

**POSSIBLE MECHANISMS FOR LEVOSIMENDANINDUCED
CARDIOPROTECTION.**

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degree

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Declaration

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ABSTRACT

Background and purpose. To limit ischaemic injury, rapid restoration of coronary blood flow is required, which will in turn reduce infarct size. However, reperfusion itself causes myocyte death – a phenomenon termed *lethal reperfusion-induced injury*, which limits protection of the ischaemic myocardium. Thus the reperfusion of irreversibly damaged myocytes may accelerate the process of cell necrosis.

Additive protection of the ischaemic myocardium in the form of adjunct therapy remains a topic of intensive research. Levosimendan, a calcium sensitizing agent with positive inotropic effects has in several studies been found to alleviate the damaging effects of reperfusion injury. Levosimendan has been shown to be a K_{ATP} channel opener. These channels have been implicated to play an important role in ischaemic preconditioning (IPC). With this knowledge, the aim of this study was to determine whether levosimendan and IPC have certain cardioprotective mechanisms in common and whether protection with pharmacological preconditioning could be elicited with levosimendan. In this study, we investigated whether: 1) the isolated guinea pig heart could be protected by ischaemic preconditioning (IPC) and postconditioning (IPostC), 2) the heart could be pharmacologically pre- and postconditioned, using levosimendan (LPC & LPostC), 3) a combination of IPC & LPC had an additive protective effect on the heart, 4) the K_{ATP} (both mitochondrial and sarcolemmal) channels are involved in this protection and 5) the pro-survival kinases of the RISK (reperfusion injury salvage kinase) pathway are involved.

Experimental approach. Isolated perfused guinea pig hearts were subjected to three different IPC protocols (1x5, 2x5 and 3x5 minutes of ischaemia) or levosimendan (0.1 μ M) preconditioning, before coronary artery occlusion (CAO – 40min@36.5°C), followed by 30 minutes of reperfusion. Hearts were also subjected to a combination of IPC & LPC, to establish whether they had additive protective effects. In addition, hearts were pre-treated with levosimendan directly before induction of sustained ischaemia (without washout of the drug – levosimendan pre-treatment (LPT)) for 10min. With the postconditioning protocol,

the hearts were subjected to 3x30second cycles of ischaemia/reperfusion or levosimendan/vehicle. In a separate series of experiments, hearts were treated with K_{ATP} channel blockers (for both sarcolemmal & mitochondrial), before LPC, LPT and LPostC. The endpoints that were measured were: cardiac reperfusion function, myocardial infarct size and RISK pathway expression and phosphorylation (PKB/Akt and extracellular signal-regulated kinase – ERK42/44).

Results. IPC, IPostC, LPC & LPostC decreased myocardial infarct size significantly compared with their controls ($21.9\pm 2.2\%$, $21.4\pm 2.2\%$, $20.6\pm 3.1\%$ and $20.6\pm 1.8\%$ respectively vs. $46.4\pm 1.8\%$ for controls, $p < 0.05$). The combination of IPC & LPC had no additive protective effect. Pre-treating the hearts with levosimendan (without washout), before index ischaemia, proved to be the most effective method of cardioprotection (infarct size: $5.8\pm 0.9\%$ vs. $46.4\pm 1.8\%$ for controls, $p < 0.001$). With LPT a significant increase ($p < 0.05$ vs. control) in phosphorylation of ER42/44 was also observed. An increase in the activity of one of the RISK pathway kinases, ERK42/44 seems to be one of the reasons for LPT's efficacy. Treating the hearts with K_{ATP} channel blockers before subjecting them to LPC, LPT & LPostC abolished the protective effects induced by levosimendan, suggesting a role for the sarcolemmal and mitochondrial K_{ATP} channels in levosimendan-induced cardioprotection.

Conclusions and implications. 1) Isolated guinea pig hearts could be pre- and postconditioned within the setting of ischaemia, 2) Hearts could be pharmacologically pre- and postconditioned with levosimendan, 3) levosimendan pre-treatment is the most effective way to reduce infarct size, possibly acting by increasing the phosphorylation of ERK42/44, 4) Myocardial protection was not increased by combining IPC & LPC (suggesting similar mechanisms of protection), 5) LPC, LPT and LPostC were abolished by both sarcolemmal and mitochondrial K_{ATP} channel blockers.

.LPC and especially LPT, could be useful before elective cardiac surgery while LPostC may be considered after acute coronary artery events.

Key words: Levosimendan; myocardial infarct; myocardial function, RISK pathway; K_{ATP} channel; reperfusion injury.

UITTREKSEL

Agtergrond en doel van studie. Ten einde iskemiese skade van miokardium te voorkom, moet herperfusie so vinnig moontlik plaasvind, om sodoende infarkt-grootte te verlaag. Herperfusie opsigself gaan egter met nog verdere beskadiging gepaard, wat, bekend staan as herperfusie-beskadiging, wat optimale herwinning van die iskemiese miokardium voorkom. Herperfusie van reeds beskadigde miosiete kan dus die proses van selnekrose bespoedig.

Addisionele beskerming van die iskemiese miokardium in die vorm van gepaardgaande terapie, bly 'n onderwerp van intense navorsing. Levosimendan, 'n kalsium sensitiseerder met positiewe inotropiese effekte, is in verskeie studies gevind om die skadelike effekte van herperfusie te verlig. Levosimendan is ook 'n K_{ATP} kanaal oopmaker, wat ook een van die belangrike meganismes in iskemiese pre-kondisionering (IPC) is. Die doel van die studie was dus om vas te stel of levosimendan en IPC die iskemiese hart op dieselfde wyse beskerm en of farmakologiese pre-kondisionering, deur middel van levosimendan bewerkstellig kan word. In hierdie studie wou ons vasstel of: 1) die geïsoleerde marmothart deur middel van iskemiese pre-kondisionering (IPC) en iskemiese postkondisionering (IPostC) beskerm kan word, 2) die hart farmakologies pre- en postkondisioneer kan word deur middel van levosimendan (LPC en LPostC), 3) die kombinasie van IPC en LPC 'n additiewe beskermende effek op die iskemiese hart het, 4) die K_{ATP} kanale (beide sarkolemmaal en mitochondriaal) by hierdie beskerming betrokke is, 5) die pro-oorlewings kinases van die RISK (*reperfusion injury salvage kinase*) seintransduksiepad betrokke is.

Materiaal en tegnieke. Geïsoleerde geperfuseerde marmotharte is aan drie verskillende IPC protokolle (1x5, 2x5 and 3x5 minute van iskemie) of levosimendan (0.1 μ M) blootgestel, voor koronêre arterie afbinding (CAO – 40min@36.5°C), gevolg deur 30 minute van herperfusie. Harte is ook aan 'n kombinasie van IPC & LPC blootgestel, om vas te stel of dit 'n additiewe beskermingseffek het. Hierbenewens is harte vir 10 minute met levosimendan voorafbehandel, direk (sonder uitwas) voor langdurige iskemie (levosimendan voorafbehandeling (LPT)). Met die postkondisionerings-protokol, is die harte aan 3x30 sekonde siklusse van iskemie/herperfusie of levosimendan/draer, met die aanvang van herperfusie, blootgestel. In 'n aparte reeks eksperimente, is harte met K_{ATP} kanaal blokkers (vir beide sarkolemmale en mitochondriale), voor LPC, LPT en LPostC behandel. Die eindpunte was: kardiaale funksie, miokardiale infarktgrötte en RISK-seintransduksiepad (uitdrukking en fosforilering van PKB/Akt en ekstrasellulêre sein-regulerende kinase – ERK42/44).

Resultate. IPC, IPostC, LPC en LPostC het miokardiale infarktgrötte beduidend verlaag in vergelyking met die kontroles (21.9 \pm 2.2%, 21.4 \pm 2.2%, 20.6 \pm 3.1% en 20.6 \pm 1.8% vs. 46.4 \pm 1.8% vir kontroles, $p < 0.05$). Die kombinasie van IPC & LPC het geen additiewe beskermingseffek gehad nie. Voorafbehandeling van die harte met levosimendan (LPT), het die mees beduidende effek op infarktgrötteverlaging gehad (5.7 \pm 0.9% ($p < 0.001$)) en ERK42/44 aktiwiteit tydens herperfusie verhoog. Behandeling met K_{ATP} kanaal blockers, voor blootstelling aan LPC, LPT of LPostC, het hierdie verlagende effek van levosimendan op infarktgrötte opgehef, wat 'n definitiewe betrokkenheid van die sarkolemmale en mitochondriale K_{ATP} kanale in levosimendan-geïduseerde kardiobeskerming aandui.

Gevolgtrekkings en implikasies. 1) Marmotharte kan deur middel van iskemie pre- en postkondisioneer word, 2) Harte kan farmakologies met levosimendan pre- en postkondisioneer word, 3) Voorafbehandeling met levosimendan het die mees beduidende effek op infarkt-grootte-verlaging gehad, moontlik deur die aktiwiteit van ERK42/44 te verhoog, 4) Harte is nie ekstra beskerm deur IPC en LPC te kombineer nie, 5) Toediening van K_{ATP} -kanaal blokkers het beide LPC, LPT en LPostC opgehef, 6) LPC en veral LPT mag van toepassing wees in elektiewe kardiaal chirurgie, terwyl LPostC behandeling ná akute koronêre arterie episodes oorweeg moet word.

Sleutelwoorde: Levosimendan; miokardiale infarkt; miokardiale funksie, RISK- seintransduksiepad; K_{ATP} kanaal; herperfusie-skade.

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Table 5.3: Data for LPostC group (top – pre-ischaemic data and bottom – post-ischaemic data). Displayed as Mean ± SEM.

Table 5.4: Aortic output recovery (%) for the LPostC protocol.

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Table 6.2: Aortic output recovery (%) for pre-treatment with levosimendan.

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Table 8.1: Data for inhibition of K_{ATP} channels with 5-hydroxydecanoic acid (5HD) or glibenclamide (GBD) in levosimendan preconditioned hearts (LPC).

Table 8.2: Data for inhibition of K_{ATP} channels with 5-hydroxydecanoic acid (5HD) or glibenclamide (GBD) in levosimendan pre-treated hearts (LPT).

Table 8.3: Data for inhibition of K_{ATP} channels with 5-hydroxydecanoic acid (5HD) or glibenclamide (GBD) in levosimendan postconditioned hearts (LPostC).

Table 8.4: Functional recovery (% aortic output recovery) in control-5HD, levosimendan preconditioning (LPC)+5HD, levosimendan pretreatment (LPT)+5HD and levosimendan postconditioning (LpostC)+5HD groups.

Table 8.5: Functional recovery (% aortic output recovery) in LPC+glibenclamide (GBD), levosimendan preconditioning (LPC)+GBD, levosimendan pretreatment (LPT)+GBD and levosimendan postconditioning (LpostC)+GBD groups.

Table 9.1: Effect of pERK42/44 inhibition WITH PD 098059 on infarct size.

LIST OF ABBREVIATIONS

Units of measurement

%	-	percentage
°C	-	degrees celcius
µg	-	microgram
µl	-	microlitre
µM	-	micromolar
ADevP	-	aortic developed pressure
ADP	-	aortic-diastolic pressure
ASP	-	aortic-systolic pressure
bpm	-	beats per minute
g	-	gram
HR	-	heart rate
kDa	-	kilodalton
l	-	litre
LVEF	-	left ventricular ejection fraction
mg	-	milligram
min	-	minute
ml	-	millilitre
mm	-	millimeters
mmHg	-	millimeters mercury
mmHg	-	millimeters mercury
Qa	-	aortic output
Qe	-	coronary flow
sec	-	second

Chemical compounds, enzymes and peptides.

ADP	-	adenosine diphosphate
ATP	-	adenosine triphosphate
CaCl ₂	-	calcium chloride
cAMP	-	cyclic adenosine monophosphate
cGMP	-	cyclic guanosine monophosphate
CK	-	creatine kinase
CoA	-	co-enzyme A
CT	-	cardiotrophin
dH ₂ O	-	distilled water
DHE	-	dihydroethidium
DMSO	-	dimethyl sulfoxide
EDTA	-	ethylenediaminetetraacetic acid
EGF	-	epidermal growth factor
EGFR	-	epidermal growth factor receptor
EGTA	-	ethylene glycol tetra-acetic acid
ERK	-	extracellular signal-regulated kinase
ET-1	-	endothelin-1
FGF	-	fibroblast growth factor
GC	-	guanylate cyclase
GLUT	-	glucose transporter
GPCR	-	G protein coupled receptor
GSK	-	glycogen synthase kinase 3
GSK-3 β	-	glycogen synthase kinase-3 β
GTP	-	guanosine-5'-triphosphate

HGF	-	hepatic growth factor
HMG	-	hydroxyl-3-methylglutaryl
IGF	-	insulin-like growth factor
IL	-	interleukin
iNOS	-	inducible nitric oxide synthase
KCl	-	potassium chloride
KH ₂ PO ₄	-	potassium dihydrogen orthophosphate
L-NAME	-	N ω -Nitro-L-arginine methyl ester
MAPK	-	mitogen activated protein kinase
MAPKK	-	mitogen activated protein kinase kinase
MDA	-	malondialdehyde
MgCl ₂ 6H ₂ O	-	magnesium chloride 6-hydrate
MgSO ₄ 7H ₂ O	-	magnesium sulphate heptahydrate
Na Pyruvate	-	sodium pyruvate
Na ₂ SO ₄	-	sodium sulphate
NaCl	-	sodium chloride
NaHCO ₃	-	sodium hydrogen carbonate
NaVO ₃	-	sodium orthovanadate
NGF	-	nerve growth factor
NO	-	nitric oxide
NOS	-	nitric oxide synthase
ODQ	-	1-H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PDE	-	phosphodiesterase
PDGF	-	platelet derived growth factor
PDK	-	phospholipids-dependant protein kinase

PFK	-	phosphofructokinase
PI3-K	-	phosphoinositide 3-kinase
PKA	-	protein kinase A
PKB/Akt	-	protein kinase B
PKC	-	protein kinase C
PKG	-	cGMP-dependent protein kinase or Protein Kinase G
PMSF	-	phenylmethylsulphonyl fluoride
PVDF	-	polyvinylidene fluoride
ROS	-	reactive oxygen species
SUR	-	sulfonylurea receptor units
TBST	-	tris-Buffered Saline — 0.1% Tween 20
TGF	-	transforming growth factor
TNF- α	-	tumor necrosis factor- α
TOR	-	target of rapamycin
Tris	-	tris(hydroxymethyl)-aminomethan
TTC	-	triphenyltetrazolium chloride
VEGF	-	vascular endothelial growth factor

Other

ADHF	-	acute decompensated heart failure
AHD	-	acute heart decompensation
AHF	-	acute heart failure
AIDS	-	acquired immune deficiency syndrome
AMI	-	acute myocardial infarction
ANOVA	-	analysis of variance
AR	-	area at risk

CAO	-	coronary artery occlusion
CASINO	-	Calcium Sensitizer or Inotrope or None in Low-Output Heart Failure Study
CEAR	-	Committee for Experimental Animal Research
CVD	-	cardiovascular disease
ECG	-	electrocardiogram
ESC	-	The European Society of Cardiology
FM	-	forward mode
GIK	-	“cocktail” containing <u>glucose</u> , <u>insulin</u> and <u>potassium</u>
ICa(L)	-	inward L-type Ca ²⁺ current
IHD	-	ischaemic heart disease
IPC	-	ischaemic preconditioning
IPostC	-	ischaemic postconditioning
IUPAC	-	International Union of Pure and Applied Chemistry
IUPAC	-	International Union of Pure and Applied Chemistry nomenclature
KCa	-	Ca ²⁺ -activated K ⁺ channels
KV	-	voltage-dependent K ⁺ channels
LD	-	Langendorff
LIDO	-	Levosimendan Infusion Versus Dobutamine
LPT	-	levosimendan pre-treatment
LV	-	left ventricular
mitoK _{ATP}	-	mitochondrial adenosine triphosphate sensitive potassium channel
mPTP	-	mitochondrial permeability transition pore
NCX	-	Na ⁺ /Ca ²⁺ exchanger
NYHA	-	New York Heart Association

PCI	-	percutaneous coronary stenting or intervention
PCWP	-	pulmonary capillary wedge pressure
REVIVE	-	Randomized Multicenter Evaluation of Intravenous Levosimendan Efficacy Versus Placebo in the Short-Term Treatment of Decompensated Heart Failure
RUSSLAN	-	Randomized Study on Safety and Effectiveness of Levosimendan in Patients with Left Ventricular Failure Due to an Acute Myocardial Infarct
sarck _{ATP}	-	sarcolemmal adenosine triphosphate sensitive potassium channel
SDS-PAGE	-	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SURVIVE	-	Survival of Patients with Acute Heart Failure in Need of Intravenous Inotropic Support
WH	-	working heart
WHO	-	World Health Organization

CHAPTER ONE

INTRODUCTION

According to World Health Organization (WHO) estimates 17.5 million people died of cardiovascular disease (CVD) in 2005 (2008 update). This amounts to 30% of all deaths globally (WHO 2008). Between 1997 and 2004, 195 South Africans died of some form of heart and blood vessel disease every day (Bradshaw *et al.*, 2000). Although deaths caused by AIDS are a major concern for the future in South Africa, actuarial projections suggest that the rate of chronic diseases, including heart disease, will also increase by 2010. This model suggests that the rate of chronic disease deaths will increase from 565 deaths per day in 2000, to 666 deaths per day in 2010 (Bradshaw *et al.*, 2000). Thus acute myocardial infarction (AMI) represents a major cause of death and heart failure in industrialized countries (McGovern *et al.*, 1996) as well as in South Africa. Ischaemic heart disease (IHD) or myocardial ischaemia, is a disease characterized by reduced blood supply to the heart muscle, usually due to coronary artery disease. Ischaemia can be due to an absolute or relative shortage of the blood supply to an organ. Relative shortage means a mismatch between the supply and demand of blood, with a consequent inadequate oxygenation of tissue. Ischaemia results in tissue damage because of a lack of oxygen and nutrients and ultimately, this causes considerable damage because of a buildup of metabolic waste.

Hearts that were subjected to periods of sustained ischaemia have been shown to become apoptotic. This form of cell death differs from other forms that occur in response to toxins, physical stimuli and ischaemia (Fliss *et al.*, 1996; Haunstetter *et al.*, 1998). Thus coronary artery disease, if severe enough, causes ischaemic damage and leads to myocardial infarction. In order to protect the ischaemic myocardium against further damage, it needs to be reperfused to reinstate blood flow, as soon as possible. It has been shown that early reperfusion improves myocardial recovery. However, reperfusion itself results in complex phenomena that appear to be deleterious and is referred to as reperfusion injury (Braunwald *et al.*, 1985). There is thus a definite need for therapy to provide better cardioprotection during reperfusion (Kloner and Kloner, 2004). The challenge lies in understanding the mechanisms of ischaemic/reperfusion injury and identifying

therapeutic interventions to minimize the damage caused to the myocardium by this phenomenon.

The concept of cardioprotection was introduced over a quarter of a century ago and since then a variety of interventions have been shown to reduce myocardial infarct size (Kloner *et al.*, 2004). For example, intravenous beta-blockers or adenosine seems to be beneficial for arterial wall AMI's and the infusion of the GIK (glucose-insulin-potassium) cocktail was particularly beneficial in diabetics and percutaneous coronary stenting or intervention (PCI) (Fath-Ordoubadi and Beat, 1997). The focus of current studies are thus to enhance the clinical outcome of existing therapies like adenosine, K_{ATP} channel openers, Na^+/H^+ exchange inhibitors and hypothermia (Kloner *et al.*, 2004). Another cardioprotective intervention, ischaemic preconditioning, was first described by Murray *et al.* in 1986. Ischaemic preconditioning is a procedure where the heart is subjected to transient non-lethal short episodes of myocardial ischaemia interspersed with equally brief periods of reperfusion that confers protection against myocardial infarction caused by a subsequent sustained period of ischaemia. The problem with this intervention is that it needs to be applied before the onset of an ischaemic event to be efficient and this is highly unpredictable in real life (Hausenloy *et al.*, 2005a). A more practical intervention would be one that could be applied after the ischaemic event, during reperfusion of the ischaemic myocardium. The phenomenon of postconditioning was recently described by Vinten-Johansen and colleagues (Zhao *et al.*, 2003). This intervention involves the application of brief intermittent episodes of alternating ischaemia and reperfusion, within the first five minutes of reperfusion directly after sustained ischaemia. This led to a significant reduction in infarct size. Subsequent studies have also shown that postconditioning also reduces apoptotic cell death, endothelial dysfunction, oxidative stress and neutrophil accumulation, all caused by reperfusion injury (Zhao *et al.*, 2003; Kin *et al.*, 2004). Despite the advances made with the above described interventions, the search for adjunct therapy is still an ongoing process and the exact mechanisms involved in cardioprotection by means of pre- and postconditioning are of the most intensely investigated topics in current cardiovascular research.

Previous studies have shown that by inhibiting the ERK42/44 component of the reperfusion injury salvage kinase (RISK) pathway, the cardioprotective effects induced by ischaemic preconditioning could be abolished (Yang *et al.*, 2004a; Yang *et al.*, 2004b). In various studies investigating the phenomenon of postconditioning, it was also shown that this intervention during reperfusion phosphorylates ERK42/44. In one of these studies performed on pigs, it was shown that although IPostC phosphorylated ERK42/44 in reperfusion, it did not protect the heart against ischaemic/reperfusion injury (Shchwartz LM and Lagranha CJ, 2005). However, in most other studies the phosphorylation of ERK42/44 by IPostC has been shown to be protective of the reperfused myocardium (Tsang *et al.*, 2004; Yang *et al.*, 2004b).

Levosimendan, a calcium sensitizing agent, has also been used to improve cardioprotection after an ischaemic event. Levosimendan is a K_{ATP} channel opener in smooth muscle cells (Yokoshiki *et al.*, 1997; Kopustinskiene *et al.*, 2001) and therefore has the ability to protect the heart against ischaemic/reperfusion injury (Cammarata *et al.*, 2006). In this study, we investigated the efficacy of levosimendan as a pre- and postconditioning mimetic. Levosimendan is a new Ca^{++} -sensitizing and positive inotropic agent and has been reported to act as a coronary vasodilator (Ng, 2004) and to protect the ischaemic myocardium (Pollesello and Mebazaa, 2004; Pollesello and Papp, 2007). Levosimendan is now clinically used for the treatment of acute decompensated heart failure and is on the market in more than 40 countries. Levosimendan is a distinct calcium sensitizer, as it stabilizes the interaction between calcium and troponin C by binding in a calcium-dependent manner to troponin C, improving inotropy. It increases the heart's sensitivity to calcium, thus increasing cardiac contractility without a rise in the intracellular calcium. The combined inotropic and vasodilatory actions result in an increased force of contraction, decreased preload and decreased afterload (Jørgensen *et al.*, 2008).

In view of the above data, we concluded that it is possible that levosimendan may mimic the significant cardioprotective actions of ischaemic pre- and postconditioning.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of cardiovascular disease.

2.1.1 Cardiovascular disease and its incidence in the Western world

The incidence of CVD in most Western countries is high and still increasing. Current data give a very clear picture of the threat that the increasing rate of CVD poses to the Western world. It is significant that heart disease kills more Americans than cancer (Chronic Disease Overview – United States, 1999). Until 2005 it was the leading cause of death in America and most European countries. In South Africa CVD is the second largest cause of deaths (AIDS being the leading cause) and in the Western Cape CVD is the highest cause of mortality (Bradshaw *et al.*, 2000). Cardiovascular disease (25%) was the leading cause of death among both men and women, followed by malignant neoplasms (16%), infectious and parasitic disease excluding HIV/AIDS (10%), intentional injuries (9.7%), HIV/AIDS (8.4%), and unintentional injuries (7.5%) (Bradshaw *et al.*, 2000).

Cardiovascular disease (CVD) involves the heart and the blood vessels (arteries and veins) and this term technically refers to any disease that affects the cardiovascular system as a whole (Maton *et al.*, 1993). Although cardiovascular disease encompasses a variety of abnormalities, the focus of this study will be on the adverse consequences of ischaemia and methods of reducing ischaemic/reperfusion injury.

2.1.2 Major risk factors for CVD

The high incidence of CVD globally and especially in South Africa, has led to a necessity of knowledge of the risk factors that would increase a person's probability for developing CVD. The major risk factors for CVD can be divided into two distinct groups: those we cannot change and those we can. Heredity (including ethnicity), gender (men have a greater risk of heart attack than premenopausal women) and increasing age (about four out of five people who die of a heart attack are over 65), fall into the former group.

However, there are several independent risk factors for the development of CVD that can be altered. They are hypercholesterolemia (specifically low-density lipoprotein (LDL) levels in the serum) (Durrington *et al.*, 2003), hypertension, hyperglycemia (as occurs in diabetes), smoking and behavioral patterns/personality types. Personality types are divided into two distinct groups: type A: time-conscious, highly competitive, direct and assertive and less relaxed; type B: not time-conscious, avoiding confrontation and easy-going (Friedman *et al.*, 1974). The type A behavioural pattern is said to be twice as likely to cause CVD than type B. Hemostatic factors, such as high levels of fibrinogen and coagulation factor VII are also associated with and increased risk for CVD (Thomas *et al.*, 1988).

2.1.3 Consequences of CVD – myocardial ischaemia and infarction

This study will focus mainly on the adverse consequences of myocardial ischaemia and methods of reducing ischaemic/reperfusion injury.

One of the adverse consequences of CVD is a myocardial infarction. An acute myocardial infarction (AMI) or in layman's terms a heart attack, occurs when the coronary blood flow to the heart is severely reduced. This can occur due to: tachycardia (abnormally rapid beating of the heart), atherosclerosis (lipid-laden plaques obstructing the lumen of arteries), hypotension (low blood pressure, e.g. in septic shock, heart failure), thromboembolism (blood clots), outside compression of a blood vessel, e.g. by a tumor, embolism (foreign bodies in the circulation, e.g. amniotic fluid embolism) or "sickle cell" disease (abnormally shaped hemoglobin) (Lynch *et al.*, 1996).

An infarction is the process resulting in a macroscopic area of necrotic tissue in some organ (in this case the heart) caused by loss of adequate blood supply and consequent ischaemia. The term *infarction* is derived from the Latin "infarcire" meaning "to plug up or cram" and it refers to the clogging of the artery (Webster's New World Medical Dictionary, 3rd Edition, 2008). If there is no intervention to reintroduce coronary blood flow, this ischaemic event can lead to tissue death due to various metabolic and ultrastructural changes. Coronary reperfusion has proven to be the only way to limit infarct size, provided it occurs soon enough after

coronary artery occlusion. However, there is also evidence that reperfusion is not without several detrimental consequences that are collectively known as “reperfusion injury”. Reperfusion injury classically manifests itself as myocardial stunning, reperfusion arrhythmias and detrimental reperfusion (Piper *et al.*, 1998).

2.1.4 Myocardial consequences of ischaemia

When blood flow to the myocardium is interrupted, several detrimental events occur. Blood is the only supplier of oxygen and substrates to the heart. In myocardial ischaemia (MI), the coronary flow decreases as a primary event and is then followed by severe cellular hypoxia as a secondary event (Opie, 1991). The major consequences of myocardial ischaemia can be attributed to insufficient oxygen supply and the poor washout of metabolites. Immediately, after the onset of ischaemia, there are a variety of complex metabolic changes that include the depletion of energy stores (Puri *et al.*, 1975; Jennings and Ganote, 1976; Schaper *et al.*, 1979) and a build-up of metabolic by-products (Neely *et al.*, 1973). These by-products include lipid metabolites (Corr *et al.*, 1984), excess intracellular Ca^{2+} (Clusin *et al.*, 1983; Nayler *et al.*, 1988) and reactive oxygen species (ROS) (Hess and Manson, 1984; Kako, 1987). There is also a loss of K^+ (Venkatesh *et al.*, 1991) and intracellular Mg^{2+} levels increase (ATP normally occurs as the magnesium complex). An ischaemia-induced increase in cytosolic Na^+ levels is in turn responsible for the increase in cytosolic Ca^{2+} via infarction of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Murphy *et al.*, 1990). The high intracellular calcium concentrations, leads to a failure of the myocardium to relax and subsequent myocardial stiffening. High cytosolic Ca^{2+} levels also activate Ca^{2+} -dependent enzymes such as calcineurin, and trigger pathological responses, such as hypertrophy (Marks, 2000).

Depending on its severity, ischaemia usually has a biphasic effect on glycolysis. Stimulation is the first step, then, as ischaemia becomes more severe, delivery of glucose decreases, glycogen becomes depleted and inhibitory metabolites accumulate (this leads to a decrease in the glycolytic rate) (Opie, 2004). During mild ischaemia, glycolysis is stimulated at several levels, including translocation of GLUT-1 and -4 to the sarcolemma, while the activity of the key enzyme

phosphofructokinase increases so that the glycolytic rate increases as the energy declines (Voldersa *et al.*, 2000).

Ischaemia has two major effects on fatty acid metabolism. First it leads to an accumulation of lipid metabolites (including intracellular free fatty acids, acyl-CoA and acylcarnitine). As the tissue contents of these metabolites increase, they inhibit various aspects of membrane function (such as mitochondrial translocase, the sodium pump and phospholipid cycles). Secondly, membrane phospholipids are broken down and high concentrations of the breakdown product accumulate to form micelles, which are highly membrane active.

All these metabolic changes lead to the activation and opening of the K_{ATP} channel to open. The opening of this channel is normally inhibited by high levels of intracellular ATP as occurs during normal conditions (Lederer *et al.*, 1989). Due to the metabolic changes described above, the sarcolemmal K_{ATP} ($sarCK_{ATP}$) channels open and there is a considerable outflow of potassium ions from the cell. This process forms the basis of membrane depolarization in the ischaemic area and can be detected as the early ECG changes induced by ischaemia (Lederer *et al.*, 1989).

2.1.5 Conventional treatment of CVD and the adoption of more novel approaches and drugs

Myocardial ischaemia and the resultant infarction, will eventually lead to the loss of cardiac muscle which will ultimately lead to heart failure (HF). The conventional treatment for acute heart failure has remained unchanged for many years. Conventional medical treatment consists of oxygen supplementation or restoration of blood flow (reperfusion of the ischaemic myocardium) and mechanical ventilatory support, as well as the administration of drugs that include diuretics, morphine, nitrates and inotropic agents. The European Society of Cardiology (ESC), recently published new guidelines regarding the diagnoses and treatment of CVD (Swedberg *et al.*, 2005). In addition, new therapies for the treatment of CVD were developed and this led to a revolutionary therapeutic approach and new concepts of CVD (Hodt *et al.*, 2006). The current role for the traditional drugs for the treatment of CVD is described. In addition the role of newer approaches such

as vasodilators, endothelin or vasopressin agonists and the new inotropic agents that include the calcium sensitizer levosimendan are discussed (Grimm *et al.*, 2006). What sets this newer inotropic agent apart from the rest? The dynamics and potential of levosimendan as a promising, more efficient treatment for patients with CVD will be discussed in more detail later in this chapter.

2.2 Effective interventions for the treatment of myocardial ischaemia.

Effective and already established interventions for the treatment of patients with cardiac ischaemia include oxygen, opioid (such as morphine or diamorphine), nitrates and diuretics administration. Traditional inotropes improve contractility by increasing intracellular calcium that can bind to cardiac troponin C at the expense of increasing myocardial energy and oxygen demand, thereby increasing the risk for arrhythmias (Ioannou and La Wyncoll, 2004). There have also been numerous studies on the effects of traditional calcium sensitizers, such as MCI 154, EMD 57033 and EMD 60263 on the function of the postischaemic heart (Abe *et al.*, 1995; De Zeeuw *et al.*, 2000; Soei *et al.*, 1994), showing an improvement in functional recovery after a severe ischaemic event. Calcium sensitizers could potentially be particularly useful in the setting of ischaemia and reperfusion if the possible pro-arrhythmic effect of these drugs can be excluded (Du Toit *et al.*, 2001).

2.2.1 Preconditioning

One of the most elaborate interventions that has been the main focus of extensive research and publications is the phenomenon of preconditioning. This phenomenon was first described in 1986 and consisted of exposure of the heart to brief periods of ischaemia/reperfusion to elicit a cardioprotective response which protected the heart against subsequent sustained ischaemia (Murray *et al.*, 1986). This resulted in a significant reduction in infarct size. The cardioprotective effect of preconditioning has been demonstrated in both models of regional and global ischaemia in the human heart (Yellon *et al.*, 1993; Jenkins *et al.*, 1997; Szmagal *et al.*, 1998; Laurikka *et al.*, 2002). Cardioprotection elicited within 1 – 3 hours after the preconditioning algorithm is applied is called classic preconditioning (Murray *et al.*, 1991). A second phase of protection, the so-called “second window of

protection” was observed (from 24-36 hours) after ischaemic preconditioning (Yamashita *et al.*, 1998). Preconditioning is not restricted to the myocardium, but can also be elicited in other tissues such as neuronal tissue and the small intestine (Yellon *et al.*, 1998). In the current study the focus was on classic preconditioning in the myocardium, initiated by one or more brief cycles of ischaemia/reperfusion or by pharmacological interventions (pharmacological preconditioning).

2.2.1.1 Ischaemic preconditioning

Several preconditioning protocols have been shown to be effective. The preconditioning protocol may involve 4 x 5 minute cycles of ischaemia, separated by 4 x 5 minute cycles of reperfusion (Murray *et al.*, 1986), 2 x 2 minute cycles of ischaemia/reperfusion or 1 x 5 minute cycle of ischaemia/reperfusion (Li *et al.*, 1990; Yellon *et al.*, 1992). A repetition of the 5 minute cycle substantially increases the protection against myocardial infarction (Yang *et al.*, 1997). It has however been noted that at least one minute of reperfusion is needed before sustained ischaemia is induced to elicit the effect of cardioprotection (Yang *et al.*, 1993).

2.2.1.2 Cardiac consequences of IPC – manifestations of protection

In the numerous studies investigating IPC, a reduction in myocardial infarct size was the most frequently used endpoint to demonstrate the protective effects of IPC (Murray *et al.*, 1986; Thornton *et al.*, 1990; Yellon *et al.*, 1992). A reduction in infarct size for the evaluation of the efficacy of preconditioning is however not always associated with improved functional recovery. Although, an improvement in functional recovery of the isolated working rat heart subjected to global ischaemia has been observed in many studies and used as alternative endpoint (Cave *et al.*, 1992; Csonka *et al.*, 1999; Goto *et al.*, 1992; Volovsek *et al.*, 1992), infarct size was found to be a more reliable and robust endpoint than functional recovery in preconditioning studies (Lochner *et al.*, 2003). Reduction in infarct size is only found after a temporary occlusion and not during a permanent occlusion (Yellon *et al.*, 1998).

Infarct size is a measure of necrotic tissue (Fishbein *et al.*, 1981). As far as the processes of apoptosis and necrosis are concerned, however IPC does not elicit a reduction in apoptotic/necrotic cell death (Gottlieb *et al.*, 1996). It has also been found that, in the isolated rat heart, IPC leads to a marked reduction in ischaemia/reperfusion induced arrhythmias (Liu *et al.*, 1992b).

Although IPC reduces infarct size and improves functional recovery during reperfusion of the globally ischaemic heart, it does not protect the heart against myocardial stunning (a post-ischaemic contractile dysfunction) (Jenkins *et al.*, 1995; Ovize *et al.*, 1992). Contractile function after prolonged coronary occlusion may be confounded by two important factors: 1) the presence of subendocardial necrosis, which influences wall motion in the surrounding viable myocardium and 2) the preconditioning regimen (i.e., repeated brief episodes of ischaemia/reperfusion) results in contractile dysfunction before sustained ischaemia and may thereby limit or mask a beneficial effect of preconditioning on wall motion (Ovize *et al.*, 1992).

2.2.1.3 The different phases of IPC

According to Downey and coworkers (2008), the preconditioning process can be divided into two phases, a trigger and a mediator phase. Traditionally the trigger phase of IPC was said to be before index ischaemia and the mediator phase during index ischaemia and reperfusion. In a recent review of Downey *et al.* (2008), the mediator phase was shown to be restricted to reperfusion only. In IPC the trigger phase comprises of events before the index ischaemic event. It is believed that PKC activation is the end of the trigger phase and its kinase activity is the first step of the mediator phase (see figure 2.1).

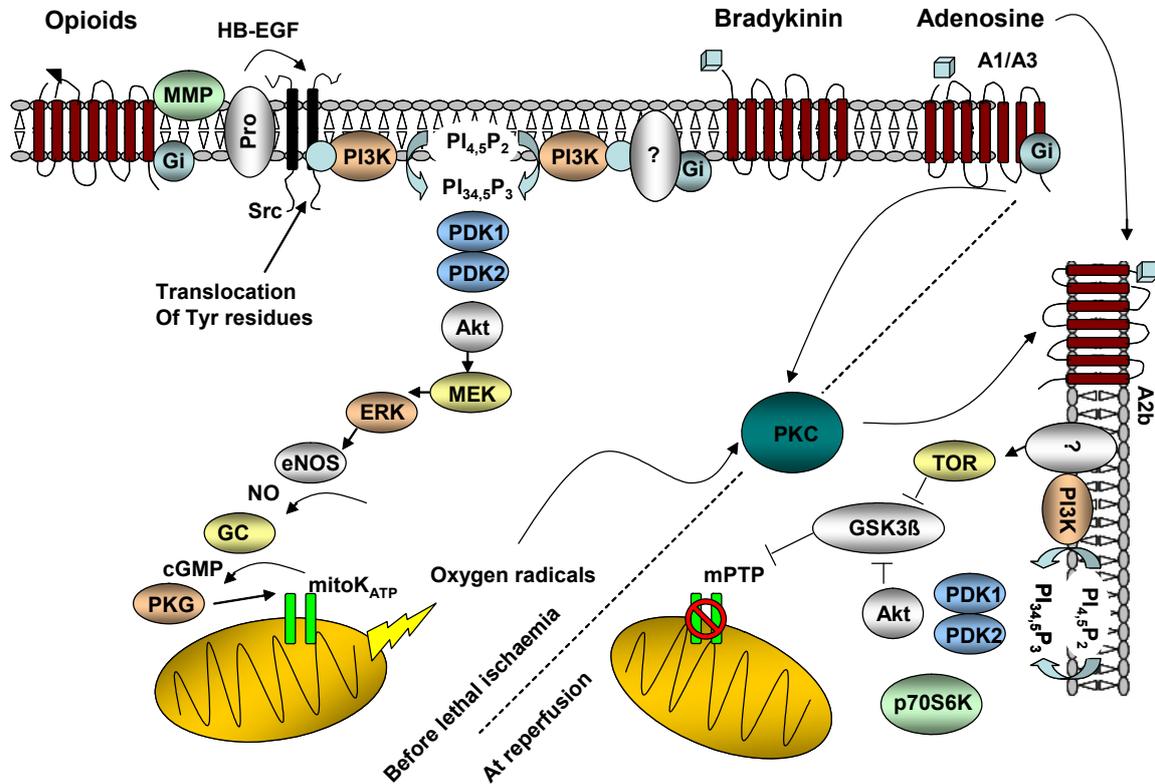


Figure 2.1: An illustration showing the sequence of signalling events involved in triggering the preconditioning state prior to the ischaemic insult and those that mediate protection in the first minutes of reperfusion (illustration adapted from reprinted illustration from Downey *et al.*, 2008; original illustration from Tissier *et al.*, 2007a).

1) The triggers of IPC

In describing the triggers of IPC it is important to note that there are two kinds of triggers, namely receptor dependent and receptor independent triggers. The triggers mentioned in this section (TNF α and ROS) can have damaging effects (depending on particular context and concentrations), but in this particular context they only act as transient initiators of signaling pathways involved in cardioprotection.

The threshold hypothesis in the triggering of IPC (Goto *et al.*, 1995) postulates that the triggers of IPC are connected to receptors, which are all coupled to G-Proteins that activate the post-receptor signalling system leading to PKC activation. All the signals from the different receptors must then reach a certain threshold in order to elicit cardioprotection. Paracrine/autocrine factors that trigger IPC include adenosine, catecholamines, bradykinin, acetylcholine, opioid peptides, endothelin and angiotensin II (Cohen *et al.*, 2000). It is also noted that cytokines can trigger

IPC. Cytokines are regulatory proteins and play an important role in the immune- and inflammatory responses. Cytokines such as tumor necrosis factor- α (TNF- α) and interleukins- 1 β and -6, trigger IPC by stimulating protein kinase C (PKC) the mitogen activated protein kinase (MAPK) cascade and the production of reactive oxygen species (ROS) (Smith *et al.*, 2002). Receptor independent triggers include oxygen-derived free radicals (Das *et al.*, 1999) and nitric oxide (NO) (Bolli, 2001; Rakhit *et al.*, 1999). Opening of the mitochondrial K_{ATP} channel has also been suggested as a trigger of IPC (Cohen *et al.*, 2000; Fryer *et al.*, 2000a), but its position in the sequence of events that leads to cardioprotection after IPC is not fully elucidated.

2) The signalling system of IPC

In studying the signal transduction pathways implicated in the protection during index ischaemia, the fact that not all the steps in PC signalling are arranged in series, confounds our ability to conclusively outline the signalling pathways. Several signalling pathways are involved, acting to attenuate ischaemic/reperfusion injury. However, in a recent study by Downey *et al.* (2008), they were able to produce a fairly comprehensive “map” of the signalling pathways that are involved in triggering IPC (see figure 2.1).

The role of mitochondrial K_{ATP} channels in IPC

For several years the mitochondrial K_{ATP} channel (mito K_{ATP}) has been the main focus of research as an end-effector of IPC (Cohen *et al.*, 2000; Fryer *et al.*, 2000a; O'Rourke, 2000). However the current viewpoint is that it plays an important role upstream of the mPTP and that it may act as a trigger, rather than end-effector. The heart contains two potassium channels that are regulated by the metabolic state of the cell, a sarcolemmal and a mitochondrial channel which have been termed the sarcolemmal (sarco K_{ATP}) and mitochondrial K_{ATP} (mito K_{ATP}) channels, respectively. (Gross and Auchampach, 1992). Both channels are regulated by the intracellular concentration of ATP and other nucleotides and have been shown to play an important endogenous protective role against irreversible tissue damage. Initially, it was hypothesized that the surface or sarcolemmal K_{ATP} (sarco K_{ATP}) channel mediated protection observed after IPC; however, subsequent

evidence suggested that the recently identified mitochondrial K_{ATP} channel (mito K_{ATP}) may be the structure mediating IPC-induced cardioprotection (Gross and Fryer, 1999). By blocking these channels with their respective blockers, the cardioprotective effect of IPC could be abolished (Jabûrek *et al.*, 1998; Janin *et al.*, 1998).

Cyclic guanosine monophosphate (cGMP) acts as a second messenger to activate the serine/threonine kinase cGMP-dependant kinase (PKG). It was shown that by incubating isolated mitochondria in a solution containing cGMP and PKG the mito K_{ATP} channel could be opened (Garlid *et al.*, 2003).

The role of PI3-kinase (PI3-K) and epidermal growth factor (EGF) in IPC

In a study by Tong *et al* (2000), it was shown that IPC no longer protects the ischaemic heart, when PI3-K is inhibited by Wortmannin. EGF binds to its receptor and causes EGFR monomers to dimerize and transphosphorylate each other's tyrosine groups (transactivation). These phosphorylated tyrosine groups leads to the assembly of a signalling complex containing activated PI3-K among other things (Downey *et al.*, 2008). PI3-K phosphorylates phosphatidylinositol in three positions. This phosphorylated lipid then activates phospholipids-dependant protein kinase (PKC 1 & 2) which in turn phosphorylates a number of target proteins, including PKB/Akt (a component of the RISK pathway). The unresolved question is how G_1 proteins activate PI3-K. This probably occurs by transactivation of the EGFR. For example, opioid receptors triggered protection by IPC through the EGFR, but not bradykinin. None of the inhibitors of the EGFR signalling affected bradykinin's ability to protect the heart (Cohen *et al.*, 2007).

A role for ROS and NO in IPC

ROS is one of the important role players in IPC. Two different studies have shown that direct administration of ROS could precondition hearts (Baines *et al.*, 1997; Tritto *et al.*, 1997). One of the targets for ROS has been assumed to be PKC as it has been shown to be directly activated by ROS (Gopalakrishna and Jaken, 2000; Korichneva *et al.*, 2002). Both studies found that the protection was PKC-dependent and must therefore be downstream of ROS.

NO plays an important role in ROS production. NO is synthesized by the activation of nitric oxide synthase (NOS). NO acts as a second messenger to activate soluble guanylyl cyclase which in turn generates cyclic GMP (cGMP) from guanosine-5'-triphosphate (GTP). Oldenburg *et al.* (2004) demonstrated that the protection induced by bradykinin could be blocked by administration of the NOS inhibitor N ω -Nitro-L-arginine methyl ester (L-NAME) and the guanylyl cyclase inhibitor 1-H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), confirming the involvement of NO in IPC.

PKA

Although PKC activation has long been advocated as the main signal transduction pathway in IPC, this was questioned in several other studies (Moolman *et al.*, 1996; Brooks *et al.*, 1996; Zimkhovich *et al.*, 1998). In a more recent study it was proved that the protection elicited by IPC may be partially dependent on activation of the β -adrenergic signalling pathway, which implies a role for PKA (Lochner *et al.*, 1999). A role for PKA is also supported by previous findings that stimulation with norepinephrine or isoproterenol mimics ischaemic preconditioning (Asimakis *et al.*, 1994). Forskolin also elicits cardioprotection by increasing cyclic adenosine monophosphate (cAMP) in a compartmentalized manner (Worthington *et al.*, 1992), which in turn leads to the activation/phosphorylation of PKA (Lochner *et al.*, 1999).

3) The mediator pathway

The trigger pathways implicated in IPC are fairly well defined, but little is known about the mediator pathways and only a few components have been identified (Downey *et al.*, 2008). It was found that activation of PI3-K and ERK42/44 is required for cardioprotection in the first few minutes of reperfusion (Hausenloy *et al.*, 2005b). These pro-survival kinases have been shown to inhibit the opening of the mitochondrial permeability transition pore (mPTP) by phosphorylation of glycogen synthase kinase-3 β (GSK-3 β) (Juhaszova *et al.*, 2004). It was found that at reperfusion, PI3-K is controlled by adenosine receptors (Solenkova *et al.*, 2006) which are in turn controlled by PKC. The adenosine receptors that play a role at reperfusion appears to be the A_{2b} subtype (Kuno *et al.*, 2007) (in contrast to sustained ischaemia when it activates PKC via the adenosine A₁ and A₃

receptors). To support this hypothesis, Eckle *et al.* (2007) showed that A_{2b}-knockout hearts could not be protected by IPC. According to Hausenloy *et al.*, (2002) IPC decreased Ca²⁺ overload and reactive oxygen species (ROS) production at reperfusion so that the probability of mPTP opening was reduced.

2.2.2 Postconditioning

In many ways postconditioning is considered to be a much more clinically relevant intervention for cardioprotection than preconditioning. Although extensive studies have been done in the area of preconditioning and its major success was in the salvaging of the ischaemic myocardium, its clinical application is limited (Verdouw and Duncker, 1995; Yellon and Downey, 2003; Riksen *et al.*, 2004; Sato *et al.*, 2007). In other words, in order for preconditioning to have a cardioprotective effect by reducing infarct size, it would have to be applied just before the patient experienced an ischaemic event. There is thus a definite need to extend the borders of research on cardioprotection in order to find a clinically applicable intervention and possible pharmacotherapies that can salvage the post-ischaemic myocardium. Reperfusion remains the definitive treatment for the ischaemic myocardium but, once perfusion is restored to the ischaemic myocardium, this in itself causes further damage that is known as reperfusion injury. Reperfusion injury prevents the optimal salvage of the ischaemic myocardium during cardiac surgery, percutaneous coronary intervention, or cardiac transplantation. Thus protecting the already vulnerable ischaemic myocardium against further damage caused by reperfusion injury has become a high priority for both cardiologists and cardiac surgeons. All strategies initiated before or at the onset of reflow including the administration of drugs at reperfusion can be classified as modifications of reperfusion.

It has been known for more than 20 years that reperfusion damage could be modified by slowly initiating reflow (Okamoto *et al.*, 1986). Infarct size was reduced, post-ischaemic contractile function was restored and edema reduced in the area at risk (Okamoto *et al.*, 1986; Vinten-Johansen *et al.*, 1992; Sato *et al.*, 1997). Postconditioning is an intervention that can modify reperfusion-induced adverse effects by simply moving the preconditioning “stimulus” from before the onset of prolonged ischaemia to the onset of reperfusion (IPostC). In initial

experiments investigating IPostC, the cycles of ischaemia/reperfusion were 5 minutes which failed to reduce infarct size (Vinten-Johansen *et al.*, 2005). Hence the phenomenon of postconditioning was not revisited for several years. In the meantime, it was found that many adverse events occurred early after the onset of reperfusion. These events include: (1) Oxygen free radicals being generated within minutes of reperfusion (Zweier *et al.*, 1987); (2) neutrophils being activated and adhering to the coronary vascular endothelium; (3) damage to the coronary vascular endothelium worsened as reperfusion continued (Tsao *et al.*, 1990); and (4) calcium dyshomeostasis which caused rapid damage to cell structures (Piper *et al.*, 2004). The reperfusion/occlusion cycles were subsequently reduced from several minutes to seconds (30 seconds in dogs and 10 seconds in rats and mice) and postconditioning was shown to be effective in reducing infarct size (Vinten-Johansen *et al.*, 2005).

In one of the first studies in postconditioning published in 2003 (Zhao *et al.*, 2003) researchers presented evidence that repetitive cycles of briefly interrupted reperfusion (3 x 30 seconds ischaemia/reperfusion) applied at the onset of coronary reflow (i.e., postconditioning) significantly reduce infarct size. This reduction in infarct size was corroborated by a similar decrease in plasma creatine kinase (CK) levels and attenuation in tissue edema. In addition, postconditioning reduced neutrophil accumulation in the “area at risk” (AR) of the myocardium and preserved post-ischaemic coronary artery endothelial function, assessed by vasodilatory responses to acetylcholine and adherence of unactivated neutrophils to the endothelial surface. Postconditioning was associated with a significant decrease in malondialdehyde (MDA)-reactive products of lipid peroxidation and with less intensity of dihydroethidium (DHE) staining (detection of superoxide) of endothelium and myocytes in the AR myocardium. In another study all experiments were conducted using the isolated buffer-perfused rabbit heart model of regional myocardial ischaemia (Darling *et al.*, 2005). Using this model, postconditioned hearts were subjected to 30 minutes of index ischaemia, followed by a 4-minute postconditioning stimulus (which consisted of four alternating 30-s cycles of reperfusion and subsequent coronary reocclusion) and were freeze-clamped after 1 minute or 3 minutes of full reperfusion. In this study, the authors have demonstrated significant cardioprotection with postconditioning. They also

reported that infarct size reduction with postconditioning was abolished by the ERK42/44 inhibitor PD-98059, but not by the PI3-K antagonist LY-294002. These findings were consistent with their observation of increased immunoreactivity of phospho-ERK42/44, but not phospho-PKB/Akt during the initial minutes of reperfusion. These data thus implicate the involvement of ERK42/44 signalling in the reduction of infarct size achieved with postconditioning in isolated rabbit heart.

The isolated buffer-perfused rat heart can also be postconditioned using a working heart model (Van Vuuren, 2007). A postconditioning protocol of 10 x 6 seconds of ischaemia/reperfusion caused a significant reduction in infarct size. However, it was found that accurate temperature control during the postconditioning phase was essential for reproducible results (Van Vuuren, 2007).

2.2.2.1 The mechanisms of ischaemic postconditioning (IPostC)

A prerequisite for IPostC is that the intervention applied must protect the ischaemic myocardium against the potential mediators of ischaemic/reperfusion injury. These mediators are discussed in the following paragraphs.

The mediators of ischaemic reperfusion injury

1) The oxygen paradox - Experimental studies have established that the reperfusion of the ischaemic myocardium generates oxidative stress, which itself can mediate myocardial injury (Zweier *et al.*, 1988). Oxidative stress is part of the oxygen paradox, in which the re-oxygenation of ischaemic myocardium generates a degree of myocardial injury that greatly exceeds the injury induced by ischaemia alone (Hearse *et al.*, 1973). Oxidative stress during myocardial reperfusion also reduces the bioavailability of the intracellular signalling molecule, nitric oxide, thereby removing its cardioprotective effects. These effects include the inhibition of neutrophil accumulation, inactivation of superoxide radicals, and improvement of coronary blood flow (Zweier *et al.*, 2006).

2) The calcium paradox - At the time of myocardial reperfusion, there is an abrupt increase in intracellular Ca^{2+} , which is secondary to sarcolemmal-membrane damage and oxidative stress-induced dysfunction of the sarcoplasmic reticulum. These two forms of injury overwhelm the normal mechanisms that

regulate Ca^{2+} levels in the cardiomyocyte and this phenomenon is known as the calcium paradox (Piper *et al.*, 1998). The result is intracellular and mitochondrial Ca^{2+} overload, and this excess Ca^{2+} induces cardiomyocyte death by causing hypercontracture of the heart cells and mPTP opening (Piper *et al.*, 1998).

3) The pH paradox - The rapid restoration of physiologic pH during myocardial reperfusion, which follows the washout of lactic acid and the activation of the sodium–hydrogen exchanger and the sodium–bicarbonate symporter contributes to detrimental reperfusion injury. This phenomenon is known as the pH paradox (Lemasters *et al.*, 1996).

4) Inflammation - After AMI, the release of chemo-attractants draws neutrophils into the infarct zone during the first 6 hours of myocardial reperfusion, and during the next 24 hours they migrate into the myocardial tissue. This process is facilitated by cell-adhesion molecules. These neutrophils cause vascular plugging and release degradative enzymes and reactive oxygen species (Vinten-Johansen *et al.*, 2004).

5) Mitochondrial PTP - Opening the channel collapses the mitochondrial membrane potential and uncouples oxidative phosphorylation, resulting in ATP depletion and cell death (Hausenloy *et al.*, 2003). During myocardial ischaemia, the mPTP remains closed, only to open within the first few minutes after myocardial reperfusion in response to mitochondrial Ca^{2+} overload, oxidative stress, restoration of a physiologic pH, and ATP depletion (Kim *et al.*, 2006; Griffiths *et al.*, 1995).

Modulators of ischaemic/reperfusion injury

1) Metabolic modulation - Several experimental and clinical studies have examined the cardioprotective potential of therapy with glucose, insulin, and potassium administered as an adjunct to myocardial reperfusion.(Jonassen *et al.*, 2001; Apstein and Opie, 2005; Fath-Ordoubadi and Beatt, 1997; Zhang *et al.*, 2005; Pache *et al.*, 2004). These studies have been conducted on the premise that ischaemic myocardium benefits more from metabolizing glucose than from

fatty acids (Opie *et al.*, 1970). Insulin also has anti-apoptotic effects (Kulik *et al.*, 1995).

2) Magnesium Therapy - Experimental studies have reported that intravenous magnesium administered during myocardial reperfusion can reduce myocardial infarct size, but the mechanism of this effect is unclear (Christensen *et al.*, 1995).

3) Therapeutic Hypothermia - Mild hypothermia (33 to 35°C) has been reported to benefit patients surviving a cardiac arrest (Nolan *et al.*, 2003).

In a study by Tsang *et al.* (2004) it was shown that the cardioprotection of the perfused myocardium by IPostC was achieved by activating the RISK pathway. This pathway is also activated by preconditioning (Tsang *et al.*, 2004; Yang *et al.*, 2004b) and will be discussed in more detail on page 26.

As is the case for preconditioning, the mPTP also plays a key role in the protection conferred by IPostC. This pore opens during the first few minutes of reperfusion in response to mitochondrial calcium overload, ATP depletion and oxidative stress (Halestrap *et al.*, 2004). IPostC protects the myocardium by the inhibition of the opening of this pore. (Argaud *et al.*, 2005).

When the mPTP opens, the permeability barrier of the inner membrane becomes disrupted with two major consequences. First, although all small molecular weight solutes move freely across the membrane, proteins do not and, as a result, they exert a colloidal osmotic pressure that causes mitochondria to swell. Although the unfolding of the cristae allows the matrix to expand without rupture of the inner membrane, the outer membrane will break and lead to the release of proteins in the intermembrane space such as cytochrome C that play a critical role in apoptotic cell death. Second, the inner membrane becomes freely permeable to protons. This uncouples oxidative phosphorylation, causing the proton-translocating ATPase to reverse direction and so actively hydrolyse ATP, rather than synthesize it. Under such conditions, intracellular ATP levels rapidly decline, leading to the disruption of ionic and metabolic homeostasis and the activation of degradative enzymes such as phospholipases, nucleases and proteases. Unless

mPTP closure occurs soon, these changes will cause irreversible damage to the cell, resulting in necrotic death (Halestrap *et al.*, 2004).

It is also noted that IPostC may be directly or indirectly involved in the beneficial effects of anti-inflammatories and anti-oxidants (Kin *et al.*, 2004).

2.2.2.2 Is postconditioning ready for clinical application?

A study by Staat *et al.* (2005) investigating the efficacy of IPostC in the human heart was said to “bring the bench to the bedside”. This study suggests that postconditioning after a coronary angioplasty protects the human heart after an acute myocardial infarction. It made all the hard work relevant done by researchers ever since the phenomenon of postconditioning was introduced. This study was aimed at and designed to test the safety and efficacy of postconditioning in a select population of patients undergoing angioplasty and stent deployment. The following conclusions were made from this study; before there is a general application of postconditioning, a large double-blinded multicenter study is not needed, but rather additional studies to confirm the efficacy of postconditioning in a larger and more diverse group of patients. In addition, studies should look sufficiently beyond the interventional event (ie, 1 to 5 years) to ensure that more subtle adverse events do not surface (Staat *et al.*, 2005). A recent study followed postconditioned patients for 6 months and concluded that postconditioning affords persistent infarct size reduction and improves long-term functional recovery in patients with acute myocardial infarction (Thibault *et al.*, 2008). People in the clinical arena now have to accept the utter importance of a clinically applicable intervention with ischaemic/reperfusion injury as its main target (Vinten-Johansen *et al.*, 2004).

2.3 The reperfusion injury salvage kinases (RISK) pathway: A mutual target in pre- and postconditioning.

Cell signalling is part of a complex system of communication that governs basic cellular activities and coordinates cell actions (Witzany, 2000). The ability of cells to perceive and correctly respond to their microenvironment is the basis of development, tissue repair, and immunity as well as normal tissue homeostasis.

The Reperfusion Injury Salvage Kinase (RISK) Pathway is a term given to a group of pro-survival protein kinases (including PKB/Akt and ERK42/44). The activation and phosphorylation of these two elements (PKB/Akt and ERK42/44) of the RISK pathway is an integral part in the study of interventions aimed at alleviating myocardial damage.

2.3.1 PKB/Akt and ERK42/44 activation in triggering of preconditioning

These kinases have been shown to be activated during the triggering phases of preconditioning, as well as during reperfusion. The first few studies on PKB/Akt and ERK42/44, did not investigate the cardioprotective effect of these pro-survival kinases at the time of reperfusion. They were only investigated as potential signalling components, participating to convey the preconditioning signal from the membrane of the cell to the inner components (mitochondria) during ischaemia. The roles of PKB/Akt and ERK42/44 were thus investigated in the first few studies in the context of ischaemia rather than reperfusion (Baines *et al.*, 1999; Baxter *et al.*, 2001; Hausenloy *et al.*, 2005b). The first study that showed the PI3K/Akt – pathway as a potential signalling component of IPC was by Tong *et al.*, (2000). They demonstrated that the IPC signal phosphorylates Akt and GSK3 β (downstream from it) and that this phosphorylation is necessary for cardioprotection by IPC (Tong *et al.*, 2002). Several components of the IPC signalling pathway (from the membrane to the mitochondria) have since been identified by further research (Oldenburg *et al.*, 2004; Krieg *et al.*, 2004a; Krieg *et al.*, 2004b), but the role of ERK42/44 as a potential signalling component in IPC is still unclear. One study suggest that there is no change in the phosphorylation of ERK42/44 in response to IPC (Behrends *et al.*, 2000) and another clearly shows a protective role for phosphorylated ERK42/44 during the index ischaemia in IPC protection (Fryer *et al.*, 2001b). Although both these studies were performed in vivo, the animal models and timepoints in reperfusion for phosphorylated ERK42/44 determination were different.

2.3.2 PKB/Akt and ERK42/44 in reperfusion

More recent studies suggest that ERK42/44 is indeed activated by the release of ROS from the mitochondria at reperfusion (in response to IPC) (Samavati *et al.*,

2002). These kinases confer powerful cardioprotection when activated at reperfusion (Hausenloy *et al.*, 2005b; Hausenloy *et al.*, 2007). The phenomena of both IPC and IPostC have been shown to be associated with activation of this protective pathway during reperfusion, after ischaemia (Tsang *et al.*, 2004; Hausenloy *et al.*, 2005a). Immediately after index ischaemia, at the onset of reperfusion, the mPTP opens in response to ROS formation and this contributes to cell death (Griffiths *et al.*, 1995; Kim *et al.*, 2006). By suppressing the opening of the mPTP at reperfusion, cardioprotection is conferred and this is central to pharmacological and ischaemic preconditioning (Hausenloy *et al.*, 2004a). Although activation of the RISK pathway and prevention of mPTP opening have both been clearly defined as essential components of cardioprotective pathways, it is not known whether they are linked, or whether activation of the RISK pathway inhibits mPTP opening. In a study by Davidson *et al.*, (2006) insulin, a cardioprotective compound, was used to investigate the link between the RISK pathway and mPTP opening. Activation of the RISK pathway was necessary for insulin stimulation protection at the level of the mPTP (Davidson *et al.*, 2006).

Adenosine (through the use of various agonists), bradykinin and opioids (Yang *et al.*, 2004a; Kis *et al.*, 2003; Park *et al.*, 2006; Bell *et al.*, 2003; Gross *et al.*, 2004) have all been shown to significantly reduce infarct size when administered at the time of myocardial reperfusion through the activation of the RISK pathway (Hausenloy *et al.*, 2007). Ligand binding at the G protein coupled receptor leads to the activation of its tyrosine receptor kinase, which then activates the PI3K-Akt and MEK1/2-ERK42/44 signalling cascades. These pro-survival kinases are thought to inhibit the opening of the mPTP by phosphorylating glycogen synthase kinase-3 β (GSK-3 β) (Juhaszova *et al.*, 2004).

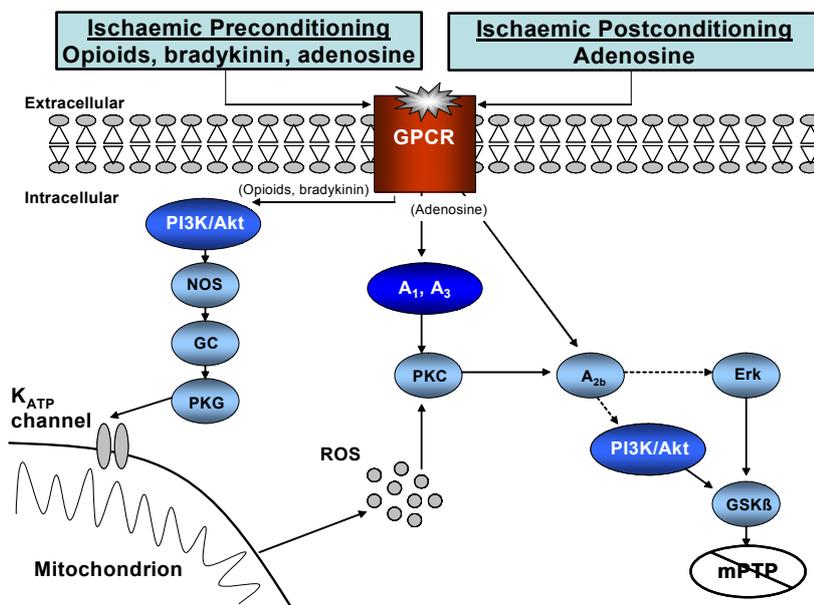


Figure 2.2: A hypothetical, schematic illustration of the signal transduction pathways involved in IPC and IPostC, including all the survival kinases that are implicated. GPCR – G protein coupled receptor, PI3K - phosphoinositide 3-kinase, AKT – Protein kinase B (PKB), NOS – nitric oxide synthase, GC – guanylate cyclase, PKG - cGMP-dependent protein kinase or protein kinase G, ROS – reactive oxygen species, PKC – protein kinase C, ERK42/44 - extracellular signal-regulated kinase, GSK - glycogen synthase kinase, mPTP – mitochondrial permeability transition pore.

2.3.3 The RISK pathway: potential activating mechanisms

Activation of the RISK pathway by several hormones and compounds has been described. Insulin activates the PI3K-Akt signalling cascade, while insulin-like growth factor-1 (IGF-1) activates both the PI3K-Akt and ERK42/44 signalling cascades (Parizzas *et al.*, 1997; Fujio *et al.*, 2000). Both transforming growth factor- β 1 (TGF- β 1) and fibroblast growth factor (FGF) activate the ERK42/44 signalling cascade and the latter modulates cell proliferation, survival and apoptosis (Szebenyi *et al.*, 1999). Cardiotrophin-1 (CT-1) (Sheng *et al.*, 1997) as well as urocortin (Brar *et al.*, 1999) activates the PI3K and ERK42/44 signalling cascades.

Other cardio-protective growth factors that activate the pro-survival kinases of the RISK pathway like: the vascular endothelial growth factor (VEGF) (Luo *et al.*, 1997) and the hepatic growth factor (HGF) (Ueda *et al.*, 1999; Nakamura *et al.*,

2000) have been shown to be cardioprotective. Similarly, epidermal growth factor (EGF) (Pillai *et al.*, 1999), nerve growth factor (NGF) (Nagy *et al.*, 2002) and platelet derived growth factor (PDGF) (Nagy *et al.*, 2002) have also been shown to be protective in various tissues, but have not yet been tested in cardiac tissue.

Other compounds capable of activating the PI3-K signalling cascade are atorvastatin (the hydroxyl-3-methylglutaryl (HMG)-co-enzyme A (CoA) reductase inhibitor) (Takemoto *et al.*, 2001), bradykinin (Hartman *et al.*, 1995; Bell *et al.*, 2003) and adenosine (Maddock *et al.*, 2002).

2.3.4 The clinical importance of the RISK pathway in cardioprotection

To activate the RISK pathway and inhibit mPTP opening to prevent further damage caused by an acute ischaemic event, the cardioprotective intervention and subsequent signalling must take place at time of reperfusion.

The Akt and ERK42/44 components of the RISK pathway appear to act as a point of convergence for the apparent diverse and unrelated cardioprotective triggers/phenomena of IPC and Ischaemic postconditioning. The opening of the mPTP is inhibited by the activation of the RISK pathway and protection against reperfusion injury is further accomplished by several other protective cellular mechanisms. The RISK pathway therefore provides a novel target for protecting the heart against lethal reperfusion injury in clinical settings of reperfusion. Interventions involving reperfusion include thrombolysis, primary coronary artery angioplasty and cardiac surgery. Components of the RISK pathway may be activated or upregulated by administering stimulatory pharmacologic agents at the time of reperfusion to confer cardioprotection.

2.4 The adenosine triphosphate sensitive potassium channel and cardioprotection.

The opening of the mPTP is central to the role that the mitochondria play in cell survival. Two conditions exist within the cytosol during early reperfusion that will influence the opening of the mPTP. They are Ca²⁺ overload and reduction or depletion of ATP (Hausenloy *et al.*, 2002; Hausenloy *et al.*, 2003; Hausenloy *et al.*, 2004a). By inhibiting the opening of the mPTP with cyclosporine-A, a well-

known mPTP-inhibitor, a marked increase in cardioprotection was observed (Shanmuganathan *et al.*, 2005).

Hausenloy proposed that mitochondrial K_{ATP} channel openers or IPC decreased Ca^{2+} overload and reactive oxygen species (ROS) production at reperfusion so that the probability of mPTP opening was reduced (Hausenloy *et al.*, 2002). The opening of the K_{ATP} channel during prolonged/index ischaemia was shown to be cardioprotective as blocking these mitochondrial K_{ATP} channels abolished cardioprotection provided by adenosine (one of the triggers of IPC) (Van Winkle *et al.*, 1994).

2.4.1 The discovery and structure of the K_{ATP} channel

The K_{ATP} channel has already been described in many tissues including pancreatic β -cells, neurons, vascular smooth muscle, skeletal muscle and cardiac myocytes (Cohen *et al.*, 2000). The ATP-sensitive potassium channel is a type of potassium channel containing Kir6.0-type subunits and sulfonylurea receptors (SUR), along with additional components (Stephan *et al.*, 2006). They can be further identified by their location within the cell as being either sarcolemmal (sarco K_{ATP} channel) or mitochondrial (mito K_{ATP} channel).

The K_{ATP} channel isoforms in the heart: Inoue *et al.* (1991) first identified an ATP-sensitive K^+ channel in the inner mitochondrial membrane (mito K_{ATP}) in rat liver by patch clamping giant mitoplasts prepared from rat liver mitochondria. Present working models of sarco K_{ATP} channels suggest that they are composed of a tetramer of core inward rectifier K^+ channels (Kir6.x) surrounded by 4 sulfonylurea receptor subunits (SUR), which confer sensitivity not only to glibenclamide but also to K_{ATP} channel openers (Babenko *et al.*, 1998; Seino *et al.*, 1999). There are 3 SUR isoforms encoded by 2 genes: SUR1 and the splice variants SUR2A and SUR2B (Aguilar-Bryan *et al.*, 1998). The predominate pancreatic isoform is thought to be SUR1 paired with Kir6.2, whereas the myocyte sarcolemmal isoform has been identified as SUR2A/Kir6.2 (Inagaki *et al.*, 1995 and 1996). In smooth muscle, SUR2B/Kir6.1 is thought to be the small-conductance, diazoxide-sensitive, ATP-insensitive isoform observed in patch-clamp recordings (Yamada *et al.*, 1997). Similarities between mito K_{ATP} and surface K_{ATP} have led to the assumption

that mitoK_{ATP} channels will also consist of SUR and Kir components. This is reinforced by the tentative identification of a 63 kDa sulfonylurea binding protein purified from mitochondria and a putative pore forming channel subunit of 55 kDa reported by Grover and Garlid (2000).

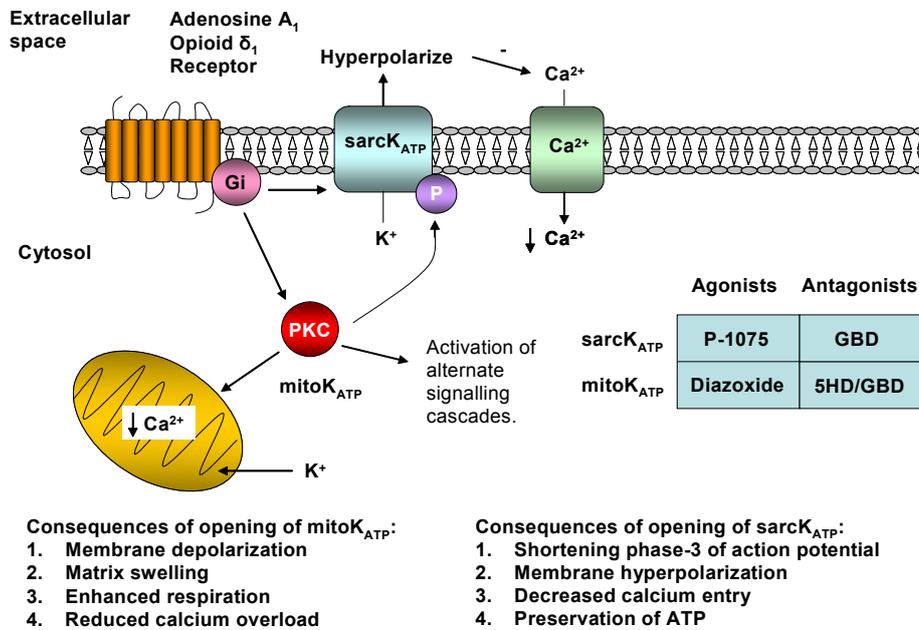


Figure 2.3: Schematic illustration demonstrating proposed mechanisms by which opening of the sarc- or mitoK_{ATP} channel might produce a cardioprotective effect and specific modulators of each channel (figure adapted from Gross *et al.*, 1999).

2.4.2 Cardiovascular K_{ATP} channels and protection from ischaemia/reperfusion injury

As discussed previously, opening of the K_{ATP} channels is an important event in the signal transduction processes of ischaemic pre- and postconditioning. K_{ATP} channels are inhibited from opening by the presence of intracellular ATP and opens as intracellular ATP levels are depleted (Trapp *et al.*, 1997). Much of the evidence of the K_{ATP} channel's involvement in cardioprotection is based on the abolition of the protective effects of ischaemic and pharmacological pre- and postconditioning by blocking the opening of the K_{ATP} channels (Grover *et al.*, 1997). In a study where the mitochondrial K_{ATP} channel blocker 5-

hydroxydecanoic acid (5-HD) was administered in the rat heart, the protective effects of preconditioning were abolished (Schultz *et al.*, 1997).

Studies dating back to 1989 showed that administration of K_{ATP} channel openers could confer profound cardioprotection against myocardial ischaemia in numerous mammalian species (Quast *et al.*, 1989). Post-ischaemic cardiac function improved after administering K_{ATP} channel openers BMS-180448 or cromakalim (Grover *et al.*, 1995). In more recent studies the effects of K_{ATP} channel openers (P-1075, Pinacidil and Diazoxide) on energetics and contractile function were investigated in the isolated rat heart (Jilkina *et al.*, 2002). This group concluded that, unlike the uncoupling effect of 2,4-dinitrophenol, an ATP-synthesize inhibitory effect of P-1075 (a K_{ATP} channel opener) is produced by uncoupling of oxidative phosphorylation through the activation of mitochondrial K_{ATP} channels.

In recent studies the importance of the activation of the subtypes of the K_{ATP} channel was highlighted. The blocking of the mito K_{ATP} channel with 5-hydroxydecanoic acid (5-HD) or MCC-134 completely abolishes the cardioprotective effect of IPC (Mubagwa *et al.*, 2001).

2.5 The treatment of cardiovascular disease and applications for levosimendan.

2.5.1 New drugs for the treatment of congestive heart failure

Heart failure is a condition where the heart is unable to maintain a normal cardiac output which leads to inadequate blood flow and oxygen delivery to peripheral tissues and organs. Under-perfusion of organs leads to reduced exercise capacity, fatigue, and shortness of breath. It can also lead to organ damage and dysfunction (e.g., renal failure) in some patients. Acute heart failure (AHF) develops rapidly and can be life threatening because the heart does not have time to undergo compensatory adaptations. Acute failure (hours/days) may result from cardiopulmonary by-pass surgery, acute infection (sepsis), acute myocardial infarction, valve dysfunction or severe arrhythmias. Acute heart failure can often be managed successfully by pharmacological or surgical interventions. Chronic heart failure is a long-term condition (months/years) that is associated with the

heart undergoing maladaptive responses (e.g., dilation, hypertrophy) to a precipitating cause. These adaptive responses, can however be deleterious in the long-term and lead to a worsening condition.

The traditional medical treatment for acute heart failure has remained unchanged for many years. Traditional medical treatment consists of oxygen supplementation and mechanical ventilatory support, as well as the administration of drugs such as diuretics, morphine, nitrates and inotropic agents. Recently (2005), The European Society of Cardiology (ESC), published new guidelines regarding the diagnoses and treatment of acute heart failure. In a review article by Grimm (2006), the current role for the traditional drugs for AHF, as well as the role of newer compounds like vasodilators, endothelin agonists or vasopressin agonists and even newer inotropic agents like the calcium sensitizer, levosimendan, are described.

2.5.2 Levosimendan as a positive inotrope

Both positive and negative inotropes are used in the management of various cardiovascular conditions, such as AMI. The choice of compound, largely depends on the specific pharmacological effects of individual compounds, depending, in turn, on the condition being treated. The inotropic drugs that are currently used to improve contractility, act by increasing the intracellular concentrations of free calcium, but also increase myocardial energy and oxygen consumption and cause arrhythmias. For this reason their clinical application seems to be limited and is not without risk (Garcia-Gonzalez *et al.*, 2006).

These positive inotropic drugs include the following:

- Cardiac glycosides (digoxin)
- Catecholamines (dopamine, dobutamine, dopexamine, epinephrine/adrenaline, isoprenaline/isoproterenol and norepinephrine/noradrenaline)
- Eicosanoids (prostaglandins)
- Phosphodiesterase inhibitors (enoximone, milrinone, theophylline)

In order to explain the mode of action of these inotropic compounds, one of them, dobutamine (a catecholamine) will be discussed briefly. Dobutamine is predominantly a β_1 - and β_2 -adrenergic agonist and are used for a variety of clinical indications in patients with a low cardiac output and systemic hypotension. It is used clinically in cases of cardiogenic shock for its β_1 inotropic effect in increasing heart contractility and cardiac output. Since it does not act on dopamine receptors to induce the release of norepinephrine (another α_1 agonist), dobutamine is less likely to induce hypertension than is dopamine. Chronotropic, arrhythmogenic, and vasodilative effects are negligible. Dobutamine is administered as a racemic mixture consisting of both (+) and (-) isomers, and the (+) isomer is a potent β_1 agonist while the (-) isomer is a α_1 agonist

Both positive and negative inotropes are used in the management of various cardiovascular conditions. One of the most important factors affecting inotropic state is the level of calcium in the cytoplasm. Positive inotropes usually increase the cytosolic calcium level, while negative inotropes decrease it (Kihara *et al.*, 1989).

Levosimendan is a new calcium-sensitizing agent and produces positive hemodynamic effects, without increasing myocardial oxygen demand or causing arrhythmias. It does so by binding in a calcium dependent manner to cardiac troponin C and thereby increasing contractility by enhancing the sensitivity of myofilaments to calcium. The systematic "International Union of Pure and Applied Chemistry" (IUPAC) name of levosimendan is: (-)-(R)-(4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl) carbonohydrizonoyl dicyanide (refer to figure 2.4 for molecular structure).

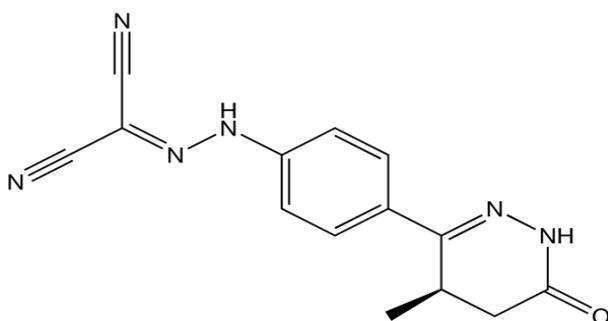


Figure 2.4: Molecular structure of levosimendan (wikipedia).

Levosimendan also has a coronary and systemic vasodilatory effect by virtue of its K_{ATP} channel opening properties. It opens both the sarcolemmal and mitochondrial K_{ATP} channels (Antoniades *et al.*, 2007). The opening of these ATP sensitive potassium channels may also give rise to direct cardioprotective effects on cardiomyocytes. Levosimendan stimulates the ATP-sensitive K^+ (K_{ATP}) channel in small resistance vessels and the Ca^{2+} -activated K^+ (KCa) channels and voltage-dependent K^+ (KV) channels in large-conductance vessels. These actions hyperpolarize the membrane, thereby inhibiting inward L-type Ca^{2+} current ($I_{Ca(L)}$), as well as promoting the forward mode (FM) of Na^+/Ca^{2+} exchanger (NCX), i.e., 3 Na^+ in/1 Ca^{2+} out. The resultant decrease in $[Ca^{2+}]_i$ would produce vasorelaxation. Levosimendan also may decrease the Ca^{2+} sensitivity of the contractile proteins directly and/or indirectly through the hyperpolarization (Yokoshiki and Sperelakis, 2003). See figure 2.5 for the proposed mechanism of vasodilation of levosimendan.

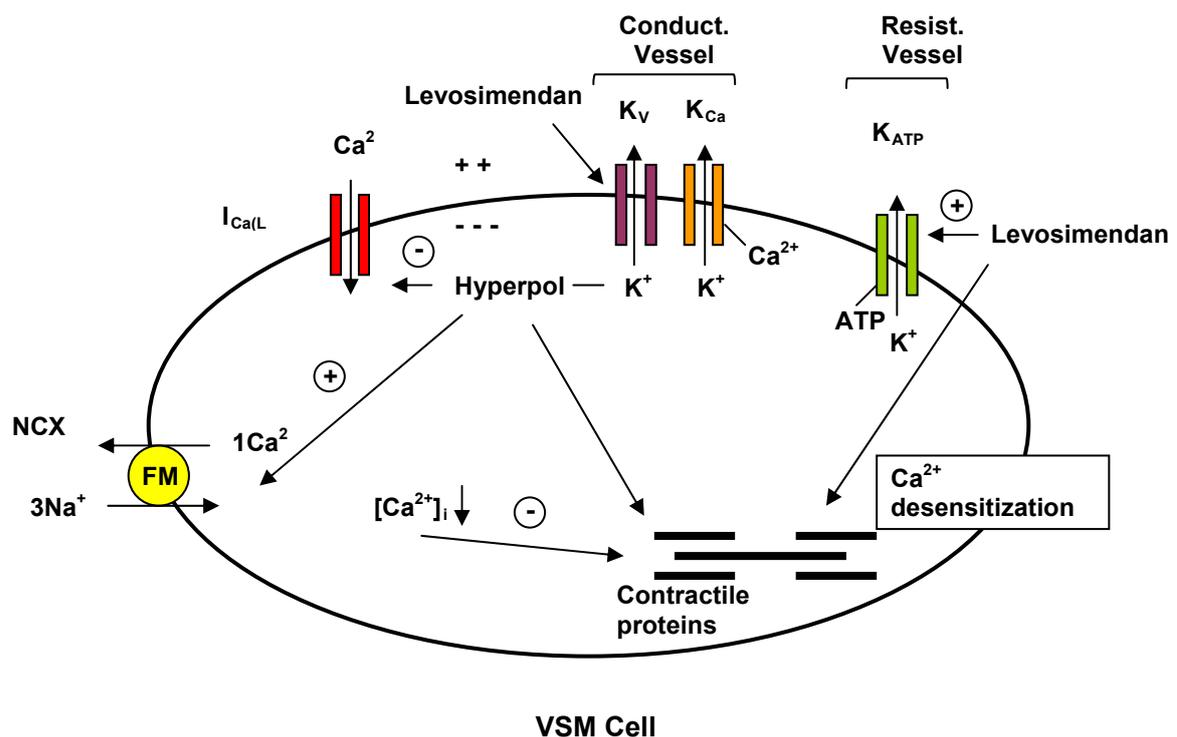


Figure 2.5: Proposed vasodilating mechanism of levosimendan. The plus signs (+) indicate stimulation and the minus signs (-) indicate inhibition. Ca^{2+} -activated K^+ (KCa) channels, voltage-dependent K^+ (KV) channels, inward L-type Ca^{2+} current ($I_{Ca(L)}$), forward mode (FM), Na^+/Ca^{2+} exchanger (NCX) and vascular smooth muscle (VSM). (Figure adapted from Yokoshiki and Sperelakis, 2003).

Furthermore, there is also evidence that levosimendan may have additional anti-inflammatory and anti-apoptotic properties, affecting important pathways in the pathophysiology of heart failure. Thus, in contrast to traditional inotropic agents, levosimendan as a calcium sensitizer and vasodilator, has been proposed to be superior to these agents (Yokoshiki *et al.*, 1997; Kersten *et al.*, 2000; Kopustinskiene *et al.*, 2001). A previous study on the isolated perfused guinea pig heart, has investigated the postcardioplegic response of the heart to levosimendan (Lochner *et al.*, 2000). They found that except for the increase in heart rate, the effect of levosimendan on functional performance during reperfusion was comparable to that of adrenaline.

2.5.3 The clinical importance and relevance of levosimendan

The combined inotropic and vasodilatory actions of levosimendan result in an increased force of contraction, decreased preload and decreased afterload. Moreover, by also opening the mitochondrial (ATP)-sensitive potassium channels in cardiomyocytes, the drug exerts a cardioprotective effect (Garcia Gonzalez *et al.*, 2006). The K_{ATP} channel has been mentioned as an important role player in IPC and IPostC (see section 2.4.2). In various studies it was indicated that by blocking these channels, the cardioprotective effect of IPC and IpostC was completely abolished (Grover *et al.*, 1997). It is possible that the same cardioprotective effect of IPC and IpostC can be mimicked by administering levosimendan instead of the short ischaemic episodes, as levosimendan potentially targets the same mechanisms.

Regarding future clinical application, levosimendan-induced preconditioning may be useful before elective cardiac surgery while levosimendan postconditioning could be applied immediately after coronary reperfusion during open heart surgery. The major experimental effect of levosimendan given as pretreatment also warrants clinical trials, particularly in those with large AMI's, where this compound may be expected to protect from both LV failure and subsequent reperfusion injury.

2.5.4 Clinical trials on levosimendan

The effects of levosimendan in patients with AHFS have been studied in 5 randomized clinical trials and are being investigated in 2 large-scale trials. These studies comprised of the following:

- a double-blind, placebo-controlled, randomized dose-ranging study was conducted in 151 patients with stable New York Heart Association (NYHA) functional class II to IV heart failure (HF) (Nieminen *et al.*, 2000),
- the Randomized Study on Safety and Effectiveness of Levosimendan in Patients with Left Ventricular Failure Due to an Acute Myocardial Infarct (RUSSLAN) was a doubleblind, placebo-controlled trial conducted in 504 patients (Moiseyev *et al.*, 2002),
- the Levosimendan Infusion Versus Dobutamine (LIDO) study was a randomized, double-blind, double-dummy, parallel-group trial conducted in 203 patients with severe low-output HF (Follath *et al.*, 2002),
- the Calcium Sensitizer or Inotrope or None in Low-Output Heart Failure Study (CASINO) was a randomized, doubleblind, double-dummy, parallel-group trial conducted in 299 patients with decompensated low-output (left ventricular ejection fraction - LVEF < 0.35) HF that compared levosimendan with dobutamine and placebo (Zairis *et al.*, 2004; Coletta *et al.*, 2004),
- the Randomized Multicenter Evaluation of Intravenous Levosimendan Efficacy Versus Placebo in the Short-Term Treatment of Decompensated Heart Failure (REVIVE) was a largescale, placebo-controlled, double-blind study (Packer *et al.*, 2003),
- the Survival of Patients with Acute Heart Failure in Need of Intravenous Inotropic Support (SURVIVE) study is the first prospective, randomized trial using mortality as the primary end point in evaluating the efficacy of intravenous drug therapy in AHFS (Mebazaa *et al.*, 2004) and
- the effects of levosimendan in patients who had recently experienced MI were examined in RUSSLAN.6. In a recent study, 24 patients undergoing angioplasty after acute coronary syndromes (ie, 23 patients with acute MI, 1 with unstable angina) were enrolled in a randomized, doubleblind, placebo-controlled study (Sonntag *et al.*, 2004).

- Results from these trials indicate that levosimendan increases cardiac output, decreases pulmonary capillary wedge pressure (PCWP), and improves symptoms in patients with AHFS. Several of these trials indicated that levosimendan provided a survival advantage compared with conventional treatments for AHFS (Moiseyev *et al.*, 2002; Follath *et al.*, 2002; Zairis *et al.*, 2004).

2.6 Objectives of this study.

The treatment of AHF with inotropic agents such as catecholamines or by phosphodiesterase (PDE) inhibition, is effective, but has the risk of compromising the oxygen demand/supply balance and being pro-arrhythmic as it acts by primarily increasing the cyclic adenosine monophosphate (cAMP) and cytosolic calcium levels (Cremers *et al.*, 2003). There is thus a shift to newer calcium-sensitizing agents that will not increase the risk of arrhythmias or worsen the imbalance in the oxygen demand/supply. Levosimendan is such an agent. With this in mind, we set out to investigate the role of levosimendan in pharmacological cardioprotection of the ischaemic myocardium. Our investigation focussed on the following areas:

- 1) Establishment of a suitable protocol for ischaemic pre- and postconditioning in the guinea pig perfused heart model (for comparison purposes – comparison with studies already carried out in the rat heart).
- 2) Determining whether levosimendan can be used to mimic the cardioprotective effects of IPC and IpostC.
- 3) Determining the effect of pre-treatment of the isolated perfused guinea pig hearts with levosimendan on recovery after myocardial ischaemia (to distinguish from levosimendan when given as a “trigger” of IPC).
- 4) Investigating whether ischaemic preconditioning and levosimendan preconditioning has an additive protective effect.
- 5) Investigating of the role of adenosine triphosphate sensitive potassium channels in the levosimendan-induced preconditioning.
- 6) Exploring the signal transduction pathways in pre- and postconditioned cardioprotection with particular attention to the role of the “survival kinases” of the RISK pathway in levosimendan-induced cardioprotection.

CHAPTER THREE

MATERIALS AND METHODS

The materials and methods described in this chapter were used throughout this study. Variations in either the materials or methods will be described in the relevant sections.

3.1 Animal model

Age and weight matched male Dunkin Hartley guinea pigs were used for this study. Guinea pigs were chosen as an animal model as the guinea pig heart displays similar inotropic responses to levosimendan as the human heart. These animals were bred and supplied by National Health Laboratory Services in Parktown, Johannesburg. At the Faculty of Health Science, University of Stellenbosch, they were housed in the Central Research Facility. A 12-hour artificial day-night cycle at a constant temperature of 22°C and 40% humidity was maintained. The South African Medical Research Council's guide for the humane treatment of laboratory animals was followed in this study. The study was approved by the Committee for Experimental Animal Research (CEAR) of the Faculty of Health Sciences, University of Stellenbosch. Male guinea pigs (\pm 300g) were randomly assigned to different experimental groups. All animals were fed on the same guinea pig chow diet and teff. Teff is used as a dietary supplement as it is high in dietary fibre and iron and provides some protein and calcium. The water was supplemented (25 mg/100 g body weight per day) with ascorbic acid (Merck) to ensure that the animals did not suffer from a vitamin C deficiency (Bates *et al.*, 1979). Although Vitamin C is known to reduce oxidative stress during ischaemia (Rojas *et al.*, 1996), the supplemented amount was below such levels as to have a significant influence on the results found in this study.

3.2 Isolated heart perfusion model.

The animals were anaesthetized by intraperitoneal injection containing 12 mg/kg pentobarbitone sodium (Euthanaze). Hearts were excised and placed in a container with 4°C modified Krebs-Heinseleit buffer (see table 3.1). The cold

buffer arrests the heart within 2-3 seconds and consequently conserves myocardial energy during the transfer to the perfusion apparatus.

<u>Modified Krebs-Heinseleit solution</u> <u>(guinea pigs)</u>		<u>Krebs-Henseleit solution</u> <u>(rats)</u>	
Chemical Compounds	Concentration (in mM)	Chemical Compounds	Concentration (in mM)
NaCl	121.5	NaCl	119
MgCl ₂ ·6H ₂ O	1.2	MgSO ₄ ·7H ₂ O	0.6
NaHCO ₃	15.5	NaHCO ₃	25
Na Pyruvate	2	Na ₂ SO ₄	0.6
Mannitol	16	KCl	4.75
KCl	3.8	CaCl ₂	1.25
CaCl ₂	2.5	KH ₂ PO ₄	1.2
KH ₂ PO ₄	1.2	Glucose	10
Glucose	11		

Table 3.1: The composition of the perfusion solutions used in isolated guinea pig hearts and rat hearts respectively. The perfusate was oxygenated with 95% O₂/5%CO₂ (pH 7.4).

The heart was mounted on the working heart perfusion apparatus, within 60 seconds of excision from the animal. This is done by picking up the heart by the aorta with fine-tipped forceps, and slipping it onto a grooved perfusion cannula. The heart is fastened to the cannula using a cotton thread. Once the aorta was cannulated, retrograde perfusion was established by initiating the flow of a perfusion buffer to the heart at a pressure of 75 cmH₂O. All excess tissue at the base of the heart was then removed and the left atrial cannula was inserted into the pulmonary vein (to allow perfusion to the left atrium in working heart (WH) mode) and tied tightly. This procedure was according to the working heart model described by Neely *et al.* (1967) and modified by Opie and coworkers (1971).

3.3 Isolated working heart perfusion protocol.

After the heart was mounted, it was subjected to a predetermined perfusion protocol. Below is an illustration (see figure 3.1) of the standard perfusion protocol

which was followed during the entire study with slight modifications before or after the index ischaemia.

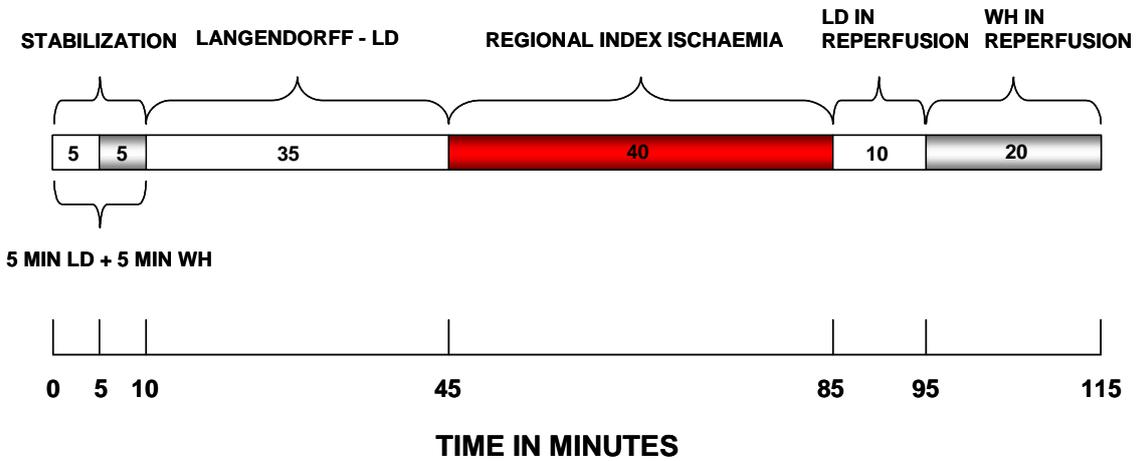


Figure 3.1: The standard perfusion protocol used throughout this study.

After the excised heart has been mounted on the perfusion apparatus, it was first stabilized by 5 minutes of Langendorff (LD) perfusion (also known as retrograde perfusion) followed by 5 minutes of working heart perfusion (at a preload of 15 cmH₂O and afterload of 75 cmH₂O). Subsequently the heart was subjected to 35 minutes of LD perfusion followed by the prolonged period of index ischaemia. In our study we made use of left anterior descending coronary artery ligation to induce regional index ischaemia. The temperature was maintained at 36.5°C and monitored constantly by placing a temperature probe in the coronary sinus. After the index ischaemia the perfusion to the heart was re-established. The LD perfusion mode was maintained for 10 minutes before hearts were subjected to 20 minutes of working heart perfusion at the end of the experimental protocol. The pre-ischaemic and reperfusion WH perfusions were performed in order to assess pre-ischaemic and reperfusion mechanical function of the hearts.

3.4 Drugs used in this study.

Levosimendan: A stock (1mM) was prepared using 50µl 1M NaOH in 10ml phosphate buffer (2.32% Na₂HPO₄ in distilled water) and adding 280.3mg levosimendan to this solution. The stock solution was diluted 1:10,000 to give a final working concentration of 0.1µM levosimendan. This concentration was

established in a previous study (Du Toit *et al.*, 1999) to have optimal protective effects. The usual dosage in clinical studies is 0.05 – 0.2µg/kg/min (a 0.2µM concentration) as a continuous infusion (Figgitt *et al.*, 2001).

5-Hydroxydecanoic acid (5HD): 5HD is a non-selective K_{ATP} channel antagonist. The stock (100mM) was prepared by dissolving 420mg in 20ml perfusion buffer. A volume of 1ml from the stock was added to 500ml of perfusion buffer to give a final working concentration of 200µM.

Glibenclamide (GBD): GBD is an antidiabetic sulfonylurea derivative and was used in this study as a non-specific K_{ATP} channel antagonist. It was prepared by dissolving 25 mg in 1ml 1% dimethyl sulfoxide DMSO and 99 ml dH₂O, giving a stock concentration of 500µM. The stock solution was diluted 1:100 with perfusate for a final working concentration of 5µM.

For selected experiments these blockers were applied in addition to the levosimendan pretreatment or preconditioning protocols.

PD 098059: PD 098059 is a specific inhibitor of the mitogen activated protein kinase kinase (MAPKK) and is used to inhibit ERK42/44 that is directly downstream from MEK. A stock solution was prepared by dissolving 5mg in 1ml of DMSO (18.71mM). A volume of 24.7µl of this stock solution was added to 50ml perfusate to give a final working concentration of 10µM.

3.5 Endpoints that were measured in this study.

Aortic pressure data was obtained using a pressure transducer in the aortic cannula of the isolated heart perfusion apparatus which was in turn connected to a PhysiTutor® data acquisition unit.

3.5.1 Mechanical function

The following measurements were documented:

Aortic output (Qa): The amount of perfusate that is pumped out by the aorta per unit time, measured in ml/min.

Coronary flow (Qe): The volume of buffer that leaves the coronary sinus per unit time, measured in ml/min.

Heart rate (HR): Heart rate was extrapolated from the aortic pressure recording and expressed as beats per minute (bpm).

Aortic systolic pressure: The aortic systolic pressure is defined as the peak pressure developed in the aorta and is measured in mmHg.

Aortic diastolic pressure: The aortic diastolic pressure is the lowest pressure in the aorta (at the resting phase of the cardiac cycle) and is measured in mmHg.

Aortic developed pressure (ADevP): This value is calculated by subtracting the diastolic pressure from the systolic pressure.

Data were obtained at specific time points during the different protocols. Coronary flow was measured at 5, 10, 40, 65, 95 and 115 minutes. Aortic output was measured at 10, 95 and 115 minutes, during working heart perfusion. Aortic developed pressure was also measured during working heart perfusion at 10, 95 and 115 minutes. For a schematic representation of the timepoints of data collection, see figure 3.2.

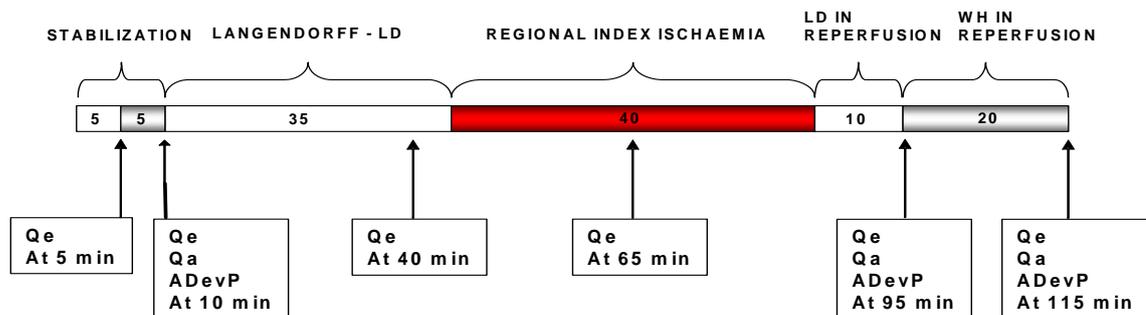


Figure 3.2: Data obtained at specific time points.

3.5.2 Infarct size

Myocardial infarct size was determined after occluding the left anterior descending coronary artery with a braided silk suture (Johnson & Johnson Medical), with a ½ circle, 25mm taper needle size (Du Toit *et al.*, 2005). This occlusion was released (and leaving the suture thread in the heart tissue) for 30 minutes of reperfusion

after the prolonged period of regional ischaemia. Myocardial infarct size was determined in the area at risk after 30 minutes reperfusion by re-occlusion of the coronary artery and injection of a 2.5% Evans blue solution into the aorta. It was decided on 30 minutes of reperfusion (rather than 120 minutes) for the infarct size determinations because our group has previously shown that infarct size does not change significantly whether we reperfused for 30 minutes or 120 minutes (Marais *et al.*, 2005).

Hearts were then stored in a freezer at -20°C (for no longer than 2 days, to prevent frostbite of the tissue) and cut into 5-7 slices (with a cross-section of about 2mm each), incubated in sodium phosphate buffer containing 1% w/v triphenyltetrazolium chloride (TTC) for 15 – 20 minutes to visualize the unstained infarcted region (appears white) and area at risk (appears red). Infarcted and areas at risk were determined by clamping the slices between two glass plates, and placing a transparent sheet on top of the glass and tracing the different areas of each slice (see Image 3.1 below). The image on the transparent sheet is then transferred to a computer by using a scanner. By using drawing software (Image Tool 2.0 – a shareware program developed by the University of Texas at San Antonio) the total pixels of the different areas of all the slices of a heart could be determined from the digital image of the tracings. The pixels were then used to calculate the infarct size and this was expressed as a percentage of the area at risk. The area at risk was determined for all experimental groups and was found to be similar. The average value for all the groups was $36.7 \pm 1.1\%$ of the volume of the left ventricle.

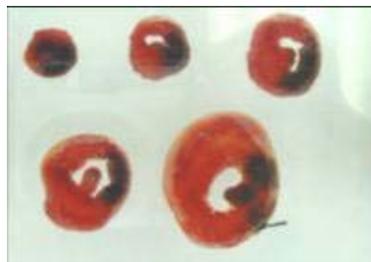
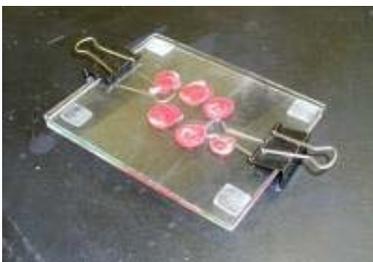


Image 3.1: Heart slices placed between two glass plates (on the left) and a close-up image of the heart slices (on the right) to show the three areas of interest. White areas = infarct, red areas = area at risk and blue areas = viable tissue (Klein *et al.*, 1989).

3.5.3 Heart tissue collection and assessment of total protein and phosphorylation of ERK42/44 and PKB/Akt by Western blot analysis.

After 5 or 10 minutes of reperfusion ischaemic and non-ischaemic areas of the hearts were separated and freeze clamped with Wollenberger clamps. The areas were separated by excising the occluded area, freezeclamping it and then freeze-clamping the rest of the heart. Samples were stored at -80°C in liquid nitrogen until Western blot analyses were performed. The proteins, cardiac ERK42/44 and PKB/Akt were extracted with a PKB lysis buffer containing (in mM): Tris-HCl 20, EGTA 1, EDTA 1, NaCl 150, β -Glycerolphosphate 1, Tetra-Na-pyrophosphate 2.5, NaVO_3 1, 10 $\mu\text{g/ml}$ Leupeptin, 10 $\mu\text{g/ml}$ Aprotinin, 1% Triton and 50 $\mu\text{g/ml}$ phenylmethyl sulfonyl fluoride (PMSF).

The amount of protein per sample was determined with a Bradford assay (Bradford, 1976). The tissue lysates were then prepared with Western sample buffer and lysis buffer mentioned above, boiled for 5 minutes and stored at -20°C . Then 10 μg of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to a Polyvinylidene Fluoride (PVDF) membrane (Immobilon™ P, Millipore supplied by Microsep).

These membranes were routinely stained with Ponceau Red for visualization of proteins. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-Buffered Saline — 0.1% Tween 20 (TBST). The membranes were then incubated overnight at 4°C with the primary antibodies that recognize phospho-specific ERK42/p44 ($\text{Thr}^{202}/\text{Tyr}^{204}$) or PKB (Ser^{473} and Thr^{308}). A duplicate of the membrane was also incubated overnight at 4°C with the primary antibodies that will recognize the total ERK42/44 and PKB. Membranes were subsequently, thoroughly washed with TBST (5 \times 5 minutes) and the immobilized antibody conjugated with a diluted horseradish peroxidase-labeled secondary antibody (Amersham LIFE SCIENCE). The secondary antibody was either diluted in TBST (for phospho and total ERK42/44) or in a 5% fat-free milk-TBST solution (for phospho and total PKB).

After thorough washing with TBST (5x5 minutes), membranes were covered with ECL detection reagents (Amersham LIFE SCIENCE) and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103 - Amersham LIFE SCIENCE) to detect light emission through a non-radioactive method (ECL Western blotting). Films were densitometrically analyzed using computer software (UN-SCAN-IT, Silkscience) and phosphorylated protein values and total protein values were determined. Ratios of these proteins were expressed graphically, as shown later in the results sections.

3.6 Statistical methods used in this study.

Infarct size was expressed as a percentage of the area at risk and for functional recovery data, reperfusion aortic output was expressed as a percentage of the pre-ischaemic value. The following formula is used to calculate this value: $\text{post-ischaemic aortic output} / \text{pre-ischaemic aortic output} \times 100/1 = \% \text{ aortic output}$. Values were presented as the mean \pm SEM.

A minimum of 6 hearts/group for all experimental groups were used. For multiple comparisons the one way analysis of variance (ANOVA) followed by the Bonferroni correction was applied. A value of $p < 0.05$ was considered significant.

CHAPTER FOUR

ESTABLISHING A PROTOCOL FOR PRE- AND POSTCONDITIONING IN THE GUINEA PIG MODEL.

4.1 Introduction

The first question to be addressed in this study was to determine whether ischaemic pre- and postconditioning (IPC and IPostC) would confer cardioprotection in the guinea pig model. Extensive studies on preconditioning had already been performed on the isolated rat heart model (Murray *et al.*, 1986; Liu *et al.*, 1992a; Asimakis *et al.*, 1992; Mitchell *et al.*, 1995; Shipolini *et al.*, 1997; Aitchison *et al.*, 2000; Javadov *et al.*, 2003). Similarly, several studies have also been done investigating the phenomenon of postconditioning in the rat heart model (Kin *et al.*, 2004; Galagudza *et al.*, 2004; Yang *et al.*, 2005; Argaud *et al.*, 2005; Penna *et al.*, 2006; Bopassa *et al.*, 2006).

Only a few studies attempted to precondition the guinea pig heart (Riess *et al.*, 2002; Kasamaki *et al.*, 1997; Jin and Chen, 2007). However, as far as we know, the phenomenon of postconditioning has not yet been studied in the isolated guinea pig heart. It was thus necessary to establish suitable protocols, with a clearly defined ischaemic time, for the guinea pig model that would induce enough damage in the control animals and leave a suitable margin for improvement after pre- and postconditioning interventions.

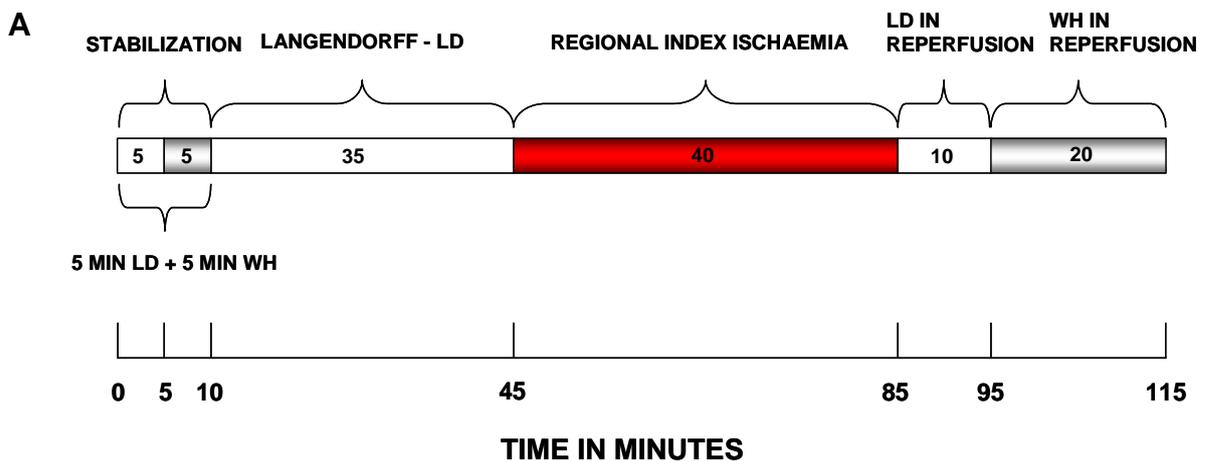
4.2 Materials and Methods

Guinea pig hearts were perfused in the working heart mode and subjected to ischaemia/reperfusion as described in chapter three. The detail of the composition of this perfusion buffer was described in chapter three (see page 35). Infarct size as a percentage of the area at risk was used as the endpoint for the different experimental groups to determine whether a protocol for cardioprotection had been established. Aortic output during reperfusion (expressed as a percentage of values obtained before ischaemia) was given as a measure of functional recovery.

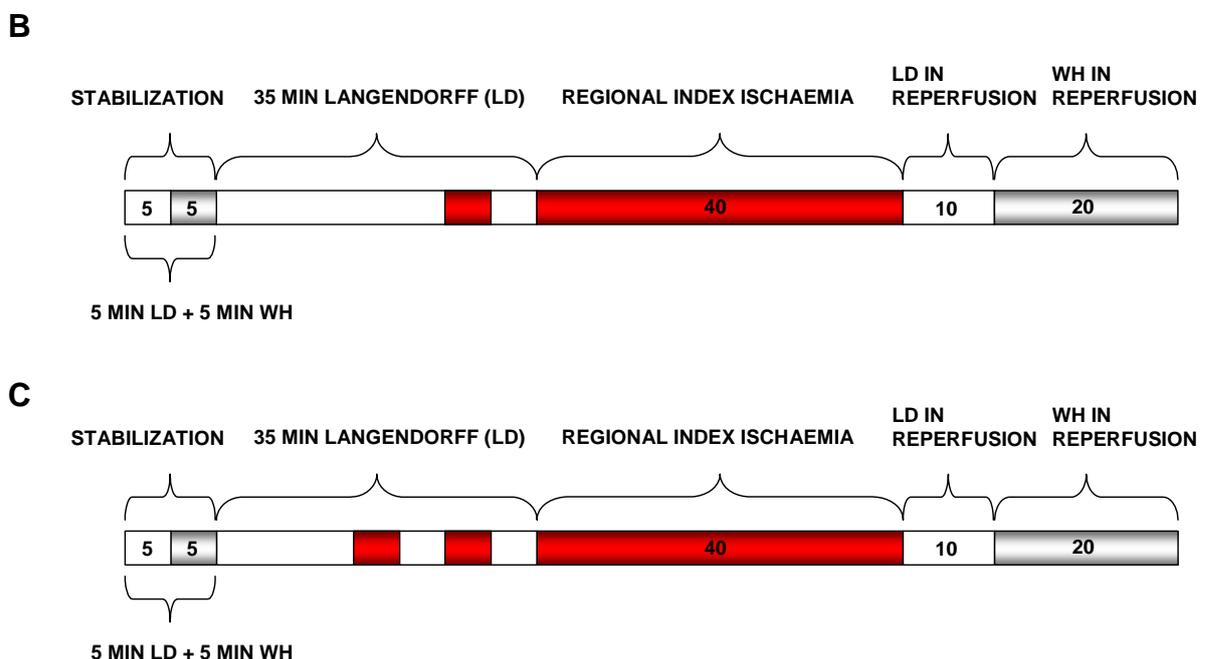
4.2.1 Establishing a preconditioning protocol.

The first challenge was to establish a suitable protocol for preconditioning that could reduce infarct size and which could be used throughout the study. The perfusion protocol for control (non-preconditioned) hearts is shown in figure 4.1 A. Three IPC protocols were used: 1 x 5 min, 2 x 5 min and 3 x 5 min of global ischaemia/reperfusion. These protocols were applied during the 35 minutes of LD perfusion preceding index ischaemia, for preconditioned hearts (see figures 4.1 B-D). A detailed description of the method of infarct size determination is given in chapter three, pages 38-39. Measurements of cardiac performance were made at specific timepoints during the perfusion protocols (see figure 3.2, page 38).

Control (non-preconditioned):



Preconditioning with ischaemia:



D

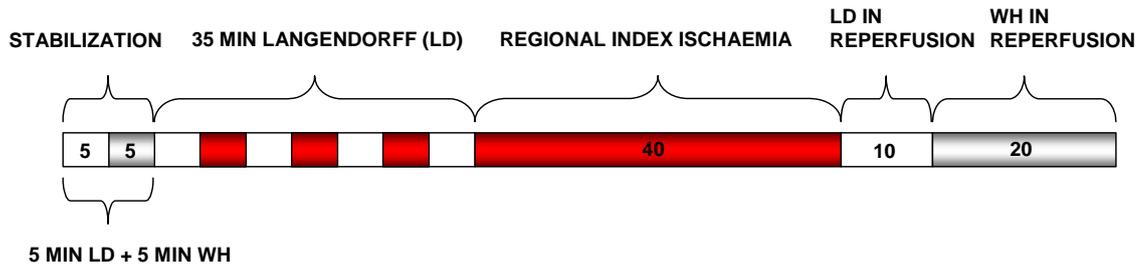


Figure 4.1: A – standard protocol for controls, B – protocol 1 for IPC (1 x 5 min ischaemia), C – protocol 2 for IPC (2 x 5 minutes ischaemia) and D – protocol 3 for IPC (3 x 5 min ischaemia).

4.2.2 Establishing a postconditioning protocol.

The standard protocol (see figure 4.1 A) was followed, with modifications during the first two minutes of reperfusion (3 x 20 seconds of ischaemia/reperfusion), for postconditioned hearts (see figure 4.2). Infarct size (method described in previous chapter) was used as an endpoint. Aortic output (% recovery) was given as a measure of functional recovery. The temperature during index ischaemia and during postconditioning was maintained at 36.5°C. Data were obtained at certain time points during the different protocols (see figure 3.2, page 38).

Postconditioning with ischaemia:

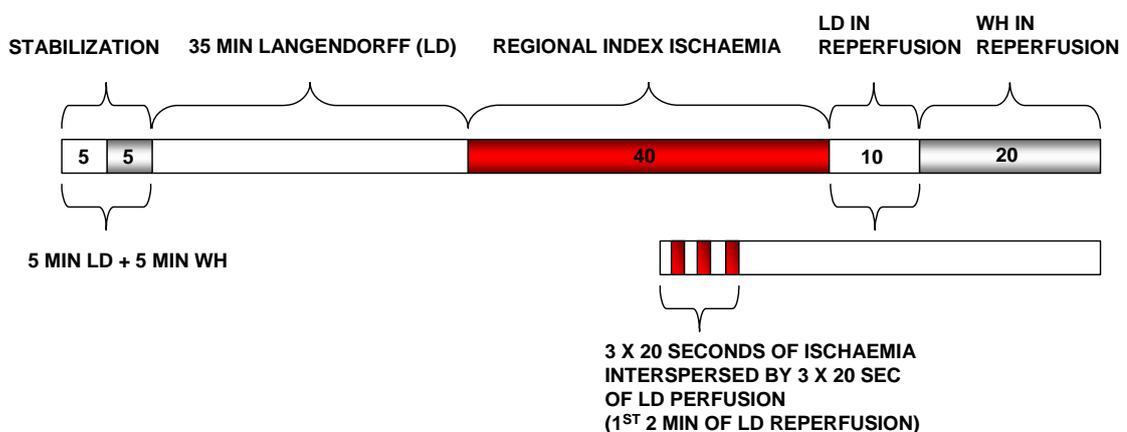


Figure 4.2: Protocol for IPostC (3 x 20 seconds ischaemia).

4.3 Results

4.3.1 Results for preconditioning protocols.

Preconditioning hearts with 1 x 5 min global ischaemia/reperfusion (IPC1 protocol) had no effect on infarct size ($42.94 \pm 2.75\%$ compared to $46.56 \pm 1.80\%$ in controls, see figure 4.3). A significant reduction in infarct size was observed in both the IPC2 (2 x 5 min) and 3 (3 x 5 min) protocols ($25.39 \pm 2.76\%$, $21.19 \pm 2.16\%$ vs. 46.56 ± 1.8 respectively, $p < 0.001$, see figure 4.3). See table 4.1 below for infarct size values for the control (non-preconditioned) group and the three IPC groups. Refer to addendum A1 – A4 for the detailed data of each experimental group.

Apart from protocol IPC1 (1 x 5 min) which had no recovery during reperfusion (0ml/min), hearts subjected to either 2 x 5 minutes ischaemia/reperfusion (IPC2) or 3 x 5 min ischaemia/reperfusion (IPC3) produced aortic output values similar to those of control, Interestingly all hearts exhibited an increase in coronary flow rate during reperfusion (except IPC2 hearts). However, no significance was discerned between the groups (see tables 4.1 and 4.2).

Table 4.1: Data for preconditioned hearts (pre-ischaemic data – top and post-ischaemic data – below). Displayed as Mean \pm SEM.

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
Control	54.78 ± 3.76	19.89 ± 1.41	31.41 ± 2.69	207.40 ± 8.51	
	21.56 ± 5.13	33.11 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80
IPC 1	41 ± 3.28	18.50 ± 1.09	16.30 ± 3.07	187.90 ± 8.82	
	0	40.50 ± 5.36	4.35 ± 1.73	135.97 ± 13.54	42.94 ± 2.75
IPC 2	58.29 ± 2.93	23.64 ± 1.36	36.77 ± 2.54	218.93 ± 10.30	
	32.21 ± 4.82	24.07 ± 1.49	27.11 ± 3.60	193.84 ± 7.04	25.39 ± 2.76 *
IPC 3	54.50 ± 6.20	20 ± 2.62	32.61 ± 1.95	191.39 ± 9.34	
	17.33 ± 5.35	37 ± 3.97	20.73 ± 2.33	170.75 ± 9.56	21.90 ± 2.16 *

* $p < 0.001$ vs. control

Table 4.2: Aortic output recovery (%) for all the IPC protocols.

Experimental Group	Control	IPC 1	IPC 2	IPC 3
Pre-ischaemic aortic output (ml/min)	54.78 ± 3.76	41.00 ± 3.28	58.29 ± 2.93	54.50 ± 6.20
Post-ischaemic aortic output (ml/min)	21.56 ± 5.13	0	32.21 ± 4.82	17.33 ± 5.35
Aortic Output (% recovery)	36.23 ± 7.69	0	54.16 ± 6.93	28.20 ± 8.57

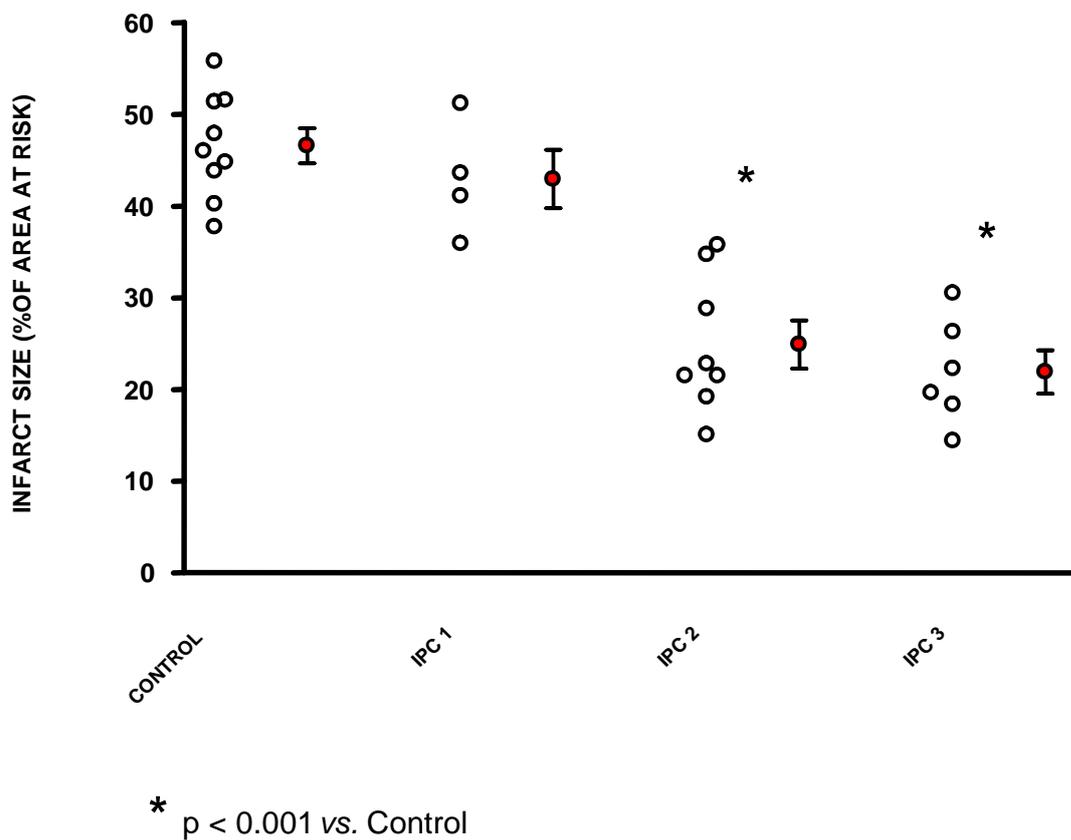


Figure 4.3: Infarct sizes for IPC 1, 2 & 3 (% of area at risk).

4.3.2 Results for postconditioning protocols.

A postconditioning protocol of 3x20 seconds was applied and found to be cardioprotective. No further protocols were tested. The infarct size (% of area at risk) has decreased from a control value of 45.56±1.80% to 21.40±2.43% (p<0.001) in the ischaemic postconditioning group (see figure 4.4). See table 4.3 below for infarct size values for the control (non-preconditioned) group and the IPostC group. Refer to addendum A1 & A5 for the detailed data of each experimental group.

As in preconditioned hearts, postconditioning did not improve functional recovery and the % recovery or aortic output was similar to that of controls.

Table 4.3: Data in postconditioned group (pre-ischaemic data – top and post-ischaemic data – below). Displayed as Mean ± SEM.

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size
Control	54.78 ± 3.76	19.89 ± 1.41	31.41 ± 2.69	207.40 ± 8.51	
	21.56 ± 5.13	33.11 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80
IPostC	53.63 ± 6.28	22 ± 1.77	22.47 ± 2.01	149.23 ± 12.48	
	31.50 ± 5.95	32.63 ± 3.29	18.57 ± 0.83	149.85 ± 7.58	21.40 ± 2.43 *

* p < 0.05 vs. control

Table 4.4: Aortic output recovery (%) for the IPostC protocol.

Experimental Group	Control	IPostC
Pre-ischaemic aortic output (ml/min)	54.78 ± 3.76	53.63 ± 6.28
Post-ischaemic aortic output (ml/min)	21.56 ± 5.13	31.50 ± 5.95
Aortic Output recovery (%)	36.23 ± 7.69	59.99 ± 9.16

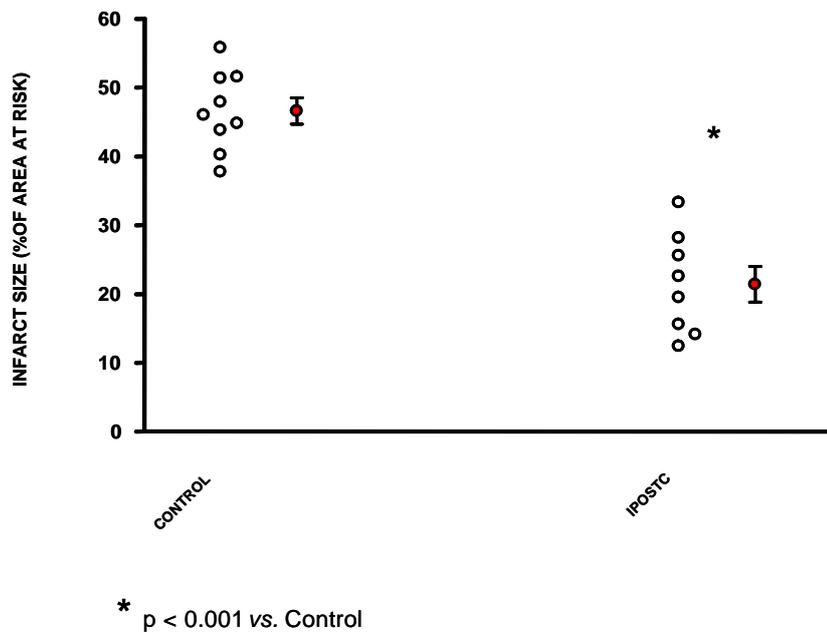


Figure 4.4: Infarct sizes for Control vs. IPostC (% of area at risk).

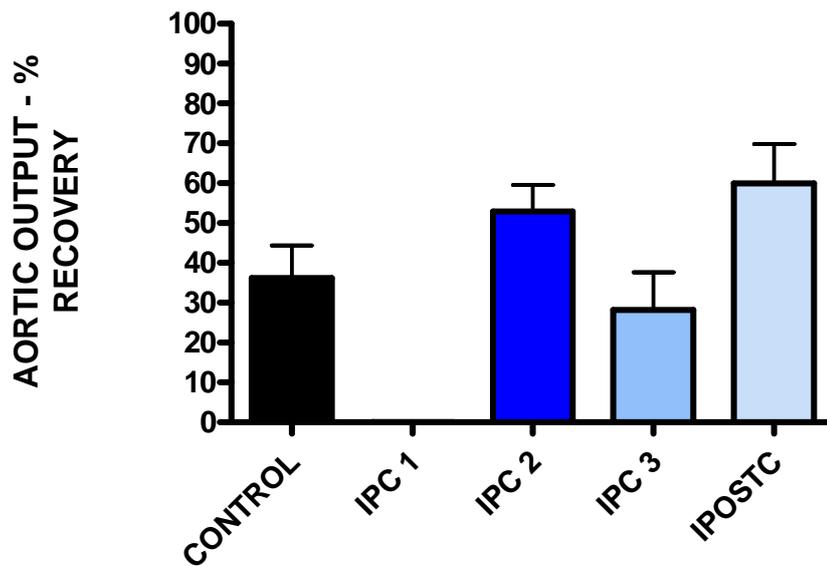


Figure 4.5: Aortic output (% recovery) for IPC 1-3 and IPostC vs. Control.

4.4 Summary of results.

Although no functional recovery was observed in these protocols for IPC and IPostC, protocols were established that could significantly reduce infarct size for IPC and IpostC in the isolated guinea pig heart. These results confirm that infarct size is a more sensitive endpoint for demonstration of cardioprotection than functional recovery after coronary artery ligation.

CHAPTER FIVE

ESTABLISHING WHETHER LEVOSIMENDAN CAN BE USED AS A PRE- AND POSTCONDITIONING MIMETIC.

5.1 Introduction

In the previous section of the study a model that could successfully pre- and postcondition with ischaemia was established. The next concept that was investigated was that of pharmacological pre- and postconditioning. There has recently been a shift in emphasis from pre- and postconditioning with ischaemia to that of pharmacological pre- and postconditioning as the latter is more practical in a clinical setting. Thus the main focus of this study is to investigate whether levosimendan confers cardioprotection against ischaemic damage by mimicking pre- and postconditioning. The pharmacological preconditioning effects of levosimendan have previously been investigated in rabbit hearts (Leprán *et al.*, 2006) and it was found to reduce infarct size significantly. However their study did not actually investigate “preconditioning” with levosimendan, but rather “pre-treatment” with levosimendan, since the drug was not washed out before initiation of index ischaemia. In the current study the brief ischaemic periods of IPC were replaced with levosimendan and the drug was washed out before the index ischaemia (levosimendan preconditioning - LPC). The phenomenon of postconditioning by replacing the ischaemic episodes in IPostC with levosimendan (levosimendan postconditioning – LpostC) was also investigated.

Thus by substituting the brief ischaemic episodes that were used in IPC and IPostC with levosimendan, we investigated whether levosimendan could confer the same (or even better) cardioprotection than IPC and IPostC. As far as we know, this is the first study to use a model of coronary artery occlusion of the isolated guinea pig heart, with infarct size as endpoint.

It is well-established that the K_{ATP} channels play an important role in conferring protection in classic preconditioning (O'Rourke, 2000). Since it has been shown that levosimendan can also open the K_{ATP} channels (Yokoshiki *et al.*, 1997;

Kopustinskiene et al., 2001), we proposed that levosimendan may be able to protect the ischaemic heart.

5.2 Materials and Methods

The data from the same group of control (non-preconditioned) hearts that were used in chapter 4 were used for comparative purposes in the study on pharmacological pre- and postconditioning with levosimendan. A dose response curve was generated in a previous study to determine the concentration of levosimendan that would be expected to have optimal cardioprotective effects during ischaemia (Du Toit *et al.*, 1999). Levosimendan at a concentration of 0.1µM was successfully used to treat the ischaemic heart in previous studies in the guinea pig model (Du Toit *et al.*, 1999; Kaheinen *et al.*, 2001). It was therefore decided to use levosimendan at this concentration in the current study.

The working heart perfusion apparatus was used as described in chapter 3. Infarct size as a percentage of the area at risk was used as the endpoint to determine whether levosimendan could successfully be used as a pre- and postconditioning mimetic. Aortic output during reperfusion (expressed as a percentage of pre-ischaemic values) was evaluated as a measure of functional recovery.

5.2.1 Pharmacological preconditioning with levosimendan

One of the protocols that was tested in the previous chapter (see figure 4.1 B) for IPC was adapted for pharmacological preconditioning with levosimendan. The two brief cycles of ischaemia (2 x 5 min) was substituted with levosimendan (see figure 5.1), each followed by 5 minutes washout. Index ischaemia was induced by occlusion of the left anterior descending coronary artery (regional ischaemia) for 40 minutes while myocardial temperature was kept constant at 36.5°C. These protocols were referred to as levosimendan preconditioning (LPC).

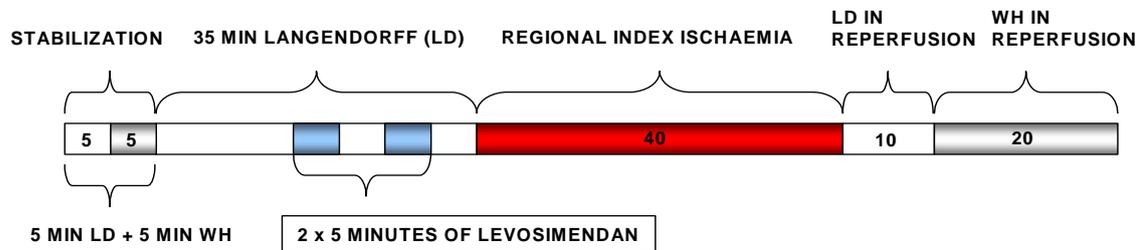


Figure 5.1: LPC protocol showing the two cycles of 5 minutes of levosimendan before index ischaemia (replacing ischaemia in IPC).

Data was obtained at specific time points during the different protocols (see figure 3.2, page 38).

5.2.2 Pharmacological postconditioning with levosimendan - LPostC.

The same protocol that was established in the previous chapter (see figure 4.2) for IPostC was adapted for pharmacological postconditioning with levosimendan. The three brief cycles of ischaemia were substituted with levosimendan (see figure 5.3).

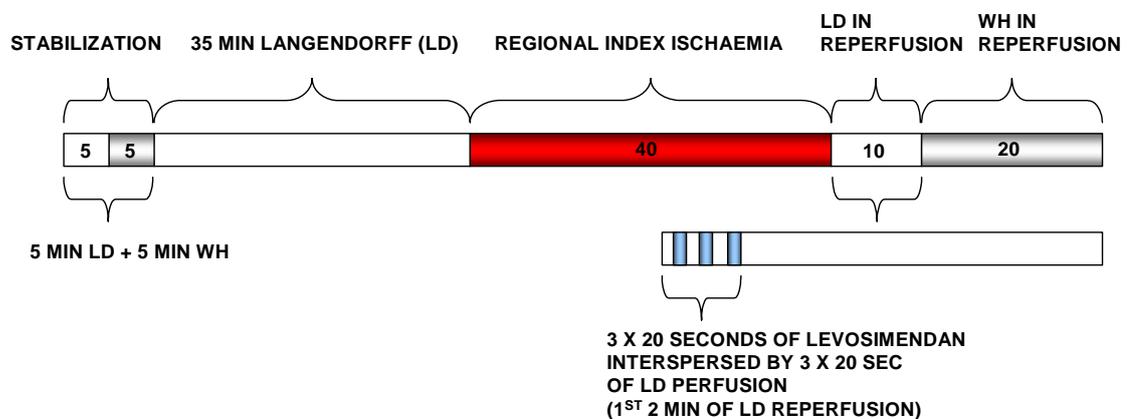


Figure 5.2: LPostC protocol showing the three cycles of 20 seconds of levosimendan (replacing ischaemia in IPostC).

Index ischaemia was induced by occlusion of the left anterior descending coronary artery (regional ischaemia) as described above in section 5.2.1. The temperature was maintained at 36.5°C during the postconditioning intervention. These protocols were referred to as levosimendan postconditioning (LPostC). Data were collected as described before (see figure 3.2, page 38).

5.3 Results

5.3.1 Pharmacological preconditioning with levosimendan.

Infarct size (% of area at risk) of the LPC group was significantly decreased ($20.64 \pm 3.13\%$, $p < 0.001$), compared to that of the control hearts ($46.56 \pm 1.80\%$) (See figure 5.3). As far as the functional recovery is concerned the LPC group displayed aortic output values (ml/min) similar to those of control hearts. Also as mentioned in the previous chapter this experimental group also had an increase in coronary flow from pre-ischaemic values (see table 5.1 & 2). Refer to Addendum A1 (control) and B1 (LPC) for detailed results for each experimental group.

Table 5.1: Data for LPC group (top – pre-ischaemic data and bottom – post-ischaemic data). Displayed as Mean \pm SEM.

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
CONTROL	54.78 ± 3.76	19.89 ± 1.41	31.41 ± 2.69	207.40 ± 8.51	
	21.56 ± 5.13	33.11 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80
LPC	56.50 ± 3.57	21.13 ± 1.19	31.40 ± 1.19	237.83 ± 4.77	
	33.88 ± 4.78	31.63 ± 3.54	19.84 ± 2.15	218.67 ± 5.18	$20.64 \pm 3.13^*$

* $p < 0.05$ vs. control

Table 5.2: Aortic output recovery (%) for the LPC protocol.

Experimental Group	Control	LPC
Pre-ischaemic aortic output (ml/min)	54.78 ± 3.76	56.50 ± 3.57
Post-ischaemic aortic output (ml/min)	21.56 ± 5.13	33.88 ± 4.78
Aortic Output recovery (%)	36.23 ± 7.69	58.61 ± 5.84

5.3.2 Pharmacological postconditioning with levosimendan.

Postconditioning with levosimendan (LPostC), caused a significant ($p < 0.05$) reduction in infarct size compared to controls ($20.55 \pm 1.83\%$ vs. $46.56 \pm 1.80\%$)(see figure 5.3). Similar trends in post-ischaemic aortic output and

coronary flow values have been observed for the LPostC hearts (see table 5.3 & 4). Please refer to Addendum A1 (control) and B2 (LPostC) for detailed data of each experimental group.

Table 5.3: Data for LPostC group (top – pre-ischaemic data and bottom – post-ischaemic data). Displayed as Mean \pm SEM.

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
CONTROL	54.78 \pm 3.76	19.89 \pm 1.41	31.41 \pm 2.69	207.40 \pm 8.51	
	21.56 \pm 5.13	33.11 \pm 3.56	21.68 \pm 3.09	196.87 \pm 18.18	46.56 \pm 1.80
LPostC	50.86 \pm 5.31	20.29 \pm 1.14	23.50 \pm 2.96	162.94 \pm 21.29	
	28.71 \pm 2.83	28.29 \pm 2.49	18.61 \pm 1.68	161.88 \pm 10.56	20.55 \pm 1.83 *

- p < 0.05 vs. control
-

Table 5.4: Aortic output recovery (%) for the LPostC protocol.

Experimental Group	Control	LPostC
Pre-ischaemic aortic output (ml/min)	54.78 \pm 3.76	50.86 \pm 5.31
Post-ischaemic aortic output (ml/min)	21.56 \pm 5.13	28.71 \pm 2.83
Aortic Output recovery (%)	36.23 \pm 7.69	63.99 \pm 13.09

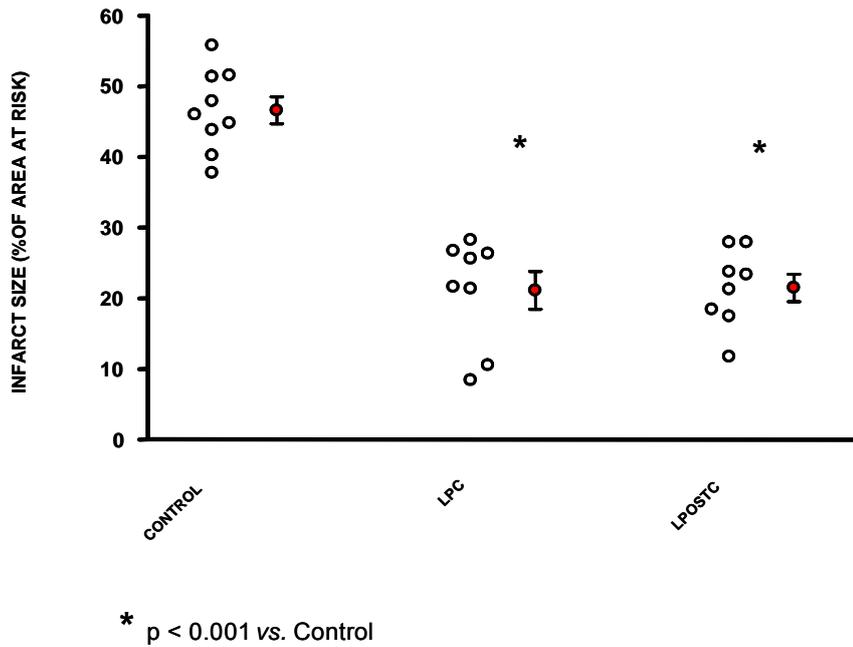


Figure 5.3 Infarct size (as % of area at risk) of LPC and LPostC vs. Control hearts.

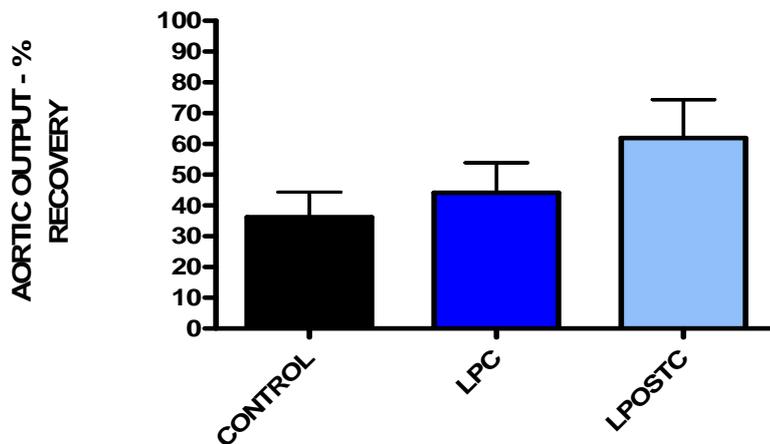


Figure 5.4: Functional recovery (aortic output - % recovery) for LPC and LPostC vs. Control.

5.4 Summary of results.

Replacing the short ischaemic episodes of IPC and IPostC with levosimendan perfusion (0.1µM) caused a significant reduction in infarct size in both instances. The isolated guinea pig heart could therefore be pharmacologically pre- and postconditioned. As observed in the case of ischaemic pre- and postconditioning, the reduction in infarct size was not associated with improved functional recovery.

CHAPTER SIX

PRE-TREATMENT OF THE ISOLATED GUINEA PIG HEART WITH LEVOSIMENDAN.

6.1 Introduction

The data presented in chapter 5, showed that levosimendan could be used to mimic pre- and postconditioning. Since the LPC protocol included a 5 minute washout period before the onset of index ischaemia, it can be assumed that the drug was not present in the heart at the beginning of ischaemia. In this next section the efficacy of levosimendan in cardioprotection during ischaemia/reperfusion, without washout was investigated. In other words, the heart was pre-treated with levosimendan for 10 minutes directly before subjecting it to index regional ischaemia. It is important to note here that in this study a distinction was made between cardioprotection by “pharmacological preconditioning” and by “pharmacological pre-treatment”. In the literature there are several studies that refers to their method of cardioprotection as “preconditioning”, when it is in actual fact “pre-treatment” (Zhao *et al.*, 2001; He *et al.*, 2001; Imamura *et al.*, 2002; Wang *et al.*, 2003)

6.2 Materials and Methods

Using the standard protocol, we substituted the last 10 minutes of Langendorff perfusion with levosimendan perfusion (see figure 6.1). This was followed by the period of index ischaemia, without wash-out of the levosimendan. This procedure was referred to as levosimendan pre-treatment (LPT).

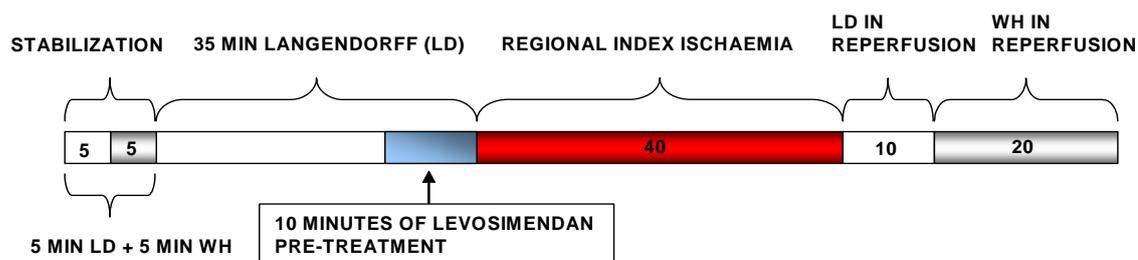


Figure 6.1: Levosimendan pre-treatment (LPT) protocol showing the 10 minutes of pre-treatment with levosimendan before subjecting the heart to 40 minutes of index ischaemia.

Data was obtained at specific time points during the different protocols as shown previously in figure 3.2, page 38.

6.3 Results

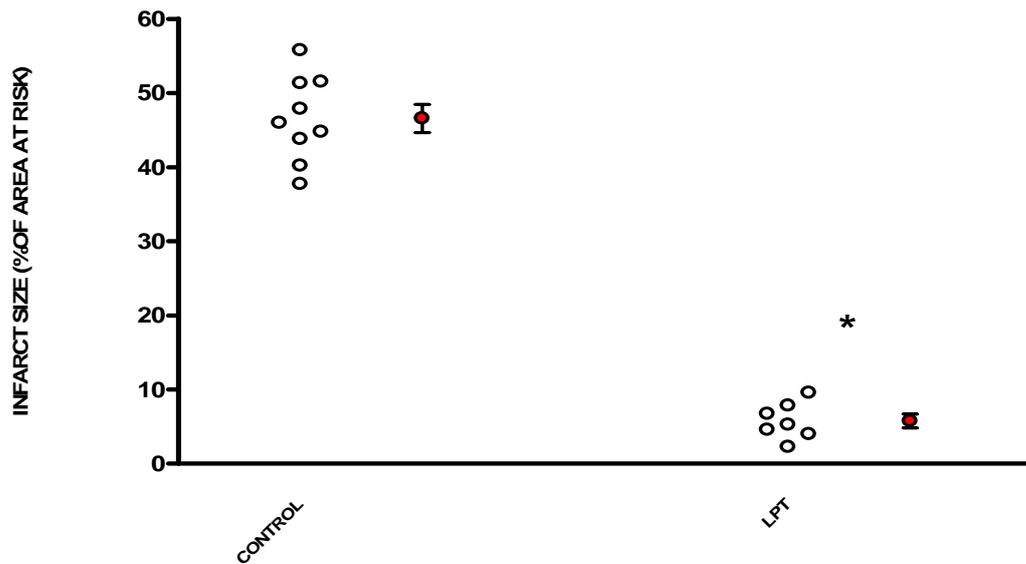
6.3.1 Pre-treatment with levosimendan – effect on infarct size.

Pre-treatment with levosimendan (LPT) without washout, resulted in a vast ($p < 0.001$) reduction in infarct size of the LPT hearts ($5.75 \pm 0.87\%$) compared to the control hearts ($46.56 \pm 1.80\%$) (see table 6.1 and figure 6.2). Judging by this marked reduction in infarct size, it was expected that the hearts should show improved functional recovery. A significant ($p < 0.05$) improvement in functional recovery ($61.32 \pm 4.91\%$) compared to that of the control hearts ($36.23 \pm 7.69\%$) (see table 6.2 and figure 6.3) was indeed observed. Refer to Addendum A1 (control) and C1 (LPT) for detailed data of each experimental group.

Table 6.1: Data for levosimendan pre-treatment (LPT) group (top – pre-ischaemic data and bottom – post-ischaemic data). Displayed as Mean \pm SEM.

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
CONTROL	54.78 ± 3.76	19.89 ± 1.41	31.41 ± 2.69	207.40 ± 8.51	
	21.56 ± 5.13	33.11 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80
LPT	62 ± 3.94	23.43 ± 0.80	30.59 ± 0.88	238.64 ± 3.55	
	38.57 ± 4.27	27.57 ± 1.31	24.68 ± 3.54	201.59 ± 9.41	$5.75 \pm 0.87^*$

* $p < 0.001$ vs. control



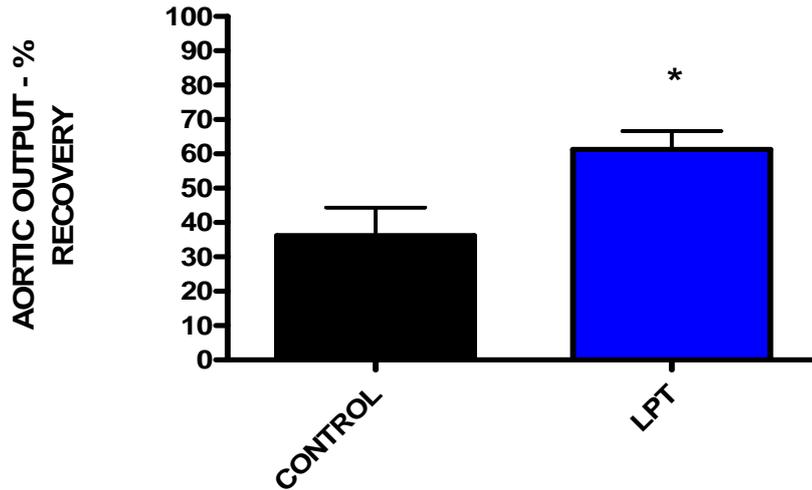
* $p < 0.001$ vs. Control

Figure 6.2: Infarct size as % of area at risk of LPT vs. Control hearts.

Table 6.2: Aortic output recovery (%) for pre-treatment with levosimendan.

Experimental Group	Control	LPT
Pre-ischaemic aortic output (ml/min)	54.78 ± 3.76	62 ± 3.94
Post-ischaemic aortic output (ml/min)	21.56 ± 5.13	38.57 ± 4.27
Aortic Output (% recovery)	36.23 ± 7.69	61.32 ± 4.91 *

* $p < 0.05$ vs. control



* $p < 0.05$ vs. Control

Figure 6.3: Aortic Output (% recovery) of levosimendan pre-treated (LPT) vs. Control hearts.

6.4 Summary of results.

It was shown in this study that pre-treating the isolated guinea pig heart with levosimendan (without washout as in our LPC protocols), resulted in the most marked reduction in infarct size observed thus far. Pre-treatment with levosimendan proved to be more effective than ischaemic or levosimendan pre- and postconditioning. In addition, this 88% reduction in infarct size was associated with a significant improvement in postischaemic functional recovery.

CHAPTER SEVEN

INVESTIGATING WHETHER ISCHAEMIC AND LEVOSIMENDAN PRECONDITIONING HAS ADDITIVE CARDIOPROTECTIVE EFFECTS IN THE HEART.

7.1 Introduction

In this section of the study the protocols for IPC and LPC were combined to investigate whether these interventions confer additive cardioprotection when compared with the IPC or LPC interventions alone. These interventions have both been found to confer significant protection in this guinea pig model. The rationale behind this section of our study was to determine whether IPC and LPC conferred cardioprotection by means of the same mechanisms. Additive beneficial effects would suggest that their mechanisms of protection probably differ.

7.2 Materials and Methods

7.2.1 IPC and LPC combined.

The following modifications were made to the standard protocol (see figure 4.2A): at 15 minutes of total perfusion time, hearts were subjected to a 1 x 5 minute cycle of ischaemia followed by 2 x 5 minute cycles of levosimendan, interspersed with LD perfusion (see figure 7.1). This protocol was referred to as IPC+LPC.

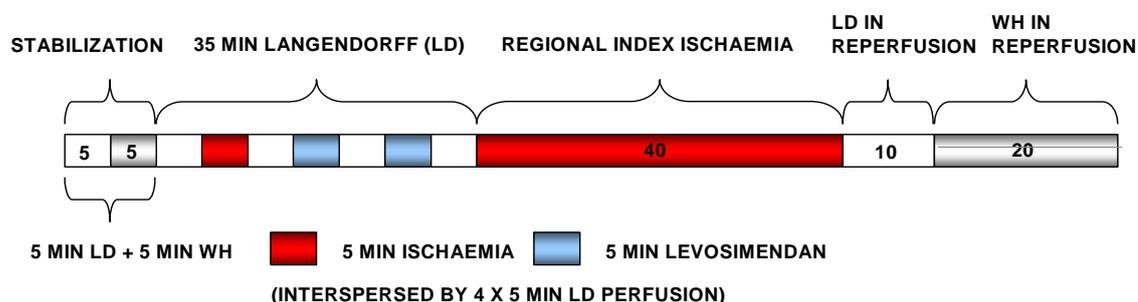


Figure 7.1: Protocol for ischaemic preconditioning and levosimendan preconditioning combined - IPC + LPC (1 x 5 minute ischaemia + 2 x 5 minutes levosimendan).

Functional measurements were taken at several, specific time points during the protocol of IPC + LPC, as shown previously in figure 3.2, page 38.

7.3 Results

Subjecting hearts to both IPC and LPC, did not result in additive protection: the infarct size obtained was similar to that obtained when hearts were subjected to IPC or LPC alone (see figure 7.2). As before, aortic output (% recovery) was similar in these groups (see figure 7.3). Refer to addendum A1 and D1 for detailed data of each experimental group.

Table 7.1: Data for additive (IPC + LPC) groups (with pre-ischaeamic values – top and post-ischaeamic values – bottom). Displayed as Mean ± SEM.

Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
CONTROL	54.78 ± 3.76	19.89 ± 1.41	31.41 ± 2.69	207.40 ± 8.51	
	21.56 ± 5.13	33.11 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80
IPC + LPC	50.17 ± 3.20	19.67 ± 1.12	24.40 ± 2.86	195.75 ± 15.30	
	23.67 ± 2.90	30.50 ± 1.10	20.08 ± 1.36	195.80 ± 9.69	21.44 ± 2.20 *

* p < 0.05 vs. control

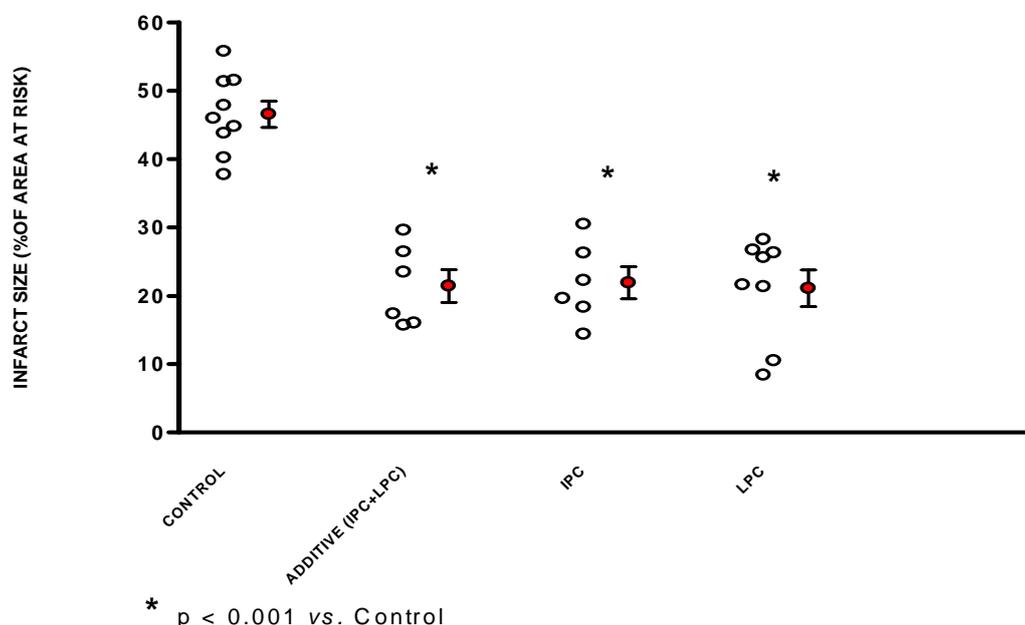


Figure 7.2: Infarct sizes for the ischaemic + levosimendan preconditioned (IPC + LPC) (% of area at risk) group. Infarct sizes of ischaemic- and levosimendan preconditioning (IPC and LPC respectively) are included to compare the efficacy of protection of the three different protocols.

Table 7.2: Functional data in ischaemic- and levosimendan preconditioning (IPC + LPC) group. Displayed as Mean \pm SEM.

Experimental Group	Control	IPC+LPC
Pre-ischaemic aortic output (ml/min)	54.78 \pm 3.76	50.17 \pm 3.20
Post-ischaemic aortic output (ml/min)	21.56 \pm 5.13	23.67 \pm 2.90
Aortic Output (% recovery)	36.23 \pm 7.69	47.04 \pm 4.88

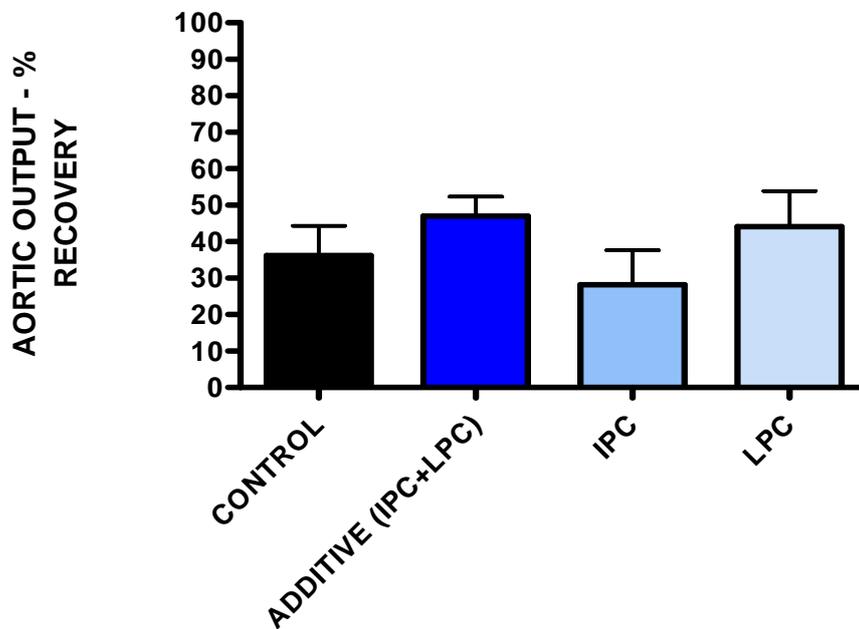


Figure 7.3: Aortic output recovery (% - as functional recovery) for ischaemic+levosimendan preconditioned (IPC + LPC) or Control, ischaemic preconditioned (IPC) or levosimendan preconditioned (LPC) hearts.

7.4 Summary of results.

Since no additive cardioprotective effect was found when the two interventions of IPC and LPC were combined, it can be concluded that the same mechanisms are probably involved in the cardioprotection conferred by both these interventions.

CHAPTER EIGHT

INVESTIGATING THE EFFECT OF THE ATP SENSITIVE POTASSIUM CHANNEL BLOCKERS ON LEVOSIMENDAN INDUCED PRE- AND POSTCONDITIONING.

8.1 Introduction

It is well established that opening of the K_{ATP} channels is an important role player in the signal transduction processes in cardioprotection. These channels are closed in the presence of adequate intracellular ATP levels and open as ATP levels are depleted within the cell, as occurs during myocardial ischaemia (Trapp *et al.*, 1997).

The K_{ATP} channel's involvement in cardioprotection is based on evidence that the protective effects of ischaemic pre- and postconditioning and pharmacological pre- and postconditioning can be abolished by blocking the opening of the K_{ATP} channels (Grover *et al.*, 1997). For example, in a study where the mitochondrial K_{ATP} channel blocker 5-hydroxydecanoic acid (5-HD) was administered to the rat heart, the protective effects of preconditioning were abolished (Schultz *et al.*, 1997). Further evidence for the involvement of the K_{ATP} channels, was the finding that pharmacological opening of the K_{ATP} channels, mimicked preconditioning. In a study on guinea pig hearts, the post-ischaemic cardiac function of the hearts improved after administering K_{ATP} channel openers BMS-180448 or cromakalim (Grover *et al.*, 1995). In more recent studies the effects of K_{ATP} channel openers (P-1075, Pinacidil and Diazoxide) on energetics and contractile function were investigated in the isolated rat heart (Jilkina *et al.*, 2002), and it was found that these K_{ATP} channel openers conferred cardioprotection by a modest activation of the mitochondrial K_{ATP} channels, which does not produce any detectable mitochondrial uncoupling. The cardioprotective pharmacology of the K_{ATP} channels was revisited in a recent review that focuses the attention on several known K_{ATP} channel openers (Aprikalim, Cromakalim, Pinacidil, BMS-180448 and Nicorandil) (Grover and Garlid. 2000).

In recent studies the importance of the activation of the subtypes of the K_{ATP} channel was highlighted. Both the $sarck_{ATP}$ and $mitoK_{ATP}$ channels are required for the intervention of IPC to have a maximal effect. The selective blocking of the $mitoK_{ATP}$ channel with 5-hydroxydecanoic acid (5-HD) or MCC-134 completely abolishes the cardioprotective effect of IPC (Mubagwa *et al.*, 2001). In a study where the sarcolemmal K_{ATP} channel's importance in cardioprotection is compared to that of the mitochondrial K_{ATP} channel, it was found that the consequences of the opening of the $sarck_{ATP}$ channel include: shortening phase-3 of the action potential, membrane hyperpolarization, decreased calcium entry and preservation of ATP (Gross *et al.*, 1999). The consequences of the opening of the $mitoK_{ATP}$ channel include: membrane depolarization, matrix swelling, enhanced respiration and reduced calcium overload (Gross *et al.*, 1999). It has been suggested that the $sarck_{ATP}$ channel is not the main effector of cardioprotection, or that it may play only a minimal, supplementary role to the $mitoK_{ATP}$ channel (Fryer *et al.*, 2000b).

8.2 Materials and Methods

In order to investigate the possible involvement of the K_{ATP} channels in the cardioprotection that was observed with levosimendan pre- and postconditioning, K_{ATP} channel blockers were administered to inhibit the opening of these channels during LPC and LPostC. For inhibition of the mitochondrial K_{ATP} channel, 5-hydroxydecanoic acid (5HD) at a concentration of 200 μ M was used. For inhibition of the sarcolemmal K_{ATP} channel, glibenclamide (GBD) at a concentration of 5 μ M was used.

8.2.1 Blocking the mitochondrial K_{ATP} channel with 5HD & GBD.

The standard protocol for the control hearts (see figure 4.1 A) was adapted and instead of the 35 minutes of LD perfusion, the heart was perfused with a blocker for 35 minutes following the stabilization phase (see figure 8.1). Three different protocols were used to study the effect of 5HD and GBD: (i) the blockers were administered for 25 minutes before the onset of index ischaemia. After 5 minutes, 0.1 μ M levosimendan was administered simultaneously for 2 x 5 minutes, interspersed with 3 x 5 minute cycles with the blocker alone (5 μ M GBD or 200 μ M 5HD)(see figure 8.2) (ii) for the second protocol 5HD or GBD was administered

30 minutes after the initiation of the experiment for a period of 5 minutes, followed by a 10 minute period of pre-treatment with levosimendan plus 200 μ M 5HD or 5 μ M GBD (see figure 8.3). (iii) in the third protocol, the standard protocol for LPostC was adapted. Modifications were made by substituting the 3 x 20 second cycles of levosimendan (at the beginning of reperfusion) with a solution of 0.1 μ M levosimendan and 200 μ M 5HD or 5 μ M GBD (see figure 8.4).

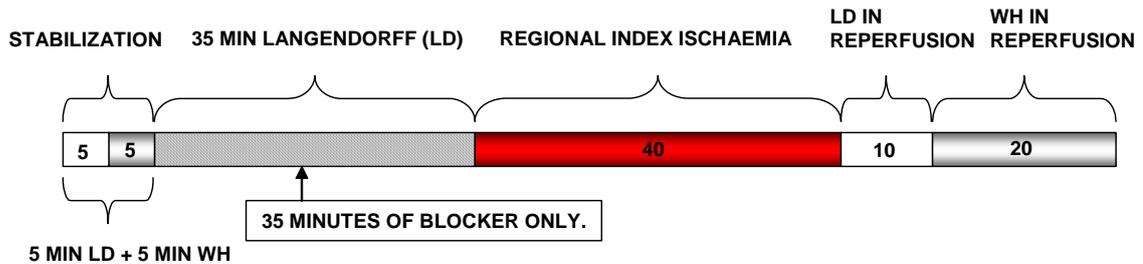


Figure 8.1: Protocol used for 5HD/GBD control hearts.

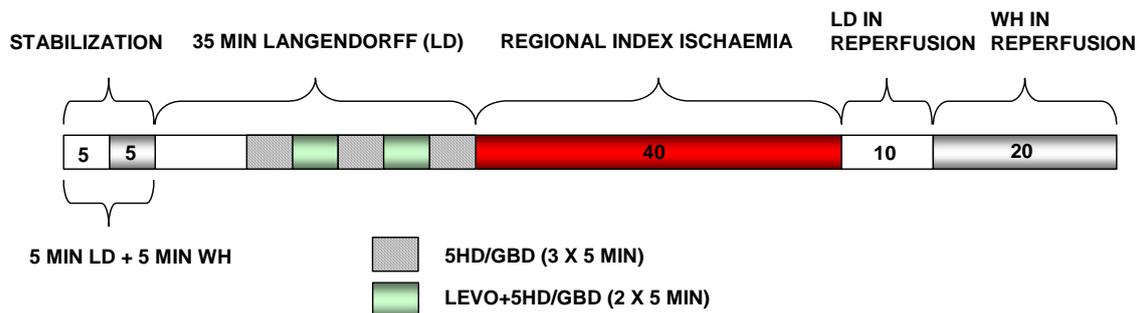


Figure 8.2: Protocol used for levosimendan preconditioning (LPC) + 5HD/GBD.

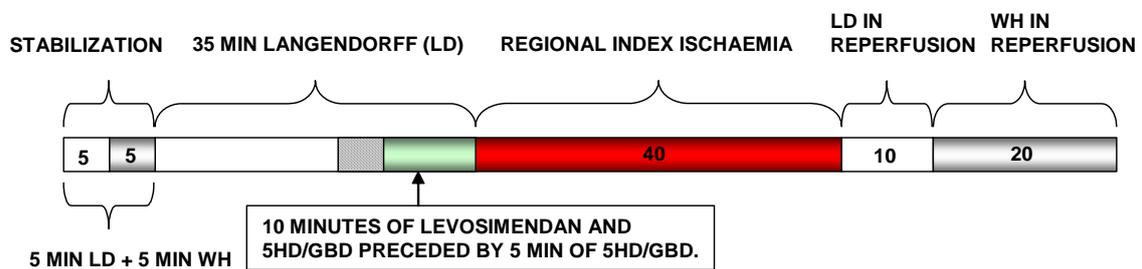


Figure 8.3: Protocol used for levosimendan pre-treatment (LPT) + 5HD/GBD.

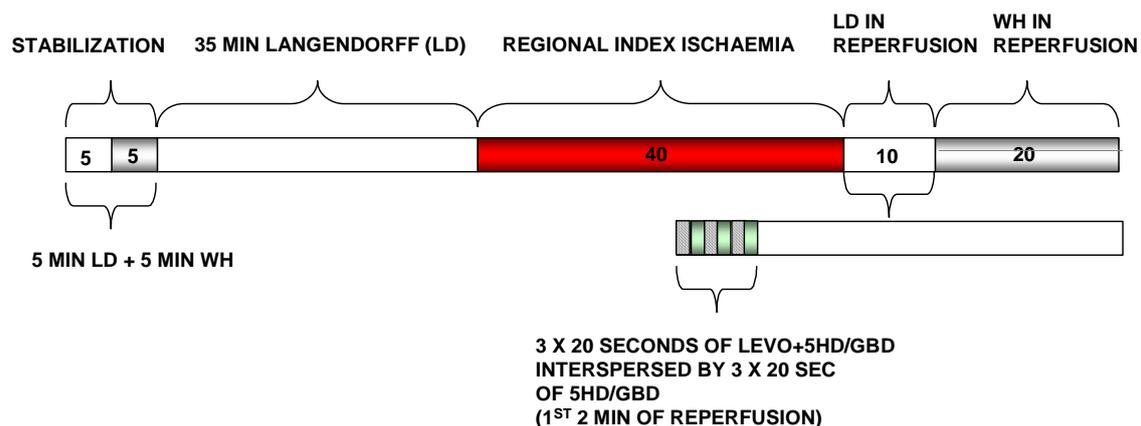


Figure 8.4: Protocol used for levosimendan postconditioning (LPostC) + 5HD/GBD.

Functional measurements were obtained at specific timepoints during the protocol, as shown in figure 3.2, page 38.

8.3 Results

The results are illustrated in tables and figures below.

8.3.1 Effect of K_{ATP} channel blockers and control hearts.

Administration of the mito K_{ATP} channel blocker, 5HD or the mito K_{ATP} and sarc K_{ATP} channel blocker, GBD in the standard protocols for control hearts, had no effect on infarct size (see figures 8.5 & 6 and table 8.1). As far as functional recovery is concerned for these control hearts, the 5HD treated group showed a significant increase ($78.15 \pm 5.99\%$) in the aortic output (% recovery) compared to control hearts (see table 8.4 and figure 8.7). GBD had no effect on the functional recovery of control hearts (see table 8.5 and figure 8.8). Refer to addendum A1, E1, E2, E5 and E6 for the detailed data of each experimental group.

8.3.2 Effect of K_{ATP} channel blockers on LPC.

Both blockers (5HD and GBD) (when administered during the LPC protocol) completely abolished protection and infarct sizes were similar to that of control hearts (see figure 8.5 and 6 and table 8.1). There was also no difference between the infarct sizes of the two groups (LPC+5HD and LPC+GBD). In both groups with control and blocker, we also observed no changes in functional

recovery (see figures 8.7/8 and tables 8.4/5). Refer to addendum A1, E1, E2, E5 and E6 for the detailed data of each experimental group.

8.3.3 Effect of K_{ATP} channel blockers on LPT.

The administration of both blockers (5HD and GBD) in conjunction with levosimendan pre-treatment (which conferred the most significant protection in our study so far) also completely abolished all protection (see figure 8.5 and 6 and table 8.2). Interestingly in the LPT + 5HD treated hearts we observed a significant improvement in functional recovery ($68.64 \pm 5.32\%$) compared to Control + 5HD (see table 8.4 and figure 8.7). However in the LPT + GBD and control hearts, similar values for functional recovery were observed (see table 8.5 and figure 8.8). Refer to addendum A1, E1, E3, E5 and E7 for the detailed data of each experimental group.

8.3.4 Effect of K_{ATP} channel blockers on LPostC.

Again the protection that was previously found with LPostC was abolished by the K_{ATP} channel blockers (see table 8.3 and figure 8.5). In both groups of blockers (LPostC + 5HD and LPostC + GBD) no changes in functional recovery have been observed when compared to controls (see tables 8.4/5 and figures 8.7/8). Refer to addendum A1, E1, E4, E5 and E8 for the detailed data of each experimental group.

Table 8.1: Data for inhibition of K_{ATP} channels with 5-hydroxydecanoic acid (5HD) or glibenclamide (GBD) in levosimendan preconditioned hearts (LPC).

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
CONTROL	54.78 ± 3.76	19.89 ± 1.41	31.41 ± 2.69	207.40 ± 8.51	
	21.56 ± 5.13	33.11 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80
CONTROL-5HD	55.75 ± 2.68	22.63 ± 0.88	25.72 ± 2.46	193.86 ± 10.33	
	43.00 ± 3.09	28.50 ± 1.70	18.67 ± 2.06	189.52 ± 8.59	33.91 ± 2.77
LPC+5HD	65.50 ± 3.97	19.83 ± 1.54	28.62 ± 2.58	191.10 ± 13.14	
	24.00 ± 3.65	36.33 ± 5.84	19.15 ± 2.32	197.83 ± 11.57	37.67 ± 1.65
CONTROL-GBD	55.25 ± 3.79	26.13 ± 3.73	26.38 ± 1.10	198.38 ± 4.47	
	22.75 ± 5.16	31.38 ± 2.95	19.91 ± 1.94	181.58 ± 13.02	46.77 ± 2.40
LPC+GBD	54.75 ± 3.16	21.38 ± 2.97	28.67 ± 1.76	217.58 ± 11.13	
	23.50 ± 4.63	31.00 ± 4.75	20.50 ± 1.37	185.95 ± 10.50	46.81 ± 1.28

Table 8.2: Data for inhibition of K_{ATP} channels with 5-hydroxydecanoic acid (5HD) or glibenclamide (GBD) in levosimendan pre-treated hearts (LPT).

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
CONTROL	54.78 ± 3.76	19.89 ± 1.41	31.41 ± 2.69	207.40 ± 8.51	
	21.56 ± 5.13	33.11 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80
CONTROL-5HD	55.75 ± 2.68	22.63 ± 0.88	25.72 ± 2.46	193.86 ± 10.33	
	43.00 ± 3.09	28.50 ± 1.70	18.67 ± 2.06	189.52 ± 8.59	33.91 ± 2.77
LPT+5HD	63.00 ± 4.18	22.50 ± 1.37	24.07 ± 2.18	196.42 ± 7.18	
	42.67 ± 3.63	36.33 ± 2.59	20.64 ± 1.44	186.96 ± 7.07	38.56 ± 2.37
CONTROL-GBD	55.25 ± 3.79	26.13 ± 3.73	26.38 ± 1.10	198.38 ± 4.47	
	22.75 ± 5.16	31.38 ± 2.95	19.91 ± 1.94	181.58 ± 13.02	46.77 ± 2.40
LPT+GBD	52.88 ± 3.12	20.63 ± 1.54	29.15 ± 1.22	214.24 ± 15.29	
	14.25 ± 4.94	24.38 ± 4.45	15.99 ± 2.88	176.85 ± 14.12	48.47 ± 2.90

Table 8.3: Data for inhibition of K_{ATP} channels with 5-hydroxydecanoic acid (5HD) or glibenclamide (GBD) in levosimendan postconditioned hearts (LPostC).

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
CONTROL	54.78 ± 3.76	19.89 ± 1.41	31.41 ± 2.69	207.40 ± 8.51	
	21.56 ± 5.13	33.11 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80
CONTROL-5HD	55.75 ± 2.68	22.63 ± 0.88	25.72 ± 2.46	193.86 ± 10.33	
	43.00 ± 3.09	28.50 ± 1.70	18.67 ± 2.06	189.52 ± 8.59	33.91 ± 2.77
LPostC+5HD	65.75 ± 3.01	22.50 ± 2.25	22.89 ± 0.80	171.99 ± 14.37	
	39.75 ± 5.24	37.25 ± 4.68	16.32 ± 1.91	176.33 ± 27.34	35.39 ± 0.01
CONTROL-GBD	55.25 ± 3.79	26.13 ± 3.73	26.38 ± 1.10	198.38 ± 4.47	
	22.75 ± 5.16	31.38 ± 2.95	19.91 ± 1.94	181.58 ± 13.02	46.77 ± 2.40
LPostC+GBD	64.25 ± 4.51	22.50 ± 2.25	21.45 ± 5.22	171.40 ± 20.10	
	21.00 ± 10.71	42.75 ± 6.05	20.40 ± 2.82	120.13 ± 12.71	41.04 ± 0.03

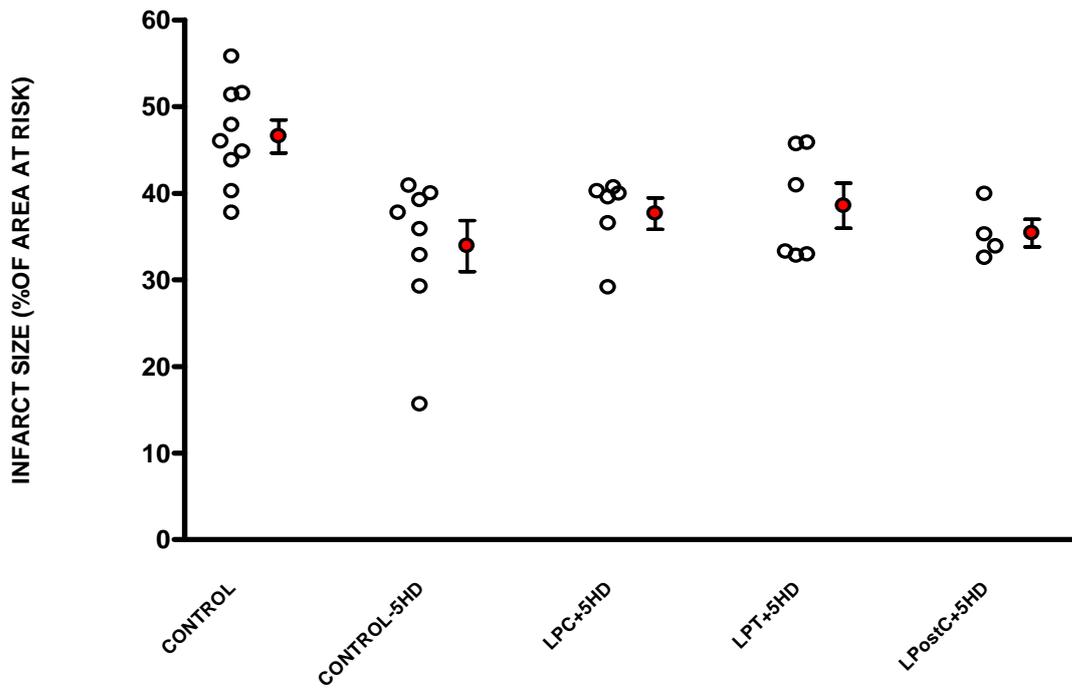


Figure 8.5: Infarct sizes for control & control-5HD vs. levosimendan preconditioning (LPC)+5HD, levosimendan pre-treatment (LPT)+5HD and levosimendan postconditioning (LPostC)+5HD.

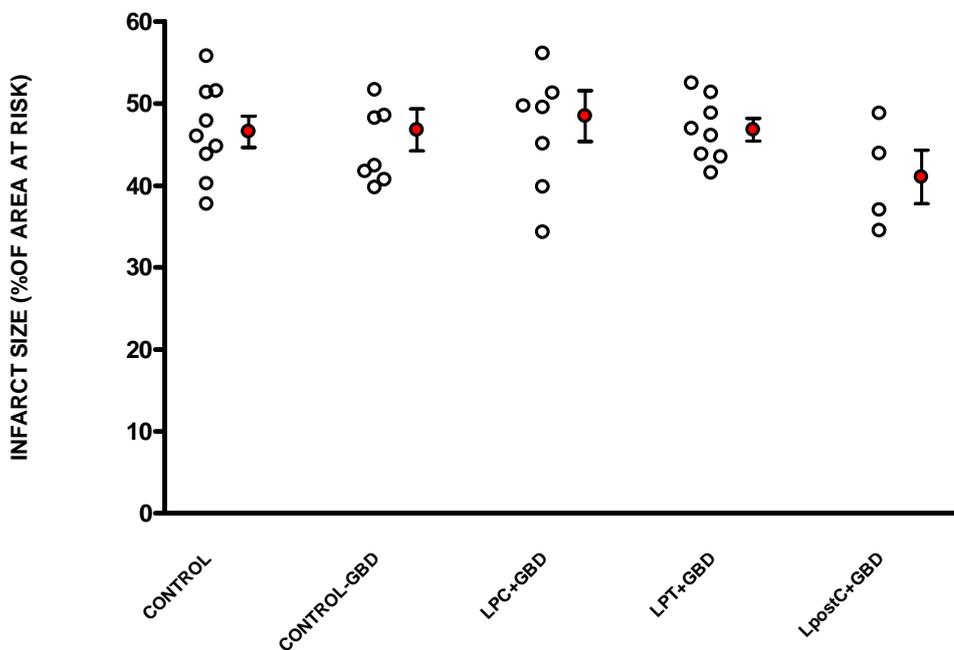


Figure 8.6: Infarct sizes for control & control-glibenclamide (GBD) vs. levosimendan preconditioning (LPC)+GBD, levosimendan pre-treatment (LPT)+GBD and levosimendan postconditioning (LPostC)+GBD.

Table 8.4: Functional recovery (% aortic output recovery) in control-5HD, levosimendan preconditioning (LPC)+5HD, levosimendan pre-treatment (LPT)+5HD and levosimendan postconditioning (LPostC)+5HD groups.

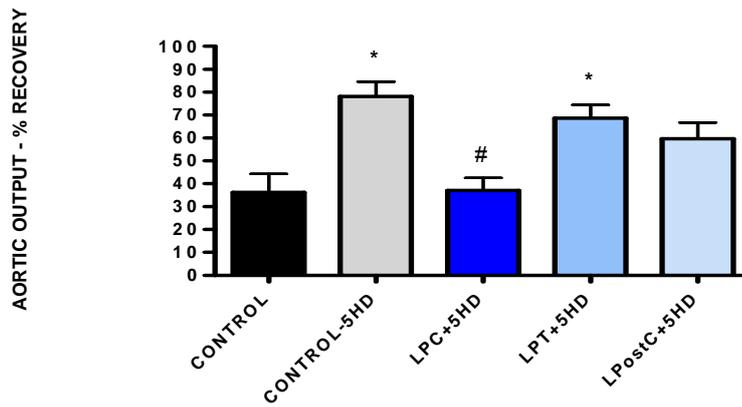
Experimental Group	CONTROL	CONTROL-5HD	LPC+5HD	LPT+5HD	LPostC+5HD
Pre-ischaemic aortic output (ml/min)	54.78 ± 3.76	55.75 ± 2.68	65.50 ± 3.97	63.00 ± 4.18	65.75 ± 3.01
Post-ischaemic aortic output (ml/min)	21.56 ± 5.13	43.00 ± 3.09	24.00 ± 3.65	42.67 ± 3.63	39.75 ± 5.24
Aortic Output (% recovery)	36.23 ± 7.69	78.15 ± 5.99 *	37.04 ± 5.03	68.64 ± 5.32 #	59.74 ± 0.06

* p < 0.05 vs. controls

p < 0.05 vs. control + 5HD

Table 8.5: Functional recovery (% aortic output recovery) in LPC+glibenclamide (GBD), levosimendan preconditioning (LPC)+GBD, levosimendan pre-treatment (LPT)+GBD and levosimendan postconditioning (LPostC)+GBD groups.

Experimental Group	CONTROL	CONTROL-GBD	LPC+GBD	LPT+GBD	LPostC+GBD
Pre-ischaemic aortic output (ml/min)	54.78 ± 3.76	55.25 ± 3.79	54.75 ± 3.16	52.88 ± 3.12	64.25 ± 4.51
Post-ischaemic aortic output (ml/min)	21.56 ± 5.13	22.75 ± 5.16	23.50 ± 4.63	14.25 ± 4.94	21.00 ± 10.71
Aortic Output (% recovery)	36.23 ± 7.69	43.00 ± 9.84	42.74 ± 6.99	24.98 ± 7.91	31.82 ± 0.16



* $p < 0.05$ vs. Control

$p < 0.05$ vs. Control-5HD

Figure 8.7: Functional recovery (aortic output - % recovery) for control-5-hydroxydecanoic acid (5HD), levosimendan preconditioning (LPC)+5HD, levosimendan pre-treatment (LPT)+5HD and levosimendan postconditioning (LPostC)+5HD.

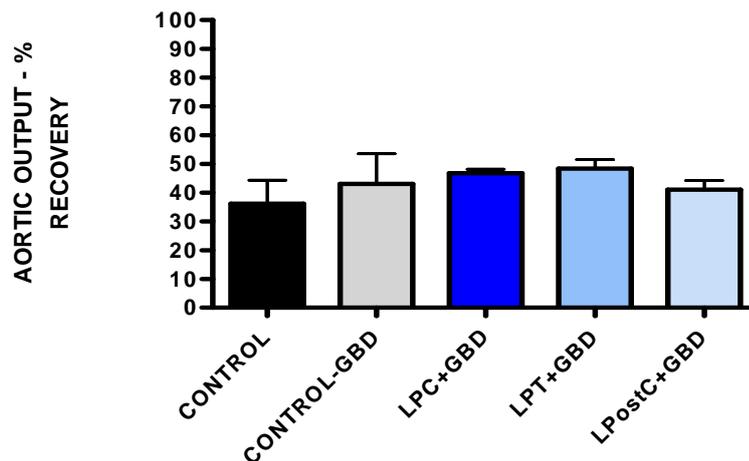


Figure 8.8: Functional recovery (aortic output - % recovery) for control-glibenclamide (GBD), levosimendan preconditioning (LPC) + GBD, levosimendan pre-treatment (LPT) + GBD and levosimendan postconditioning (LPostC) + GBD.

8.4 Summary of results.

From the data it is concluded that administration of K_{ATP} channel blockers (both sarcolemmal and mitochondrial channels), 5HD and GBD abolished the protection induced by levosimendan in all three protocols (LPC, LPT and LPostC). This data suggests that it is essential for the K_{ATP} channels to be open for levosimendan to exert its cardioprotective actions.

CHAPTER NINE

INVESTIGATING THE POSSIBLE INVOLVEMENT OF THE RISK PATHWAY IN LEVOSIMENDAN INDUCED CARDIOPROTECTION.

9.1 Introduction

The Reperfusion Injury Salvage Kinase (RISK) pathway refers to a group of pro-survival protein kinases (PKB/Akt and ERK42/44) which confer powerful cardioprotection, when activated at the time of reperfusion (Hausenloy *et al.*, 2005b; Hausenloy *et al.*, 2007). Several interventions, conferring cardioprotection, has been shown to significantly reduce infarct size through the activation of the RISK pathway when administered (Hausenloy *et al.*, .2005a).

Adenosine (through the use of various agonists), bradykinin and opioids (Yang *et al.*, 2004a; Kis *et al.*, 2003; Park *et al.*, 2006; Bell *et al.*, 2003; Gross *et al.*, 2004) has been shown to significantly reduce infarct size through the activation of the RISK pathway when administered at the time of myocardial reperfusion (Hausenloy *et al.*, 2007). Ligand binding at the G protein coupled receptor (GPCR), leads to the activation of its tyrosine receptor kinase, which then activates the PI3K-Akt and MEK1/2-ERK42/44 signaling cascades.

Recent studies have shown that IPC also leads to the phosphorylation of both PKB/Akt and ERK42/44 at the time of reperfusion (Hausenloy *et al.*, 2005b). The significance of activation of these kinases in cardioprotection was demonstrated by the use of appropriate inhibitors. PKB/Akt can be indirectly inhibited by the inhibition of PI3-K activation by Wortmannin (Kandel and Hay, 1999). ERK42/44 can be inhibited by administering the MEK1/2 inhibitor, PD 098059 (Hausenloy *et al.*, 2005b). In doing so, the protective effect of IPC was abolished, suggesting an essential role for these kinases in cardioprotection by IPC (Hausenloy *et al.*, 2005b).

9.2 Materials and Methods

Hearts were perfused as described in chapter 3. After 5 or 10 minutes of reperfusion ischaemic and non-ischaemic areas of the hearts were separated and

freeze-clamped. Samples were stored at -80°C in liquid nitrogen until Western blot analysis were performed. The phosphorylation of cardiac ERK42/44 and PKB/Akt was investigated. Infarct size as a percentage of the area at risk was used as the endpoint for the experimental groups where we inhibited the activation of ERK42/44 with PD 098059 (the inhibitor of MEK, upstream of ERK42/44) at a concentration of $10\mu\text{M}$.

9.2.1 The role of the phosphorylation of PKB/Akt & ERK42/44 in levosimendan induced cardioprotection.

Hearts were subjected to the same protocols as described for control, LPC and LPT studies. Hearts were then freeze-clamped at 5 or 10 minutes of reperfusion (see figures 9.1 – 9.3).

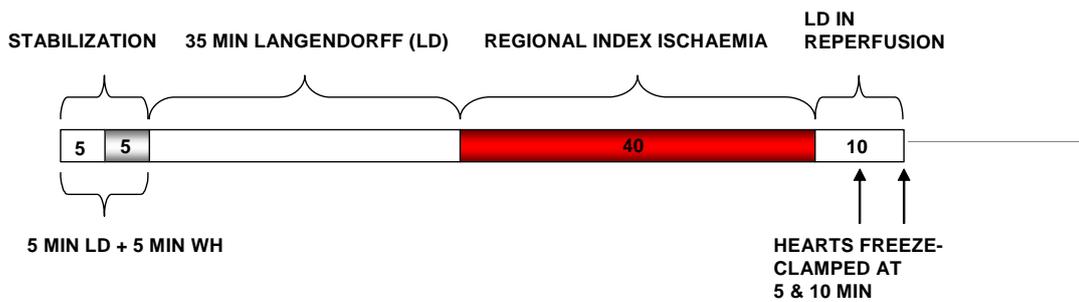


Figure 9.1: Protocol for the control group, with hearts freeze-clamped at 5 or 10 minutes of reperfusion for Western blot analysis.

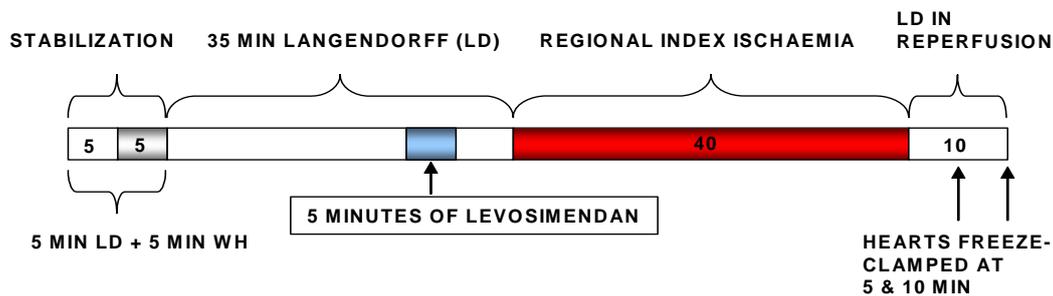


Figure 9.2: Protocol for the levosimendan preconditioned (LPC) group, with hearts freeze-clamped at 5 or 10 minutes of reperfusion for Western blot analysis.

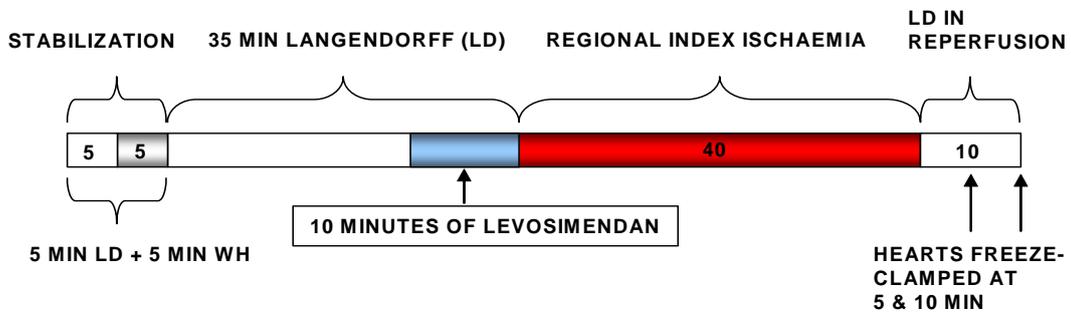


Figure 9.3: Protocol for the levosimendan pre-treated (LPT) group, in which hearts were freeze-clamped at 5 or 10 minutes of reperfusion for Western blot analysis.

9.2.2 The effect of the K_{ATP} channel blockers during LPostC on phosphorylation of PKB/Akt and ERK42/44 in levosimendan-postconditioned hearts (LPostC).

The effect of the K_{ATP} channel blockers on the phosphorylation of the kinases of the RISK pathway, were investigated in the setting of postconditioning only. Hearts were subjected to the protocols used previously in control, IPostC, IPostC+5HD, LPostC and LPostC+5HD (see figure 9.1 and 9.4 – 9.7). To investigate the role of the mitochondrial K_{ATP} channel blockers on the phosphorylation of PKB/Akt and ERK42/44 in LPostC and IPostC, 5HD/GBD was added as shown in figures 9.5 and 9.7. Hearts were freeze-clamped at 5 minutes reperfusion and kept in liquid nitrogen until further experimentation.

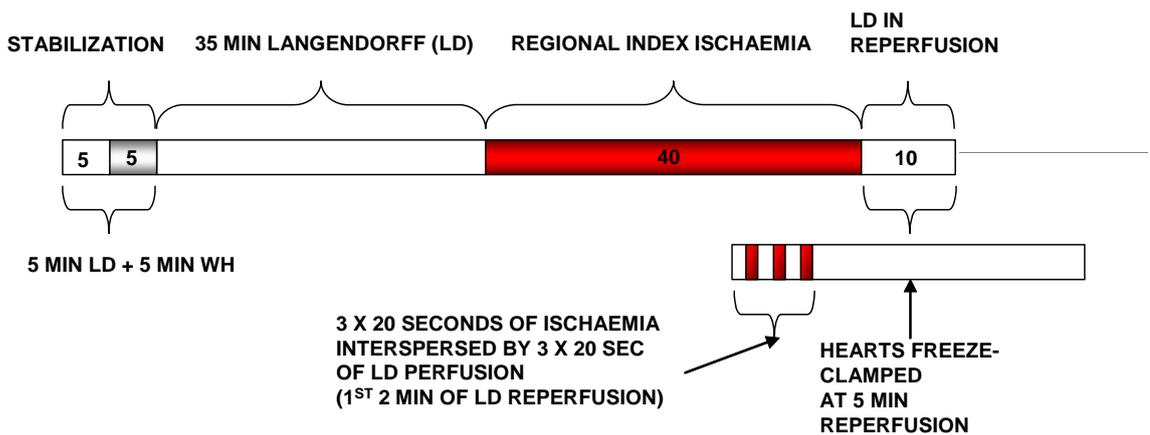


Figure 9.4: Protocol for the ischaemic postconditioned (IPostC) group, in which hearts were freeze-clamped at 5 minutes of reperfusion for Western blot analysis.

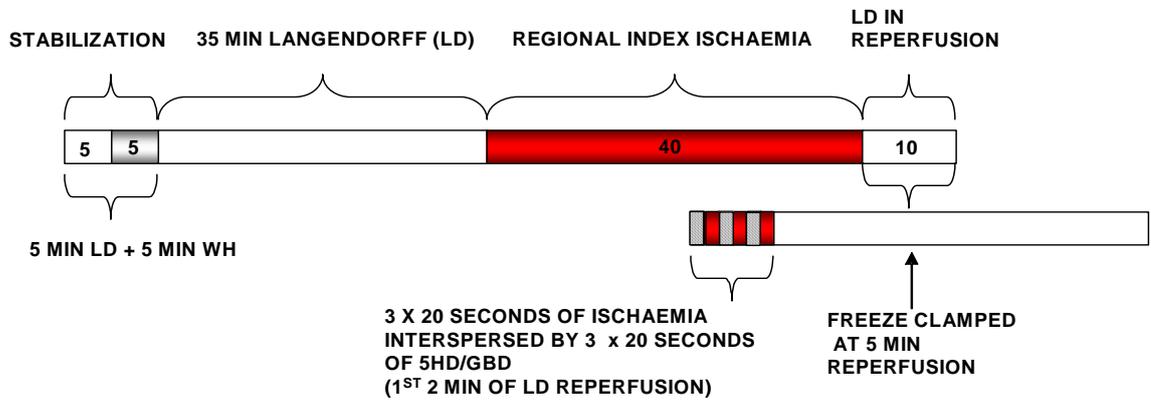


Figure 9.5: Protocol for the ischaemic postconditioning + 5-hydroxydecanoic acid (IPostC+5HD) or glibenclamide (IPostC+GBD) group, in which hearts were freeze-clamped at 5 minutes of reperfusion for Western blot analysis.

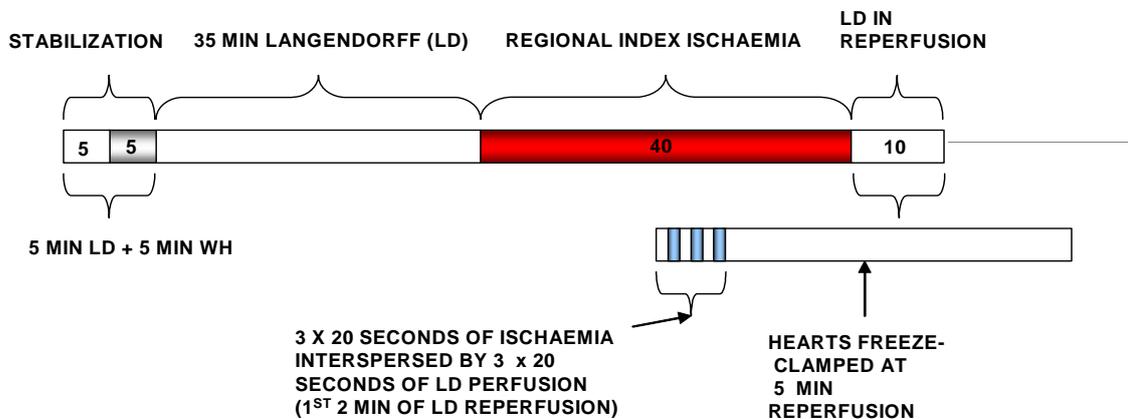


Figure 9.6: Protocol for the levosimendan postconditioning (LPostC) group, in which hearts were freeze-clamped at 5 minutes of reperfusion for Western blot analysis.

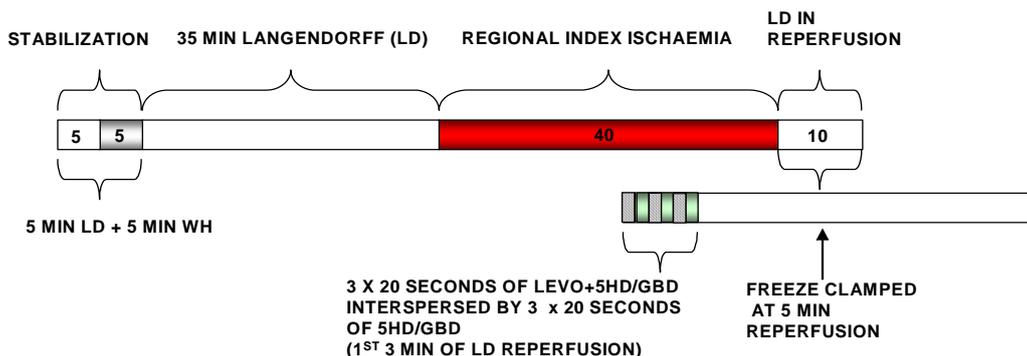


Figure 9.7: Protocol for the levosimendan postconditioning + 5-hydroxydecanoic acid (IPostC+5HD) or glibenclamide (IPostC+GBD) group, in which hearts were freeze-clamped at 5 minutes of reperfusion for Western blot analysis.

9.2.3 The effects of the mitogen activated protein kinase (MAPK) MEK-inhibitor PD 098059 on the phosphorylation of ERK42/44.

To investigate the effect of PD 098059 on the phosphorylation of one of the RISK pathway components (ERK42/44), hearts were subjected to the protocols described for control, control + PD 098059 and levosimendan + PD 098059 (see 9.1, 9.8 and 9.10). Hearts were freeze-clamped at 5 or 10 minutes of reperfusion and stored in liquid nitrogen for Western blot analysis. We performed Western blot analysis to ensure that we were able to significantly reduce the phosphorylation of ERK42/44 with the inhibitor of MEK, PD 098059 in our model. Subsequently we investigated the effect of the inhibition of ERK42/44 on infarct size (see figure 9.9 and 9.11).

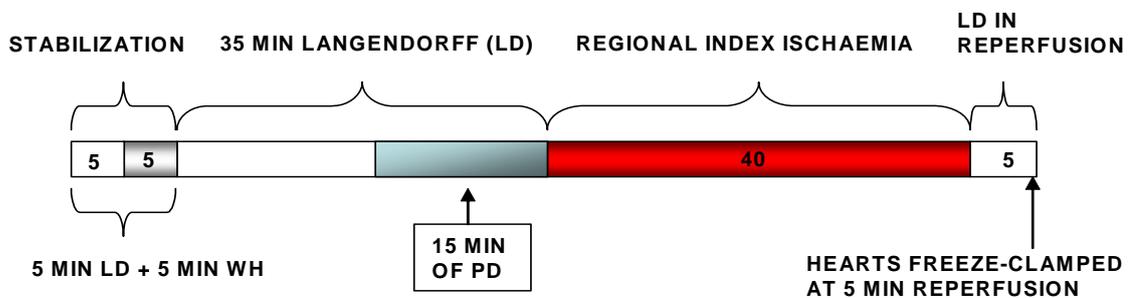


Figure 9.8: Protocol for the control + PD 098059 group, with hearts freeze-clamped at 5 minutes of reperfusion for Western blot analysis.

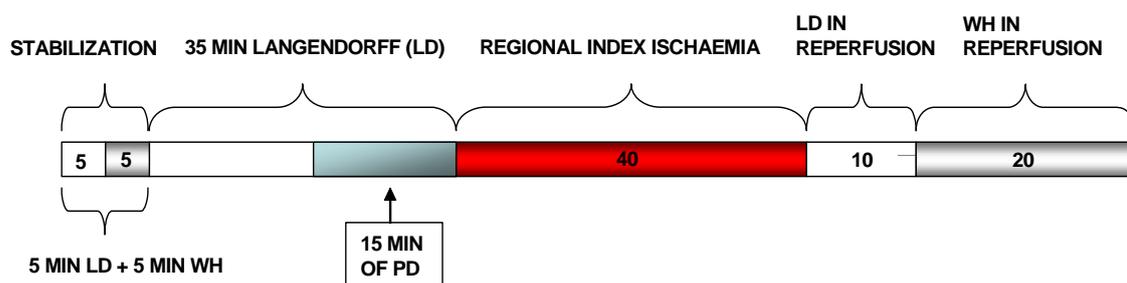


Figure 9.9: Protocol for the control + PD 098059 group used for the determination of infarct size.

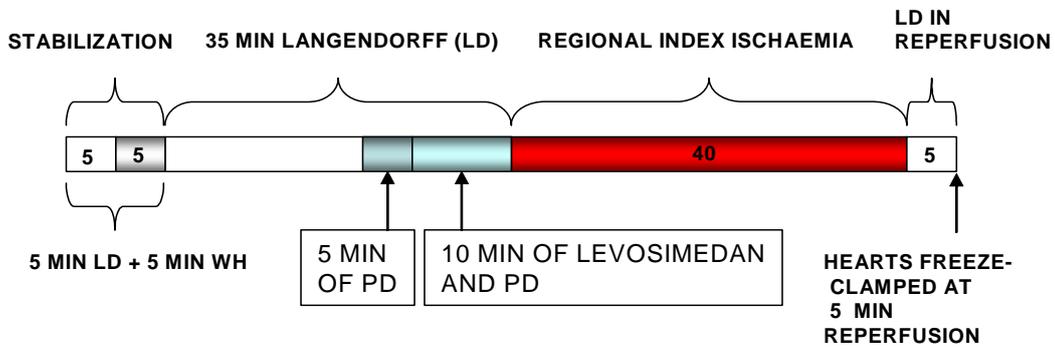


Figure 9.10: Protocol for the levosimendan + PD 098059 group, with hearts freeze-clamped at 5 minutes of reperfusion for Western blot analysis.

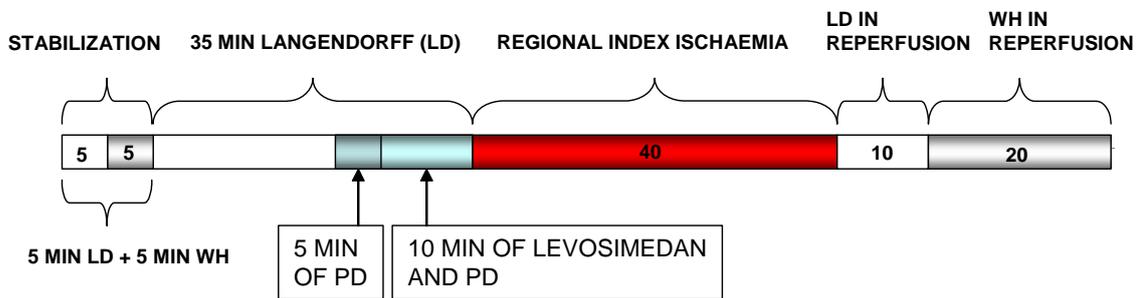


Figure 9.11: Protocol for the levosimendan + PD 098059 group used for the determination of infarct size.

9.3 Results

9.3.1 Phosphorylation of PKB/Akt in levosimendan induced cardioprotection.

From Western blot analyses data on the levosimendan induced cardioprotection groups (LPC, LPT and LPostC), no difference in phosphorylation of PKB/Akt was observed when comparing control group hearts to the levosimendan preconditioning or pre-treatment (see figure 9.13), at either 5 or 10 minutes reperfusion. LPostC was investigated at 5 minutes reperfusion only and the phosphorylation of PKB/Akt was found to be similar to that of the other groups.

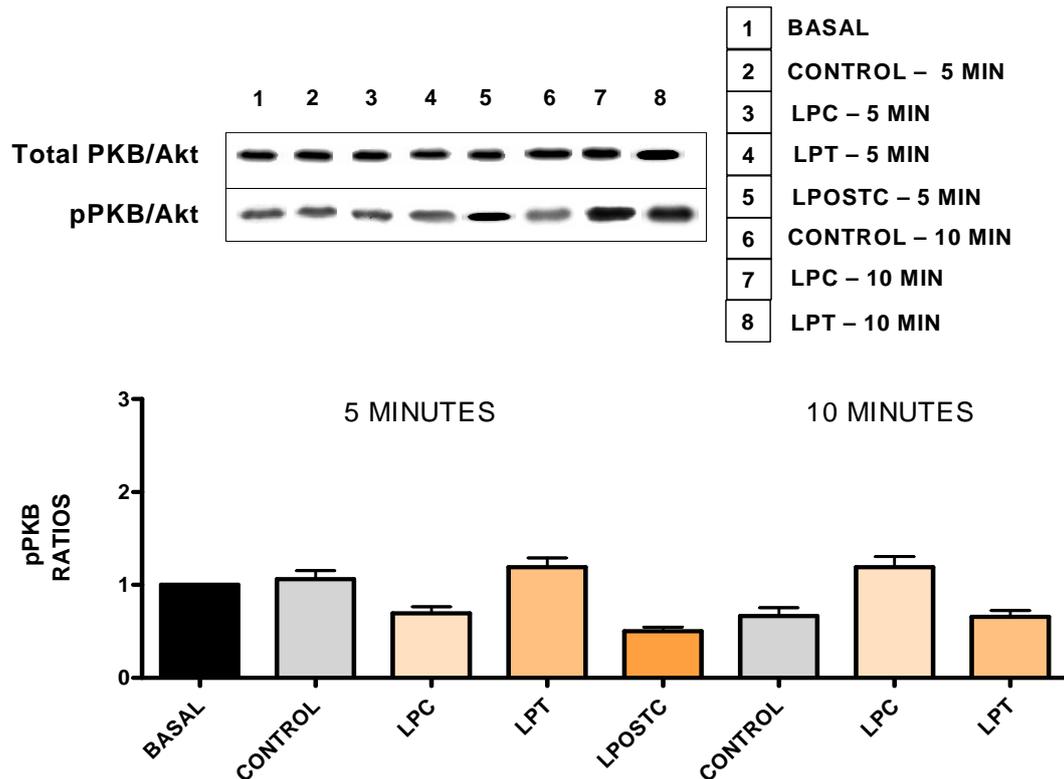
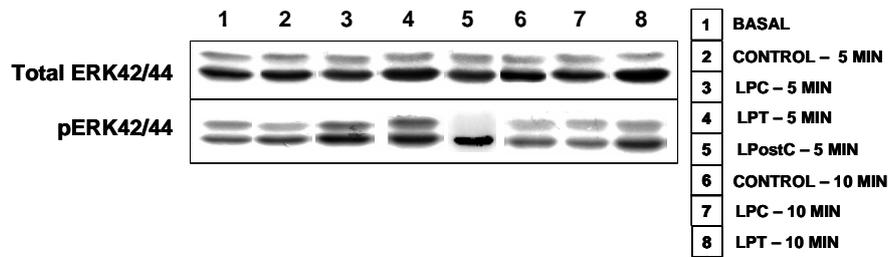


Figure 9.12: Phosphorylation of PKB/Akt under basal, control, LPC and LPT experimental conditions at 5 and 10 minutes reperfusion and LPostC at 5 minute reperfusion. Values expressed as a ratio of the basal value = 1. Basal hearts were not subjected to any intervention and were freeze-clamped before index ischaemia.

9.3.2 Effect of levosimendan preconditioning & pre-treatment on the phosphorylation of ERK42/44.

When the Western blot analyses were performed to investigate the phosphorylation of ERK42/44 the following was observed. At 5 minutes reperfusion there was a significant increase in ($p < 0.05$) phosphorylation of both ERK 44 and ERK 42 in the LPT group vs. the control hearts (see figure 9.13). At 10 minutes reperfusion, a similar significant increased ($p < 0.05$) phosphorylation of both ERK 44 and ERK42 in the LPT group was observed (see figure 9.13). A significant phosphorylation of ERK42 in the LPC hearts, was also observed at 10 minutes reperfusion ($p < 0.05$).



A

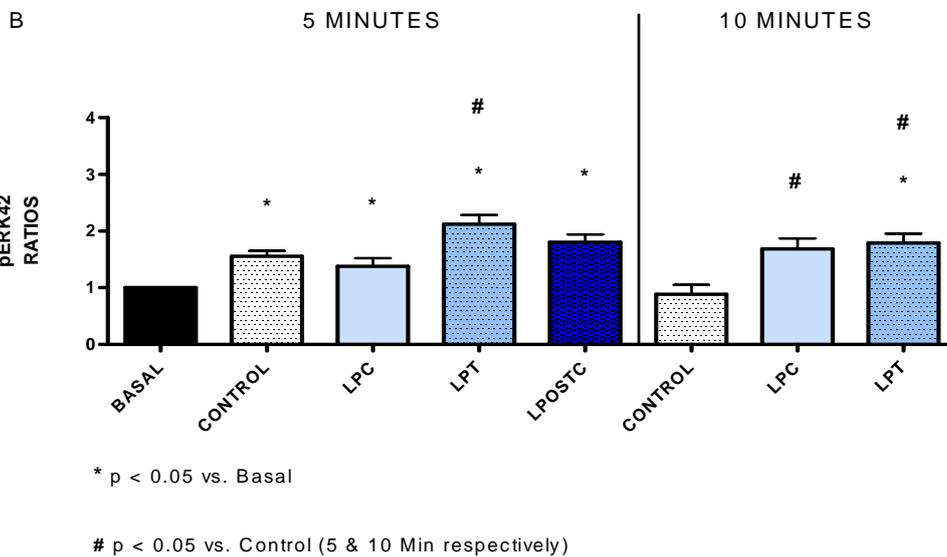
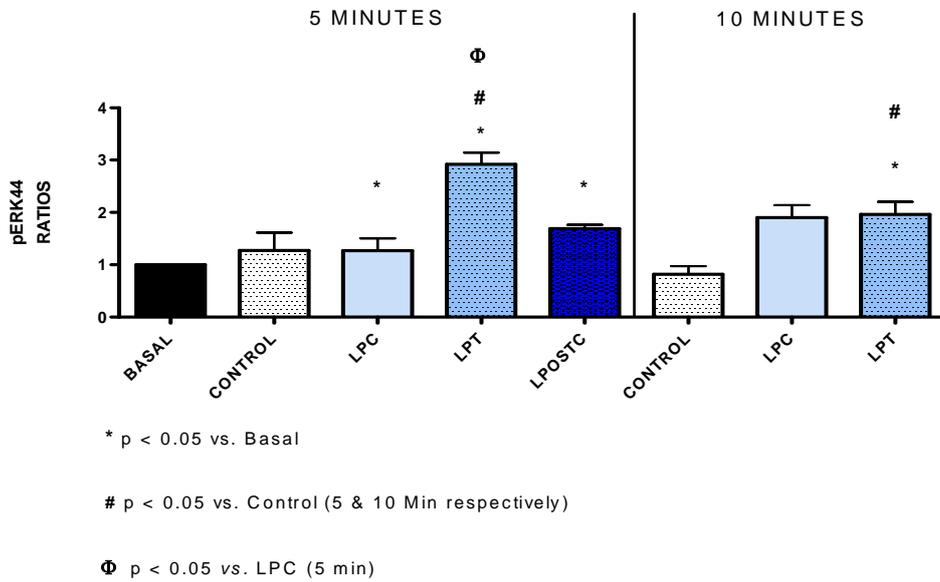


Figure 9.13: A: Phosphorylation of ERK44 under basal, control, LPC, LPT and LPostC (only at 5 minutes) experimental conditions at 5 and 10 minutes reperfusion. B: Phosphorylation of ERK42 in Basal, Control, LPC, LPT and LPostC (only at 5 minutes) experimental conditions at 5 and 10 minutes reperfusion. Values expressed as a ratio of the basal value = 1. Basal hearts were not subjected to any intervention and were freeze-clamped before index ischaemia.

9.3.3 Effect of the K_{ATP} channel blockers on the phosphorylation of PKB/Akt in LPostC hearts.

Due to limited time and resources, the effect of blockers on the phosphorylation of the kinases of the RISK pathway was only investigated in postconditioning. Hearts were freeze-clamped at 5 minutes reperfusion only as this time point was found to give the most significant results in the preconditioning experiments.

From the data obtained for the postconditioning groups with the K_{ATP} channel blockers there was no evidence of phosphorylation of PKB/Akt in either ischaemic postconditioned or levosimendan-postconditioned hearts (see figure 9.14).

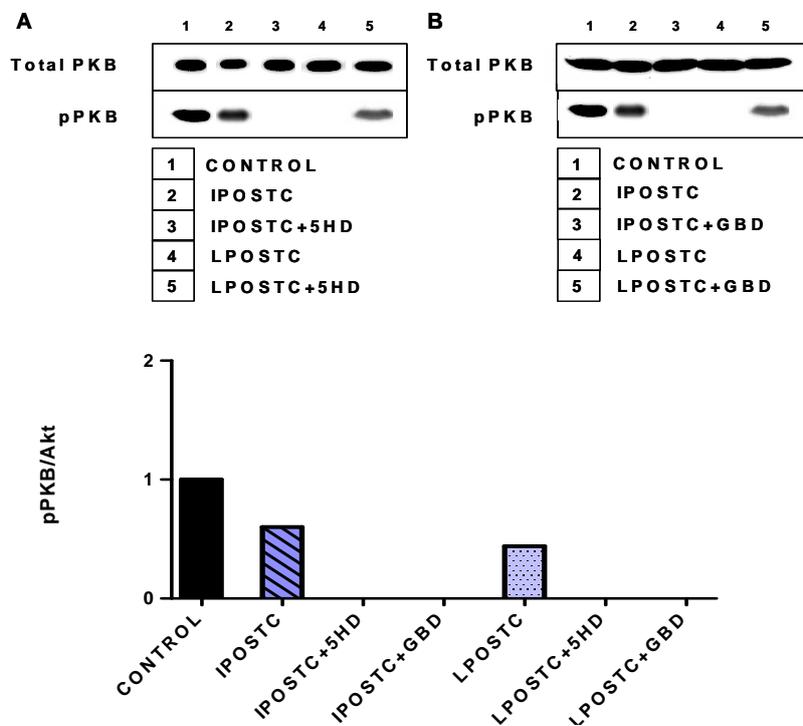


Figure 9.14: A: Phosphorylation of PKB/Akt under control, IPostC+5HD, LPostC and LPostC+5HD experimental conditions at 5 minutes reperfusion. B: Phosphorylation of PKB/Akt in control, IPostC+GBD, LPostC and LPostC+GBD experimental conditions at 5 minutes reperfusion. Note the total inhibition of PKB/Akt activation when GBD or 5HD is administered. Values expressed as a ratio of the basal value = 1. Basal hearts were not subjected to any intervention and were freeze-clamped before index ischaemia.

9.3.4 Effect of the K_{ATP} channel blockers on the phosphorylation of ERK42/44 in LPostC hearts.

When the K_{ATP} channel was blocked with GBD and Western blot analyses were performed on the hearts, there was a significant increase ($p < 0.05$) in phosphorylation of ERK42, but not ERK44 vs. control in IPostC and LpostC hearts (see figure 9.15 and 9.16). There is no change in the phosphorylation of these MAPK when the mito K_{ATP} channel blocker 5HD is administered together with these interventions (see figure 9.14).

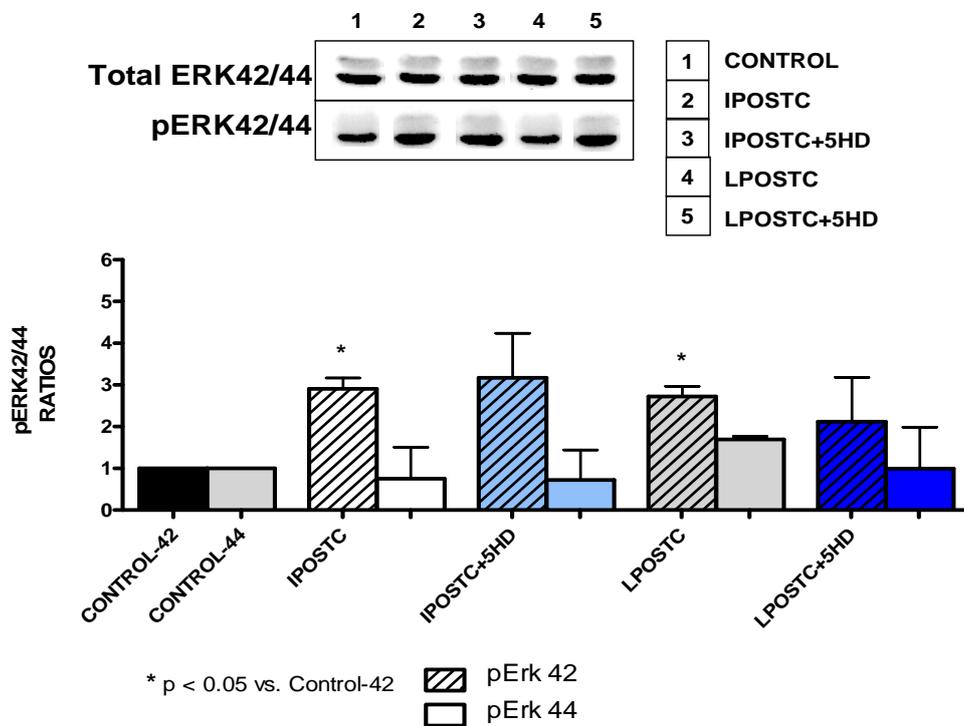


Figure 9.15: Phosphorylation of ERK42/44 under control, IPostC, IPostC+5HD, LPostC and LPostC+5HD experimental conditions at 5 minutes reperfusion. Values expressed as a ratio of the control value = 1.

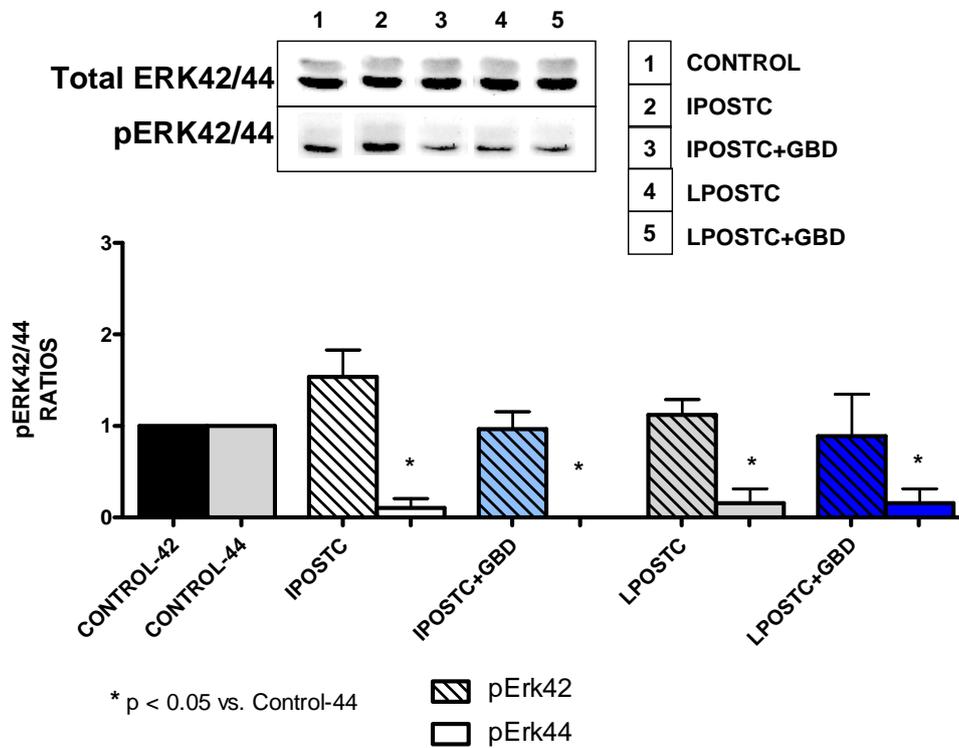


Figure 9.16: Phosphorylation of ERK42/44 under control, IPostC+GBD and LPostC+GBD experimental conditions at 5 minutes reperfusion. Values expressed as a ratio of the control value = 1.

9.3.5 Effect of the MEK inhibitor – PD 098059 on the phosphorylation of ERK42/44.

In this study, only an inhibitor of ERK42/44 was used as there was no phosphorylation of PKB/Akt observed in the levosimendan preconditioning, pre-treatment or postconditioning (LPC, LPT and LPostC) hearts. Since the most significant effect of levosimendan was obtained when hearts were pre-treated with levosimendan (LPT), the effect of the MEK inhibitor PD 098059 on ERK42/44 activation was studied in these hearts only. A significant ($p < 0.05$) reduction in phosphorylation of ERK42/44 was observed when we administered the PD 098059 alone. At 5 minutes reperfusion, PD abolished the increase in ERK42/44 phosphorylation seen in LPT hearts (see figure 9.17). Since PD 098059 abolished ERK42/44 phosphorylation during reperfusion, it was decided to evaluate its effect on infarct size. PD alone, in the absence of levosimendan, had no effect on infarct size and values similar to the controls were obtained. However, when administered in the presence of levosimendan (LPT), it caused a significant reduction, but not abolition of protection, since the infarct size increased from

5.75±0.87% to 17.12±1.51% (p < 0.05) (from LPT to PD+LPT)(see figure 9.17 and table 9.1).

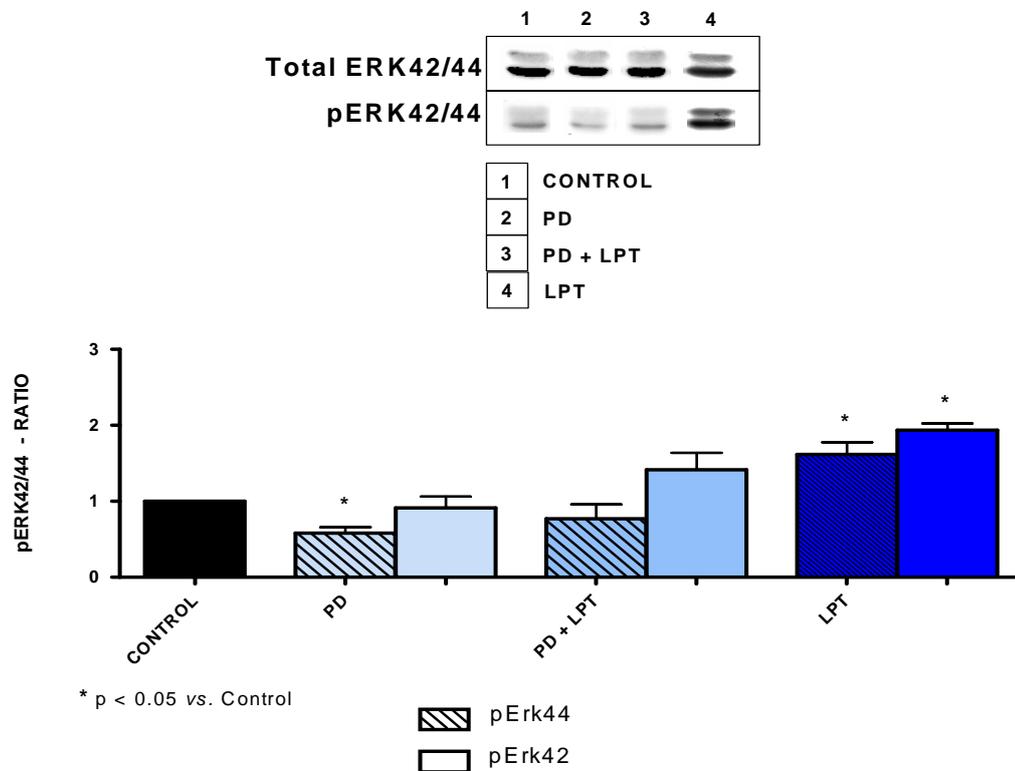


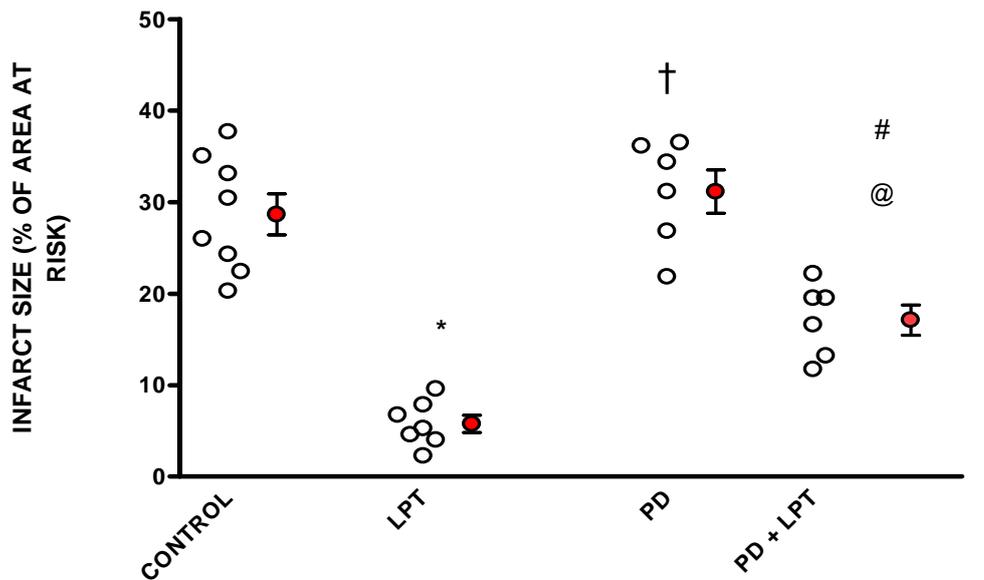
Figure 9.17: Phosphorylation of ERK42/44 under control, PD and PD+LEVO experimental conditions at 5 minutes reperfusion. Values expressed as a ratio of the control value = 1.

Table 9.1: Effect of pERK42/44 inhibition WITH PD 098059 on infarct size.

Refer to addendums F1 and F2 for detailed data of each experimental group.

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
CONTROL	54.78 ± 3.76	19.89 ± 1.41	31.41 ± 2.69	207.40 ± 8.51	
	21.56 ± 5.13	33.11 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80
PD	66.83 ± 2.93	23.17 ± 1.80	22.75 ± 1.86	199.78 ± 12.71	
	34.50 ± 7.13	33.67 ± 6.38	15.69 ± 1.87	200.43 ± 12.06	31.15 ± 2.17
PD + LEVO	70.50 ± 1.54	24.50 ± 1.65	22.97 ± 1.26	177.27 ± 5.95	
	52.67 ± 2.21	27.33 ± 1.71	20.92 ± 0.80	178.59 ± 7.78	17.12 ± 1.51 *

* p < 0.05 vs. control



* p < 0.001 vs. Control

p < 0.05 vs. Control

† p < 0.001 vs. LPT and PD + LPT

@ p < 0.001 vs. LPT

Figure 9.18: Infarct sizes for control, LPT, PD and PD + LEVO treated hearts. (Infarct sizes for control and LPT included from chapter 6, page 73).

9.4 Summary of results.

From these results we propose that PKB/Akt does not play any role in the protective effect that was observed in the interventions with levosimendan. However, the increased phosphorylation of ERK42/44 after 5-10 minutes reperfusion showed that the RISK pathway is involved in levosimendan induced cardioprotection. It was also observed that a K_{ATP} channel blocker which abolished the protection afforded by an levosimendan postconditioning (LPostC + blocker) also reduced phosphorylation of ERK42/44. The significance of ERK42/44 activation during reperfusion was further evaluated by administering the MEK1/2 inhibitor to levosimendan pre-treated hearts (LPT). It caused a significant inhibition in ERK42/44 activation and this was associated with partial abolition of levosimendan protection.

CHAPTER TEN

DISCUSSION AND CONCLUSIONS

10.1 Establishing a pre- and postconditioning protocol in the guinea pig model.

There are numerous studies on ischaemic preconditioning of the heart in the literature (Murray *et al.*, 1986; Liu *et al.*, 1992a; Asimakis *et al.*, 1992; Mitchell *et al.*, 1995; Shipolini *et al.*, 1997; Aitchison *et al.*, 2000; Javadov *et al.*, 2003). These studies have primarily investigated the cardioprotective effects of IPC in the rat heart. Several reviews on the topic of ischaemic preconditioning have also been published (Rubino and Yellon, 2000; Bolli R, 2000; Cohen *et al.*, 2000; Eisen *et al.*, 2004; Antonio *et al.*, 2006). A number of studies have also investigated the phenomenon of postconditioning in the rat heart model (Kin *et al.*, 2004; Galagudza *et al.*, 2004; Yang *et al.*, 2005; Argaud *et al.*, 2005; Penna *et al.*, 2006; Bopassa *et al.*, 2006). The first objective of this study was to establish a protocol for IPC and IPostC for the isolated guinea pig heart. This model was chosen since the guinea pig heart displays similar inotropic responses to levosimendan as the human heart.

The IPC protocol (3 x 5 minutes global ischaemia) that was chosen to use in the guinea pig model, elicited a significant reduction in infarct size when compared with those of control experimental hearts. Despite the reduction in infarct size, functional recovery after exposure of the heart to 35 minutes of regional ischaemia remained unchanged. In contrast to functional recovery, infarct size appears to be a robust technique which has been used successfully to demonstrate a reduction in damage by preconditioning in all species tested thus far (Lochner *et al.*, 2003). It is thus not uncommon for a reduction in infarct size not to correlate positively with an improvement in functional recovery and similar observations were made in the rat heart (Murray *et al.*, 1986; Liu *et al.*, 1992a; Asimakis *et al.*, 1992; Mitchell *et al.*, 1995; Shipolini *et al.*, 1997; Aitchison *et al.*, 2000; Javadov *et al.*, 2003). From this data we concluded that the isolated guinea pig heart can indeed be preconditioned, as reflected by a reduction in infarct size, but not improvement in functional recovery. This fact was previously proven by other studies where

researchers managed to precondition the isolated guinea pig heart (Lochner and Genade, 1998; Jin and Chen, 2007; Ravingerova *et al.*, 1998). All these studies investigated ischaemic preconditioning in the isolated guinea pig heart (the same as in this current study), but applied global ischaemia and measured functional recovery as the end-point. The current study applied a model of regional ischaemia and measured infarct size as an end-point and, as far as we know, this is the first study to use infarct size to assess the efficacy of IPC in the isolated guinea pig heart. It does appear that functional recovery is a less sensitive indicator of protection as was the case in the rat heart studies (Lochner *et al.*, 2003). However, one may speculate that these hearts were merely stunned and that they had reversible suppression of mechanical function, that may have improved with prolonged reperfusion. In the current study, we have performed a prolonged reperfusion (1 hour) on a separate series of hearts, to rule out this possibility.

With the postconditioning protocol applied in this study, the infarct size was also significantly decreased from the control hearts, but functional recovery was again unchanged. From these we concluded that a model of postconditioning has been successfully established. More or less similar studies were performed in rat hearts (Tsang *et al.*, 2004; Galagudza *et al.*, 2004; Serviddio *et al.*, 2005). In each of these studies the isolated rat hearts were subjected to 35 minutes of regional ischaemia, 30 minutes of regional ischaemia and 45 minutes of global ischaemia respectively. All these researchers used a different number and/or length of ischaemia (all global ischaemia) after reperfusion, to postcondition the hearts. Several studies were performed using in-vivo dog models (Zhao *et al.*, 2002; Halkos *et al.*, 2004; Sato *et al.*, 1997). All the researchers in these studies used coronary artery ligation to induce different lengths of regional index ischaemia and different postconditioning interventions were used after the index ischaemia. In the current study a model of 40 minutes of regional ischaemia was used and the ischaemic cycles after reperfusion (3 x 20 seconds) were also regional ischaemia. However, although these studies differ from one another and from the current study in terms of animal models, form of index ischaemia and postconditioning interventions, all algorithms of postconditioning managed to significantly reduce infarct size compared to the control hearts. To my knowledge, this is the first study

to investigate the phenomenon of postconditioning in the guinea pig heart. With suitable protocols for both pre- and postconditioning with ischaemia established in the guinea pig model, the focus shifted to the next area of the study.

10.2 Establishing whether levosimendan can be used as a pre- and postconditioning mimetic.

10.2.1 Conventional uses for levosimendan.

The majority of clinically used inotropes act by increasing cytosolic calcium levels which may cause arrhythmias and worsen reperfusion stunning. The finding that levosimendan acts as a calcium sensitizing agent without these harmful effects, led to evaluation of its clinical potential. So far several clinical trials have been performed to investigate the efficacy of levosimendan as a function-maintaining compound in patients with heart failure due to an AMI (Follath *et al.*, 2002; Cleland *et al.*, 2004; Nieminen *et al.*, 2000; Michaels *et al.*, 2005; Duygu *et al.*, 2007; Givertz *et al.*, 2007; Sargento *et al.*, 2007; Pollesello and Papp, 2007). Previous laboratory studies have shown levosimendan to have cardioprotective effects in different animal models. It decreased infarct size in dog hearts (Kersten *et al.*, 2000), improved reperfusion function in the isolated guinea pig heart (Du Toit *et al.*, 1999) while in the rabbit heart it was found to decrease infarct size and improve reperfusion cardiac function (Lepran *et al.*, 2006). In the current study, the possible pre- and postconditioning mimetic effects of levosimendan were investigated as we had previously proposed that levosimendan would improve reperfusion function without promoting arrhythmias (Du Toit *et al.*, 1999).

10.2.2 Levosimendan as a possible preconditioning mimetic.

Although Lepran and co-workers referred to their intervention as “pharmacological preconditioning” when they used levosimendan in isolated rabbit hearts (Lepran *et al.*, 2006), this was not true preconditioning since the drug was not washed out before sustained ischaemia. Their studies should rather be referred to as “pre-treatment”. Several studies were performed on other drugs to test their preconditioning mimetic abilities, for example resveratrol (Hattori *et al.*, 2002), desflurane, sevoflurane, isoflurane and halothane (Piriou *et al.*, 2002) and

morphine (Ludwig *et al.*, 2003). The first and last mentioned studies were performed on isolated rat hearts and the middle one on rabbit hearts. All these researchers demonstrated reduced infarct sizes in their experimental models, similar to that of preconditioning. In the current study, levosimendan was also shown to be a preconditioning mimetic. Other studies on levosimendan's effects on the isolated guinea pig heart were either done by infusion of levosimendan into the heart during low-flow index ischaemia or reperfusion (Du Toit *et al.*, 1999) or levosimendan was administered after 45 minutes of normothermic or 180 minutes of hypothermic cardioplegic arrest (Lochner *et al.*, 2000). In the current study the protocols for preconditioning with levosimendan (LPC), included washout of the drug before subjecting the heart to index ischaemia which resulted in a reduction in infarct size, while cardiac function was unchanged. It was concluded that levosimendan could indeed be used as a mimetic for preconditioning

10.2.3 Levosimendan as a possible postconditioning mimetic

In a study by Tissier *et al.*, 2007b rabbit hearts were postconditioned with the phytoestrogen genistein which reduced infarct size. By substituting the ischaemic episodes in the IPostC protocol with levosimendan, infarct size was significantly reduced compared to the control experimental group. As was the case for levosimendan preconditioning, there was no change observed in functional recovery. Thus it was also concluded that levosimendan could mimic the cardioprotective effects of IPostC. Although other studies tested the postconditioning mimetic effects of other drugs, as far as we know, this is the first study to demonstrate that levosimendan can mimic the cardioprotective effects of ischaemic pre- and postconditioning in the isolated guinea pig heart.

10.3 Pre-treatment of the isolated guinea pig heart with levosimendan

Preconditioning should not be confused with pre-treatment. Numerous studies have been carried out on different animal models by pre-treating the heart with a compound (without washout), before subjecting it to index ischaemia. For example, pre-treatment of isolated rabbit hearts with angiotensin II (Liu *et al.*, 1995) or the isolated rat heart with endothelin-1 (ET-1)(Wang *et al.*, 1995) or the

isolated rat heart with tumor necrosis factor- α (TNF- α) (Eddy *et al.*, 1992) all resulted in a significant reduction in infarct size.

In the previous section of the study the focus was on levosimendan preconditioning of the myocardium against the adverse consequences of ischaemia. In this section of the study, levosimendan was administered for 10 minutes without washout before the heart was subjected to index ischaemia. When the guinea pig heart was pre-treated with levosimendan, the greatest reduction in infarct size was induced compared to un-treated controls and for the first time in our study a concurrent improved functional recovery as a result of an intervention was observed. Thus pre-treatment with levosimendan confers greater cardioprotection against ischaemic/reperfusion injury than LPC.

The pre-treatment data in the current study confirmed observations by other research groups (Kersten *et al.*, 2000; Du Toit *et al.*, 1999; Lepran *et al.*, 2006). These data demonstrated that levosimendan has greater efficacy as a protective compound against ischaemia when administered directly prior to index ischaemia. This is probably due to the fact that during LPC, the drug is washed out during the 5 minutes of perfusion prior to index ischaemia and may therefore be less effective. Thus the drug seems to have greater efficacy if it is still present in the heart during index ischaemia. This may be due to the fact that levosimendan also directly opens the K_{ATP} channels which would, during index ischaemia, be expected to be beneficial to the heart.

10.4 Investigating whether ischaemic- and levosimendan preconditioning has additive effects.

In order to establish whether the two interventions of IPC and LPC shared the same mechanisms of protection, IPC and LPC were combined, to investigate whether they would have an additive protective effect in the ischaemic/reperfused myocardium. No difference was found in infarct size when IPC and LPC were combined. The functional recovery of the hearts was not changed by the combination of IPC and LPC. In a study done on adult male rats to investigate whether hypoxic and ischaemic preconditioning had an additive protective effect,

Neckář and co-workers (2002) found no additive effect, suggesting a common mechanism of protection for hypoxic and ischaemic preconditioning.

It can therefore be concluded that IPC and LPC probably share common mechanisms of protection.

10.5 The effects of the K_{ATP} channel blockers on levosimendan-induced pre- and postconditioning.

The mitochondrial and sarcolemmal K_{ATP} channels have been implicated to play a major role as an end-effector in IPC (Gross & Auchampach, 1992; Auchampach *et al.*, 1992; Garlid *et al.*, 2003). According to a recent review by Cohen *et al.* (2006), the focus has shifted from these channels as end-effectors in preconditioning to the mPTP as the primary end-effector. However, convincing evidence exists for an important role for these channels in cardioprotection. Much of the evidence for the mitochondrial and sarcolemmal K_{ATP} channel's involvement in cardioprotection is based on the observation that the protective effects of ischaemic or pharmacological pre- and postconditioning can be lost by blocking/inhibiting the opening of these channels (Grover *et al.*, 1997). In a study on the isolated rat heart, the heart was protected by mimicking the effects of IPC with morphine and was blocked by the administration of glibenclamide (GBD) (Schultz *et al.*, 1996), a blocker of both the mitochondrial and sarcolemmal K_{ATP} channels. Similarly, hearts could be protected with K_{ATP} channel openers such as diazoxide and this protection was also lost when these hearts were perfused with either 5HD or GBD (Wang *et al.*, 2001).

Since levosimendan has K_{ATP} channel opening properties (Yokoshiki *et al.*, 1997; Kopustinskiene *et al.*, 2001), it was decided to investigate the possible role levosimendan and its K_{ATP} channel opening properties as a trigger or an end-effector in LPC, LPT and LPostC.

Several studies investigated the effect of the mitochondrial channel blocker on the intervention of preconditioning (Lim *et al.*, 2004; Cohen *et al.*, 2001; Hide and Thiemermann, 1996). In all these studies, the protective effect that was seen with preconditioning was abolished by mito K_{ATP} channel blockers. The same

observations were made in the current study, although the intervention of LPT and LPostC were included and where previous studies were on the isolated rat heart, the current study was performed on the isolated guinea pig heart.

Firstly the non-specific mitochondrial K_{ATP} channel blocker, 5-hydroxydecanoic-acid (5HD), was used: with co-administration of 5-HD and levosimendan, the significant infarct size reduction of LPC alone, was abolished. This finding suggested that the K_{ATP} channel may be involved in the cardioprotection induced by levosimendan, when administered as preconditioning stimulus (trigger).

Similar observations were made when 5HD was administered during levosimendan pre-treatment. This intervention abolished the very pronounced cardioprotection that was observed with levosimendan pre-treatment, suggesting an important role for the K_{ATP} channels in cardioprotection with LPT. This data suggests that the cardioprotective effects that were found with LPT were partially due to the fact that the K_{ATP} channels were opened before the heart was subjected to index ischaemia. The protection obtained with levosimendan as trigger may also be due to its effects on these channels before the onset of index ischaemia.

Co-administration of levosimendan and 5HD during postconditioning, also caused abolishment of postconditioning, as shown by the increased infarct size and lack of functional recovery (see figure 8.5). The opening of the K_{ATP} channels in the initial stages of reperfusion thus seems to be a necessity for salvaging of the ischaemic/reperfused myocardium.

However, it must be noted that 5HD were found to have additional effects on mitochondrial function that may undermine their use as a specific mito K_{ATP} channel blocker (Lim *et al.*, 2002; Hanley *et al.*, 2002).

In the literature, there are also studies that investigated the effect of glibenclamide (a non-specific K_{ATP} channel blocker) on the protective effect of ischaemic preconditioning (Tomai *et al.*, 1994; Toombs *et al.*, 1993; Schultz *et al.*, 1996). These researchers also demonstrated that the administration of GBD abolished the protection as was found in the current study.

Lastly, a non-specific (mitochondrial and sarcolemmal) K_{ATP} channel blocker, glibenclamide (GBD), was also used. This non-specific K_{ATP} channel blocker was chosen, as a specific sarcolemmal K_{ATP} channel blocker, HMR 1098, has been shown to have questionable outcomes in conditions of metabolic stress (Rainbow *et al.*, 2005). In another study, direct evidence was provided that glibenclamide alone (10 μ M or 100 μ M) causes mitochondrial oxidation (Hu *et al.*, 1999). The observations are consistent with the reported uncoupling effect of glibenclamide on mitochondria (Szewczyk *et al.*, 1997). All the protocols that were mentioned above for the co-administration of levosimendan and 5HD were repeated and it was found that GBD abolished the infarct size lowering effect of both LPC and LPostC.

In summary, these results also strongly suggest the involvement of the K_{ATP} channels in cardioprotection by levosimendan-induced as it did in the case of 5HD co-administration with levosimendan. This is also the first study to investigate the role of these channels in protection in the isolated guinea pig heart.

10.6 The role of the RISK pathway in levosimendan-induced cardioprotection.

Ischaemic/reperfusion injury leads to both apoptotic and necrotic cell death and recent studies have demonstrated that preconditioning-induced or pharmacological activation of the RISK pathway during reperfusion can reduce both apoptosis and necrosis and thus, infarct size (Yellon & Baxter, 1999; Hausenloy & Yellon, 2004b). The exact mechanisms whereby activation of the RISK pathway induces cardioprotection have not yet been established but probably involve closing the mPTP during reperfusion (Hausenloy *et al.*, 2005b). The reperfusion induced opening of these mitochondrial transition pores is thought to be secondary to mitochondrial calcium overload, oxidative stress and ATP depletion after an ischaemic event (Hausenloy & Yellon, 2003). Closing of these pores possibly occurs through the phosphorylation of ERK, which is in turn associated with the phosphorylation and activation of eNOS, phosphorylation and inactivation of GSK3 β or phosphorylation and mitochondrial translocation of PKC ϵ (Hausenloy *et al.*, 2005b). To investigate the possible role of the RISK pathway in levosimendan-induced cardioprotection, the phosphorylation of both PKB/Akt and ERK42/44 was monitored during the first 10 minutes of reperfusion in LPC and LPT hearts. No

change in total or phosphorylated PKB/Akt could be found in LPC or LPT. No differences were found in the total protein of ERK42/44 for the different experimental groups. However, in contrast to PKB/Akt, phosphorylation of both ERK42 and ERK44 was significantly increased in LPC and LPT at 5 and 10 minutes reperfusion.

Although a protective role for RISK pathway activation has already been demonstrated in previous studies in ischaemic pre- and postconditioning (Hausenloy *et al.*, 2005a) our data suggests that the RISK pathway, and more specifically ERK42/44, can also be phosphorylated by pharmacological preconditioning and pre-treatment with levosimendan. To our knowledge, this is the first study to demonstrate a strong association between the cardioprotective effects of pharmacological pre-treatment of the heart with levosimendan and the phosphorylation of the cardiac ERKs.

Several studies have reported increased ERK42/44 activity during reperfusion after postconditioning (Darling *et al.*, 2005; Schwartz and Lagranha, 2005). However, in the current study, there was a significant increase in phosphorylation of ERK42, but not for ERK44 and PKB/Akt in IPostC and LpostC.

To further assess the significance of ERK phosphorylation in levosimendan induced cardioprotection, we inhibited the phosphorylation of ERK42/44 with the MEK inhibitor – PD 098059: no differences were found in the total protein expression of ERK42/44 for the different experimental groups. It was decided not to make use of a PKB/Akt inhibitor, as no phosphorylation of PKB/Akt was observed in any of the investigated interventions. As expected, the phosphorylation of ERK42/44 was significantly reduced at 5 minutes of reperfusion with the administration of PD alone. To investigate the effect that the inhibition of the phosphorylation of ERK42/44 has on cardioprotection, all the above protocols (PD and PD+LPT) were repeated and infarct size was determined. For the experimental group which was perfused with PD only, infarct size was similar to that of untreated controls. However, the administration of PD significantly reduced the protection afforded by LPT, but the infarct size was still significantly less than that of the control group. This suggests that the cardioprotective effects of levosimendan are not solely

dependent on phosphorylation of the RISK pathway, but that other factors may also be involved.

It must also be noted that this is the first study to investigate the effects of levosimendan on the RISK pathway components (PKB/Akt and ERK42/44).

10.7 Limitations of this study.

In this study, the effects of two K_{ATP} channel blockers only, namely 5HD and GBD, were investigated. More specific K_{ATP} channel blockers should have been included in this study, in order to eliminate a wider range of effects that these drugs may have on cardioprotection afforded by the specific interventions that were investigated in this study. Due to price and availability of these channel blockers, it was decided to only use 5HD and GBD as these blockers were readily available. However, conclusive results were obtained with both 5HD and GBD showing the involvement of the K_{ATP} channels in levosimendan-induced cardioprotection.

In the Western blot analysis the phosphorylation of PKB/Akt and ERK42/44 were only evaluated at two timepoints in reperfusion (5 and 10 minutes). The data would perhaps have been more complete if a few more timepoints were included (both earlier and later during reperfusion). It is possible that optimal phosphorylation of these kinases might have occurred at other timepoints than the two used in this study. However, other studies in our laboratory showed no activation of PKB/Akt occurred after 10 minutes of reperfusion.

10.8 Future directions.

The major experimental cardioprotective effect of levosimendan pre-treatment warrants further clinical trials, especially in those patients with large AMIs when this compound may be expected to protect against both LV failure and subsequent reperfusion injury. Future work should include investigating the possible link between levosimendan pre-treatment, ERK42/44 phosphorylation and prevention of mPTP opening.

ADDENDUM A

A1: Pre- and post ischaemic data for control hearts.

A1.1: Pre-ischaemic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 20	36	18	13.49	195.48
MH 35	60	16	28.54	184.04
MH 38	56	16	30.16	202.67
MH 39	56	20	36.71	224
MH 40	48	20	28.95	214.14
MH 41	40	14	28.42	171.99
MH 42	72	26	44.11	260.22
MH 43	69	27	37.86	226.96
MH 70	56	22	34.49	187.06
Mean & SEM	55 ± 3.76	20 ± 1.41	31.41 ± 2.69	207.40 ± 8.51

A1.2: Post-ischaemic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 20	0	46	0.27	180.2	51.54
MH 35	32	30	18	190.08	37.74
MH 38	6	56	12.76	156.15	44.79
MH 39	16	32	27.89	175.14	55.76
MH 40	6	26	24	336.49	40.22
MH 41	16	36	26.43	156.05	51.34
MH 42	36	21	26.42	233.62	43.8
MH 43	46	27	30.94	154.96	45.99
MH 70	36	24	28.45	189.14	47.88
Mean & SEM	22 ± 5.13	33 ± 3.56	21.68 ± 3.09	196.87 ± 18.18	46.56 ± 1.80

A2: Pre- and post ischaemic data for IPC 1 hearts.

A2.1: Pre-ischaemic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 13	40	18	16.32	190.17
MH 14	52	22	26.22	175.19
MH 15	36	16	10.11	215.74
MH 19	36	18	12.53	170.49
Mean & SEM	41 ± 3.28	19 ± 1.09	16.30 ± 3.07	187.90 ± 8.82

A2.2: Post-ischaeamic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 13	0	22	7.58	165.12	51.18
MH 14	0	46	7.98	158.53	43.56
MH 15	0	48	1.53	99.24	41.09
MH 19	0	46	0.3	120.98	35.91
Mean & SEM	0	41 ± 5.36	4.35 ± 1.73	135.97 ± 13.54	42.94 ± 2.75

A3: Pre- and post ischaemic data for IPC 2 hearts.

A3.1: Pre-ischaeamic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 36	64	18	34.7	177.56
MH 37	48	18	24.22	197.16
MH 46	54	26	32.37	210
MH 47	50	27	37.29	254.99
MH 48	60	26	41.38	220
MH 49	72	26	46.56	259
MH 50	60	26	40.89	213.83
MH 36	64	18	34.7	177.56
Mean & SEM	58 ± 3.30	24 ± 1.36	36.77 ± 2.54	218.93 ± 10.30

A3.2: Post-ischaeamic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 36	28	24	26.12	167.44	21.5
MH 37	12	20	15.75	177.32	35.74
MH 46	44	27	30.69	177.46	22.8
MH 47	23	27	10.86	210.83	34.71
MH 48	27	27	36.33	195.9	28.78
MH 49	52	27	32.01	220.57	19.17
MH 50	40	17	38.04	207.36	15.05
MH 36	28	24	26.12	167.44	21.5
Mean & SEM	32 ± 4.82	24 ± 1.49	27.11 ± 3.60	193.84 ± 7.04	25.39 ± 2.76 *

p < 0.001 vs. control

A4: Pre- and post ischaemic data for IPC 3 hearts.

A4.1: Pre-ischaemic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 23	22	8	28.39	221.63
MH 29	54	16	31.23	216.53
MH 69	62	22	35.44	183.59
MH 71	69	28	41.46	196.66
MH 72	60	22	32.05	157.65
MH 73	60	24	27.09	172.26
Mean & SEM	55 ± 6.20	20 ± 2.62	32.61 ± 1.95	191.39 ± 9.34

A4.2: Post-ischaemic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 23	0	44	14.2	193.79	14.41
MH 29	18	32	23.21	181.57	22.28
MH 69	34	26	28.45	184.13	26.29
MH 71	26	28	25.29	165.09	30.49
MH 72	26	38	20.62	177.93	19.61
MH 73	0	54	12.58	121.96	18.34
Mean & SEM	17 ± 5.35	37 ± 3.97	20.73 ± 2.33	170.75 ± 9.56	21.90 ± 2.16 *

p < 0.001 vs. control

A5: Pre- and post ischaemic data for IPostC hearts.

A5.1: Pre-ischaemic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 224	36	15	17.66	214.14
MH 226	20	15	25.36	106.45
MH 229	60	30	34.12	177.8
MH 233	60	24	16.47	160.51
MH 236	46	21	16.92	130.59
MH 238	66	24	21.74	99.96
MH 241	81	20	27.04	142.44
MH 243	60	27	20.48	161.96
Mean & SEM	54 ± 6.28	22 ± 1.77	22.47 ± 2.04	149.23 ± 12.48

A5.2: Post-ischaeamic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 224	21	24	21.81	184.3	19.48
MH 226	12	18	16.66	163.33	33.29
MH 229	39	42	19.16	172.23	22.57
MH 233	45	27	18.71	158.27	28.15
MH 236	45	42	20.03	122.32	25.57
MH 238	45	45	19.49	131.7	15.58
MH 241	45	36	19.3	140.59	14.12
MH 243	0	27	13.43	126.02	12.43
Mean & SEM	32 ± 5.95	33 ± 3.29	18.57 ± 0.83	149.85 ± 7.58	21.40 ± 2.43 *

p < 0.05 vs. control

ADDENDUM B

B1: Pre- and post ischaemic data for LPC hearts.

B1.1: Pre-ischaeamic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 52	54	26	29.01	250.64
MH 54	75	23	30.71	235.46
MH 56	54	17	31.73	230.65
MH 57	69	24	31.59	256.32
MH 58	52	21	28.18	240.99
MH 136	40	15	30.5	225.77
MH 138	54	22	29.7	213.35
MH 140	54	21	39.8	249.48
Mean & SEM	5.50 ± 3.57	21.13 ± 1.19	31.40 ± 1.19	237.83 ± 4.77

B1.2: Post-ischaeamic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 52	45	24	13.57	232.16	26.65
MH 54	56	26	31.32	199.87	20.87
MH 56	34	23	12.61	222.75	33.6
MH 57	48	27	22.68	234.63	23.55
MH 58	25	21	12.92	220.34	29.16
MH 136	21	45	20.3	192.17	9.36
MH 138	27	39	22.72	214.95	14.79
MH 140	15	48	22.63	232.50	7.14
Mean & SEM	33.88 ± 4.78	31.63 ± 3.54	19.84 ± 2.15	218.67 ± 5.18	20.64 ± 3.13 *

p < 0.05 vs. control

B2: Pre- and post ischaemic data for LPostC hearts.

B2.1: Pre-ischaemic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 225	50	24	29.17	116.03
MH 227	60	18	37.87	97.62
MH 230	36	15	24.51	116.15
MH 232	74	24	25.56	135.32
MH 235	60	21	17.45	197.21
MH 237	30	19	13.7	236.28
MH 239	46	21	16.27	241.98
Mean & SEM	51 ± 5.31	20 ± 1.14	23.50 ± 2.96	162.94 ± 21.29

B2.2: Post-ischaemic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 225	24	42	20.02	126.91	27.91
MH 227	27	21	25.23	116.53	23.75
MH 230	30	27	23.18	156.89	11.73
MH 232	24	27	19.63	167.95	21.27
MH 235	36	21	15.8	193.02	18.41
MH 237	42	30	14.75	185.67	23.35
MH 239	18	30	11.66	186.21	17.43
Mean & SEM	29 ± 2.83	28 ± 2.49	18.61 ± 1.68	161.88 ± 10.56	20.55 ± 1.83 *

p < 0.05 vs. control

ADDENDUM C

C1: Pre- and post ischaemic data for LPT hearts.

C1.1: Pre-ischaemic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 61	56	20	31.89	231.85
MH 62	75	24	32.65	250.17
MH 63	72	24	30.71	246.05
MH 64	48	21	26.64	247.59
MH 65	69	27	32.55	222.52
MH 66	66	24	32.25	232.17
MH 67	48	24	27.46	240.14
Mean & SEM	62 ± 3.94	23.43 ± 0.80	30.59 ± 0.88	238.64 ± 3.55

C1.2: Post-ischaeamic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 61	34	30	12.51	215.84	6.74
MH 62	50	26	24.97	224.85	4.01
MH 63	50	30	28.4	209.62	4.58
MH 64	16	20	8.73	181.70	9.58
MH 65	48	30	33.11	237.08	7.84
MH 66	36	30	33.86	173.05	2.27
MH 67	36	27	31.16	169.01	5.26
Mean & SEM	38.57 ± 4.27	27.57 ± 1.31	24.68 ± 3.54	201.59 ± 9.41	5.75 ± 0.87 *

p < 0.001 vs. control

ADDENDUM D

D1: Pre- and post ischaemic data for IPC + LPC hearts.

D1.1: Pre-ischaeamic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 207	50	20	30.00	207.05
MH 209	63	20	43.00	115.93
MH 211	40	21	19.00	227.26
MH 213	56	15	41.00	226.01
MH 216	50	18	32.00	199.49
MH 217	42	24	18.00	198.78
Mean & SEM	50.17 ± 3.20	19.67 ± 1.12	24.40 ± 2.86	195.75 ± 15.30

D1.2: Post-ischaeamic:

Heart Number	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 207	33	36	20.82	168.8	16.03
MH 209	28	30	24.50	174.49	17.38
MH 211	21	30	22.06	173.49	23.47
MH 213	30	30	20.95	214.14	29.61
MH 216	15	27	13.9	222.24	26.43
MH 217	15	30	18.24	221.62	15.71
Mean & SEM	23.67 ± 2.90	30.50 ± 1.10	20.08 ± 1.36	195.80 ± 9.69	21.44 ± 2.20 *

p < 0.05 vs. control

ADDENDUM E

E1: Pre- and post ischaemic data for CONTROL-5HD hearts.

E1.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 151	54	24	36.35	156.91
MH 153	54	24	12.1	194.77
MH 155	60	24	29.13	192.37
MH 157	50	20	28.39	137.38
MH 159	60	26	28.78	222.03
MH 161	48	18	24.76	220.26
MH 165	72	24	27.99	211.53
MH 167	48	21	18.25	215.59
Mean & SEM	55.75 ± 2.68	22.63 ± 0.88	25.72 ± 2.46	193.86 ± 10.33

E1.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 151	54	30	10.66	180.68	29.21
MH 153	51	21	11.09	171.83	15.6
MH 155	32	28	23.12	158.33	39.2
MH 157	36	26	28.52	165.75	32.85
MH 159	32	28	21.73	213.84	37.32
MH 161	40	26	16.46	201.63	39.97
MH 165	54	30	22	234.9	40.86
MH 167	45	39	15.74	189.18	35.83
Mean & SEM	43.00 ± 3.09	28.50 ± 1.70	18.67 ± 2.06	189.52 ± 8.59	33.91 ± 2.77

E2: Pre- and post ischaemic data LPC+5HD hearts.

E2.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 154	70	18	26.15	216.57
MH 156	72	22	30.89	191.12
MH 158	72	26	39.34	174.33
MH 163	50	18	24.17	130.01
MH 169	75	21	31.75	226.76
MH 160	54	14	19.42	207.83
Mean & SEM	65.50 ± 3.97	19.83 ± 1.54	28.62 ± 2.58	191.10 ± 13.14

E2.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 154	36	24	18.97	199.9	39.52
MH 156	36	28	23.97	195.39	36.54
MH 158	20	34	28.91	156.41	40.2
MH 163	20	66	12.71	177.74	29.12
MH 169	12	40	13.96	209.11	40.67
MH 160	20	26	16.4	248.44	39.95
Mean & SEM	24.00 ± 3.65	36.33 ± 5.84	19.15 ± 2.32	197.83 ± 11.57	37.67 ± 1.65

E3: Pre- and post ischaemic data for LPT+5HD hearts.

E3.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 162	66	18	17.79	201.24
MH 170	78	24	31.75	226.76
MH 171	54	24	24.57	204.63
MH 172	48	18	17.39	174.35
MH 174	72	24	23.58	177.69
MH 177	60	27	29.31	193.83
Mean & SEM	63.00 ± 4.18	22.50 ± 1.37	24.07 ± 2.18	196.42 ± 7.18

E3.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 162	45	30	16.27	204.34	33.26
MH 170	36	45	24.77	179.19	40.91
MH 171	45	33	24.83	154.38	32.76
MH 172	34	28	16.14	204.63	45.83
MH 174	60	42	20.49	194.47	32.93
MH 177	36	40	21.34	184.75	45.67
Mean & SEM	42.67 ± 3.63	36.33 ± 2.59	20.64 ± 1.44	186.96 ± 7.07	38.56 ± 2.37

E4: Pre- and post ischaemic data for LPostC+5HD hearts.

E4.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
AG 119	66	24	21.58	144.92
AG 122	69	27	24.21	216.51
AG 124	56	15	21.05	148.5
AG 125	72	24	24.73	178.02
Mean & SEM	65.75 ± 3.01	22.50 ± 2.25	22.89 ± 0.80	171.99 ± 14.37

E4.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
AG 119	33	50	13.16	144.92	33.86
AG 122	54	24	22.11	270.64	32.53
AG 124	27	40	12.64	137.73	35.24
AG 125	45	35	17.37	152.04	39.91
Mean & SEM	39.75 ± 5.24	37.25 ± 4.68	16.32 ± 1.91	176.33 ± 27.34	35.39 ± 0.01

E5: Pre- and post ischaemic data for CONTROL-GBD hearts.

E5.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 180	50	51	20.15	209.35
MH 183	40	21	23.98	188.32
MH 186	66	24	27.14	210.97
MH 190	75	24	30.62	194.07
MH 194	56	33	28.58	202.63
MH 198	45	20	24.7	209.41
MH 208	60	15	26.81	200.92
MH 210	50	21	29.05	171.33
Mean & SEM	55.25 ± 3.79	26.13 ± 3.73	26.38 ± 1.10	198.38 ± 4.47

E5.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 180	0	21	11.00	189.59	42.42
MH 183	26	36	17.58	167.25	48.2
MH 186	0	30	13.90	229.19	41.71
MH 190	30	24	22.20	195.5	40.71
MH 194	45	33	29.97	168.72	39.75
MH 198	30	50	23.39	173.06	48.53
MH 208	30	30	20.15	225.12	61.21
MH 210	21	27	21.08	104.19	51.66
Mean & SEM	22.75 ± 5.16	31.38 ± 2.95	19.91 ± 1.94	181.58 ± 13.02	46.77 ± 2.40

E6: Pre- and post ischaemic data for LPC+GBD hearts.

E6.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 185	66	21	32.14	145.14
MH 188	60	18	31.48	227.07
MH 192	60	15	25.01	236.95
MH 196	40	42	30.36	238.64
MH 199	50	15	26.35	253.44
MH 201	60	24	37.34	203.94
MH 202	60	21	26.94	229.88
MH 205	42	15	19.76	205.56
Mean & SEM	54.75 ± 3.16	21.38 ± 2.97	28.67 ± 1.76	217.58 ± 11.13

E6.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 185	48	33	20.09	188.44	48.83
MH 188	10	40	15.35	212.37	46.93
MH 192	16	12	14.56	190.85	51.33
MH 196	18	60	24.70	161.87	43.81
MH 199	24	21	21.01	211.86	46.08
MH 201	42	24	26.88	209.75	43.49
MH 202	12	28	20.82	119.22	41.56
MH 205	18	30	20.55	193.2	52.47
Mean & SEM	23.50 ± 4.63	31.00 ± 4.75	20.50 ± 1.37	185.95 ± 10.50	46.81 ± 1.28

E7: Pre- and post ischaemic data for LPT+GBD hearts.

E7.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
MH 179	60	18	32.74	111.6
MH 182	66	30	28.78	221.75
MH 189	56	21	30.17	245.2
MH 191	45	18	31.94	184.91
MH 195	45	15	27.73	233.94
MH 200	46	21	21.08	246.56
MH 203	63	24	31.61	223.79
MH 204	42	18	29.11	246.19
Mean & SEM	52.88 ± 3.12	20.63 ± 1.54	29.15 ± 1.22	214.24 ± 15.29

E7.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
MH 179	12	27	17.79	189.11	49.7
MH 182	42	30	24.24	232.67	45.08
MH 189	0	0	1.97	156.83	34.32
MH 191	15	33	21.15	111.15	49.54
MH 195	18	33	20.22	198.07	56.11
MH 200	0	33	16.13	199.53	51.27
MH 203	27	33	23.25	205.42	39.38
MH 204	0	6	3.16	122.05	61.94
Mean & SEM	14.25 ± 4.94	24.38 ± 4.45	15.99 ± 2.88	176.85 ± 14.12	48.47 ± 2.90

E8: Pre- and post ischaemic data for LPostC+GBD hearts.

E8.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
AG 102	66	21	24.21	151.58
AG 106	66	30	28.42	206.59
AG 108	50	18	3.69	212.27
AG 115	75	21	29.47	115.17
Mean & SEM	64.25 ± 4.51	22.50 ± 2.25	21.45 ± 5.22	171.40 ± 20.10

E8.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
AG 102	36	35	18.95	148.5	37.01
AG 106	48	55	23.15	132.67	34.49
AG 108	0	27	27.37	79.86	48.78
AG 115	0	54	12.11	119.49	43.89
Mean & SEM	21.00 ± 10.71	42.75 ± 6.05	20.40 ± 2.82	120.13 ± 12.71	41.04 ± 0.03

ADDENDUM F

F1: Pre- and post ischaemic data for PD hearts.

F1.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
AG7	66	18	23.67	209.87
AG9	80	24	28.07	179.05
AG11	60	30	27.52	195.34
AG13	60	18	16.51	208.66
AG15	72	22	23.64	152.04
AG19	63	27	17.07	253.69
Mean & SEM	90.00 ± 3.30	23.17 ± 1.80	22.75 ± 1.86	199.78 ± 12.71

F1.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
AG7	18	21	12.11	194.94	31.17
AG9	54	34	18.16	219.47	36.5
AG11	30	45	18.72	172.65	21.84
AG13	33	60	13.76	196.3	26.83
AG15	60	30	22.57	165.53	34.39
AG19	12	12	8.81	253.69	36.14
Mean & SEM	34.50 ± 7.13	33.67 ± 6.38	15.69 ± 1.87	200.43 ± 12.06	31.15 ± 2.17

F2: Pre- and post ischaemic data for PD + LPT hearts.

F2.1: Pre-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)
AG8	66	21	20.36	156.75
AG10	66	21	20.91	163.08
AG12	75	24	28.08	175.45
AG14	72	21	20.37	199.09
AG18	69	30	21.67	178.93
AG20	75	30	26.42	190.33
Mean & SEM	94.67 ± 2.88	24.50 ± 1.65	22.97 ± 1.26	177.27 ± 5.95

F2.2: Post-ischaemic:

Experimental Group	Aortic Output (ml/min)	Coronary Flow (ml/min)	Aortic Developed Pressure (mmHg)	Heart Rate (bpm)	Infarct Size (%)
AG8	48	30	19.27	179.23	13.2
AG10	59	21	22.02	195.72	16.61
AG12	60	27	20.37	137.55	22.18
AG14	45	23	19.27	183.99	11.7
AG18	52	33	19.82	185.37	19.52
AG20	52	30	24.77	189.7	19.52
Mean & SEM	52.67 ± 2.21	27.33 ± 1.71	20.92 ± 0.80	178.59 ± 7.78	17.12 ± 1.51 *

p < 0.05 vs. control

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