trial in human subjects tested the effect of GLP-1 infusion on brachial artery blood flow (Nystrom et al. 2004). This study clearly

demonstrated changes in brachial artery diameter in GLP-1 treated type 2 diabetic subjects compared to non diabetic control subjects suggesting an additional beneficial effect in endothelial dysfunction. This group also found that GLP-1 had no affect on insulin resistance in their study. A role in endothelial function has also been demonstrated in the pulmonary vasculature where receptors for GLP-1 have also been identified, mediated possibly through a nitric oxide dependent pathway (Golpon et al. 2001).

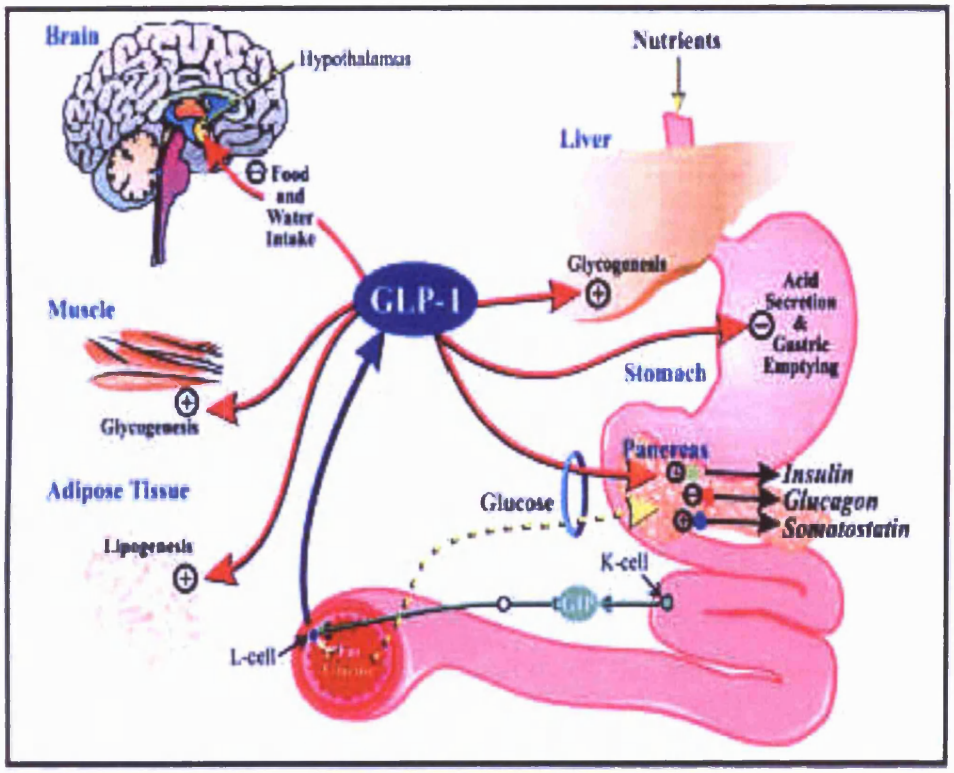
The exact physiological role for GLP-1 in the myocardium has yet to be clearly identified. However, of specific interest is the fact that GLP-1 has been also shown to promote the activity of phosphoinositide 3-kinase (PI3K) in β cells (Buteau et al. 1999). This kinase has been clearly associated with myocardial protection in the setting of ischemic-reperfusion injury (Hausenloy et al. 2004) as well as myocardial preconditioning (Tong et al. 2000 and Mocanu et al. 2002)

Recent data has suggested that GLP-1 can exert a direct cytoprotective effect via inhibition of apoptosis, either directly in target cells expressing the GLP-1 R, or possibly via the activation of survival factors (Redondo et al. 2003). GLP-1 has also been shown to protect against apoptosis in insulinoma cell lines through both c-AMP and PI3K (Hui et al. 2003). In this context, it is important to bear in mind that insulin has been shown to activate pro survival kinases such as PI3K (Jonassen et al. 2001), which have been proposed as integral components of anti-apoptotic cascades involved in myocardial protection.

1.5 Summary and Main Objectives of the Thesis

This thesis explores the actions of GLP-1 in the myocardium exposed to ischaemic-reperfusion injury. The aim of this study was to examine the direct effect of GLP-1 on the ischaemic myocardium in both the in vivo (insulin dependent) and in vitro (insulin independent) rat myocardium, assessing myocardial infarct size as an end-point of injury in both models. Furthermore, an additional aim was to investigate the cellular mechanisms underlying the protective effect of GLP-1 against myocardial ischaemia-reperfusion injury.

Figure 1.6 Known actions of GLP-1



This scheme illustrates the physiological interactions of GLP-1 on the Brain, Gastrointestinal tract, muscle and adipose tissue

Chapter 2 General Methods

2.1 Introduction

The effects of ischaemia and ischaemic-reperfusion injury have been studied in numerous settings including the whole organism, the isolated whole myocardium, and myocardial cellular preparations. Preliminary investigations to test concepts are commonly performed in animal models as ethical implications preclude the use of the human heart model in most scientific research. Fragments of knowledge put together from different types of experimental models create the pieces of the jigsaw for the design of successful potential treatment. Regional ischaemia, infarct size limitation and heart function in the rodent isolated perfused heart as well as in vivo are the basis of the experimental approach used here.

Experiments described in this thesis have been performed in the laboratory of The Hatter Institute and Centre for Cardiology, University College London Hospitals and Medical School, London, UK. These studies were conducted in accordance with The Home Office Guidance on the operation of Animals (Scientific Procedures) Act, 1986.

2.2 Choice of animal model

The rat is an established experimental species, and both the in vivo and isolated heart preparation has been extensively characterised. The ease of breeding and maintenance of these animals is favourable compared to other species. Sprague-Dawley rats were used in our experiments and were purchased from Charles River, Bicester, Oxon. Male animals were consistently used as gender has been shown to influence infarct size in models of ischaemic-reperfusion injury in the rat recently (Brown et al. 2005,

Pitcher et al. 2005, Wang et al 2005). The animals were housed in groups of four to a cage in the animal house and were fed a standard pellet chow. They were allowed to acclimatise for a minimum of four days prior to use, during which time a 12 hour light-dark cycle was maintained at 19-22° C, and 55 ± 10% humidity.

2.3 In Vivo Procedure

The aim of the in vivo procedure was to produce a dynamic model of myocardial ischaemia in which the effects of pharmacological agents on the myocardium as well as their systemic effects could be measured. In particular as GLP-1 has been identified as a potent incretin hormone it was important to use a model in which its action on insulin levels and hence glucose homeostasis could theoretically be observed.

2.3.1 Anaesthesia

All rats used for in vivo experiments were anaesthetised with a mixture of Hypnovel (midazolam 5 mg/ml, Roche, Welwyn Garden City, UK) and Hypnorm (0.315 mg/ml fentanyl and 10 mg/ml fluanisone, Jansen Animal Health, High Wycombe, UK). This mixture was made by adding 1ml of Hypnovel with 2ml sterile water for injection followed by 1ml of Hypnorm and giving 0.27ml per 100g bodyweight (Flecknell 1996) together with heparin (1IU/g) given by intra-peritoneal (i-p) injection. Consciousness was usually lost within 5 minutes and no longer than 10 minutes after injection. The animal was then weighed and transferred to the operating area. Adequate depth of anaesthesia was confirmed by the loss of the pedal withdrawal reflex before thoracotomy was performed.

Further doses of anaesthesia were given intra-venously (iv) at 20 minute intervals or earlier as required for maintenance of anaesthesia.

2.3.2 Surgical Procedure

A longitudinal midline incision was made in the neck to expose the strap muscles. The trachea was identified and exposed to allow a tracheosotomy to be performed. An endotracheal tube placed under direct vision to allow ventilation and secured in position with silk ties. The endotracheal tube was connected to a small animal ventilator from Harvard Apparatus, (Edenbridge, UK) and ventilated with oxygen at a rate of 70-80 breaths per minute and a tidal volume of 1.0 to 1.5 mls. Next the right or left external jugular vein was dissected and isolated. Central venous access to the jugular vein was gained and a line inserted, allowing infusions to be set up. Further doses of anaesthesia were given intra-venously (iv.) at 20 minute intervals or earlier as required for maintenance of anaesthesia. To allow transduction of blood pressure and heart rate, the left carotid artery was isolated and cannulated. The arterial line was connected to a pressure transducer (Oxnard, CA), and readings taken through a pen chart recorder-Multitrace2 (Lectromed, Letchworth, UK) to give continuous blood pressure (BP) and Heart rate (HR) / pulse measurement. Intermittent arterial blood gas analysis with a blood gas analyser ABL-705 was performed (Radiometer Medical Instruments, Copenhagen, Denmark). Ventilation was adjusted (rate or tidal volume) to maintain a physiological pH (7.35-7.45), pCO2 and pO2.

A left parasternal thoracotomy was performed to gain access to the heart. The pericardium was fully opened. The left anterior coronary artery was identified and a 6.0 suture was placed carefully around it, near its origin, from the left main coronary artery. The suture was then used to thread a plastic snare to permit reversible occlusion of the coronary artery. The animal was then allowed to stabilise for 15 to 20 minutes. To induce ischaemia, the snare was tightened around the coronary artery. Ischaemia was confirmed by regional ischaemic pallor, hypokinesia and a fall in BP. ECG recordings were taken to confirm the presence of myocardial ischaemia. The snare was relaxed at the end of the 30 minute ischaemic time period to allow reperfusion of the myocardium for 120 minutes.

2.3.3 Exclusion Criteria

Procedural exclusion criteria included blood loss of more than approximately 1 ml during the operative procedure, a mean arterial pressure < 90 mmHg during stabilisation, poor temperature control with rectal temperature under or above 36-37.5 °C, excessive bleeding from the epicardial surface on tightening of the snare and induction of ischaemia, and metabolic acidemia with a pH < 7.35. Animals with a weight range between 300-450g were used for in vivo experiments.

2.3.4 Parameters measured

1) Electrocardiogram

This was recorded using a three lead ECG capture system (ADI Instruments, Oxfordshire, UK).

2) Blood pressure and Heart rate

After placement of an intra-arterial cannula, this is connected to a pressure transducer and then onto the pen chart recorder (Lectromed, Letchworth, UK).

3) Ventilator rate and tidal volume

The small animal ventilator used allows the rate and tidal volume to be adjusted and hence recorded (Harvard Apparatus, Edenbridge, UK).

4) Arterial blood gases and Blood glucose

The arterial cannula allows blood samples to be taken and analysed for blood gases and glucose using the blood gas analyser described.

5) Insulin levels

To measure insulin levels, serum was separated from fresh blood samples and frozen at -20°C. Samples were packed in dry ice and couriered to be batch processed in at NovoNordisk, Bagsvaerd, Denmark.

Preparation of drugs

GLP-1

GLP-1in an anhydrous crystalline form was kept frozen at -20°until required. The GLP-1 was then dissolved in pure water to form a solution of 5 μmol and infused at the rate of 4.8 $\rho molkg^{-1}min^{-1}$.

Valine pyrrolidide

VP obtained from Novo Nordisk was kept as a powder at room temperature. The appropriate amount was weighed out depending on the weight of the animal to give a dose of 20 mg kg^{-1} and dissolved in 0.5 ml of normal saline and given by subcutaneous injection 30 minutes prior to the commencement of the experiment.

2.4 Isolated heart studies

2.4.1 The Langendorff heart preparation

2.4.1.1 Principle

The underlying principle is to force blood, or any other oxygenated fluid appropriate to maintain cardiac activity, towards the heart through a cannula inserted into the ascending aorta in a technique described by Oscar Langendorff (1853-1908). Retrograde perfusion closes the aortic valve – just as in the in situ heart during diastole – and the perfusate is displaced through the coronary arteries into the coronary sinus and opened right atrium. The cardiac cavities remain basically empty throughout the experiment and do not perform pressure -volume work.

The Langendorff preparation is appropriate for all warm blooded animals with a coronary vascular system. Human hearts have been kept alive for several hours during heart surgery using this principle. The preparation can be operated with either a constant flow or constant pressure. From a physiological standpoint, perfusion at constant pressure should be given preference, because whenever possible mean perfusion is maintained constant in the in intact circulation (Sutherland et al. 2000).

2.4.1.2 Measuring parameters

Mechanical parameters of the working myocardium (contractile force, volume and ventricular diameter), mean coronary flow, bioelectrical parameters

(electrocardiogram, monophasic injury potentials) and cardiac rhythm can be measured and recorded (Sutherland et al. 2000).

The intact function of the working myocardium and coronary vessels can be examined using various simple tests regarding the myocardium, the smooth musculature and the endothelium of the coronary vessel. Due to technical ease, a variety of parameters can be measured, providing information on the state of the myocardium and coronary vessels. Further these experiments can be easily repeated and are economically feasible. The heart tends to remain devoid of central neuronal stimulation / depression, which makes it ideal for studying the direct effects of treatments.

2.4.2 Chemicals and drugs

Chemicals used for Krebs-Henseleit buffer [containing in (mmol/l) 118 NaCl, 25 $NaHCO_3$, 11 glucose, 4.7 KCl, 1.2 $MgSO_4$. $7H_20$, 1.2 KH_2PO_4, and 1.8 $CaCl_2.2H_2O$], were purchased from BDH Laboratory Supplies (Merck Eurolab, UK). Carbogen (95% O_2 / 5% CO_2) to aerate and maintain the buffer pH was obtained from our local hospital supplies. GLP-1 and Valine Pyrrolidide were supplied by Novo Nordisk, Bagsvaerd, Denmark. Rapamycin, U0126 and LY294002 were obtained from Tocris, UK. Exendin 9-39 from Bachem, UK and Rp-cAMP from Calbiochem, UK. Triphenyl-tetrazolium chloride (TTC) and formaldehyde were purchased from Sigma (Poole, UK).

2.4.3 Perfusion equipment

The Langendorff apparatus used in these studies were based on the same principle as originally pioneered by Oscar Langendorff in 1895. A schematic representation of the apparatus and the actual apparatus Powerlab ML 176 (ADI Instruments, Oxfordshire, UK) used in these experiments are shown in figure 2.1 and 2.2 respectively. The buffer was maintained at a pH 7.3-7.5 and was monitored using a blood gas analyser (ABL-705, Radiometer Medical Instruments, Copenhagen, Denmark). Left ventricular pressures were transduced through a saline filled latex balloon.

Figure 2.1

Schematic representation of the apparatus

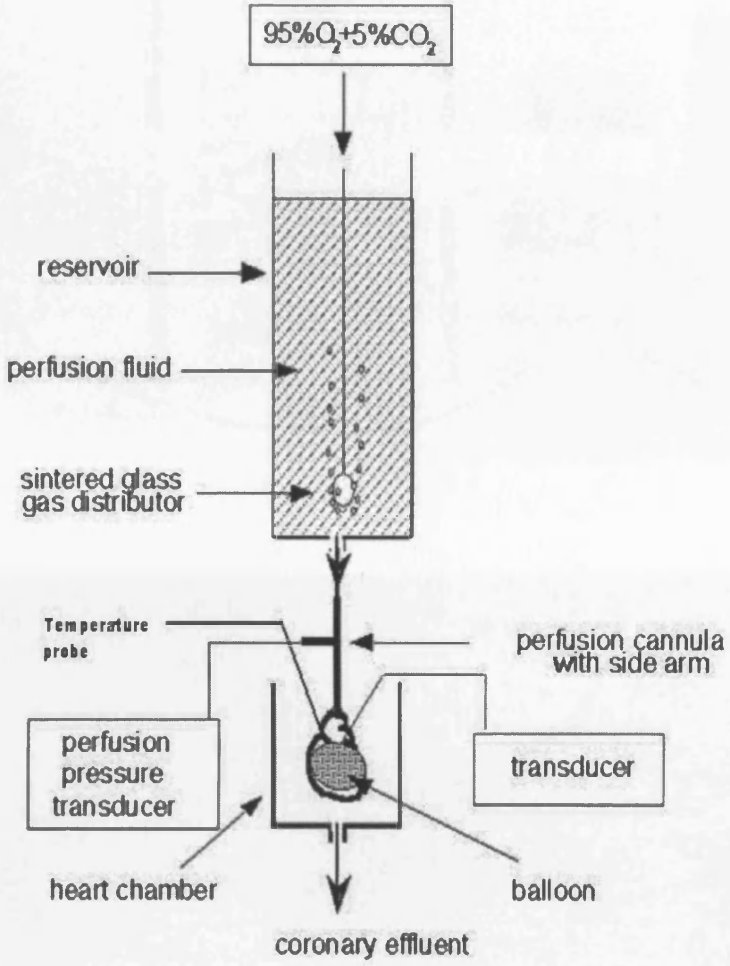
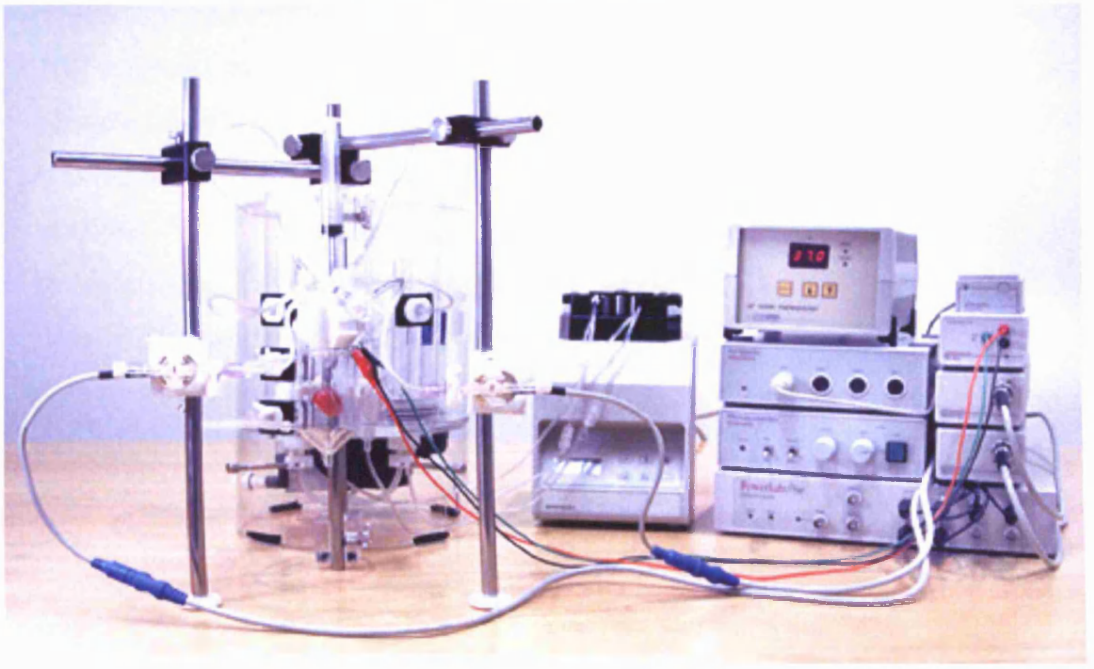


Figure 2.2 Actual apparatus used for in vitro experiments



2.4.4 Anaesthesia

All animals used for in vitro experiments were anaesthetised with sodium phenobaritone (Sagatal 50 mg/kg) obtained from Rhone Merieux, Dublin, Eire. This was administered intra-peritonealy (ip) and concomitantly with heparin (1 IU/g) obtained from Leo Laboratories Ltd (Bucks, UK) to prophylactically prevent thrombosis in the coronary vasculature or ventricular chambers. Consciousness was usually lost within 5 minutes and no longer than 10 minutes after injection. The animal was then weighed and transferred to the operating area. Adequate depth of anaesthesia was confirmed by the loss of the pedal withdrawal reflex before thoracotomy was performed.

2.4.5 Isolated heart preparation

To optimise the field of view a subcoastal incision was performed to gain access to the xiphisternal process, from whence the thorax was opened to expose the mediastinum and its contents. The heart is excised quickly by transecting the great vessels superiorly and the inferior vena cava inferiorly and immediately transferred to ice cold buffer solution to arrest contraction. The heart was then mounted onto the Langendorff apparatus by carefully holding the aorta with forceps and introducing the perfusion cannula into the aortic lumen. With practice this procedure can be completed safely without damaging the aortic valve or coronary ostia and without preconditioning the heart by exposing it to a prolonged ischaemic insult (Minhaz et al. 1995; Awan et al.1999). Perfusion should ideally be restored as quickly as possible and is achievable in 1-2 minutes. Buffer flow was adjusted automatically by the perfusion apparatus to maintain a constant perfusion pressure which was fixed at a constant 75 mmHg. A saline filled latex balloon connected to a pressure transducer was inserted into the left ventricle (LV), and baseline end-diastolic pressure was set at 5-10 mmHg. Heart rate, LV end-diastolic pressure and developed pressure were recorded continuously. A 3.0 suture was placed around the left main coronary artery and threaded through a plastic snare to permit reversible occlusion of the coronary artery. Myocardial temperature was monitored by introducing a probe into the right ventricular outflow tract and maintained at 36-37°C with the aid of a thermostat

controlled water bath around the buffer reservoirs. A heating lamp and warming chamber were also used in thermoregulation. A bubble trap in the system reduces the probability of an air embolism to heart from the perfusate.

2.4.6 Regional Ischaemia

A period of 15 minutes stabilisation was used during which the hearts were allowed to equilibrate prior to the induction of ischaemia. Regional ischaemia, was induced, by tightening a plastic snare on the suture around the left anterior descending coronary artery. Figure 2.3 and 2.4 Coronary occlusion was maintained for 35 minutes by clamping the snare onto the heart. Ischaemia was confirmed by regional ischaemic pallor, hypokinesia and a fall in rate pressure product and coronary flow. Reperfusion was achieved by releasing the snare which was often accompanied by reperfusion arrhythmias and a gradual improvement in rate pressure product and coronary flows. When regional ischaemia has been employed it becomes necessary to delineate the field of the occluded artery. This field is referred to the region at risk or the risk zone, as it is at risk of infarction. At the end of 120 minutes reperfusion, the left main coronary artery was re-ligated using the 3.0 suture already placed and the risk zone delineated with Evans Blue dye infused via the aortic root. Figure 2.5 demonstrates a heart with the risk zone demarcated by Evans Blue.

Figure 2.3 In Vitro heart in stabilisation

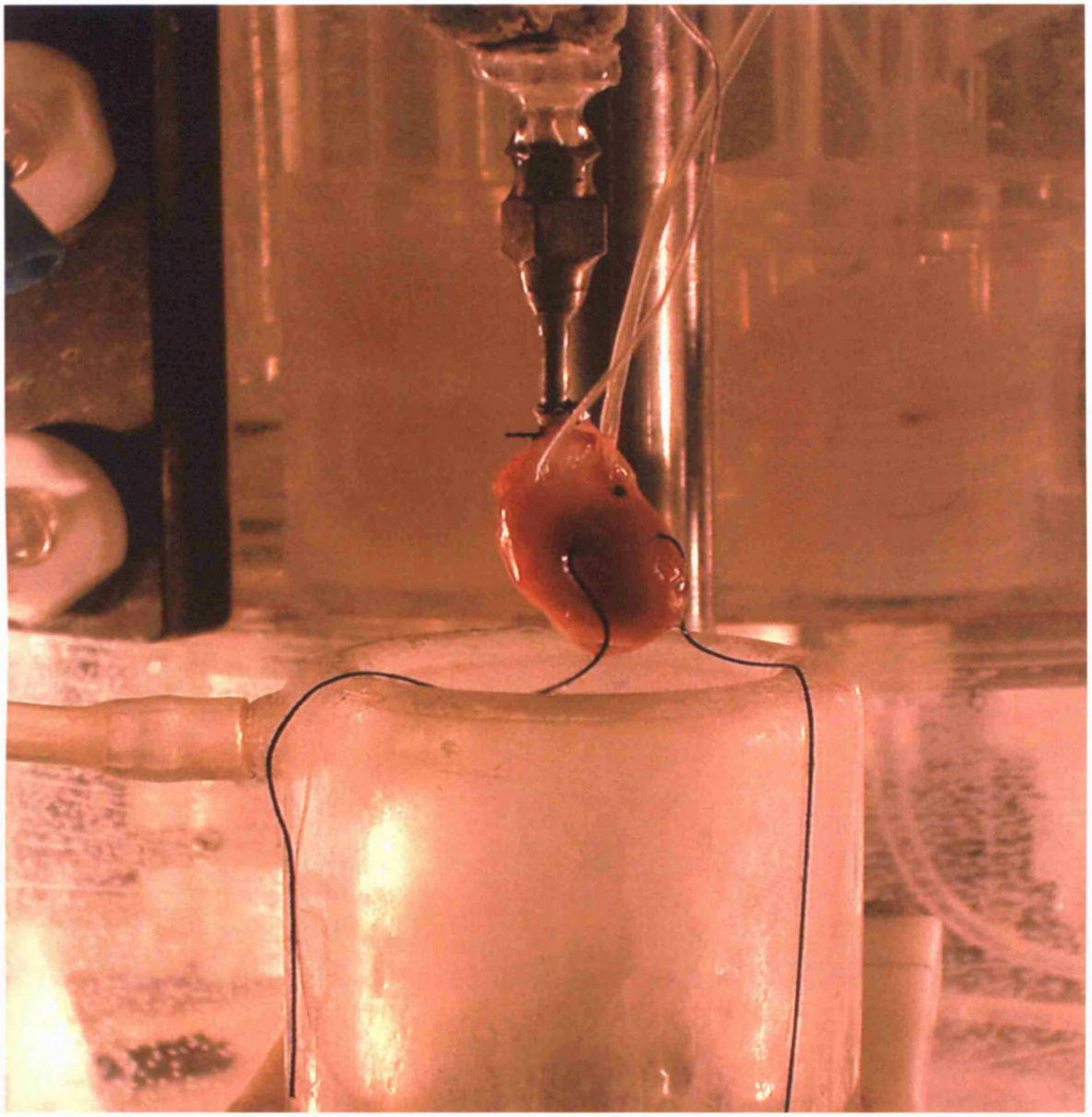


Figure showing heart in stabilization with perfusion cannula tied in the proximal aorta, temperature probe in right ventricular outflow tract, pressure transducer line entering left atrial appendage excision site and suture placed widely around left anterior descending coronary artery

Figure 2.4 In vitro heart in ischaemia

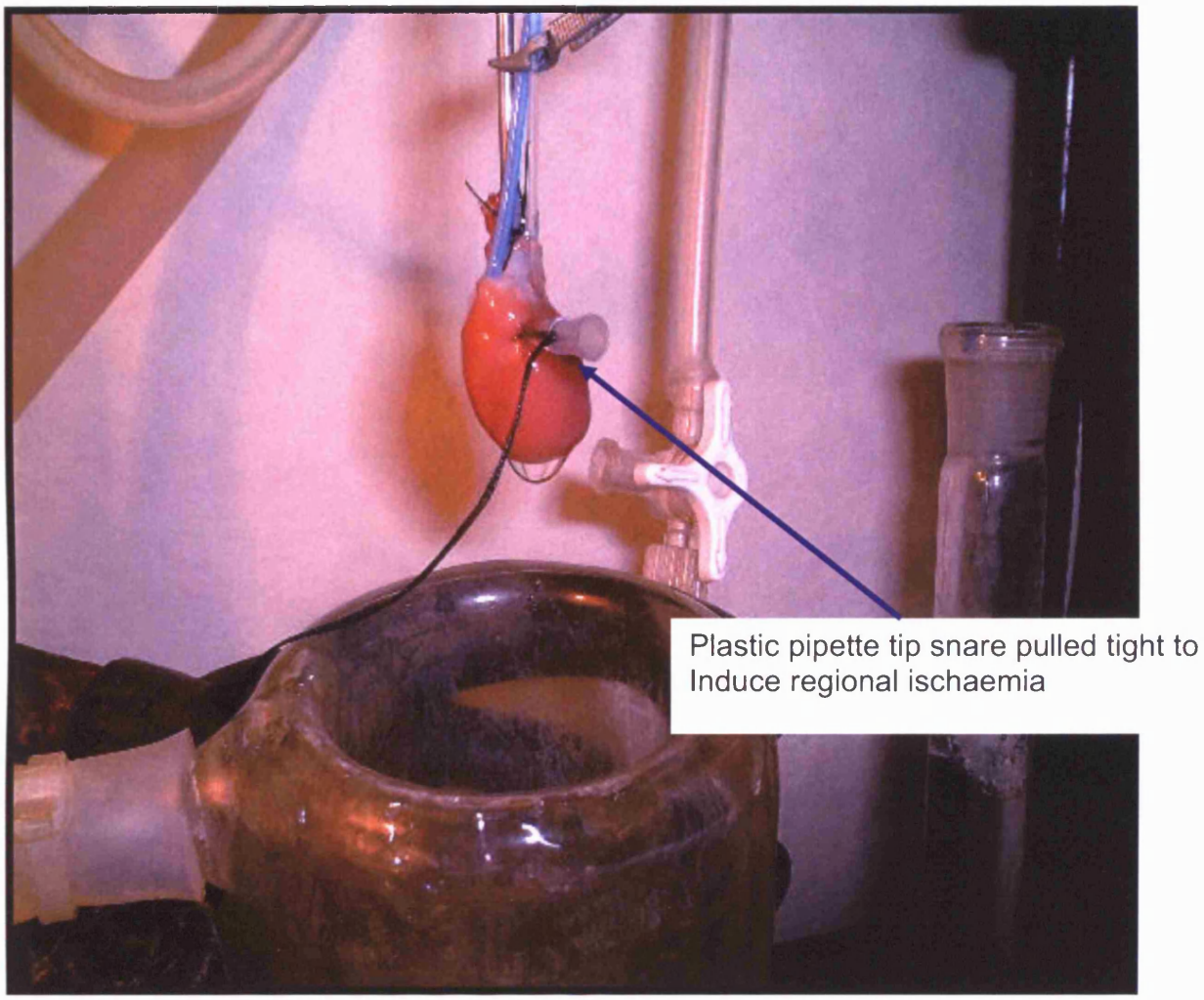


Figure showing snare pulled tight on suture around left anterior descending coronary artery to induce regional ischaemia

Figure 2.5 Evans Blue demarcation of area at risk

Figure showing area at risk or risk zone demarcated with Evans Blue dye after the suture around the left anterior coronary artery has been tied down.

2.4.7 Parameters measured

2.4.7.1 Coronary flow rate

The Langendorff hearts were perfused at a constant pressure, hence coronary flow was linked to the vascular resistance of the coronary arterial beds, as determined by heart contraction and arteriolar vascular smooth muscle tone. Coronary flow was measured by a pressure transducer built into the ADI perfusion apparatus which relays into the Powerlab recording software. An electronic feed-back circuit controls a peristaltic pump for constant-pressure perfusion of isolated hearts (Shattock et al. 1997). Coronary flow was monitored at regular intervals throughout the experiment. In control hearts not subjected to ischaemia coronary flow was found to diminish with time.

2.4.7.2 Temperature

Hypothermia has been shown to play an important role in the attenuation of ischaemia-reperfusion injury (Hale et al. 1997). The maintenance of a controlled temperature within strict limits is therefore crucial. Therefore temperatures were maintained carefully between 36-37° C and recorded continuously by the Powerlab apparatus.

2.4.7.3 Rate pressure product

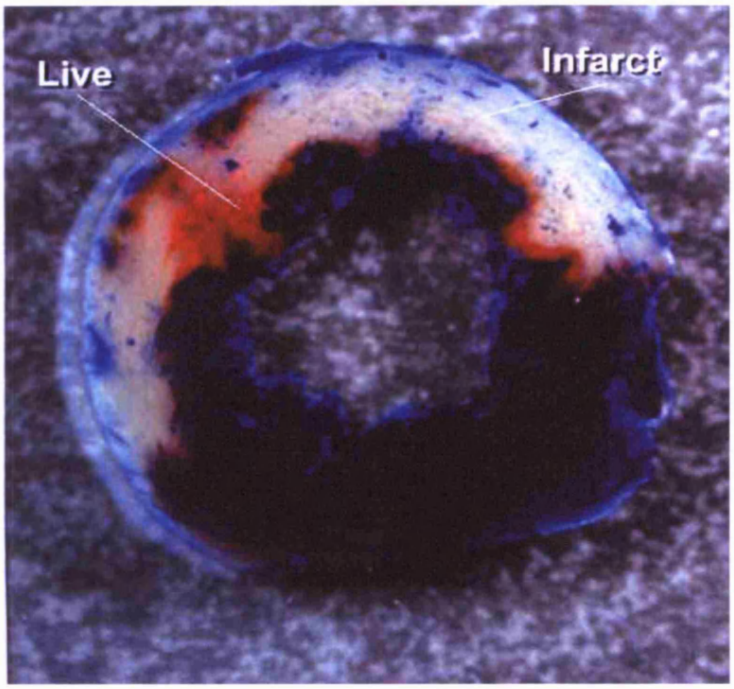
The rate-pressure product (RPP) is a good and reliable index of cardiac workload and function. It is calculated as the product of the ventricular heart rate and developed pressure at a given time and is expressed as millimetres of mercury per minute (mmHg min^{-1}). Continuous recordings of the RPP were made.

2.4.8 Infarct size evaluation

At the end of the 120 minute reperfusion protocol, perfusion through the aortic cannula was terminated. The suture around the left coronary artery used to induce ischaemia, is then tied off to effectively halt any flow down the coronary artery. Evans Blue dye was then retrogradely infused into the aortic cannula and hence into the coronary arteries to delineate the risk zone as described above (section 2.4.6). Hearts were rapidly frozen, then sectioned (2 mm) and incubated in 1% triphenyl-tetrazolium chloride (TTC) in phosphate buffer (pH 7.4, 37°C) for 12 minutes to stain viable tissue red as opposed to the non-stained white necrotic tissue. TTC staining is a widely recognised, validated method of staining for myocardial infarction (Ito et al. 1997; Schwarz et al, 2000). TTC in-fact stains non infracted viable tissue due its reduction by NADH and other cofactors producing a formazan pigment which is visible as a red stain. This is distinct from non stained white infracted tissue. Fig.2.6, 2.7 and 2.8 The tissue was then fixed in 4% formalin for 24 hours.

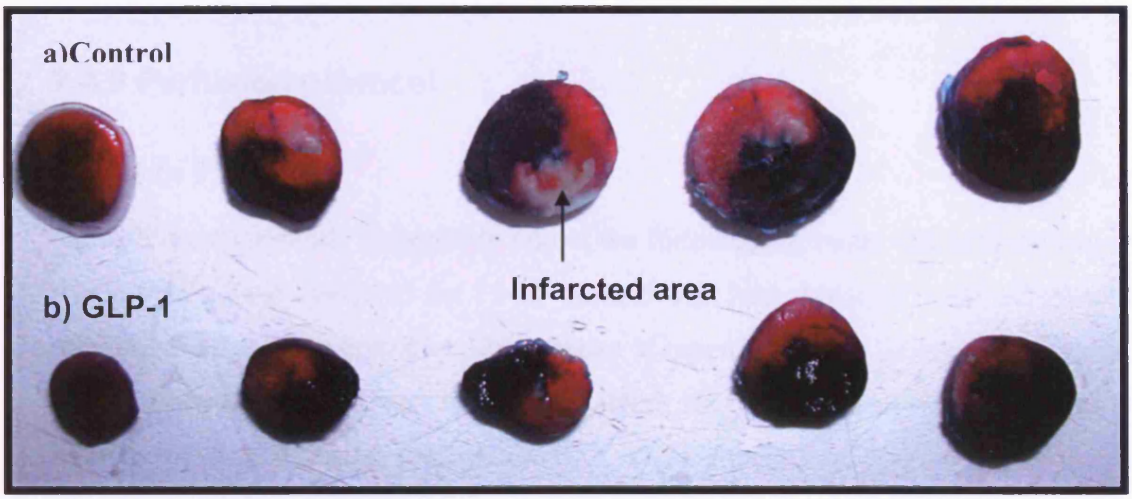
The formalin fixed and stained heart slices were mounted onto a glass plate. A cover glass plate was then placed over the tissue. Two millimetre thick shims in the corners hold the glass plates away from each other. The risk area, infracted and non infracted areas were demarcated. Images of the sections were drawn, by a blinded operator, on to an acetate sheet. The risk zone areas and infarct to risk ratios were determined by computerized planimetry. Fig 2.8 (Planimetry + version 1.0 for Windows Boreal Software Inc)

Figure 2.6 Stained heart slice showing Infarction in area at risk



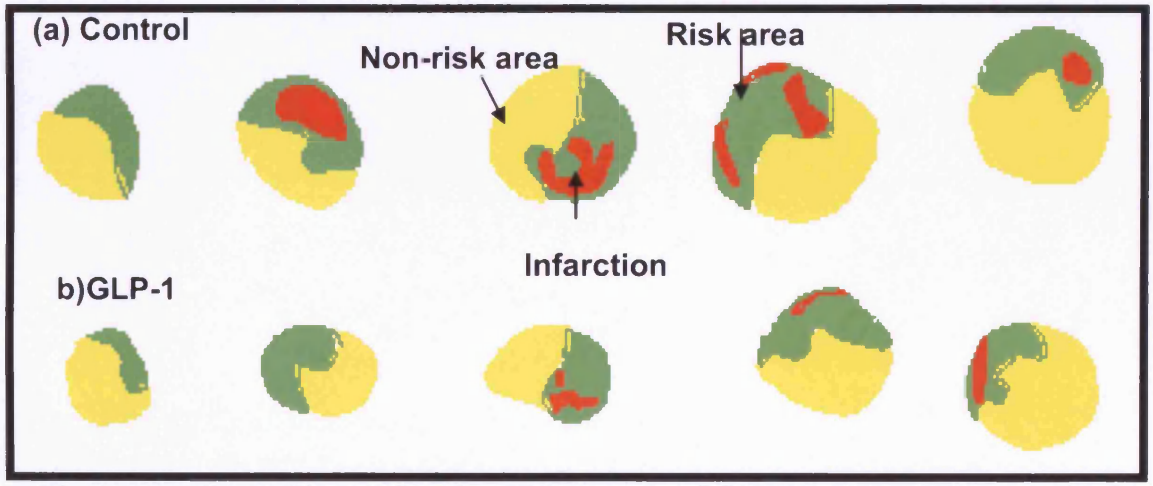
TTC stained heart slice showing infarction as white and live tissue as pink within the risk zone. The non risk area is stained blue.

Figure 2.7



Tetrazolium-Stained Heart Slices: *taken from (a) control hearts) and (b) GLP-1 treated hearts. The slices demonstrate areas of infarction (white), non-infarcted risk area (red) and non-risk area (blue). Note the smaller infarct area in the GLP-1 treated hearts vs. the control hearts.*

Figure 2.8



Planimetry Heart Slices: taken from (a) control and (b) GLP-1 treated hearts stained depicting infarcted areas (red), non-infarcted risk area (green) and non-risk area (yellow).

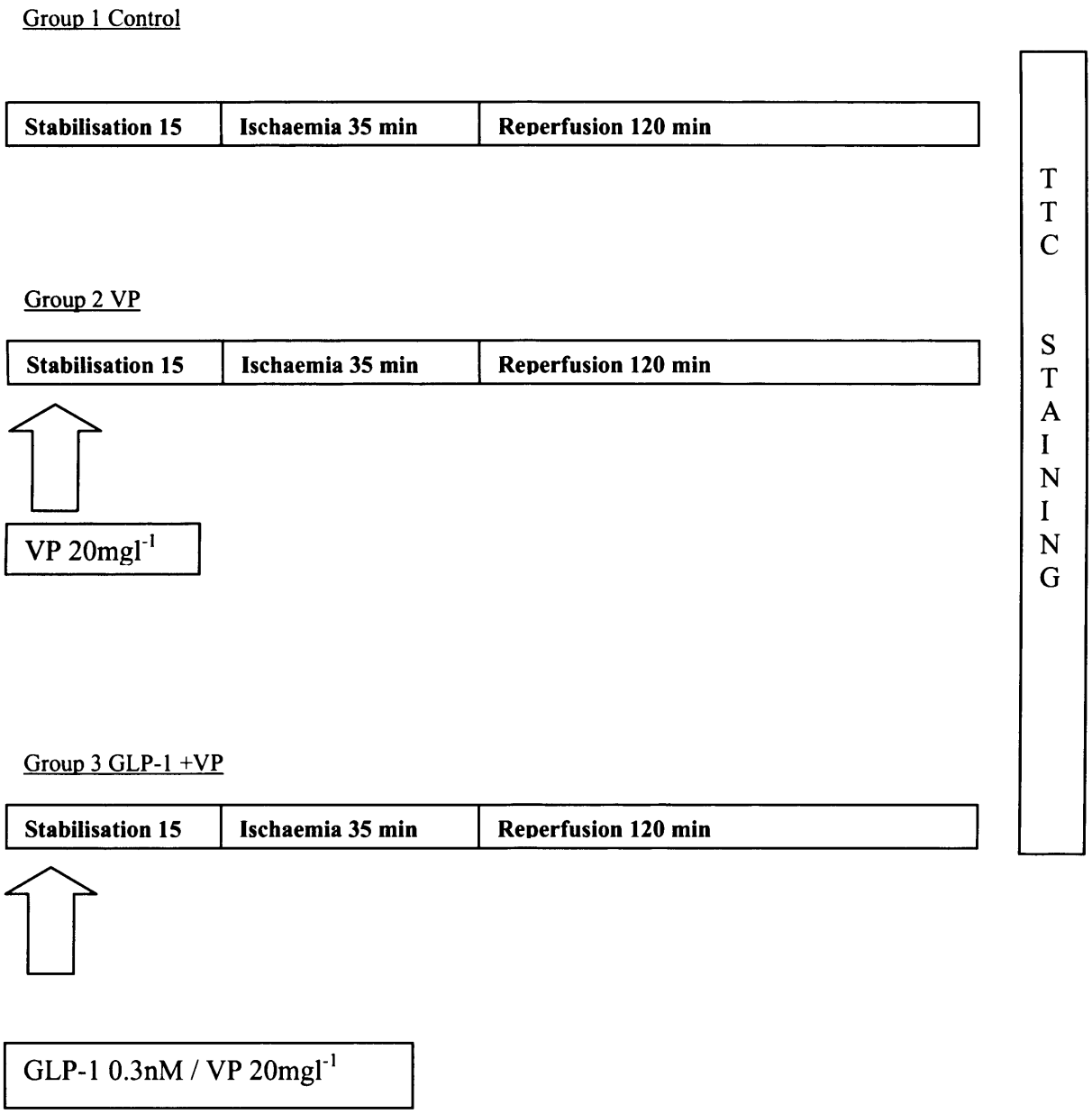
2.4.9 Perfusion protocol

2.4.9.1 In Vitro

Animals were randomly assigned to one of the followings groups: 1) Control group in which hearts were stabilised for 15-20 minutes and then subjected to 35 minutes of regional ischaemia, followed by 120 minutes of reperfusion, and 2) treatment groups, which received drugs added to buffer starting during stabilisation and continued throughout the experiment until termination of the experiment. Fig 2.9 Our initial in vitro treatment experiments were performed with GLP-1 at a concentration of 0.3 nM in combination with VP at the concentration 20mg l^{-1} of buffer, as an inhibitor of DPPIV. This was in essence to repeat the in vivo experiments in an in vitro setting to explore the effects of GLP-1 in isolation and in the absence of other circulating factors such as insulin. Further experiments were then performed as detailed in chapter 5 and 6 to explore the mechanism of the action of GLP-1 in myocardial ischaemic-reperfusion injury.

Figure 2.9 Schematic of in vitro experimental protocols

Hearts in all groups are stabilised for a minimum of 15-20 minutes. In Group 2 and Group 3 treatments (VP 20mgl^{-1} and GLP-1 0.3 nmol) were added to the perfusate during stabilisation and continued throughout the experiment.



2.5 Western blot analysis

Western blotting is useful in detecting a protein of interest that may be mixed with others (such as in a cell lysate) or verifying the identity of a protein on a gel. In general, a mixture of proteins is resolved using a denaturing acrylamide gel and then transferred from gel onto a nitrocellulose membrane. The proteins of interest are probed by incubating the membrane with specific antibodies. The membrane is next incubated with a secondary antibody which recognises any bound primary antibody, and allows eventual identification. This technique is limited by the success of the gel separation, the transfer step and specificity of the antibodies.

Chemicals used for Western blotting include sodium dodecyl sulphate (SDS), Trizma base (Tris), ammonium persulphate (APS), TEMED, bromophenol blue, Ponceau solution, reagents for suspension /sample buffers were purchased from Sigma (Poole, UK). Methanol and glycine were purchased from BDH Laboratories (Merck Eurplab, Dorset, UK) and Acrylamide (protogel) from National diagnostics (Hull, UK). The anti-BAD (rabbit polyclonal Ig G), phospho-Akt, phospho-eNOS, phospho-ERK 1/2, phospho-BAD (Ser 136), phospho-BAD (Ser 112) and horseradish peroxidase (HRP)-linked anti-rabbit Ig G (H&L) were purchased from Cell Signalling technology (Hertfordshire, UK). Nitrocellulose membrane and ECL plus detection reagents were obtained from Amersham Biosciences (Buckinghamshire, UK).

2.5.1 Tissue Preparation

All samples for Western blot analysis were collected from Langendorff perfused hearts rather than in vivo studies. All hearts were stabilised for a period of 15 minutes prior to treatment. From the literature it is apparent that GLP-1 causes a very brief enhancement in the phosphorylation and hence activation of anti-apoptotic factors and therefore a number of different time points were used for the collection of tissue samples in order to try and capture the point of phosphorylation as detailed in chapter

5. Control hearts were run for the same length of time. Experiments were performed with and without ischaemia. Ischaemia was achieved by cessation of perfusion and was global rather then regional. Again careful attention was paid to temperature control during the preparation.
At the termination of the Langendorff period, perfusion was stopped and the heart freeze-clamped with a pre-cooled Wollenberger clamp in liquid nitrogen after excision of both atria. Samples were stored at -80° C.

2.5.2 Protein Extraction

The tissue samples were extracted in a lysis buffer containing (in mM): 0.1 NaCl, 10 Tris (pH = 7.6), 1 EDTA, 2 Na pyrophosphate, 2 NaF, 2 β-glycerophosphate, 0.5 AEBSF and "Sigma Cocktail" protease inhibitor mixture. They were homogenized at 4 °C with IKA-Ultra-Turrax homogenizer (Staufen, Germany) and sonicated twice for 10 seconds, then centrifuged at 14,000 rpm for 5 min at 4 °C. The purified protein was then further diluted in sample buffer (Tris 100-pH 6.8), DTT 200 mM, SDS 2%, bromophenol blue 0.2% and glycerol 20%) and subsequently boiled for 10 min at 100° C. The supernatant was stored for protein quantification and the remainder of the sample for electrophoresis.

2.5.3 Protein quantification

Protein content was determined with BCA Protein Assay Reagent kit (Pierce, Rockford, USA). This assay is based on the formation of a purple coloured BCA-copper complex as a consequence of the reduction of ionised cooper, which is measured by optical densitometry at 562 nm.

A standard curve for known concentrations of bovine serum albumin (BSA) using optical density and a photospectrometer (Janway model 6405 UV/Vis, Dunmow, UK) was constructed. The protein in the samples were then quantified using this method,

and the protein content compared against the standard curve to provide an estimate of the protein concentration (μg/μl) to enable equal loading of the polyacrylamide gel.

2.5.4 Polyacrylamide gel electrophoresis

2.5.4.1 Gel preparation

12.5% acrylamide gels were made using 36ml deionised water, 16 ml acrylamide, 18 ml running gel base (1.5 M Tris and 0.4% SDS in deionised water, pH 8.8) 80 μl TEMED and 400 μl 10 % ammonium sulphate). The running gel is poured between two glass plates separated by spacers and sealed with Vaseline. A stacking gel made with 14 ml deionised water, 6 ml stacking gel base (0.5 M Tris, 0.4 % SDS in deionised water, pH 6.8), 4 ml 30 % acrylamide, 40 μl 8 % bromophenol blue, 48 μl TEMED and 240 μl 10 % APS was then introduced above the running gel and protein loading wells were constructed by placement of a plastic insert.

2.5.4.2 Electrophoresis

A total of 30 μg of protein for each sample was loaded into the gel. To provide control banding 10 μl of a molecular weight rainbow marker and 30 μg protein from human endothelial cell lysate were also loaded. The gel was run for 4 hours at 125 Volts constant.

2.5.5 Protein Transfer

Following electrophoresis, a similar size of Hybond ECL nitrocellulose membrane was apposed to the gel and both were sandwiched between sheets of filter paper, carefully displacing any air bubbles that may have been introduced. A transfer tank containing transfer buffer, - 200 ml methanol, 700 ml deionised water and 100 ml 10x transfer buffer (glycine, Tris and deionised water). Transfer proceeded at 140 mA constant overnight (12-14 hours). The next morning, after removal of the membranes,

equal protein loading was confirmed with Ponceau red staining (Sigma Chemicals Poole, UK) of the membranes.

2.5.6 Immunoblotting

The membranes were then washed for 15 minutes (3 x 5 minute cycles) with a washing buffer (50 ml 10x Tris-buffered saline (TBS)., 450 ml deionised water and 0.5 ml Tween-20) To prepare 1 litre of 10x TBS:24.2g Tris base, 80g NaCl: Adjust pH to 7.6 with HCl. Then incubate on a rocking platform in blocking buffer (washing buffer and 5 % non-fat milk) for 3 hours.

2.5.7 Primary antibody

The membranes were subsequently, washed in washing buffer and one incubated in primary antibody in a concentration of 1:1000 for 2 hours.

2.5.8 Secondary antibody

A secondary antibody was prepared with an-anti rabbit secondary antibody (1:2500). Before application of the secondary antibody for 1 hour, the primary antibody was washed off with washing buffer. Proteins were detected by using enhanced chemiluminescence ECL Western blotting reagent (Amersham Biosciences, Amersham, UK) and bands were visualised by autoradiography onto Kodak film.

2.5.9 Quantification

Autoradiography films and Ponceau stained membranes were scanned on to a flat-bed picture scanner and the digital image saved to disc. Images may be calibrated against densitometer values if one of the lanes has been so measured. Results are expressed as

a percentage of wild-type control expression (± SEM) and corrected for Ponceau red (Sigma) determined protein loading.

2.6 Statistical analysis

Statistical analysis was performed using Apple Macintosh software package StatView Version 4.5. Data was expressed as means ± SEM. One way analysis of variance between group means was performed using an ANOVA factorial method.

Chapter 3 Glucagon Like Peptide-1 In Vivo

3.1 Introduction

The aim of this thesis was to explore the actions of GLP-1 on the myocardium and in particular to identify any affect that GLP-1 has on ischaemic-reperfusion injury. Our initial hypothesis, that GLP-1 may activate known myocardial preservation pathways such as the reperfusion injury salvage kinase or RISK pathway (Hausenloy et al. 2004) was constructed around its potent action as an incretin (a substance that leads to increased insulin secretion or production). Insulin has previously been shown to be able to protect the ischaemic myocardium both experimentally (Jonassen et al. 2001) and in the clinical setting. (Diaz et al.1998). Insulin has also been put forward as an anti-inflammatory and antiatherosclerotic hormone (Dandona et al. 2003). To test the hypothesis that GLP-1 may protect the myocardium against ischaemic-reperfusion injury through its incretin action, we needed to use a whole body model of myocardial ischaemic reperfusion injury with an intact pancreatic insulin secreting apparatus on which GLP-1 could act as an incretin. The role of GLP-1 in myocardial ischaemic-reperfusion injury was, therefore, studied initially in the in vivo rat model. This whole animal model allowed us to evaluate the actions of GLP-1 on myocardial ischaemic-reperfusion injury with myocardial infarction as an end point but to also study the haemodynamic cardiovascular physiological effects of GLP-1 in a well described and accepted model.

The whole animal model also gave us the opportunity to measure circulating levels of insulin and dipeptidyl peptidase IV (DPPIV), the enzyme involved in the degradation of GLP-1 as well as products of GLP-1 degradation. GLP-1 as described in chapter 1, is cleaved from a precursor peptide sequence. The fragments of GLP-1 degradation may have a metabolic action such as the inactive or antagonistic GLP-1 (9–36) amide (Pauly et al. 1996). Therefore, the ability to measure the levels of insulin, intact GLP-1, DDPIV levels and GLP-1 fragments whilst determining the cardiovascular actions of GLP-1 in terms of ischaemic-reperfusion injury which the whole animal model allows had distinct advantages as an initial tool.

A number of previous studies have looked at individual aspects of GLP-1 function in the cardiovascular setting. From previous isolated myocardial cell studies (Vila Petroff et al. 2001) GLP-1 has been shown to have actions in the cardiac setting causing increases in c-AMP levels. This action of GLP-1 is thought to be compartmentalized, with the c-AMP produced as a result of GLP-1 treatment being locked in particular micro domains with a consequent restriction of the c-AMPs' actions. There have been some studies have shown that GLP-1 has haemodynamic effects, whilst others have failed to demonstrate any effects on blood pressure or heart rate. In addition a possible central role in the actions of GLP-1 on blood pressure and heart rate has also been demonstrated. These experiments were devised to try and characterise the actions of GLP-1 on myocardial infarction as the primary end point. Secondary end points included the metabolic and haemodynamic effects of GLP-1.

3.2 Materials and Methods

Male adult Sprague-Dawley rats (350-450g) were used for these studies.

3.3 Anaesthesia

For a detailed explanation of the anaesthetic protocol used see chapter two section 2.3.1

3.4 Surgical Procedure

A tracheosotomy was performed using a longitudinal midline incision. The trachea was identified and exposed. An endotracheal tube placed under direct vision to allow

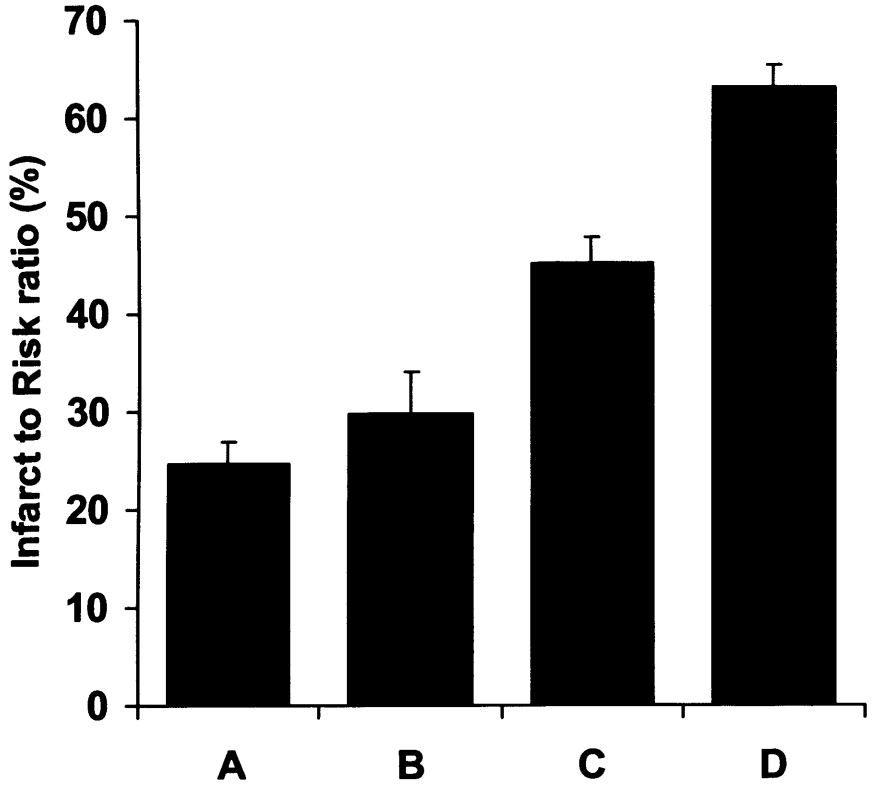
ventilation and secured in position with silk ties. For a detailed description see Section 2.3.2

3.5 Characterisation of the in vivo model

Initial experiments were performed to characterise and define the in vivo rat model of myocardial ischaemic reperfusion injury used in these studies. The in vivo model has been previously used and characterised extensively in the Hatter Institute, however, further experiments were performed to repeat the experience of previous investigators and to establish consistency on this investigators part using this experimental model. After stabilisation for approximately 20 minutes, control experiments were performed using initially 20 minutes of ischaemia. Further experiments were then performed using 25 minutes, 30 minutes and lastly 35 minutes of ischaemia to explore the effect of the duration of the ischaemia on the percentage of infarction in the area at risk. In each group the ischaemic period was followed by release of the snare around the coronary artery and reperfusion for two hours.

The results of these experiments are shown in figure 3.1

Figure 3.1 Myocardial Infarction as a percentage in the area at risk with different periods of ischaemia



Legend

In vivo myocardial infarct size expressed as a percentage of the risk zone. A= 20 minutes ischaemia , B= 25 minutes ischaemia , C= 30 minutes ischaemia, D= 35 minutes ischaemia *n=6 per group.*

Results are presented as means ± SEM

3.6 Ischaemic protocols

From the result of these experiments we found that 30 minutes of ischaemia produced the most reliable and reproducible degree of infarction whilst coupled with a good success rate in completing the reperfusion period of two hours.

3.7 Infarct size evaluation

On completion of the 120 minute reperfusion period a lethal dose of anaesthesia was given and the heart was rapidly harvested from the animal. The heart is quickly mounted onto a Langendorff apparatus with a cannula placed in the aorta and tied in place holding the heart. The suture around the left coronary artery used to induce ischaemia, is then tied off to effectively halt any flow down the coronary artery. Evans Blue dye was then retrogradely infused into the aortic cannula and hence into the coronary arteries to delineate the risk zone as described in chapter 2 (section 2.4.8). Hearts were rapidly frozen, then sectioned (2 mm) and incubated in 1% triphenyl-tetrazolium chloride (TTC) in phosphate buffer (pH 7.4, 37°C) for 12 minutes to stain viable tissue red as opposed to the non-stained white necrotic tissue. TTC staining is a widely recognised, validated method of staining for myocardial infarction (Ito et al. 1997; Schwarz et al. 2000). TTC in-fact stains non infracted viable tissue due its reduction by NADH and other cofactors producing a formazan pigment which is visible as a red stain. This is distinct from non stained white infracted tissue. The tissue was then fixed in 4% formalin for 24 hours.

The formalin fixed and stained heart slices were mounted onto a glass plate. A cover glass plate was then placed over the tissue. Two mm thick shims in the corners hold the glass plates away from each other. The risk area, infracted and non infracted areas were demarcated as shown in figure 2.7. Images of the sections were drawn by a

blinded operator on to an acetate sheet. Fig 2.8 The risk zone areas and infarct to risk ratios were determined by computerized planimetry. (Planimetry + version 1.0 for Windows

3.8 Treatment Groups

Rats were randomly assigned to either control or treated groups. Two groups were used for in vivo treatment experiments. Treated animals were divided into two groups; those given only Valine pyrrolidide and those given both GLP-1 and Valine pyrrolidide. VP, at the dose of 20 mg kg^{-1}, was administered as a subcutaneous injection 30 minutes prior to the commencement of anaesthesia and the subsequent in vivo experiment. GLP-1 was infused into the central venous line, placed during preparation of the model, at a dose of 4.8 ρmol$kg^{-1}min^{-1}$. Fig 3.2 Blood samples were taken prior to the commencement of the GLP-1 infusion to estimate blood glucose, insulin levels, DPPIV activity and native GLP-1 levels. Further blood samples were taken through out the experimental protocol to measure arterial blood gases and blood glucose at 30 minute intervals. A terminal blood sample was taken to measure insulin, GLP-1 and DPPIV activity at the end of the reperfusion period.

3.8.1 Group1 Control

These animals were anaesthetised, without pre-medication, but were given a subcutaneous injection of 0.5 ml of normal saline, the vehicle used for dissolving VP, in order to standardise the procedure. After a stabilisation period of 15-20 minutes they were subjected to 30 minutes of regional myocardial ischaemia followed by two hours of reperfusion prior to termination of the experiment and estimation of the degree of myocardial infarction using TTC staining.

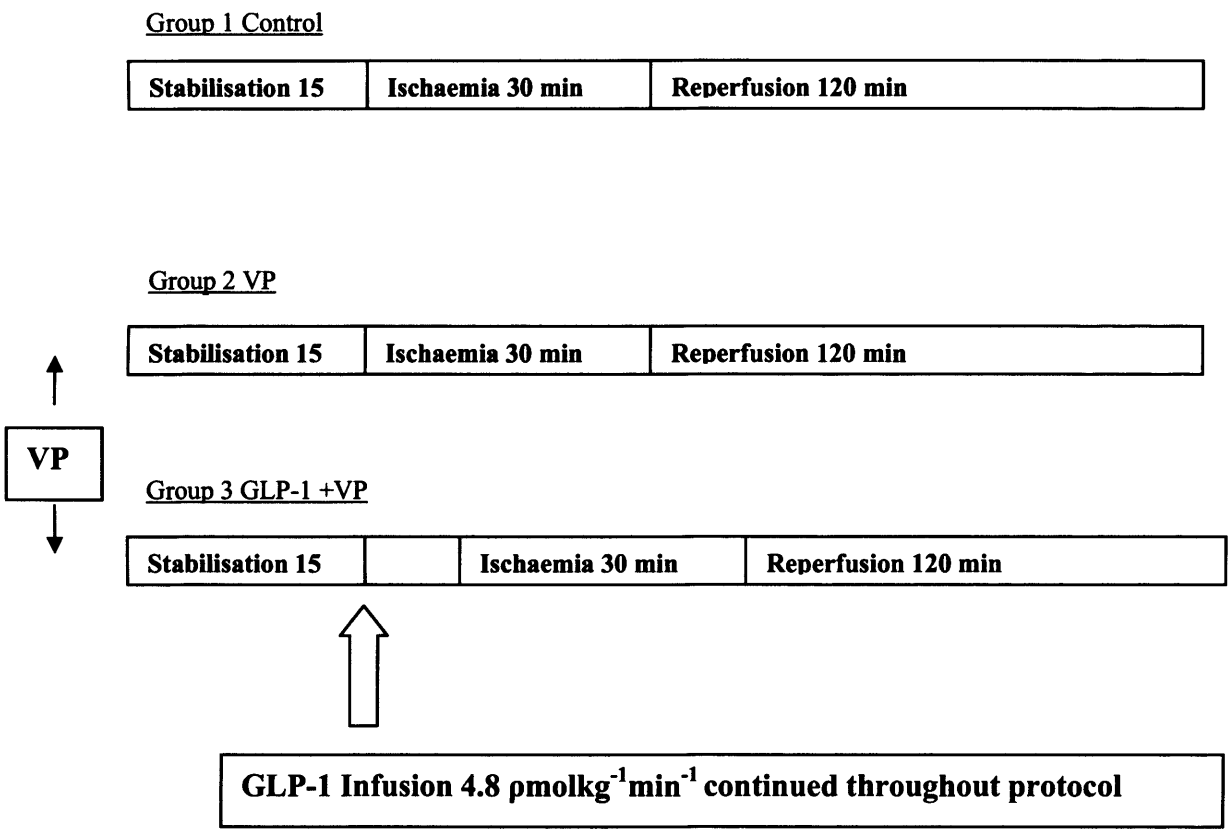
3.8.2 Group 2 VP

This group was given a subcutaneous injection of VP thirty minutes prior to the commencement of anaesthesia. They were stabilised for a period of 15-20 minutes and then subjected to 30 minutes of regional myocardial ischaemia followed by two hours of reperfusion prior to termination of the experiment and estimation of the degree of myocardial infarction.

3.8.3 Group 3 GLP-1 and VP

Again this group were given a subcutaneous injection of VP 30 minutes prior to the commencement of the experiment. After a stabilisation period of 15-20 minutes, the infusion of GLP-1, at 4.8 $\rho molkg^{-1}min^{-1}$, into the jugular vein was started and allowed to run for 10 minutes prior to the start of ischaemia, following which they underwent the same protocol as the groups mentioned above.

Figure 3.2 Schematic of in vivo experimental protocols



Hearts in all groups were stabilised for a minimum of 15-20 minutes. Those in the GLP-1 group were stabilised for a further 10 minutes following commencement of the GLP-1 infusion.

3.9 Statistical analysis

Statistical analysis was performed using Apple Macintosh iMac computers and the statistical package StatView Version 4.5. Data was expressed as means ± SEM. One way analysis of variance between group means was performed using an ANOVA factorial method. *P* values of less than 0.05 were taken to show statistical significance.

3.10 Results

3.10.1 Technical exclusions

A total of fifty eight animals were used in the in vivo experiments. Ten animals were excluded for technical reasons which included a high mortality rate during the initial procedures. The initial high mortality was ascribed to a learning curve prior to obtaining technical proficiency. The most common reason for exclusion was the development of ventricular fibrillation during ischaemia and early reperfusion which led to the exclusion of 7 experiments. This was almost inevitably a terminal event when occurring in reperfusion. A number of experiments did not complete the required two hours of reperfusion due to blood loss from the epicardial surface where the snare had caused local trauma. The number of experiments performed to characterise the model was twenty four, whilst the comparative study was performed with a further twenty four experiments.

3.10.2 Infarct size and risk zone

There were no significant differences between the groups for body weight, cardiac weight and risk volume Table 3.1.The infarct to risk ratio in the control and VP

treated groups was 44.3 ± 2.4% and 47.3 ± 4.3% respectively. GLP-1 + VP was seen to protect the myocardium with an infarct to risk ratio of 20.0 ± 2.8%,
from ischaemia-reperfusion injury in the in vivo open chest rat heart model, demonstrating a significant reduction in infarction compared to the VP or control groups, $P < 0.001$, *n=8 per group* respectively. Fig. 3.3

3.10.3 Heart rate and blood pressure

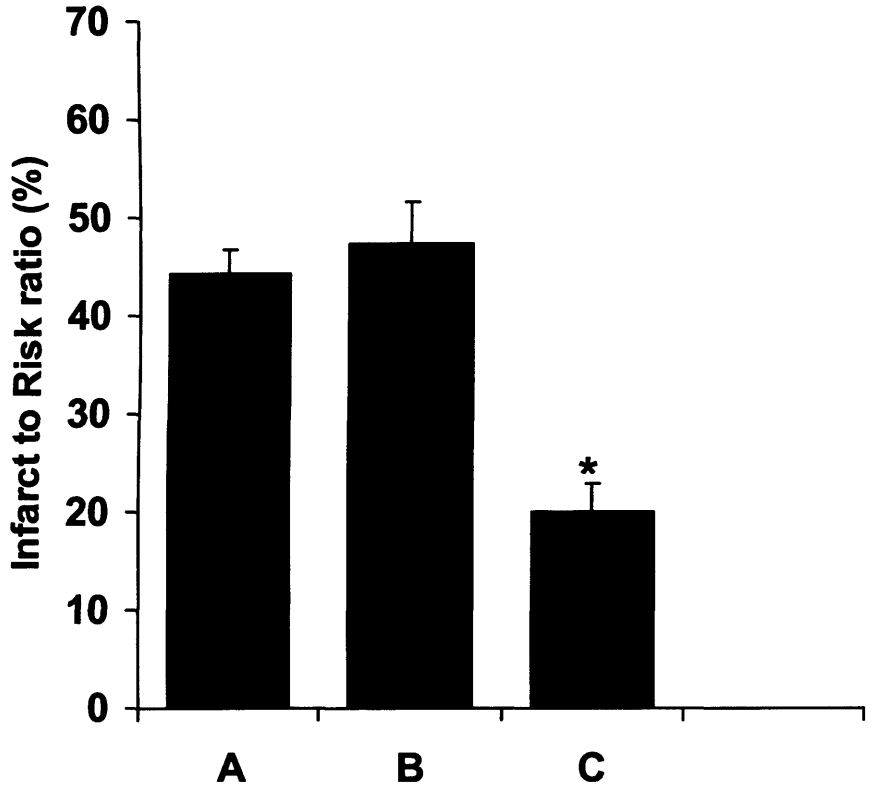
As expected the blood pressure in all the groups declined after the initiation of ischaemia. The initial reduction in blood pressure was characteristically between 30-50% of the stabilisation values. During the early phase of reperfusion the blood pressure would improve but this would be followed by a gradual reduction throughout ischaemia. The results for the three groups are represented in Table 3.2

Table 3.1 In vivo body weight, heart weight and risk volume

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	8	386±8	1.83±0.06	0.533±0.041
2. VP	8	400±5	1.88 ±0.02	0.568±0.038
3. GLP-1	8	390±8	1.86±0.20	0.562±0.046

Values are mean ± SEM. There was no significant statistical difference between the groups.

Figure 3.3 In vivo myocardial infarction



In vivo myocardial infarct size expressed as a percentage of the risk zone. A= Control *n*=8, B= Valine Pyrrolidide *n*=8, C=. Glucagon-like peptide-1 *n=8*

Results are presented as means ± SEM (* = $P<0.001$ vs. Control and VP)

Table 3.2 : In vivo Mean Arterial Blood Pressure (MAP) and Heart Rate (HR)

	Stabilisation	5 min Ischaemia	20 min Ischaemia	15 min Reperfusion
Control				
MAP	120 ± 4	80 ± 7	73 ± 4	76 ± 3
HR	400 ± 8	368 ± 4	364 ± 10	364 ± 16
n=8				
Valine Pyrrolidide				
MAP	127 ± 4	79 ± 6	77 ± 5	78 ± 7
HR	404 ± 8	360 ± 5	354 ± 9	358 ± 12
n=8				
GLP-1				
MAP	125 ± 3	74 ± 7	78 ± 4	75 ± 4
HR	406 ± 5	356 ± 9	362 ± 12	354 ± 14
n=8				

Values are means ± SEM.

3.10.4 Glucose

Glucose readings were taken at regular intervals using an arterial blood gas analyser (ABL-705, Radiometer Medical Instruments, Copenhagen, Denmark).The results shown in Table 3.3 demonstrate that there were no statistically significant differences between absolute blood glucose levels in any of the groups at the two time points used for sampling. Statistical significance was found, however if the reduction in blood glucose was compared between groups over the time course of the experiment with the GLP-1 treated group showing the greatest reduction in glucose levels compared to the other two groups. This is demonstrated in figure 3.4

3.10.5 Insulin levels

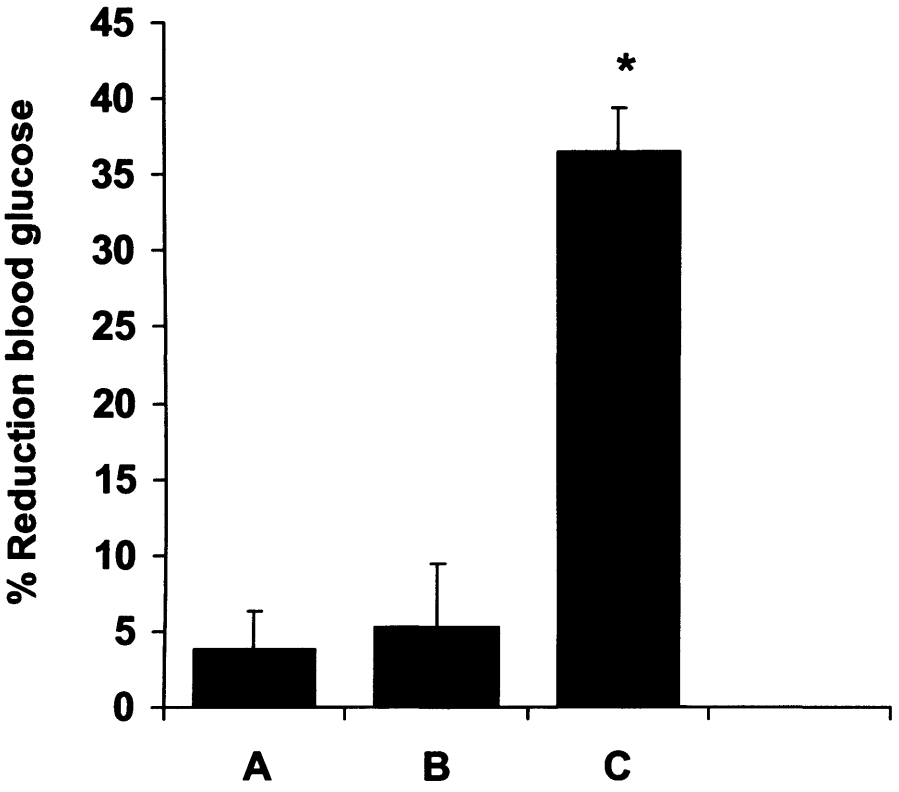
Table 3.4 demonstrates the different values obtained for insulin in all the in vivo experiments in which the insulin assay was performed. As can be seen from the table there was wide variability in the insulin levels, which is discussed further in the next section. There were no statistically significant differences between the three groups.

Table 3.3
In Vivo Plasma glucose levels

	Blood Sugar Baseline	Terminal Blood Sugar
A: control	7.100	8.200
A: control	11.400	8.900
A: control	9.200	7.400
A: control	7.600	6.300
A: control	8.000	9.100
A: control	9.900	9.300
B: Valine	9.400	10.000
B: Valine	16.200	5.800
B: Valine	10.000	8.400
B: Valine	10.300	6.900
B: Valine	9.600	6.900
B: Valine	10.500	7.400
B: Valine	10.700	7.700
B: Valine	11.300	6.900
C: GLP-1	15.600	9.300
C: GLP-1	15.800	10.100
C: GLP-1	10.700	8.800
C: GLP-1	11.800	7.900
C: GLP-1	9.300	5.400
C: GLP-1	8.000	6.800
C: GLP-1	9.900	5.000
C: GLP-1	7.400	3.700

Values are expresses as mmol/l

Figure 3.4. In vivo reduction in blood glucose



In vivo terminal Blood glucose reduction expressed as a percentage. A= Control n=6, B= Valine Pyrrolidide n=8, C=. Glucagon-like peptide-1 n=8

Results are presented as means ± SEM (* = P<0.05 vs. Control and VP)

Table 3.4
In Vivo Plasma insulin levels

	Baseline Insulin	Terminal Insulin
A: control	234	83
A: control	416	260
A: control	223	83
A: control	315	7194
A: control	132	251
A: control	619	275
B: Valine	319	191
B: Valine	428	400
B: Valine	433	230
B: Valine	405	962
B: Valine	3743	608
C: GLP-1	412	2331
C: GLP-1	262	250
C: GLP-1	427	121
C: GLP-1	964	4999
C: GLP-1	413	576
C: GLP-1	250	263
C: GLP-1	419	209
C: GLP-1	1099	398

Values are expresses as pmol/l

3.11 Discussion

In this part of the study we demonstrated that GLP-1 provided significant protection against myocardial ischaemic-reperfusion injury in an in vivo rat model compared to control experiments. Further we demonstrated that in isolation VP failed to have any effect on the primary outcome measure of myocardial infarction. Interestingly our data revealed no change in haemodynamic parameters between GLP-1 treated and control experiments.

The major end point of the in vivo study was myocardial infarction in the risk zone as measured using TTC staining. In this regard, there was no statistically significant difference between the control group and those given just VP, suggesting that VP has no effect on myocardial ischaemic-reperfusion injury in this setting. Importantly, the GLP-1 treated group, which also received VP to prevent rapid enzymatic breakdown of GLP-1 by DPPIV, showed a statistically significant reduction in myocardial infarction as compared to both the control and VP groups. Fig 3.3

Our original hypothesis was that GLP-1, as a potent incretin, could induce myocardial protection against ischaemic-reperfusion injury by producing increased plasma insulin levels. Insulin has been shown to be protective in an in vivo rat model of myocardial injury (Jonassen et al. 2000). Although GLP-1 was strongly protective against ischaemic-reperfusion injury this appears to be unrelated to the serum insulin levels in our experiments. As can be seen in table 3.4 insulin levels in all the in vivo experiments showed wide variability not only between experiments but also within each individual experiment with values for measured insulin ranging from 83 ρmol/l to 7194 ρmol/l. This may be due to a number of reasons. Handling and induction and maintenance of anaesthesia both induce a stress response, which is variable between each experiment. Stress will lead to sympathetic activity, which will alter insulin homeostasis. Therefore, our experimental model may be unstable in terms of the insulin levels due to a variable stress response. Apart from the model-induced variability the method of insulin assay may be an additional cause of variability. The

blood samples were taken fresh and spun at 4°C 6,000 rpm for 10 minutes with a buffer solution. The resulting plasma was then stored at -20° C. The samples were sent by courier on dry ice to Novo Nordisk, Bagsvaerd, Denmark from The Hatter Institute, London to be batch processed. It is possible that the samples were defrosted during transit leading to degradation of insulin, altering our results.

Blood glucose levels were measured at regular intervals throughout the experimental protocol and showed no statistically significant difference between the groups at any of the time points used for measurements. However, nearly all experiments showed a reduction in blood glucose when comparing the starting blood glucose level to the terminal blood glucose level. An analysis of the reduction in blood glucose levels showed statistical significance in the GLP-1 treated group as compared to both the control and VP group Figure 3.3. VP inhibits the breakdown of GLP-1 by DPPIV, prolonging the bioavailability and half-life of circulating, intact and hence active GLP-1. However, again the VP treated group as with the infarct data showed no difference from the control experiments in regard to glucose levels. The consistency in the blood glucose levels is at odds with our insulin data and would support the suggestion that the insulin sample collection method or assay were the source of variability rather than the model itself.

Although a rise in insulin and decrease in glucose is possible it must be remembered that these studies were undertaken in non diabetic animals which although not fasted, were not post prandial, suggesting any GLP-1 mediated stimulation of insulin release is likely to be small, because the insulinotropic effects of GLP-1 are glucose dependent.

GLP-1 receptors have been identified in the myocardium. Isolated cardiomyocytes studies have demonstrated an increase in c-AMP levels with no chronotropic or lusitropic effects (Vila Petroff et al, 2001). Our in vivo study demonstrated that GLP-1, at a dose of 4.8 $\rho molkg^{-1}min^{-1}$, has no haemodynamic effects Table 3.2.

GLP-1 has been shown to increase blood pressure and heart rate in rats (Barragan et al. 1994). This difference may be due to the different dose of GLP-1 used and method

of delivery. A study using a similar dose of GLP-1 in a porcine model failed to note any change in blood pressure or heart rate (Deacon et al. 1996). In our in vivo experiments the degree of anaesthesia required to produce a stable open-chest experimental model, which causes myocardial depression, must also be taken into account. GLP-1 has been shown to have effects on the central control of blood pressure and pulse (Barragan et al. 1999).

In summary GLP-1 in vivo produced significant reduction in myocardial ischaemic-reperfusion injury as measured by histological infarct staining, without any effect on heart rate or blood pressure.

Chapter 4 Glucagon-Like Peptide-1 In Vitro

4.1 Introduction

The in vivo experiments described in chapter 3 show that GLP-1 administration in a open-chest rat model is associated with a significant protection against myocardial ischaemic-reperfusion injury. This protection may be attributed to GLP-1's potent incretin actions, such as an insulinotropic effect on the pancreas and possibly an increased sensitivity to insulin in the tissues. Insulin has been shown to be protective in an experimental model of ischaemic-reperfusion injury in an isolated rat heart (Jonassen et al. 2001). In the clinical setting there remains intense debate, with some studies demonstrating protection against ischaemia (Krljanac et al. 2005) while others failing to show any benefit as part of GIK infusions against myocardial injury (Mehta at al. 2005). The initial hypothesis driving these clinical studies was not based on the anti-apoptotic actions or activation of the RISK pathway but on the presumed metabolic effects of insulin as part of a metabolic cocktail. More recent evidence has shifted the focus away from the metabolic cocktail to the individual actions of insulin as a pro-survival agent activating the RISK pathway (Sack et al. 2003) with a possible reduction in apoptosis (Zhang et al 2005).

An alternative hypothesis, for our study, was that the protection seen against myocardial ischaemia-reperfusion injury may have been a direct effect of GLP-1 on the myocardium, possibly transduced by the recently identified GLP-1 receptor in the heart rather than its actions as an incretin. To isolate the mode of action of GLP-1 it was necessary to perform experiments in a setting where insulin could, in effect, be excluded from the model. To do this it is necessary to establish an in vitro model of myocardial ischaemia where the myocardium is in effect isolated from circulating peptide mediators. This would allow GLP-1 to be used in the absence of circulating insulin, thereby clarifying whether insulin was required in the tissues for GLP-1 to be protective in myocardial ischaemic-reperfusion injury. In vitro experiments using isolated perfused (Langendorff) hearts, also allow the use of a controlled glucose level. The concentration of glucose in the perfusate used in the experiment is

predetermined and therefore any effect of fluctuating glucose seen in the in vivo setting is avoided.

A great deal of experience in the use of isolated perfused (Langendorff) hearts has been accumulated at the Hatter Institute where these experiments were performed. The in vitro model of myocardial ischaemic-reperfusion injury has been thoroughly validated and used extensively to explore the mechanisms involved in the reperfusion injury salvage kinase (RISK) pathway.

4.2 Materials and Methods

Male adult Sprague-Dawley rats (350-450g) were used for these studies. See chapter 2, Section 2.4 for a detailed description of methods and materials used.

GLP-1

GLP-1 0.3nmol was added to the buffer of treated hearts.

VP

VP ($20mgl^{-1}$) is stored as an anhydrous powder and added to buffer to provide a concentration of 20 mgl^{-1}.

4.2.1 In Vitro Infarct Model in Rat Heart

The isolated crystalloid-perfused rat heart model of ischaemic-reperfusion was employed for this study to simulate the pathological condition of myocardial ischaemic-reperfusion injury for several reasons: (1) it is a highly reproducible robust preparation which permits the measurement of: contractile function, morphological markers (such as infarct size) and electrophysiological markers (such as arrhythmias); (2) it allows the study of large numbers in a relatively short period of time and provides reproducible data; (3) it allows the examination of various pharmacological agents in the setting of ischaemic reperfusion injury, (4) it removes the confounding effects of the systemic circulation (neuro-hormonal factors) and therefore allows one to examine the direct effect of interventions on the myocardium (Sutherland et al. 2000).

The disadvantages of this preparation include: (1) the heart being isolated from blood-borne factors and neuronal innervation; (2) the preparation is constantly deteriorating (Sutherland et al. 2000).

4.3 Parameters Recorded

4.3.1 Temperature

Temperature was continuously monitored by a thermo-probe inserted into right ventricle by an incision made at the base of the pulmonary artery, and was maintained between 37.0°C and 37.5°C. Temperature was controlled by the use of a thermostatically-controlled water-jacketed system in which all glass reservoirs and the heart perfusion chamber are surrounded by rapidly flowing warmed water at 37.0-37.5°C, using a water circulator.

4.3.2. Rate Pressure Product and Coronary Flow

A latex, fluid-filled, iso-volumic balloon was introduced into the left ventricle via the left atrium, through a hole made by removing the left atrial appendage, to monitor contractile function of the left ventricle (figure 4.1). The balloon was inflated with distilled water to give a left ventricular end diastolic pressure of 4-8 mmHg, which was displayed on the PowerLab monitor. Left ventricular developed systolic and diastolic pressures, heart rate and coronary flow were noted at regular intervals.

4.3.3 Infarct Size Evaluation

The risk zone and percentage infarction of the area at risk were measured as described in detail in chapter 2, section 2.4.8

4.3.4 Biochemical Buffer Analysis

Samples of the buffer were tested at regular intervals to check the pH, oxygen and carbon dioxide content as well as the glucose levels within the perfusate.

4.3 Ischaemic protocols

Our primary goal was to repeat the experiments performed in vivo in an in vitro model, so in essence, the same protocols as used in the in vivo study were repeated in the in vitro study. The major difference was that the ischaemic period was increased from thirty minutes as used in the in vivo to thirty-five minutes in the vitro study based on the experience already gained whilst characterization experiments were performed and from the vast experience of the Hatter Institute. This experience had suggested that thirty five minutes of ischaemia would produce the most effective ischaemic insult whilst still allowing some myocardium to be salvageable at reperfusion. The reperfusion period of one hundred and twenty minutes was maintained for both in vitro and in vivo protocols. . At the end of reperfusion the hearts were subjected to Evans Blue demarcation of prior to TTC staining of the infracted tissue.

4.4 Statistical analysis

Statistical analysis was performed using Apple Macintosh iMac computers and the statistical package StatView Version 4.5. Data was expressed as means ± SEM. One way analysis of variance between group means was performed using an ANOVA factorial method.

4.5 Results

4.5.1 Technical exclusions

A total of thirty seven animals were used in the in vitro experiments described in this chapter. Five animals were excluded for the following technical reasons, including poor left ventricular developed pressure during stabilisation, aortic wall damage at the time of cannulation and persistent or recurrent ventricular fibrillation during ischaemia or reperfusion. Arrhythmias were a common occurrence during early reperfusion and were usually self-limiting or easily terminated.

4.5.2 Infarct size and risk size

There were no significant differences between the groups in regard to cardiac weight and risk volume (Table 4.1). The infarct to risk ratio in the control and VP treated groups was 60.5 ± 3 % and 52.5 ± 4 % respectively. The GLP-1 and VP treated group achieved an infarct to risk ratio of 29.3 ± 3 % which was statistically significant $p<0.0001$ in comparison to the control and VP groups. This is demonstrated graphically in figure 4.1

4.5.3 Coronary flow and LV function

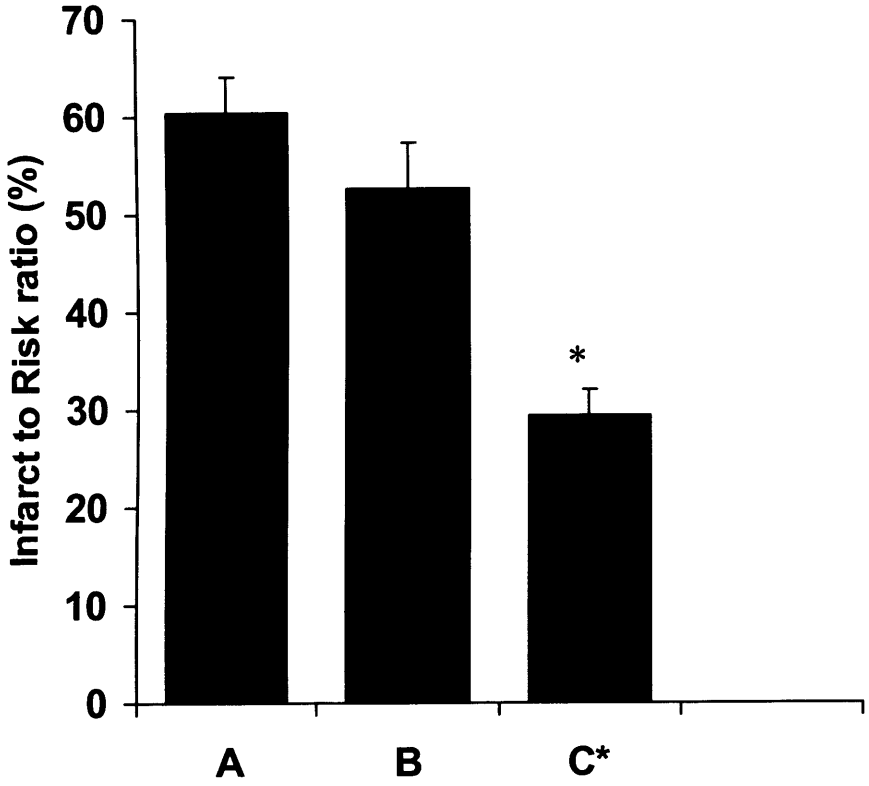
There were no statistically significant differences in heart rate or developed left ventricular pressure between the groups during stabilisation, ischaemia or reperfusion. The rate pressure product (RPP) for the groups is demonstrated in Table 4.2. Ischaemia produced a fall in both the coronary flow and RPP when compared to stabilisation. At reperfusion there were improvements in both parameters to values below that obtained during stabilisation followed by a time related decline in both parameters until termination of the experiment.

Table 4.1 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
1. Control	9	389±19	1.90±0.06	0.541±0.017
2 VP.	10	387±20	1.98±0.07	0.555±0.037
3. GLP-1 + VP	13	380±12	1.89±0.11	0.530±0.029

Values are means ± SEM. There was no significant statistical difference between the groups.

Figure 4.1 In vitro myocardial infarct size expressed as a percentage of the risk zone



A= Control *n*= 9, B= Valine Pyrrolidide *n=10*, C=. Glucagon-like peptide-1 *n=13.*

Results are presented as means ± SEM (* = *P*<0.001 vs. Control and VP)

Table 4.2 Rate pressure product analysis of groups throughout the ischaemia-reperfusion protocol

Group	Stabilisation	Ischaemia		Reperfusion	
		5min	30min	15min	120 min
Control	27 533	12 340	19 037	21 528	16 320
N=9	± 3356	± 1763	± 1549	± 2129	± 2329
Valine Pyrrolidide	26 450	13 967	18 655	20 365	16 589
N=10	± 3674	± 2278	± 1233	± 1945	± 1429
GLP-1	28 469	12 424	18 005	19 246	15 823
n=13	± 3225	± 2362	± 2046	± 1667	± 1129

Values are means ± SEM. The rate pressure product analysis for the in vitro groups is mmHg · $beat^{-1}$ · min.

4.6 Discussion

This part of the study confirmed the direct protective effect of GLP-1, independent of its incretin actions, against myocardial ischaemic-reperfusion injury with statistically significant reduction in myocardial infarction in the GLP-1 treated group in comparison to the control group.

At the time these experiments were performed, no previously published studies had looked at the actions of GLP-1 on the isolated perfused heart. There had been a previously published study looking at the actions of GLP-1 on isolated cardiomyocytes where GLP-1 was used at the concentration of 10nmol/L (Vila Petroff et al. 2001) and other cellular studies had used GLP-1 at 10nmol/L (Hui et al. 2003). We chose to use an initial dose of 0.3nmol GLP-1 based very broadly on the concentration of GLP-1 used in the in vivo experiments. To achieve this we dissolved 1μg of GLP-1 in each litre of buffer. In vivo we had given 4.8 pmol/kg/min as infusion intravenously. As we achieved significant protection with this first attempted dose we chose to move forward with the study to investigate the mechanisms of the protective effect seen. If time had allowed then a full dose response curve would have been performed, however, with the time limits of this was deferred.

The aim of this part of the study was to confirm the protective effect of GLP-1 against myocardial ischaemic-reperfusion injury in the isolated perfused heart. This would establish that the protective effect of GLP-1 on the myocardium must be mediated in the absence of circulating insulin. Our isolated perfused heart model of myocardial ischaemic-reperfusion injury is devoid of circulating blood borne factors and by allowing the model to stabilise for a period of 15 to 20 minutes any residual insulin in the myocardial tissue would be washed out and no longer biologically active.

These in vitro experiments confirmed that GLP-1 was able to provide protection against myocardial ischaemic-reperfusion injury in the absence of circulating insulin and with a glucose that was held constant. The glucose in our perfusate was found to remain constant at 10.8 mmol/l.

The in vitro findings were similar to the in vivo results reducing infarct to risk ratio by approximately 32% compared to control and 22% compared to the VP treated group Figure 4.6. The infarct to risk ratio in the control and VP treated groups was 60.5 ± 3

% and 52.5 ± 4 % respectively. The GLP-1 and VP treated group achieved an infarct to risk ratio of 29.3 ± 3 % which was statistically significant $p<0.0001$ in comparison to the control and VP groups.

Interestingly GLP-1 appears to have no effect on myocardial function. There were no significant changes in the heart rate, developed pressure and hence the RPP. GLP-1 was quite obviously protecting the myocardium from ischaemic injury when given prior to the index ischaemia without affecting cardiac contractility or inotropy. This was at odds with the findings of some of the research previously published looking at the haemodynamic effects of GLP-1 as discussed in chapter 3, section 3.10. The isolated perfused heart model produced results on myocardial function that were in keeping with our findings in the in vivo model. VP in isolation again failed to show any changes in myocardial function in comparison to control.

Some researchers had demonstrated a vasodilator action for GLP-1 in vascular endothelium. A recently published clinical paper demonstrated a significant improvement in endothelial dysfunction in diabetic patients but failed to show any endothelial action in normal subjects suggesting that this action may be mediated by an insulin sensitisation, that is increasing the sensitivity of the tissues to circulating insulin, as well as GLP-1 incretin actions. (Nystrom et al. 2004). In experimental studies a vasodilator action has quite clearly been shown in the pulmonary vasculature (Golpon at al. 2001). However, we failed to show any significant change in coronary flow which should reflect changes in endothelial function. Other parameters measured showed no statistical difference between the three groups again confirming our findings from the in vivo setup with GLP-1 having no significant effect on RPP, the in vitro surrogate for haemodynamic function, or coronary flow.

In summary, GLP-1 in vitro provides significant protection against myocardial ischaemia-reperfusion injury as measured by the degree of myocardial infarction. This is in the absence of circulating insulin, when given prior to the index ischaemia. This would support the hypothesis that GLP-1 was acting directly on GLP-1 receptors located on myocardial cells leading to activation of the RISK pathways.

Chapter 5 Mechanisms of Cardioprotection in Vitro

5.1 Introduction

In the previous chapters we have demonstrated that GLP-1 was able to induce myocardial protection against ischaemic-reperfusion injury both in vivo and in vitro when given prior to the onset of ischaemia. In both models GLP-1 was continued throughout ischaemia and reperfusion until the termination of the experiments. In addition, the preceding in vitro studies described in chapter 4 showed that GLP-1 produced significant protection against myocardial ischaemia-reperfusion injury in the absence of circulating insulin with the knowledge that any insulin present in the tissues would be washed out during the 15 minute period of stabilisation used in the model. We now moved from establishing whether GLP-1 can provide protection, to the mechanisms underlying this protection. We investigated the possible role of GLP-1 receptor activation as well as the prosurvival kinases PKA, PI3K/AKT, ERK 1/2 MAPK and the possible downstream targets eNOS and BAD.

5.1.2 GLP-1 Receptor inhibition

The aim of this part of the study was to determine whether GLP-1 induced myocardial protection is transduced by the GLP-1 cell surface receptor.

The existence of a myocardial GLP-1 receptor (Bullock et al. 1996) provided the first focus for further investigation. The GLP-1 receptor has been investigated thoroughly by other groups and specific agonists and antagonists identified. Exendin (9-39) is a potent GLP-1 receptor antagonist whilst exendin-4 (Macdonald et al. 2003) is a receptor agonist. Exendin (9-39) has also been described as an antagonist of the putative exendin receptor. This peptide blocks the stimulatory action of GLP-1 and of exendin-4, a GLP-1 receptor agonist, on cAMP production in the pancreatic acini. We investigated the role of the myocardial GLP-1 receptor in transducing the protective effects of GLP-1 on myocardial ischaemic-reperfusion injury. By antagonising the

GLP-1 receptor with exendin (9-39) if GLP-1 still provided protection against myocardial ischaemic-reperfusion injury we would be able to surmise that the action of GLP-1 in attenuating ischaemic-reperfusion injury was independent of the specific GLP-1 receptor.

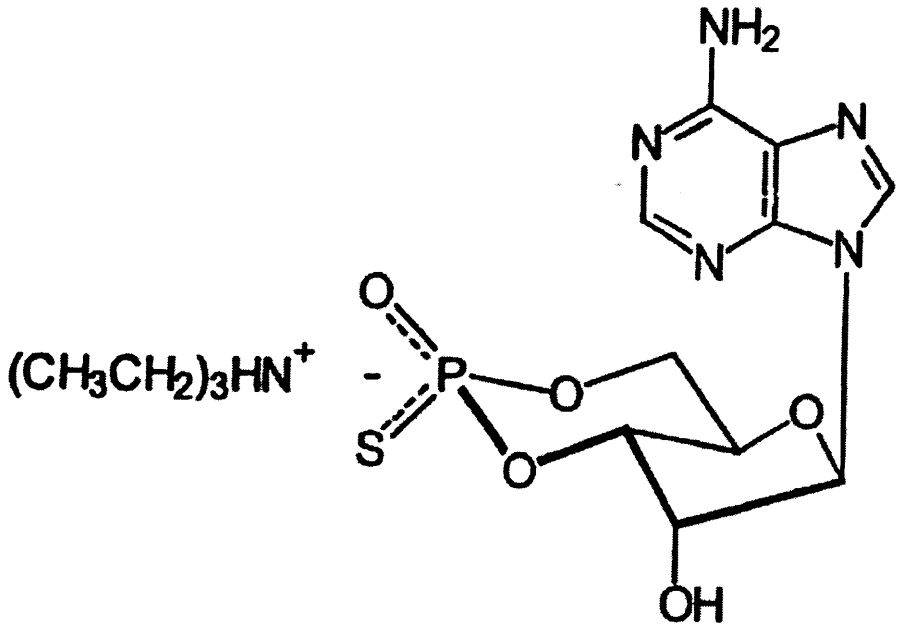
To isolate whether GLP-1 induced myocardial protection was transduced through the cell surface GLP-1 R we used the specific GLP-1 R, antagonist Exendin (9-39). The lizard Heloderma species, including H. horridum and H. suspectum are native to several American states and are poisonous. Lizard venom contains a number of highly bioactive peptides including the peptides exendin-3 and exendin-4. These peptides were named exendin (Eng et al. 1992 and Raufman et al. 1992) in that they were isolated from an exocrine gland and were subsequently shown to have endocrine actions. Although exendin-4 was originally found to stimulate amylase secretion from pancreatic acinar cells, subsequent experiments demonstrated that exendin-4 was a potent agonist for the mammalian GLP-1 receptor. Exendin-4 is much more potent than native GLP-1, largely due to its resistance to DPP-IV mediated inactivation. In contrast to GLP-1 which contains an alanine at position 2, exendin-4 has a position 2 glycine, hence it is not a substrate for DPP-IV and has a much longer half life in vivo. A truncated version of exendin-4, exendin (9-39) binds to but does not activate the GLP-1 receptor, and functions as a GLP-1 receptor antagonist.

5.1.3 GLP 1 and PKA inhibition

GLP-1 acts in several tissues via the GLP-1 R leading to intracellular calcium influx and increased production of c-AMP which in turn leads to the c-AMP dependent activation of Protein Kinase A (PKA). This is thought to be one of the key signal cascades by which GLP-1 acts in cardiac tissues (Vila Petroff et al. 2001). On the other hand PKA has been demonstrated to play a role in protecting the ischaemic reperfused myocardium (Inserte et al. 2004). Therefore, we thought it would be interesting to investigate the involvement of this cell signaling pathway in GLP-1 mediated myocardial protection by blocking the activation of PKA. To achieve this we used a cell permeable inhibitor of PKA, named Rp-cAMP (Adenosine 3',5'-cyclic

Monophosphorothioate, Rp-isomer, Triethylammonium salt) which is resistant to hydrolysis by phosphodiesterases which are responsible for the degradation and clearance of cAMP (Yokozaki et al. 1992 and Kaji et al. 1992). Rp-cAMP is a permeable cyclic adenosine monophosphate (cAMP) isomer which inhibits c-AMP dependent PKA.

Figure 5.1 Chemical structure of Rp-cAMP



5.1.4 GLP-1 and PI3Kinase Inhibition

Phosphatidyl inositol 3-OH kinase (PI3K) is central to the control of cell growth, proliferation and survival (Wymann et al. 2003). GLP-1 has been shown to activate PI3K in insulin secreting cells (Hui et al. 2003. PI3K is thought to be intimately involved in the transduction of anti apoptotic cell signals. Myocardial IPC has also been shown to involve activation of PI3K (Mocanu et al. 2002). We aimed to identify

whether GLP-1 mediated myocardial protection was blocked by inhibition of this known pro-survival pathway using the PI3K inhibitor, LY-294002 or 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one. LY-294002 is a reversible, cell-permeable, potent, and specific PI3K inhibitor (IC_{50} = 1.4 μM) that acts on the ATP-binding site of the enzyme. It also inhibits non-homologous DNA end-joining (NHEJ) in the 460 kDa phosphatidylinositol 3-like kinase DNA-PKc_s, which is the catalytic subunit of DNA-activated protein kinase. LY 294002 does not affect the activity of EGF receptor kinase, MAP kinase, PKC, PI 4-kinase, S6 kinase even at 50 μM.

5.1.5 GLP-1 and ERK 1/2 MAPK Inhibition

Mitogen activated protein kinases (MAPK) are thought to play a vital role in the cellular adaptation to various stressful stimuli (Bogoyevitch et al. 1996) and there is mounting evidence that MAPKs are activated following ischaemic-reperfusion injury (Knight et al. 1996). To inhibit the ERK 1/2 MAPK we used the potent and selective non competitive inhibitor of ERK 1/2, U0126 (Favata et al. 1998) or 1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene.

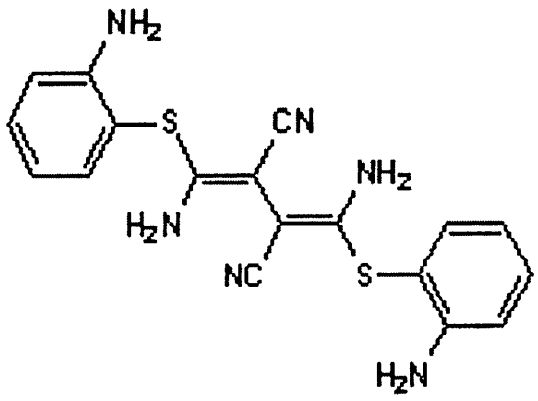


Figure 5.2 Molecular structure of U0126.

5.1.7 GLP-1 and p70s6Kinase Inhibition

PI3K activation has been shown to lead to the activation of downstream p70s6K which phosphorylates members of the initiation complex of protein translational machinery. The p70s6K pathway is thought to regulate translational protein synthesis and is central in mammalian cellular growth and cell survival (Harada et al. 2001). We inhibited this pathway using rapamycin, a specific and potent inhibitor of mTOR, the upstream activator of p70s6K.

Rapamycin (its name is derived from the native word for Easter Island, Rapi Nui) is a triene macrolide antibiotic, which demonstrates anti-fungal, anti-inflammatory, anti-tumour and immunosuppressive properties. Rapamycin has been shown to block T-cell activation and proliferation, as well as, the activation of p70s6K kinase and exhibits strong binding to FK-506 binding proteins. It also inhibits the activity of mTOR, (mammalian target of rapamycin) which functions in a signaling pathway to promote tumour growth. Rapamycin binds to a receptor protein (FKBP12) and the rapamycin/FKB12 complex then binds to mTOR and prevents interaction of mTOR with target proteins in this signaling pathway.

5.2 Materials and Methods

Male adult Sprague-Dawley rats (350-450g) were used for these studies. See chapter 2, Section 2.4.5 for a detailed description of methods and materials used. When necessary, reagents were dissolved in dimethyl sulphoxide DMSO, (Sigma-Aldrich). Final solutions were no greater than 0.01% DMSO a concentration which has previously been shown to have no effect on cardiac function or infarct size in this model (Mocanu et al. 2000) and control solutions contained the same were appropriate. Exendin (9-39) 3nmol/L from Bachem (Merseyside UK), LY294002 15μmol/L, U0126 10μmol/L and Rapamycin 0.5nmol/ L were obtained from Tocris (Bristol, UK), Rp-cAMP 1.5μmol/L from Calbiochem (Nottingham, UK),

5.2.1 In Vitro Infarct Model in Rat Heart

For a detailed description see chapter 2, section 2.4.8 and chapter 4 section 4.2.1 to 4.3. This model allowed us to explore the mechanisms of the GLP-1 protection against myocardial infarction, using specific agonists and antagonists.

5.2.2 Ischaemic protocols

All experiments were subject to the same period of index ischaemia that is 35 minutes of regional ischaemia as described in chapter 4 section 4.3, followed by 120 minutes of reperfusion.

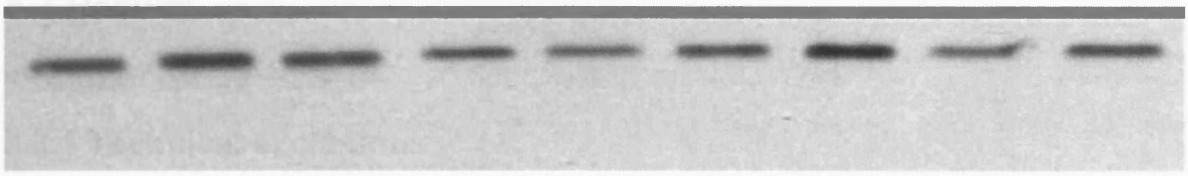
5.2.3 *Western Blotting*

For a detailed description of the materials and methods used to perform Western blots please see Chapter 2, Section 2.5

5.2.4 *Quantification of Protein Bands*

The developed films were scanned on a flat-bed picture/document scanner, and the digital image was assessed using the National Institutes of Health (NIH) Shareware program, NIH Image (version 1.63). The relative densitometry for individual protein bands was determined by the grey scale technique, using the supplied program 'Gel plotting macro'. The values were corrected if required for equal protein loading as determined by probing for β-actin.. Figure 5.3

Figure 5.3 Western Blot β Actin



Legend

Equal Protein loading was confirmed by blotting for β Actin. This is an example blot taken for βActin

5.2.5 *Western Blot Sample Collection*

The phosphorylation status of various proteins, known to be integral components of proven prosurvival pathways, were examined. The proteins examined included phospho–BAD (Ser 136), phospho-BAD (Ser 112), p70s6K(Thr389) and phospho-p70S6 kinase (Thr421/Ser424), Akt (Ser 473), eNOS and ERK 1/2. Ischaemic preconditioning was used as a positive control in a number of the blots.

Treated hearts were stabilised for 10 minutes followed by 5 min of drug administration with GLP-1 (0.3nmol) and VP (20mg/l). A further set of experiments were performed with hearts exposed to GLP-1 for 10 minutes. Control experiments were performed as well ischaemic preconditioning experiments as a positive control in a number of Western blots.

5.2.6 Statistical Analysis

Statistical analysis was performed using Apple Macintosh iMac computers and the statistical package StatView Version 4.5. Data was expressed as means ± SEM. One way analysis of variance between group means was performed using an ANOVA

factorial method. *P* values of less than 0.05 were taken to show statistical significance.

5.3 Results

5.3.1 Technical exclusions

A total of 82 animals were used in the in vitro experiments described in this chapter. Four experiments were excluded for technical reasons; including poor left ventricular developed pressure during stabilisation, aortic wall damage at the time of cannulation and persistent or recurrent ventricular fibrillation during ischaemia or reperfusion.

5.3.2 Risk Volume and Weight

There were no significant differences between the groups in regard to cardiac weight and risk volume (Table 5.1 results expressed as mean with SEM).

Table 5.1 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
Exendin(9-39) +GLP-1	6	389±19	1.90±0.06	0.411±0.017
Exendin Control	6	387±20	1.98±0.07	0.555±0.037
Rp-cAMP+GLP-1	6	380±12	1.89±0.11	0.490±0.029
Rp-cAMP Control	6	396±12	1.94±0.3	0.411±0.014
Control	6	406±18	1.98±0.5	0.4860±0.03
LY 294002+GLP-1	6	410±12	1.90±0.4	0.474±0.013
LY 294002 Control	6	404±12	1.92±0.3	0.515±0.015
Rapamycin+GLP-1	6	398±12	1.97±0.4	0.496±0.019
Rapamycin Control	6	402±12	1.93±0.2	0.479±0.016

All values are presented as means ± SEM

5.3.3 Infarct size and GLP-1 receptor inhibition

Exendin (9-39), a well known inhibitor of the GLP-1 receptor abolished the GLP-1 mediated myocardial protection (57.3 ± 3.8 % vs. 26.7 ± 2.7% P <0.001).There was no significant difference between Control and GLP-1 with Exendin(9-39).Figure 5.4

5.3.4 Infarct size and PKA inhibition

In our experiments (Fig.5.5) when the PKA inhibitor Rp-cAMP was administered in vitro concomitantly with GLP-1+VP and throughout the experiments, it abolished the protection seen with GLP-1 alone. (57.5 ± 5.0% vs. 26.7 ± 2.7% $P < 0.001$). There was no significant difference between Control and GLP-1 with PKA.

5.3.5 Infarct size and inhibitors of the pro-survival pathways

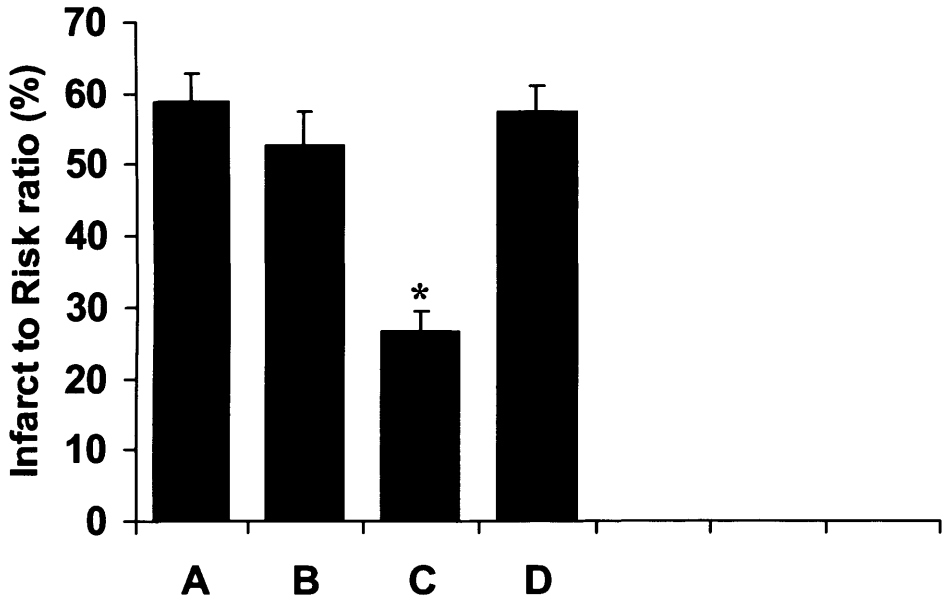
In our studies, the protection that we observed was also abolished by the PI3K inhibitor LY294002 (43.4 ± 3.9% vs. 26.7 ± 2.7% $P < 0.05$) and by the ERK 1/2 MAPK inhibitor U0126 (48.3 ± 8.6% % $P < 0.01$). The p70s6K inhibitor rapamycin also abolished protection (57.1 ± 4.9% vs. 26.7 ± 2.7% $P < 0.05$). Neither of these antagonists or inhibitors when given alone had any effect upon infarct size. Figure 5.5 and Figure 5.6 There was no significant difference between Control and GLP-1 with these inhibitors.

5.3.6 Coronary Flow and Rate Pressure Product

There were no statistically significant differences in heart rate or developed left ventricular pressure between the groups during stabilisation, ischaemia or reperfusion. The coronary flow rate (ml min^{-1}) and rate pressure product (RPP) is demonstrated in Figure 5.7 Ischaemia produced a fall in both the coronary flow and RPP when compared to stabilisation. At reperfusion there were improvements in both parameters

to values below that obtained during stabilisation followed by a time related decline in both parameters until termination of the experiment

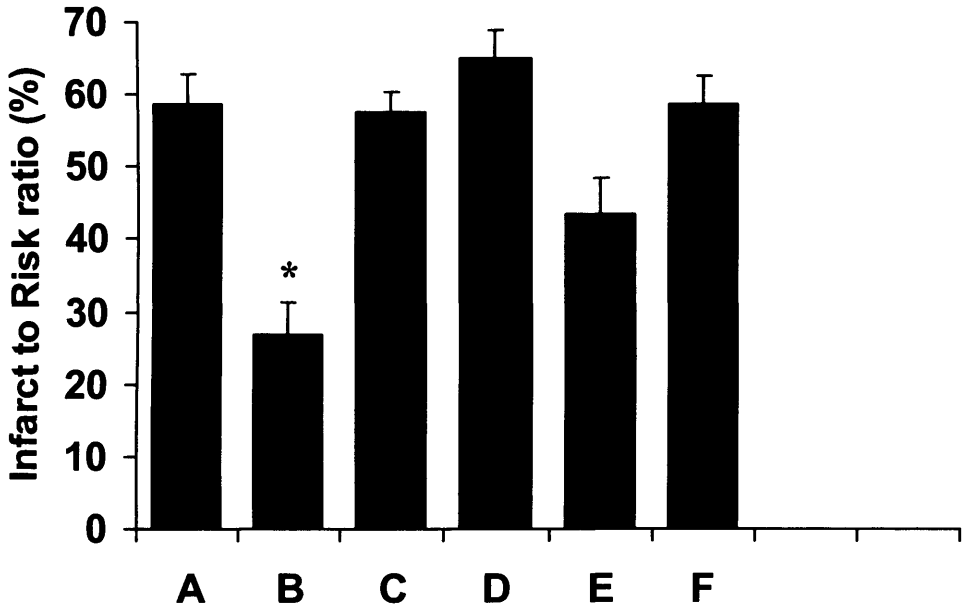
Figure 5.4 GLP-1 and the GLP-1 Receptor Inhibitor



Legend

In vitro myocardial infarct size expressed as a percentage of the risk zone. A= Control *n*= 6, B= VP *n=10*, C=. GLP-1 *n=13,* D= Exendin(9-39) and GLP-1 *n=6.* Results are presented as means ± SEM (* = $P<0.0001$ vs control)

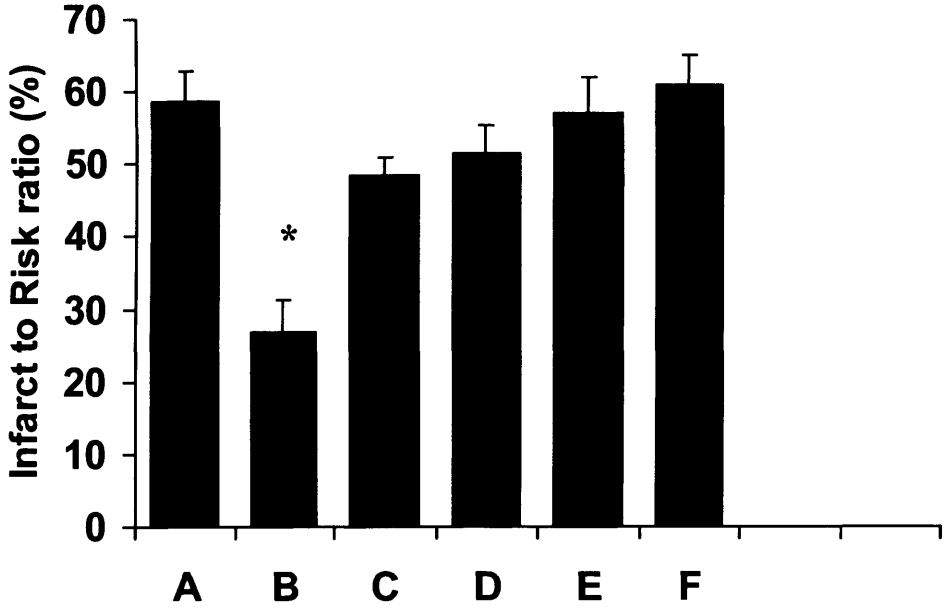
Figure 5.5 GLP-1 and Inhibitors of Pro-survival kinases Rp-cAMP and LY 294002



Legend

In vitro myocardial infarct size expressed as a percentage of the risk zone. A=Control, B=GLP-1, C= GLP-1 and Rp-cAMP *n*= 6, D= Rp-cAMP control *n=6*, E= LY 294002 and GLP-1 *n=6,* F= LY 294002 control *n=6,* Results are presented as means ± SEM (* = $P<0.0001$ vs control)

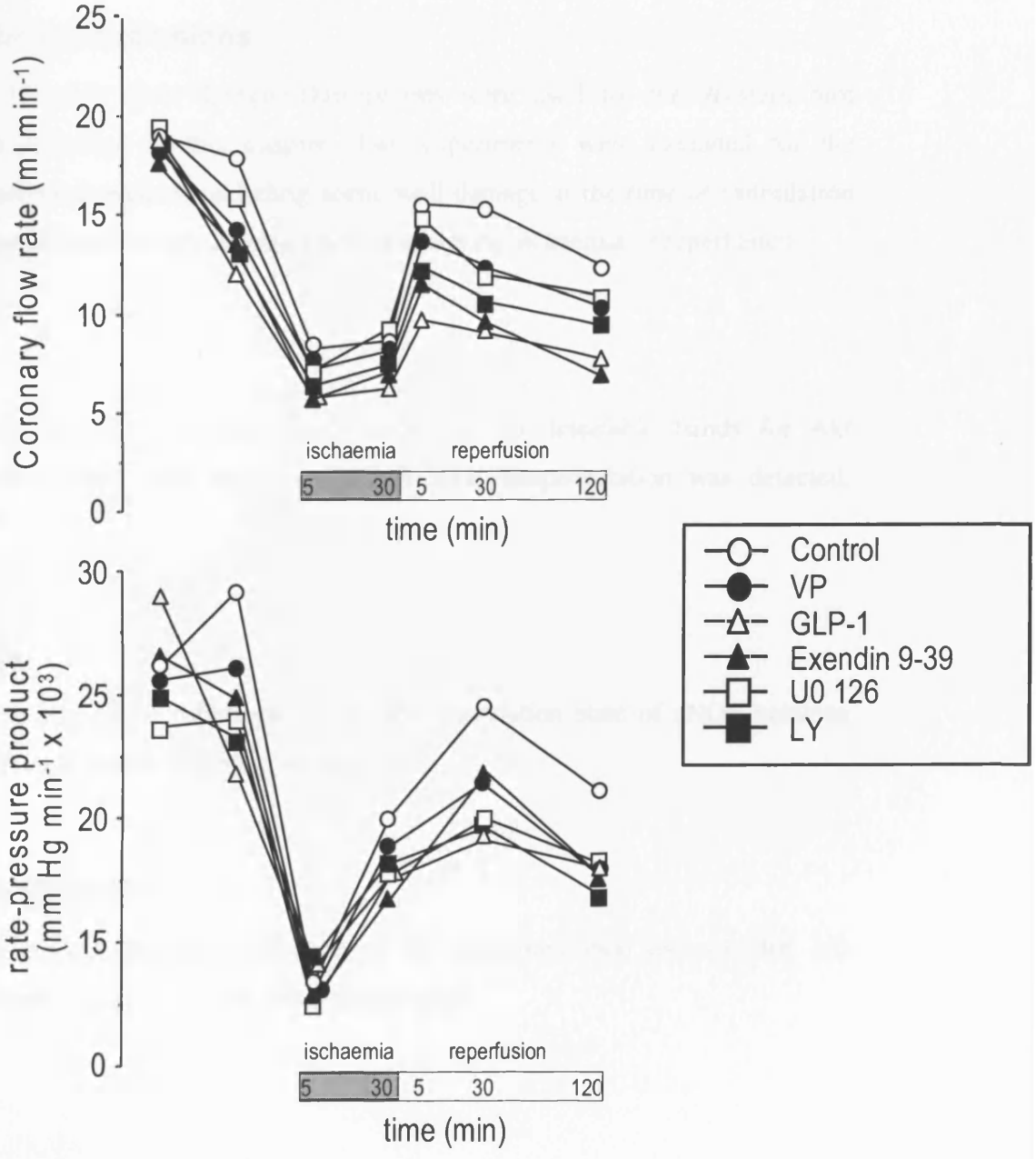
Figure 5.6 GLP-1 and Inhibitors of Pro-survival kinases U0 126 and Rapamycin



Legend

In vitro myocardial infarct size expressed as a percentage of the risk zone. A = Control, B = GLP-1, C= U0126 and GLP-1 *n=6,* D=U0126 control *n=6,* E= Rapamycin and GLP-1 *n=6,*F= Rapamycin control *n=6.* Results are presented as means ± SEM (* = $P<0.0001$ vs control)

Figure 5.7 Coronary Flow and Rate Pressure Product



5.4 Western Blot Results

5.4.1 Technical exclusions

A total of 18 adult male Sprague-Dawley rats were used for the Western blot experiments described in this chapter. Two experiments were excluded for the following technical reasons, including aortic wall damage at the time of cannulation and persistent or recurrent ventricular fibrillation during ischaemia or reperfusion.

5.4.2 Akt

With Control or GLP-1 treated hearts there was no detectable bands for Akt phosphorylation. With IPC hearts significant Akt phosphorylation was detected. Figure 5.8

5.4.3 eNOs

There was no significant difference in the phosphorylation state of eNOS between control and treated hearts. Data not included.

5.4.4 ERK 1/2 MAPK

Again there was no significant difference in the phosphorylation state of ERK 1/2 between control and treated hearts. Data not included.

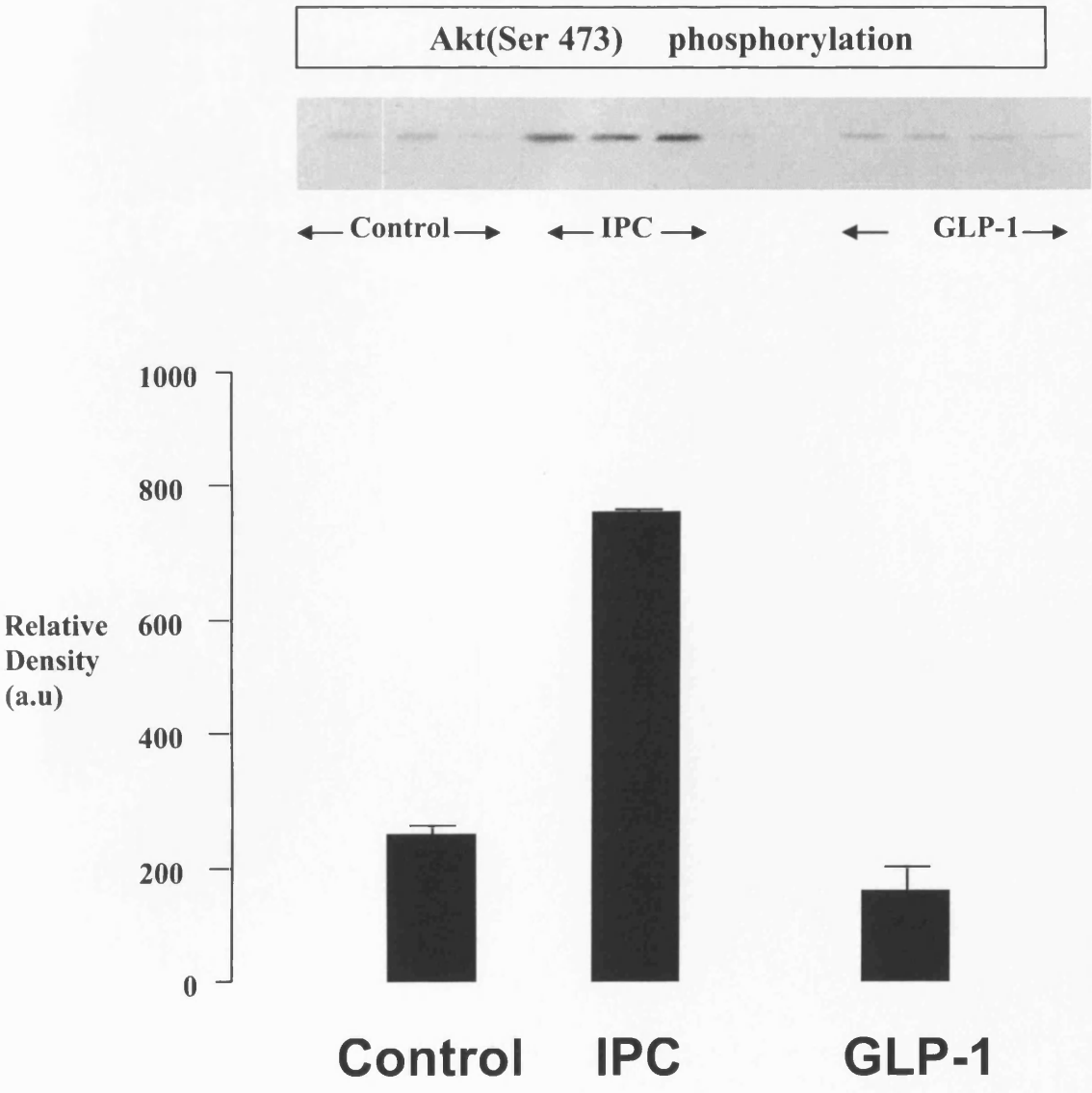
5.4.5 BAD (Ser 136)

We found no significant difference in the content of total BAD between control and GLP-1 treated hearts, whereas chemiluminescence demonstrated the presence of phospho-BAD (Ser 136) in GLP-1 treated hearts compared with control hearts. Figure 5.9

5.4.6 BAD (Ser 112)

We found no significant difference in the content of total BAD between control and GLP-1 treated hearts or the presence of phospho-BAD (Ser 112) on chemiluminescence.

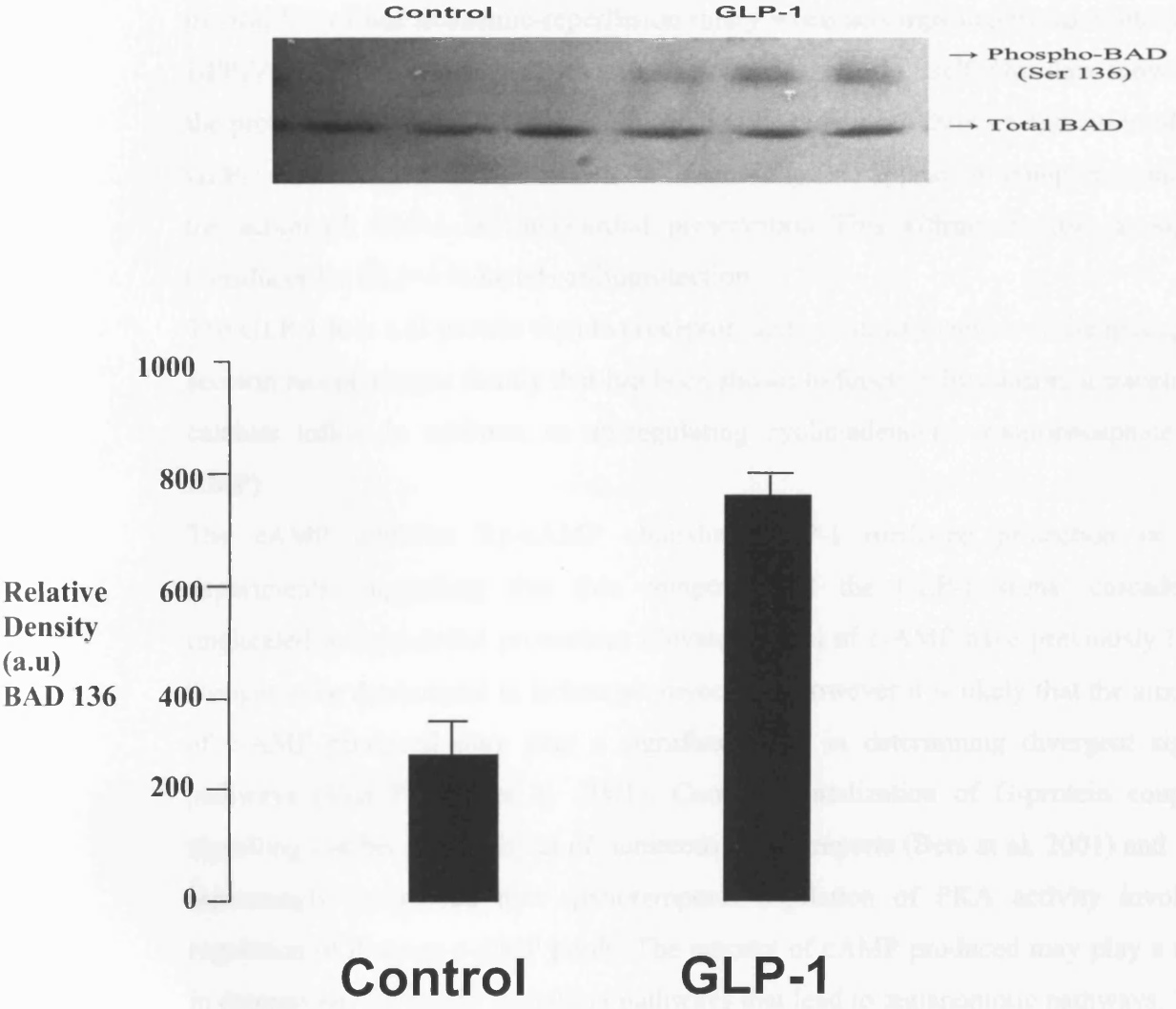
Figure 5.8 Western Blots of Akt Phosphorylation.



Legend

Representative Western blots and relative densitometry demonstrating that Control and GLP-1 treated hearts had no detectable Akt bands whilst IPC produced significant phosphorylation. N=3 per for control and IPC, n=6 for GLP-1. The relative density shown below in the bar graphs.

Figure 5.9 Western blot showing total BAD and phospho-BAD



Legend

This is a single western blot showing Phospho-BAD (Ser 136) in control and isolated hearts (n=3 each) The band size is 28 kDA for phospho-BAD. The relative density shown below in the bar graphs.

5. Discussion

5.5.1 Infarct Studies

The in vitro infarction studies demonstrate clearly that GLP-1, is able to protect the myocardium from ischaemic-reperfusion injury when accompanied by an inhibitor of DPPIV namely VP, which appears to confer no benefit by itself. Our data show that the protective action of GLP-1 would seem to be conducted through activation of the GLP-1 R, demonstrated by the fact that exendin (9-39) appears to completely inhibit the action of GLP-1 on myocardial preservation This affirms its role as signal transducer for GLP-1 induced cardioprotection.

The GLP-1 R is a G protein coupled receptor, and a distinct member of the glucagon-secretin receptor super family that has been shown to function by causing intracellular calcium influx in addition to up-regulating cyclic-adenosine monophosphate (c-AMP).

The cAMP inhibitor Rp-cAMP abolished GLP-1 mediated protection in our experiments, suggesting that this component of the GLP-1 signal cascade is implicated in myocardial protection. Elevated levels of c-AMP have previously been thought to be detrimental in ischaemic myocytes. However it is likely that the amount of c-AMP produced may play a significant role in determining divergent signal pathways (Vila Petroff et al. 2001). Compartmentalization of G-protein coupled signalling has been the subject of numerous recent reports (Bers at al. 2001) and it is increasingly recognised that spatiotemporal regulation of PKA activity involves regulation of discrete c-AMP pools. The amount of cAMP produced may play a role in determining divergent signalling pathways that lead to antiapoptotic pathways. The cAMP produced is thought to be locked in particular micro domains, known as compartmentalization, which restrict its actions.

Myocardial protection by GLP-1 is abolished by the PI3K inhibitor LY294002 and by the p44/42 MAPK inhibitor U0 126 implicating both these well demonstrated prosurvival pathways in the cardioprotection mediated by GLP-1.

Each of these pathways appears to be essential for the protection afforded by GLP-1, as inhibiting them individually abrogates the entire protection, suggesting that they may act in parallel. We were unable to identify any significant difference between the

inhibitors when individually added to GLP-1 treated hearts, with protection appearing to be an all or none phenomenon.

The p70s6K inhibitor rapamycin abolished GLP-1 mediated cardioprotection. Activation of the p70s6K may mediate IPC-induced protection via an anti-apoptotic mechanism, in which it phosphorylates and inactivates the pro-apoptotic protein BAD (Majewski et al.1999). In this regard, Jonassen and colleagues have demonstrated that the protection induced by insulin at the time of reperfusion is mediated by activation of the Akt-p70s6K-BAD pathway (Jonassen et al. 2001). Studies in haematopoietic cells, have also observed the requirement for both kinases cascades to be activated to induce cellular protection (Shelton et al. 2003).

The findings of these experiments can be summarised as follows:

1) GLP-1 mediated protection is transduced by the GLP-1 Receptor

2) This protection appears to involve several previously described constituents of the RISK pathway namely PI3K, ERK 1/2 and p70s6K, as well as c-AMP which has not previously been considered as a beneficial cell signal within the ischaemic myocardium.

5.5.2 Western blot data

From our infarct data we would have expected to find positive Western blot results for the RISK kinases implicated by the inhibition of myocardial protection by their specific blockers. For example, GLP-1 mediated myocardial protection is blocked by the ERK 1/2 MAPK kinase inhibitor U0 126, so we hoped to find a positive Western blot for ERK 1/2 in GLP-1 treated hearts. However, for all the RISK kinases we failed to find a positive Western blot result for GLP-1 treated hearts. In other words there were no significant differences between the levels of phosphorylated AKT, ERK 1/2, PKA or p70s6K between control and treated hearts with or without inhibitors.

This may be a result of the time point at which hearts were harvested for sampling. The transitory moment of the kinase activation by GLP-1 may have been missed. This is supported by other investigators research showing that GLP-1 induces a time-related phosphorylation of ERK 1/2 MAPK fractions in rat hepatocytes after two and

five minutes exposure to GLP-1 which is not detectable after 10 minutes (Redonado et al. 2003). In the protocol we used, hearts were harvested after five minutes exposure to GLP-1 and further experiments were performed with ten minutes exposure to GLP-1. A major limitation of our study was the failure to use additional time points for this investigation which would have addressed this question.

The IPC experiments, used as a positive control, showed Akt and ERK 1/2 phosphorylation, proving that our antibodies and methodology for performing Western blots was effective and used correctly.

We hypothesised from our findings and the observations of Redonados group, that the phosphorylation of the prosurvival kinases, even though short lived and difficult to assess, may have activated persistent downstream protective mechanisms which would explain the increased pancreatic cell survival seen in the literature and our own experience in the myocardium. We turned our attention to two such downstream targets, namely, eNOS and BAD and found interesting results concerning the latter.

Bad is a proapoptotic member of the Bcl-2 family that can displace Bax from binding to Bcl-2 and Bcl-xL, resulting in cell death. Survival stimuli can lead to the inhibition of the apoptotic activity of Bad by activating intracellular signal pathways that lead to the phosphorylation of Bad. There are a number of phosphorylation sites on Bad and interestingly PKA, Akt or ERK 1/2 MAPK can phosphorylate these sites. The result is the binding of Bad to 14-3-3 proteins preventing Bad from binding to Bcl-2 and Bcl-xL and promoting cell death. Akt has been shown to promote cell survival via its ability to phosphorylate Bad at Ser 136 (She et al. 2005). Ser 112 has been shown to be the substrate in vivo and in vitro of p90RSK and mitochondria-anchored PKA (Bonni et al. 1998 and Harada et al. 1999). The extracellular signal regulated kinases ERK 1/2 are thought to act as a catalyst for the phosphorylation of p90RSK (Seger and Krebs 1995). Our Western blots demonstrated phosphorylation of Bad at Ser 136 with no evidence of phosphorylation at Ser 112. Moreover, we were not happy with the quality of the phospho-Bad antibodies and more investigations are needed with respect of GLP-1 inducing Bad inactivation. However, the finding that GLP-1 results in the phosphorylation (hence inactivation) of Bad at least at Ser 136 gave us an insight into the possible end effectors responsible for GLP-1 mediated protection against cell death in the myocardial ischaemia-reperfusion injury.

Using specific inhibitors we have been able to demonstrate that GLP-1 induced myocardial protection involves multiple pathways, which may induce in the end the inactivation of important antiapoptotic factors as Bad.

Chapter 6 GLP-1 administered either before ischaemia or at reperfusion

Introduction

We have established that GLP-1 is able to protect the myocardium in both the in vivo and in vitro rat model against ischaemic-reperfusion injury when given throughout the experimental protocol. This effect is probably mediated by activation of the reperfusion injury salvage kinases (RISK) (Hausenloy and Yellon 2004) as suggested by the results of our experiments with blockade of specific known constituents of the RISK pathway. The activation of these pathways has been demonstrated to play a role in preconditioning as well as in reducing reperfusion injury. Therefore, we tested the hypothesis that GLP-1 can activate these prosurvival pathways when given prior to ischaemia or at reperfusion.

6.1 GLP-1 at Reperfusion

To salvage the ischaemic myocardium from infarction requires reperfusion. Reperfusion is therefore a prerequisite for cell survival, however, it is associated a cascade of deleterious effects, such as the generation of free radicals or oxidative stress and abrupt metabolic changes, known as reperfusion injury (Yellon 2003) Indeed some studies investigating reperfusion injury have demonstrated cellular injury in the absence of ischaemia. (Vanden Hoek et al 1996). In all our experiments to date GLP-1 had been given during stabilisation of the model prior to any ischaemic insult and continued throughout both ischaemia and reperfusion until the hearts were harvested for histological examination. We sought to clarify whether GLP-1 would induce myocardial protection when given after the index ischaemia but prior to reperfusion. In these experiments GLP-1 and VP were added to the perfusate and

circulated through the apparatus ready to be delivered to the isolated perfused heart. Two sets of experiments were performed to investigate the role of GLP-1 at reperfusion. The first group R1, were performed with the GLP-1/VP treatment commenced 5 minutes prior to the end of the ischaemic period and continued for 15 minutes into the reperfusion period, giving a total of 20 minutes treatment with GLP-1/ VP. For the rest of reperfusion period the hearts were switched back to plain buffer with no added GLP-1 or VP. A further set of experiments, Group R2, were performed with GLP-1/ VP commenced one minute prior to reperfusion and continued for 15 minutes into reperfusion. Two groups were used based on the experience of previous investigators at the Hatter Institute. Figure 6.1

6.2 GLP-1 as a Preconditioning Mimetic

Ischaemic preconditioning is considered one of the most powerful forms of myocardial protection as discussed in chapter 1. We sought to identify whether GLP-1 would act as a preconditioning mimetic. In these experiments classical ischaemic preconditioning experiments (two periods of 5 minutes global normothermic ischaemia interspaced with 10 minutes reperfusion before the 35 minutes lethal ischaemia and 120 minutes reperfusion) were compared to GLP-1 treated and control experiments. To test the hypothesis that GLP-1 could act as a pharmacological mimetic of classical ischaemic preconditioning, experiments were performed with GLP-1 given prior to index ischaemia for a period of 10 minutes followed by a washout period of 5 minutes prior to index ischaemia for 35 minutes followed by 120 minutes reperfusion as in the rest of the experiments. Figure 6.2

6.3 Materials and Methods

Male adult Sprague-Dawley rats (350-450g) were used for these studies. See chapter 2, Section 2.4.5 for a detailed description of methods and materials used.

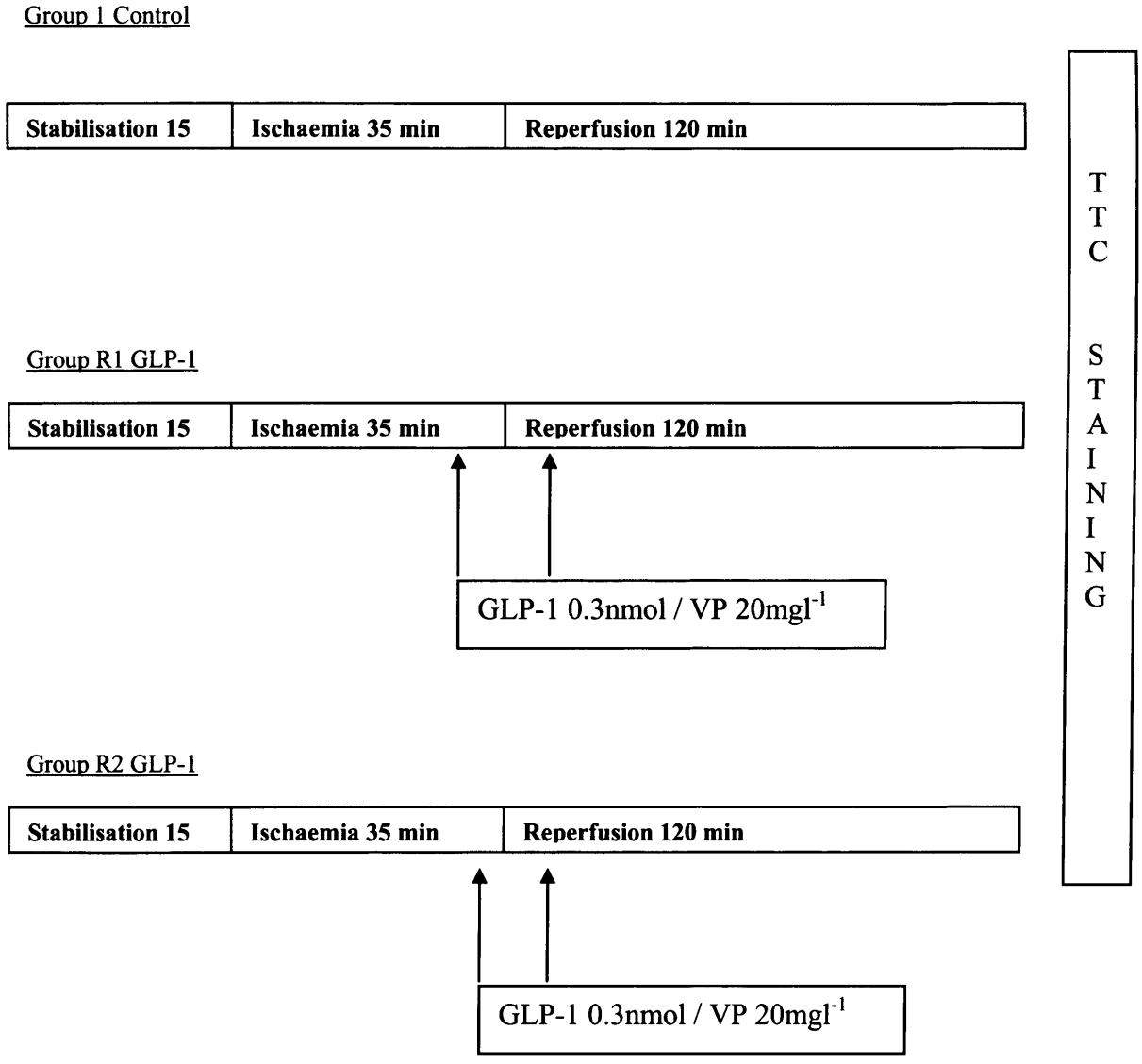
6.3.1 In Vitro Infarct Model in Rat Heart

For a detailed description see chapter 2, section 2.4.8 and chapter 4 section 4.2.1 to 4.3. This model allowed us to explore the mechanisms of the GLP-1 protection against myocardial infarction by altering the time point of exposure to GLP-1.

6.3.2 Statistical Analysis

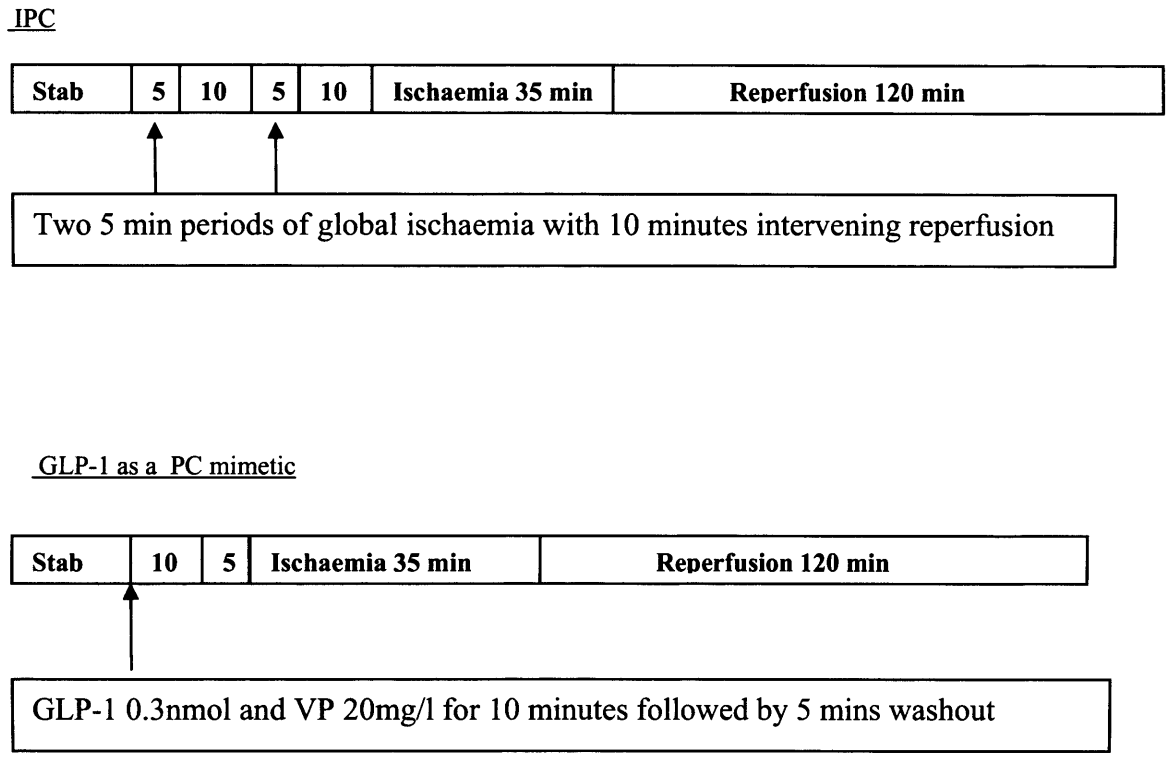
Statistical analysis was performed using Apple Macintosh iMac computers and the statistical package StatView Version 4.5. Data was expressed as means ± SEM. One way analysis of variance between group means was performed using an ANOVA factorial method. P values of less than 0.05 were taken to show statistical significance.

Figure 6.1 Scheme of in vitro GLP-1 at reperfusion experimental protocols



Hearts in all groups are stabilised for a minimum of 15-20 minutes. In Group R1, GLP-1 and VP were perfused through the hearts after 30 minutes of ischaemia., that is five minutes prior to the onset of reperfusion. In Group R2, GLP-1 and VP were perfused into the heart after 34 minutes of ischaemia, that is one minute prior to reperfusion, and continued into reperfusion for a total of 20 minutes respectively.

Figure 6.2 Schematic of in vitro Ischaemic preconditioning experimental protocols and GLP-1 as a preconditioning mimetic



Hearts in all groups are stabilised for a minimum of 15-20 minutes. IPC = Ischaemic preconditioning

6.4 Results

6.4.1 Infarct size and GLP-1 given at reperfusion

In group R1 ,GLP-1 given at reperfusion also reduces the infarct in the risk zone to 30.6 % +/- 3.4. ($p<0.0001$ vs. control). However in group R2 with GLP-1 given one minute prior to reperfusion there was an absence of protection against ischaemic reperfusion injury. Figure 6.3

6.4.2 Infarct size and GLP-1 as a preconditioning mimetic

Preconditioning reduced the infarction in the risk zone to 26.7 % +/- 2.1 compared to control 58.7 % +/- 3.5 ($p<0.0001$). GLP-1 given as a preconditioning mimetic reduced infarction in the risk zone to 29.8 % +/- 6.8. Figure 6.4

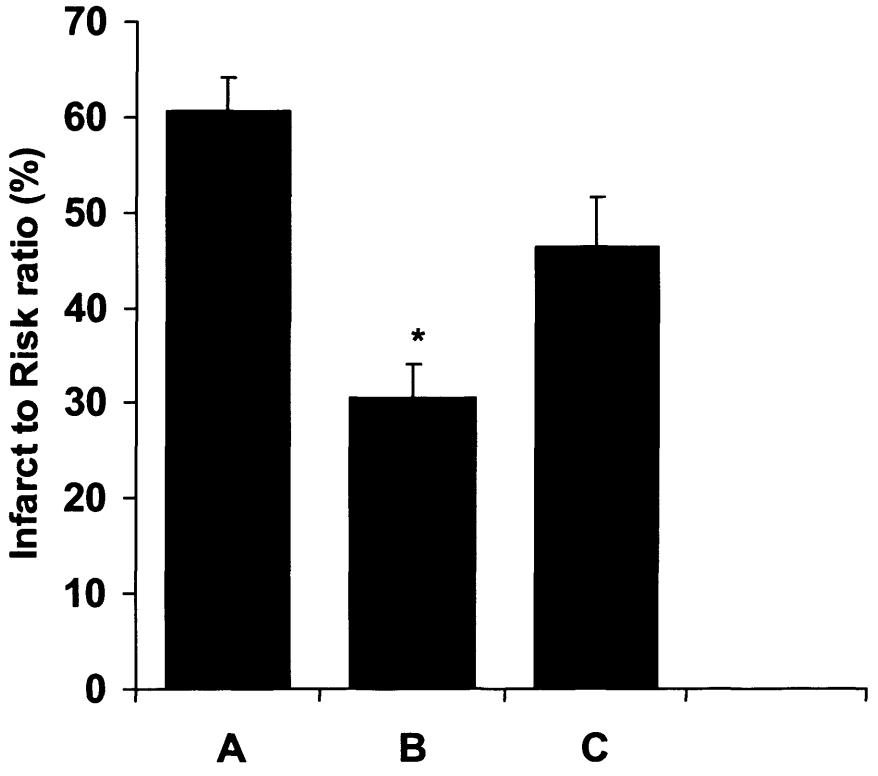
6.4.3 Coronary Flow and Rate Pressure Product

There were no statistically significant differences in heart rate or developed left ventricular pressure between the groups during stabilisation, ischaemia or reperfusion. Ischaemia produced a fall in both the coronary flow and RPP when compared to stabilisation. At reperfusion there were improvements in both parameters to values below that obtained during stabilisation followed by a time related decline in both parameters until termination of the experiment

6.4.4 Risk Volume and Weight

There were no significant differences between the groups in regard to cardiac weight and risk volume. Table 6.1

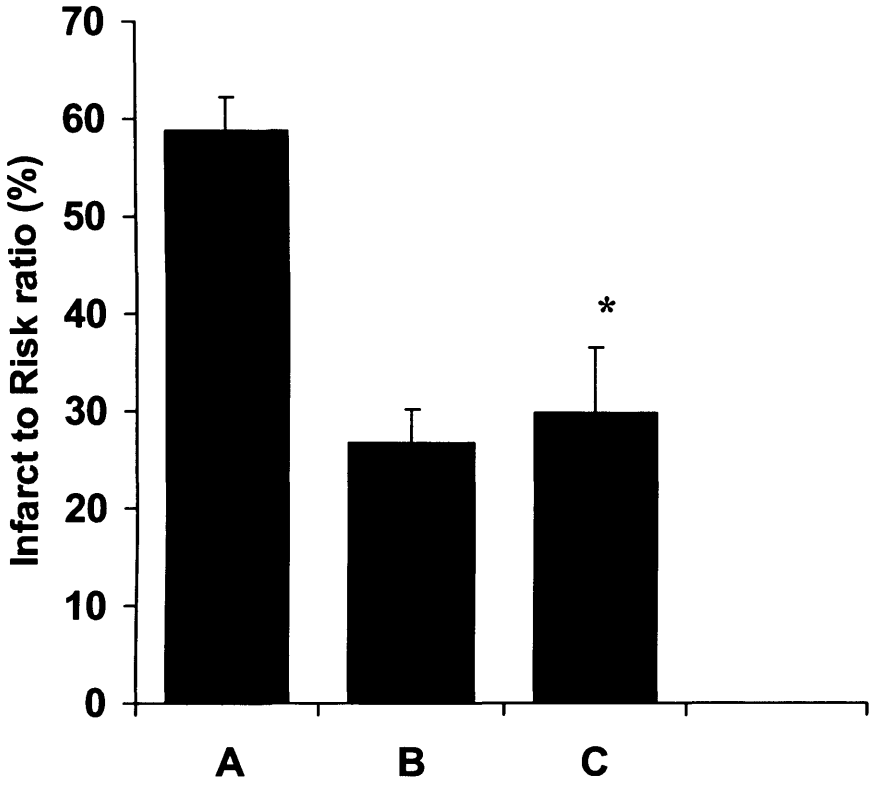
Figure 6.3 GLP-1 at Reperfusion



Legend

In vitro myocardial infarct size expressed as a percentage of the risk zone. A= Control *n*= 6, B= GLP-1/VP 5 minutes prior to reperfusion *n=7*, C=. GLP-1/VP 1 minute prior to reperfusion *n=7*. Results are presented as means ± SEM (* = $P<0.001$ vs control

Figure 6.4 GLP-1 as a Preconditioning Mimetic



Legend

In vito myocardial infarct size expressed as a percentage of the risk zone. A= Control *n*= 6, B= IPC Ischaemic Preconditioning *n=6*, C= GLP-1 as a preconditioning mimetic. *n=4*. Results are presented as means ± SEM (* = $P<0.0001$ vs control)

Table 6.1 Characteristics of Animals in Treatment Groups

Groups	Number	Body Weight (g)	Heart Weight (g)	Risk volume (cm^3)
GLP-1 Reperfusion	7	412±18	2.04±0.12	0.525±0.018
GLP-1 Reperfusion Control	7	402±16	1.98±0.10	0.496±0.019
GLP-1 Preconditioning Mimetic	4	386±14	1.89±0.06	0.464±0.017
Ischaemic Preconditioning	6	392±12	1.92±0.08	0.515±0.037
Control	6	406±18	1.94±0.08	0.4860±0.03

All values are presented as means ± SEM

6.5 Discussion

Ischaemic preconditioning (IPC) has been clearly demonstrated to be strongly cardio-protective by numerous of investigators across a variety of different animal models as well as in man as discussed in chapter 1. The RISK pathway is activated by IPC and a number of pharmacological agents (Hausenloy and Yellon 2004). The hypothesis that GLP-1 could mimic classical ischaemic preconditioning and provide protection against myocardial injury was tested by giving GLP-1 for a short period of 10 minutes immediately after stabilisation prior to any index ischaemia. GLP-1 given as a preconditioning mimetic protected against myocardial ischaemic-reperfusion injury with a significant decrease in myocardial infarction in comparison to control experiments. The degree of protection was not statistically different from the protection achieved by IPC or by giving GLP-1 throughout the index ischaemia and reperfusion or in comparison to giving GLP-1 five minutes prior to reperfusion.

Our results showed a surprising outcome when GLP-1 was given one minute prior to reperfusion when compared to the second set of experiments when GLP-1 was given five minutes prior to reperfusion. There was an absence of protection against myocardial injury in the group treated from one minute prior to reperfusion whilst the five minute group produced a similar degree of protection to our initial in vitro GLP-1 treated experiments. This could be explained by GLP-1 failing to activate the reperfusion injury salvage kinase or RISK pathways in time. When given one minute prior to reperfusion GLP-1 may fail to result in the RISK pathway kinases activation prior to the deleterious effects of reperfusion as a result of not reaching the tissues in adequate concentrations just at the moment of reperfusion. This could in turn be a consequence of the experimental set up in the Langendorff apparatus. Attempts are made to achieve the least possible dead space in the tubing carrying the GLP-1 treated buffer so as to reach the heart as quickly as possible when the buffers are switched over. This theory is supported by the finding that the second group in which GLP-1 was started five minutes prior to the onset of reperfusion is protected.

In summary these experiments confirm that in an in vitro rat model GLP-1 protects the myocardium against ischaemic reperfusion injury when given five minutes prior to the onset of reperfusion or as a preconditioning mimetic.

Chapter 7 Final considerations

7.1 Summary and Discussion

Myocardial infarction and coronary artery disease are amongst the most important and sadly prevalent pathologies dealt with by medical and surgical specialists in the healthcare field. Diabetes is considered one of the most important risk factors for the development of coronary artery disease. Indeed outcomes in terms of morbidity and mortality for those with diabetes and coronary artery disease are considerably worse in comparison to non diabetic individuals.

In this context the investigation of novel compounds such as GLP-1, with potential beneficial effects on the control and treatment of diabetes in relation to their possible actions in the myocardium, holds a great deal of interest. This thesis investigates the possibility that GLP-1 protects the myocardium against ischaemia-reperfusion injury and further seeks to identify the mechanisms by which this novel peptide hormone acts in relation to previously well described cell signal cascades, such as the RISK pathway.

GLP-1 possesses a number of properties, which makes it a potentially ideal anti-diabetic agent (see chapter 1). Furthermore this peptide also possesses other properties which have the potential to exert a direct cardioprotective effect. In this regard GLP-1 has also been shown to reduce pancreatic beta cell apoptosis (Urusova et al. 2004). Moreover the presence of the GLP-1 receptor (GLP-1R) in the heart, and the evidence that GLP-1 promotes the activity of PI3K in beta cells (Hui et al. 2003), a kinase that has been clearly associated with myocardial protection in the setting of ischaemic reperfusion injury (Hausenloy et al. 2004) as well as preconditioning (Mocanu et al. 2002), allows one to hypothesise a novel and independent action of GLP-1 in the setting of ischaemia-reperfusion damage.

GLP-1 induces an increased level of cAMP in cardiomyocytes, which, in turn, activates Protein Kinase A (PKA) (Vila Petroff et al. 2001). GLP-1 has an anti apoptotic action on insulin secreting cells mediated by c-AMP, and PI3K. Activation

of PI3 kinase leads to the phosphorylation and inactivation of the pro-apoptotic BAD by causing it to bind to 14-3-3 proteins (Zha et al. 1996). BAD is a proapoptotic member of the Bcl-2 family that can displace Bax from binding to Bcl-2 and Bcl-xl, resulting in cell death. Elevated levels of cAMP have previously been thought to be detrimental in ischemic cardiomyocytes. However, recent work in beta cells has shown that GLP-1 produced lower concentrations of cAMP compared to other cAMP increasing agents. The amount of cAMP produced may play a role in determining divergent signalling pathways that lead to antiapoptotic pathways. The cAMP produced may also be locked in particular micro domains, known as compartmentalization, which restrict its actions (Bers at al. 2001). GLP-1 mediated increases in cAMP (comparable to isoproterenol) failed to cause any inotropic or lusitropic effect (Vila Petroff et al. 2001), supporting the suggestion for such compartmentalization. Compartmentalization of G protein-coupled signalling has been the subject of numerous reports, and it is increasingly recognized that spatiotemporal regulation of PKA activity involves regulation of discrete cAMP pools.

GLP-1 has been shown to increase blood pressure and heart rate in rats (Barragan et al. 1996). In our study we failed to demonstrate any significant change in blood pressure or heart rate, both, in vivo and in vitro. This difference may be due to the different dose of GLP-1 used and method of delivery. A study using a similar dose of GLP-1 in a porcine model failed to note any change in blood pressure or heart rate (Deacon et al. 1996). In our in vivo experiments the degree of anaesthesia required to produce a stable open chest experimental model, which causes myocardial depression, must also be taken into account. GLP-1 has been shown to have effects on the central control of blood pressure and pulse (Barragan et al. 1999 and Isbil-Buyukcoskon et al. 2004). This central mechanism is excluded in the in vitro setting.

GLP-1 is a potent incretin, which could suggest a possible mechanism to explain our initial findings in the in vivo study. However, although a rise in insulin and decrease in glucose is possible, it must be remembered that these studies were undertaken in non diabetic animals which although not fasted, were not post-prandial, suggesting any GLP-1 mediated stimulation of insulin release is likely to be small, because the insulinotropic effects of GLP-1 are glucose dependent. Furthermore in our additional

studies using the in vitro, isolated perfused heart rat heart, we obtained a similar degree of myocardial protection

The myocardial protection observed in this study is reproduced in both the in vivo and in vitro models; the latter being specifically relevant, as in this setting, there is an absence of circulating insulin. Any residual insulin in these hearts at the time of harvesting from the rats would be lost from the tissues during the stabilisation period on the Langendorff apparatus. Since insulin has been shown to be cardioprotective, activating PI3K and reducing post ischaemic myocardial apoptotic death (Baines et al. 1999 and Jonassen et al. 2001), the ability of GLP-1 to protect against ischaemia reperfusion injury in this model demonstrates that it acts independently of its incretin properties.

Recombinant GLP-1 has been shown to prevent the accumulation of pyruvate and lactate in a porcine model of myocardial ischaemia, but failed to show any decrease in the infarction (Kavianipour et al. 2003). Species differences, methodology, the long duration of the ischaemic insult and the lack of an inhibitor of the rapid enzymatic degradation of GLP-1 may account for the abolition of myocardial protection.
Our results demonstrate that GLP 1 confers myocardial protection when administered together with an inhibitor of DPP-IV, valine pyrrolidide which, appears to confer no benefit by itself.
Exendin 9-39, a GLP 1 R antagonist, appears to completely inhibit the protective action of GLP-1 on myocardial preservation, confirming the role played by this receptor as a signal transducer for GLP-1 induced cardioprotection. The c-AMP inhibitor Rp-cAMP abolished protection, confirming this known GLP-1 pathway as a possible downstream mechanism. The protection was also abolished by the PI3K inhibitor LY294002, by the ERK 1/2 MAPK inhibitor U0 126 and by Rapamycin an inhibitor of p70s6K, implicating these well demonstrated pro-survival pathways in the cardioprotection mediated by GLP-1.

Recent studies have demonstrated a direct endothelial relaxant action for GLP-1 (Golpon et al. 2001 and Nystrom et al. 2005). Our in vitro model showed no significant change in coronary artery flow. This does not exclude a vascular endothelial role for GLP-1 in the heart, as our experimental model – the isolated

retroperfused Langendorff heart– is not the best model for assessing myocardial endothelial function.

We have shown, for the first time, that GLP-1 is able to protect the myocardium against ischaemia-reperfusion injury, not only in vivo, but also in vitro. GLP-1 appears to protect via activation of its receptor and the downstream signalling prosurvival pathways PKA/cAMP, PI3K/Akt, ERK 1/2 MAPK. Amongst different targets of these pathways our data confirm p70s6K (as rapamycin inhibits GLP 1 protection) and Bad (as Western blots confirm its phosphorylation and hence inactivation, in GLP 1 treated hearts). Figure 7.1

Each of these appears to be essential for the protection afforded by GLP-1, as inhibiting them individually, abrogates the protection, suggesting that they may act in parallel. However it is worth mentioning that inhibiting PI3K seems to only partially abrogate protection, a result which can be interpreted as due to a possible crosstalk between these pathways. More investigations in the area are needed.

7.2 Summary of findings

- GLP-1 acts to limit myocardial ischaemia-reperfusion injury in both a rat in vivo and in vitro model.
- GLP-1 appears to have no measurable haemodynamic actions in vivo or in vitro in our models.
- The protective effect of GLP-1 against myocardial cell death implicates the activation of the GLP-1 receptor and downstream signalling cascades cAMP/PKA, PI3K/Akt and ERK 1/2
- GLP-1 affords myocardial protection whether given prior to ischaemia or at the time of reperfusion
- GLP-1 results in the phosphorylation and hence deactivation of the pro-apoptotic factor BAD.
- GLP-1 can act as a preconditioning mimetic.

7.3 Limitations

Our study investigated the influence of GLP-1 upon myocardial infarction as a measurable end point of ischaemic-reperfusion injury. Once a positive reduction in the degree of infarction was confirmed we proceeded to try and characterize the mechanisms by which GLP-1 achieved this protection.

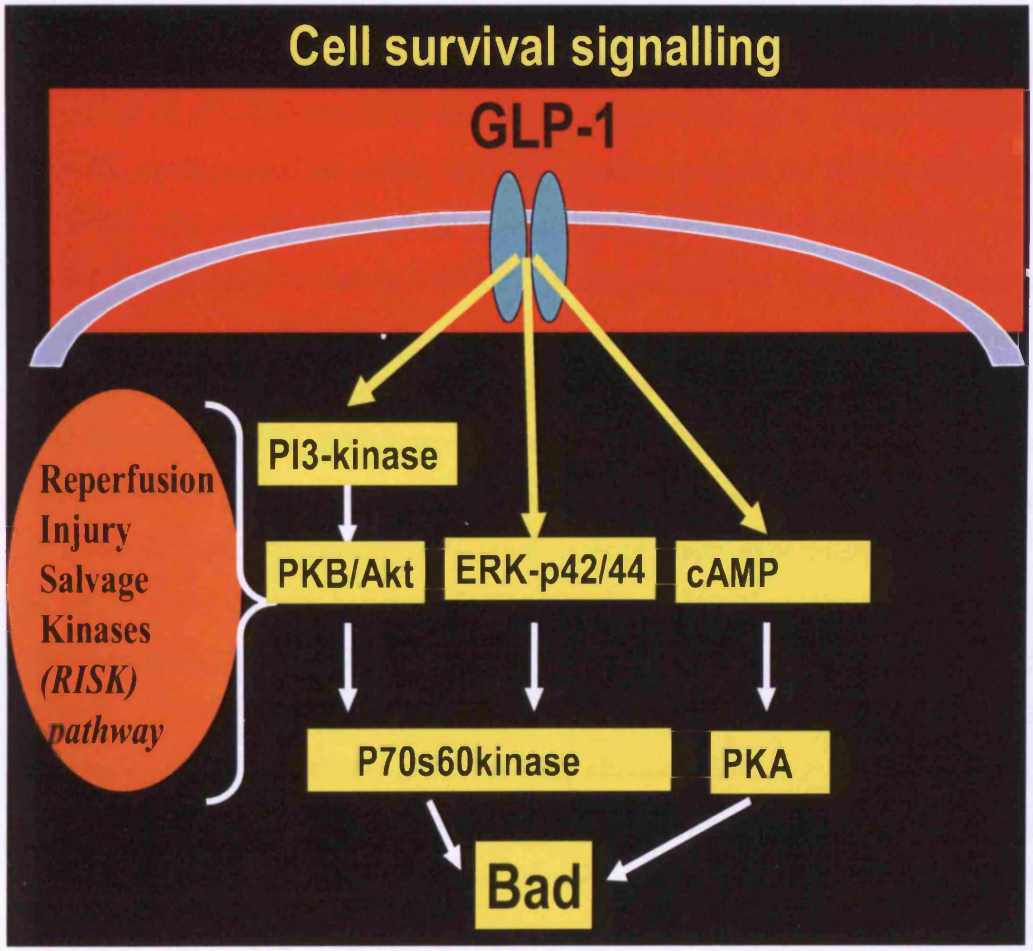
We did not perform experiments in vivo where the glucose level was standardised or clamped. Our initial in vivo protocol involved the measurement of intact GLP-1 as well as its degradation products and DPPIV activity. However, due to circumstances beyond our control these samples have never been processed so this data is unavailable. It would have been interesting to investigate the effect of different glucose levels in the perfusate upon the GLP-1 protection in the in vitro settings. All our in vitro work was done using standardised glucose content.

GLP-1 is known to have anti-apoptotic actions in β-cells in the pancreas. Myocardial infarction is known to be a composite of necrosis and apoptosis. Our experiments did not look at the contribution of apoptosis and necrosis in the resultant myocardial infarction and whether this was altered by GLP-1.

Our quest for the identification of a mechanism of action for GLP-1 examined components of the RISK pathway. Although we were able to inhibit GLP-1 mediated myocardial protection with inhibitors of the RISK pathway we failed to capture phosphorylation of these components in our Western blot experiments. This may be attributed to our protocol missing the time points at which GLP-1 phosphorylated these components, This is supported by other investigators research showing that GLP-1 induces a time-related phosphorylation of ERK 1/2 MAPK fractions in rat hepatocytes after two and five minutes exposure to GLP-1 which is not detectable after 10 minutes (Redonado et al. 2003). In the protocol we used hearts were harvested after five minutes exposure to GLP-1 and further experiments were performed with ten minutes exposure to GLP-1. A major limitation of our study was the failure to use additional time points for this investigation. The transitory moment of the kinase activation by GLP-1 may have been missed. Further experiments would be needed in order to try and tease out the time points of GLP-1 activity on these factors.

We did, however, confirm the phosphorylation of BAD by GLP-1, however more investigations regarding other members of Bcl-2 family would have been ideally performed. Our investigations have been carried out in a normoglycaemia rat models, for validation these should be repeated in a diabetic model.

Figure 7.1 GLP-1 cell signalling in the ischaemic myocardium



7.4 Clinical Implications

Our data brings a new insight into the possible therapeutic potential for this class of drugs currently undergoing trials in the treatment of non-insulin dependent diabetes.

7.5 Future Directions

Valine pyrrolidide is a prototype DPPIV inhibitor and GLP-1 analogues are stabilised forms of GLP-1 (optimised for once daily administration). It is interesting to observe that the cardioprotective effect of GLP-1 was not shared by DPPIV inhibition alone (VP had little or no effect). GLP-1 analogues and DPPIV inhibitors are both novel therapeutic approaches to the treatment of type 2 diabetes – a condition characterised by increased risk of acute myocardial infarction. Our data suggests, therefore, that GLP-1 analogues may possess an additional benefit (i.e. cardioprotection) which is not shared by DPPIV inhibitors – a property that may help distinguish between these newly emerging therapeutic approaches. Furthermore it may be of interest, therefore, to study the effects of stable GLP-1 analogues in future experiments.

A great deal of interest has been focused on the metabolic consequences of ischaemia in the myocardium and its modification with glucose, insulin and potassium (GIK) has reaped rewards (Sodi Polares 1962 and Opie 1970). The actions of GLP-1 on myocardial metabolism have yet to be fully elucidated.

It would be interesting to speculate whether the benefits reported in the clinical trials (such as the DIGAMI (Malmberg et al. 1995) and ECLA (Diaz et al. 1998) and other trials (Doenst et al. 2003)), reporting benefits from the administration of GIK therapy at the time of reperfusion, following an acute myocardial infarction, were due to the activation of the reperfusion injury kinase pathway.

The CREATE-ECLA randomized controlled trial reported in 2005 (Mehta et al. 2005). In this large, international randomized trial, high-dose GIK infusion had a neutral effect on mortality, cardiac arrest, and cardiogenic shock in patients with acute STEMI. This study cast a shadow over the role of GIK in acute myocardial infarction.

The proponents of the GIK have pointed to the limitations of this study in over twenty thousand patients with acute myocardial infarction and suggest that the critical factor may be the timing of therapy which should ideally be initiated either prior to or at the time of reperfusion (Apstein at al. 2005).

Recent studies have shown that fluoroscopic-guided intramyocardial injection in the pig model is a feasible and safe procedure for targeting the delivery of therapeutic agents to the area of myocardium at risk from ischaemia-reperfusion injury (Gwon et al. 2001). Therefore the local delivery of therapeutic agents such as GLP-1 themselves or the adenoviral vectors (carrying mutated genes which over-express growth factors) may provide a potential method for targeting and up-regulating the RISK-pathway in the clinical setting of reperfusion. Alternatively for patients undergoing an anticipated episode of ischaemia-reperfusion injury, such as during CABG surgery or elective coronary angioplasty, gene transfer may be a possible method of delivering growth factors to myocardium at risk of lethal reperfusion-induced injury.

Further studies are required to verify and further investigate the action of GLP-1 and its metabolites on the myocardium.

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